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# Oxidative stress activates a positive feedback between the γ- and β-secretase cleavages of the β-amyloid precursor protein

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## Abstract

Sequential cleavages of the β-amyloid precursor protein cleaving enzyme 1 (BACE1) by β-secretase and γ-secretase generate the amyloid β-peptides, believed to be responsible of synaptic dysfunction and neuronal cell death in Alzheimer's disease (AD). Levels of BACE1 are increased in vulnerable regions of the AD brain, but the underlying mechanism is unknown. Here we show that oxidative stress (OS) stimulates BACE1 expression by a mechanism requiring γ-secretase activity involving the c-*jun* N-terminal kinase (JNK)/c-*jun* pathway. BACE1 levels are increased in response to OS in normal cells, but not in cells lacking presenilins or amyloid precursor protein. Moreover, BACE1 is induced in association with OS in the brains of mice subjected to cerebral ischaemia/reperfusion. The OS-induced BACE1 expression correlates with an activation of JNK and c-*jun*, but is absent in cultured cells or mice lacking JNK. Our findings suggest a mechanism by which OS induces BACE1 transcription, thereby promoting production of pathological levels of amyloid β in AD.

### Keywords

Alzheimer's Disease; BACE1; JNK pathway; oxidative stress; γ-secretase

The central pathological event in Alzheimer's disease (AD) is an accumulation of amyloid β (Aβ)-peptide in the brain, the product of two sequential endoproteolytic cleavages of the β-amyloid precursor protein (APP), a transmembrane type 1 protein. The β-secretase (β-site amyloid precursor protein cleaving enzyme 1, BACE1) cleaves the ectodomain of APP, producing an APP C-terminal fragment. This is further cleaved within the transmembrane domain by a γ-secretase, resulting in the release of Aβ peptides, with two C-terminal variants, at residue 40 (Aβ40) or at residue 42 (Aβ42) (Selkoe 2001). Presenilin 1 (PS1) is the catalytic subunit of the γ-secretase. Altered activities of both γ-secretase and BACE1 are involved in the pathogenesis of AD. PS1 gene mutations linked with early-onset familial AD increase the production of Aβ42, which aggregates and accumulates faster than Aβ40 (Lemere *et al.*

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1996; Citron *et al.* 1997). The expression and the activity of PS1 and BACE1 are elevated in the brain of late-onset sporadic AD patients (Fukumoto *et al.* 2002; Holsinger *et al.* 2002; Yang *et al.* 2003; Matsui *et al.* 2007). The mechanisms responsible for the increased PS1 expression as well as of BACE1 activity in sporadic AD are unknown.

We and others have shown that the expression and activity of BACE1 is increased by oxidants and by the lipid peroxidation product 4-hydroxynonenal (HNE) (Tamagno *et al.* 2002, 2005; Kao *et al.* 2004; Tong *et al.* 2005), and that there is a significant correlation of BACE1 activity with oxidative markers in sporadic AD brain tissue (Borghi *et al.* 2007). Oxidative stress (OS) increases during normal ageing and is believed to be an early event in AD pathology (Nunomura *et al.* 2001; Cutler *et al.* 2004), which may contribute to membrane damage, cytoskeletal alterations and cell death (Perry *et al.* 2000a,b). OS and Aβ production are proportionally linked to each other because Aβ induces OS *in vivo*, and *in vitro* (Hensley *et al.* 1994; Mark *et al.* 1997; Murakami *et al.* 2005; Tabner *et al.* 2005), and OS increases the production of Aβ (Paola *et al.* 2000; Tamagno *et al.* 2005; Tong *et al.* 2005).

In the present study, we first found that OS increase the γ-secretase activity. We then demonstrated that the increased expression of BACE1 induced by OS is regulated by the γ-secretase activity, which in turn is mediated by the *c-jun* N-terminal kinase (JNK)/c-*jun* pathway.

## Materials and methods

### Cell culture, treatments and transfection

SK-N-BE neuroblastoma cells and wild-type, PS1/PS2-deficient, APP deficient and JNK-deficient mouse embryonic fibroblasts (MEFs) were generated and cultured as described previously (Herreman *et al.* 1999, 2003; Heber *et al.* 2000; Leissring *et al.* 2002; Tamagno *et al.* 2003). SK-N-BE cells and MEFs were left for 16 h in serum-free medium before any treatments. Cells were incubated for 1, 3 and 6 h with HNE (Calbiochem, Darmstadt, Germany) at a concentration of 5 μmol/L, or $H_2O_2$ (Sigma Chemical Company, St. Louis, MO, USA) at a concentration of 20 μmol/L. APPko–MEFs were incubated with 1 μmol/L Aβ 1–40 and Aβ 1–42 (Bachem, Weil am, Rhein, Germany) for 1 h. The Aβ peptides were dissolved in water at 1 mg/mL and then immediately added to the cells to avoid aggregation. Five microlitres of the solutions were applied to Formvar-coated grids, negatively stained with 5% uranyl acetate, and observed under a Philips CM10 transmission electron microscope at 80 kV. Actinomycin D (Sigma Chemical Company) was used at 1 μmol/L concentration 30 min before pro-oxidants. The BACE1 inhibitor IV was added at the concentration of 15 nmol/L.

The γ-secretase inhibitor L685,458 (Bachem) was added (at a final concentration of 1 μmol/L) to SK-N-BE cells, 8 h before treatments with pro-oxidants. The cell permeable JNK-inhibitor peptide (JIP)/JIP-1-HIV-TAT peptide (Phoenix Pharmaceuticals, Karlsruhe, Germany) was injected intraperitoneally to mice at a dose of 0.3 mg/kg 30 min before ischaemic injury or sham operation. Fusion constructs were amplified by PCR and cloned into pcDNA3, as described previously (Passer *et al.* 1999a,b; Cao and Sudhof 2001). Transient transfection of cDNAs into cells was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

### Animals and surgery

BalbC mice (Harlan Italy, San Pietro al Natisone, Udine, Italy) weighing 20 g were housed in a controlled environment at 25 ± 2°C with alternating 12 h light and dark cycles. They were provided with Piccioni pellet diet (no. 48, Gessate Milanese, Milano, Italy) and water *ad libitum*. All mice were acclimatized in our animal facility for at least 1 week before

experiments. Animal care was in compliance with Italian regulations on the protection of animals used for experimental and other scientific purposes (n. 86/609/Comunita Economica Europea) as well as with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health.

Animals were anaesthetized through intraperitoneal injection (20 mg/kg) of Zoletil 100 (Laboratoires Virbac, Carros, France). Anaesthetized mice were placed onto a thermostatically controlled heating pad, a rectal temperature probe was inserted and body temperature was monitored and maintained at 37°C. Half of the animals were intraperitoneally injected with the cell permeable JIP/JIP-1-HIV-TATpeptide (Phoenix Pharmaceuticals) at a dose of 0.3 mg/kg, 30 min before ischaemic injury or sham operation.

To provoke transient middle cerebral artery occlusion (MCAO), a monofilament surgical suture was inserted into the exposed external carotid artery, advanced into the internal carotid artery and wedged into the cerebral arterial circle to obstruct the origin of the middle cerebral artery (Huang *et al.* 1994). Ischaemic injury was maintained for 30 min. During ischaemia the animals were monitored for body temperature, respiration pattern, loss of righting reflex and unresponsiveness, corneal reflexes and fixed and dilated pupils. Reperfusion was administered for 1, 3 and 6 h. At the end of the reperfusion, the anaesthetized mice were killed by decapitation. After decapitation, brains were rapidly dissected at 0°C, removed and transferred to an appropriate ice-chilled homogenizing medium consisting of 50 mmol/L Tris, pH 7.6, 5 mmol/L EDTA, 150 mmol/L NaCl and protease inhibitors for biochemical assays.

## Tissue and cell extracts

Cytosolic and nuclear extracts of mouse brain tissues were prepared by the Meldrum *et al.* (1997). Briefly, cerebral cortical tissues were homogenized at 10% (w/v) in a Potter Elvehjem homogenizer (Wheaton, Millville, NJ, USA) using a homogenization buffer containing 20 mmol/L HEPES, pH 7.9, 1 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, 1% Nonylphenyl-polyethylene glycol, 1 mmol/L EGTA, 1 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin and 2.5 μg/mL leupeptin. Homogenates were centrifuged at 1000 *g* for 5 min at 4°C. Supernatants were removed and centrifuged at 105 000 *g* at 4°C for 40 min to obtain the cytosolic fraction, The pelleted nuclei were resuspended in extraction buffer containing 20 mmol/L HEPES, pH 7.9, 1.5 mmol/L $MgCl_2$, 300 mmol/L NaCl, 0.2 mmol/L EDTA, 20% glycerol, 1 mmol/L EGTA, 1 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin and 2.5 μg/mL leupeptin. The suspensions were incubated on ice for 30 min for high-salt extraction followed by centrifugation at 15 000 *g* for 20 min at 4°C. The resulting supernatants containing nuclear proteins were carefully removed and protein content was measured using a commercially available assay (Bio-Rad, Segrate, Italy). Preparation of cell lysates and nuclear extracts were performed as described previously (Tamagno *et al.* 2002, 2005). To determine the γ-secretase activity and the protein levels of full-length *N*-cadherin, membrane fractions were isolated as described by Kim *et al.* (2005).

## Oxidative stress determinations

Reactive oxygen species (ROS) were measured in cytosolic fractions using the probe 2′,7′ dichloro-fluorescin diacetate. It is a stable, non-fluorescent molecule that is hydrolyzed by intracellular esterases to non-fluorescent 2′,7′-dichlorofluorescein, which is rapidly oxidized, in the presence of peroxides, to a highly fluorescent adduct which is measured fluorimetrically (Ravindranath 1994). Antioxidant levels in the cytosolic fractions were evaluated in terms of GSSG/GSH ratio, by the Owens and Belcher (1965) method. A mixture was directly prepared in a cuvette: 0.05 mmol/L Na phosphate buffer, pH 7.0; 1 mmol/L EDTA, pH 7.0; and 10 mmol/L 5-5′-Dithiobis-(2-nitrobenzoic acid) plus an aliquot of the sample. GSH content was

evaluated after 2 min at 412 nm and expressed as μg/mg protein. Suitable volumes of diluted GSH reductase and of NADPH were then added to evaluate the total GSH level. The ratio between GSSG and GSH content is considered a measure of antioxidant status.

### Antibodies and immunoblot analysis

The following antibodies were used: polyclonal anti-BACE1 antibody (Chemicon, Temecula, CA, USA); polyclonal anti-PS1, anti-PS2, anti-pJNK, anti-JNK, anti-pc-*jun*, anti-c-*jun* antibodies (Cell Signalling Technology); monoclonal β-actin and polyclonal *N*-cadherin antibodies (Sigma Chemical Company, Beverly, MA, USA); monoclonal 2C11, that recognizes the APP N-terminus (Boehringer, Mannheim, Germany). Lysates, nuclear and membrane fraction extracts were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis on 9.3% (pJNK, JNK, pc-*jun*, c-*jun*, BACE1 and β-actin) or 7.5% (full-length APP, *N*-cadherin) acrylamide gels using the mini-PROTEAN II electrophoresis cell (Bio-Rad). Proteins were transferred onto nitrocellulose membranes (Hybond-C extra Amersham Life Science, Arlington Heights, IL, USA). Non-specific binding was blocked with 50 g/L non-fat dry milk in 50 mmol/L Tris–HCl, pH 7.4, containing 200 mmol/L NaCl and 0.5 mmol/L Tween-20 (Tris-buffered saline Tween). The blots were incubated with different primary antibodies, followed by incubation with peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins in Tris-buffered saline Tween containing 20 g/L non-fat dry milk. Reactions were developed with an enhanced chemiluminescence system according to the manufacturer's protocol (Amersham-Pharmacia Biotech Italia, Cologno Monzese, Italy).

### Enzyme-linked immunosorbent assay

The levels of Aβx-40 and Aβx-42 were measured by sandwich ELISA method following the manufacturer's instructions (IBL, Gunma, Japan). Samples were analysed following the manufacturer's instructions. The Aβ concentration was detected using a Benchmark Microplate Reader and evaluated by ‘microplate manager’ v. 5.1 software (Bio-Rad). ELISA analysis of all samples was performed in two different experiments.

### BACE1 activity

The activity of BACE1 was determined using a commercially available secretase kit from R&D Systems (Wiesbaden, Germany) according to the manufacturer's protocol, as previously described (Tamagno *et al.* 2006). To confirm the specificity of BACE1 cleavage a BACE1 cell permeable inhibitor IV (Calbiochem) was added together with the fluorescent substrate. Data were expressed as a percentage of the activity level of control cells.

### Analysis of gene expression

For the quantitative SYBR Green (2× iQ YBR Green PCR Super Mix; Bio-Rad Laboratories) real-time PCR, 40 ng of cDNA was used per reaction. Primer sequences, designed with PRIMER 3 software, were:

- **i.** Murine BACE1: 5′-GCATGATCATTGGTGGTATC-3′ and 5′-CCATCTTGAGATCTTGACCA-3′.
- **ii.** Murine β actin: 5′-AGCTATGAGCTGCCTGACGGC-3′ and 5′-CATGGATGCCACAGGATTCCA-3′.
- **iii.** Human BACE1: 5′-CATTGGAGGTATCGACCACTCGCT-3′ and 5′-CCACAGTCTTCCATGTCCAAGGTG-3′.
- **iv.** Human β actin: 5′-GGCACTCTTCCAGCCTTCCTTC-3′ and 5′-GCGGATGTCCACGTCACACTTCA-3′.

v. Murine PS1: 5′-TGCGGCCA TCATGATCAGTGTC-3′ and 5′-ATAAGCCAGGCGTGGATGAC-3′.

vi. Murine presenilin enhancer-2 (PEN-2): 5′-GTTGAACCTGTG CCGGAAGTAC-3′ and 5′-TGTAGGCTGGGGCGAGGAAC-3′.

vii. Human PS1: 5′-TAATAAGCCAGGCATGGATGAC-3′ and 5′-CATGGCCCTGGTGTTTATCAAG-3′.

viii. Human PEN2: 5′-GGAGAAATTGAACCTGTGCCGG-3′ and 5′-CCTCTCGGAAGAACCAGAAGATG-3′.

Quantitative PCR was performed on a real-time iCycler sequence detector instrument (Bio-Rad Laboratories). After 3 min of initial denaturation, the amplification profile included 30 s denaturation at 95°C and extension at 72°C. Primer annealing was carried out for 30 s at 60° C. The results were obtained with the comparative Ct method using the arithmetic formula $2^{-\Delta\Delta Ct}$. Samples obtained from a least three independent experiments were used to calculate the means and SD.

### RNA interference

Neuroblastoma cells were transfected with short hairpin RNA (shRNA) directed against human PS1 and PS2 or non-silencing shRNA, with Arrest-In transfection reagent, according to the manufacturer's instructions. The following sequences were used. PS1: NM_000021 : TRCN000061738 PS2: NM_000447 : 2HS_93093. The Arrest-In transfection reagent and non-silencing shRNA and shRNA sequences for PS1 and PS2 were purchased from Open Biosystems (Huntsville, AL, USA). The cells were harvested 48 h after shRNA transfection for sample preparation.

### Luciferase reporter gene assay

Neuroblastoma cells were plated onto 96-well plate, in triplicate, and co-transected with APP-Gal4, a fusion protein of $APP_{695}$ and the binding and activating domains of Gal4, pG5BLuc and promoter cytomegalo virus Renilla. As positive control of the induction of the γ-secretase activity, constructs coding for the wild-type PS1 and BACE1 were transfected together with the luciferase triplet plasmids. After 24 h cells were incubated for 3 h with 5 μmol/L HNE or with 20 μmol/L $H_2O_2$ (all in pyruvate and serum-free medium). Following incubation with pro-oxidant agents, cells were lysed according to the manufacturer's protocol (Promega, Italia, Milano, Italy) and Luciferase and Renilla signals were acquired with a Luminometer (Perkin-Elmer, Wallac MicroBeta TriLux, Waltham, MA, USA) (Minopoli *et al.* 2006).

### *In vitro* peptide cleavage assay for γ-secretase activity measurement

To evaluate γ-secretase activity, membrane enriched fractions were isolated as described by Kim *et al.* (2005). Briefly, brains were homogenized in a hypotonic buffer containing 10 mmol/L Tris–HCl (pH 7.4), 1 mmol/L EGTA and 1 mmol/L EDTA. To extract the dissolved proteins from the crude membranes, the supernatants were centrifuged at 12 000 *g* for 20 min. The pellets were dissolved in 300–500 μL of a hypotonic buffer containing 0.2% CHAPS (Sigma Chemical Company) at 4°C for 30 min. The supernatants were collected after centrifugation. To measure the enzyme activity, 20 μg proteins were incubated with 20 μmol/L of a fluorescent conjugated peptide substrate [NMA-GGVVIATVK (DNP)-DRDRDR-$NH_2$, Calbiochem] at 37°C for 2 h (Kim *et al.* 2005). The degree of substrate cleavage was measured by the emitted fluorescence using a reader (Perkin-Elmer LS-55) with an excitation wavelength of 355 nm and an emission wavelength of 440 nm. To confirm the specificity of γ-secretase cleavage the L685,458 inhibitor was added together with the fluorescent substrate.

### Activator protein 1 transcription factor determination

The activity of protein 1 (AP-1) was determined using a commercially available kit (Active Motif, Rixensart, Belgium), with a 96-well plate on which an oligonucleotide that contains a TPA (12.0-Tetradecanoyl phorbol 13-acetate) response element (5′-TGAGTCA-3′) was immobilized. AP-1 contained in nuclear extracts specifically binds to this oligonucleotide. The primary antibodies used in the kit recognize accessible epitopes on c-*fos*, *fos*-b, fos related antigen-1, fos related antigen-2, c-*jun*, *jun*-b, *jun*-d proteins upon DNA binding.

### Statistical analysis

Statistical analysis was performed using the unpaired Student's *t*-test or ANOVA, followed by the Bonferroni *post hoc* test.

## Results

### Oxidative stress up-regulates PS1/PEN-2 expression and γ-secretase activity

We previously showed that oxidant agents and HNE up-regulate BACE1 expression and activity, as well as the production of Aβ in differentiated $NT_2$ cells (Tamagno *et al.* 2005). To test the effect of the same agents on the γ-secretase, we determined the expression levels of PS1 and PEN-2 and also the activity of the γ-secretase on APP.

Real-time PCR in SK-N-BE neuroblastoma cells revealed that treatment with HNE or $H_2O_2$ induced a 1.5- to twofold increase of PS1 and PEN-2 mRNA levels, when compared with untreated cells (Fig. 1a and b). Pre-treatment of cells with actinomycin D blocked the over-expression of PS1 (Fig. 1a) and PEN-2 (Fig. 1b) induced by the stress agents.

We evaluated the γ-secretase activity on APP using an APP-Gal4 luciferase reporter assay, after exposure of SK-N-BE neuroblastoma cells to the same agents (Fig. 1c). The level of luciferase gene expression in SK-N-BE cells treated with HNE and $H_2O_2$ increased markedly after a 3 h incubation with HNE and $H_2O_2$, by 50% and 120%, respectively (Fig. 1c). A control value was obtained, based on data from transfection with an empty vector (pcDNA3), or from untransfected cells, and gave the same results (data not shown). As a positive control, the expression of wild-type PS1 resulted in a 50% increase of luciferase gene expression (Fig. 1c). To evaluate the effect of the increased β- and γ-secretase activities in SK-N-BE neuroblastoma cells treated with HNE and $H_2O_2$, we quantified the concentrations of Aβx-40 and Aβx-42 in cell media by ELISA assay. The data reported in Fig. 1(d and e) indicate that HNE and $H_2O_2$ treatments lead to a significant increase of 40% and 90% in the steady-state concentrations of intracellular Aβx-40 and Aβx-42 respectively (Fig. 1d). Secreted levels of Aβx-40 and Aβx-42 were increased of 20% and 100% respectively (Fig. 1e). Thus, OS resulted in a higher increase of Aβx-42 when compared with that of Aβx-40, that is more intense for secreted than intracellular Aβ levels, as shown by the comparison of Aβ42/40 ratios (intracellular ratio Aβ42/40: 0.38 ± 0.02 control vs. 0.50 ± 0.05 OS $p < 0.05$; secreted ratio Aβ42/40: 0.10 ± 0.008 control vs. 0.24 ± 0.007 OS $p < 0.02$).

### The γ-secretase cleavage regulates the activation of BACE1 induced by oxidative stress

The $H_2O_2$ and HNE induce an increased expression and activity of BACE1 and PS1/PEN-2, we therefore investigated whether the induced activation of BACE1 requires the γ-secretase and APP processing. It was found that HNE and $H_2O_2$ induced significant increases in the levels of BACE1 mRNA, protein and enzyme activity in SK-N-BE human neuroblastoma cells, as expected (Fig. 2a–c). Treatment with cell permeable inhibitor IV (Calbiochem) inhibited BACE1 and blocked the increase of activity induced by HNE and $H_2O_2$ (Fig. 2c). The anti-BACE1 antibody recognized a 75 kDa band as seen in the gel that corresponded to the mature,

*N*-glycosylated, form of the protein. A lower 70 kDa band was observed that corresponded to pro-BACE1, a precursor of the mature BACE1 (Capell *et al.* 2000) (Fig. 2b).

Pre-incubation of cells with L685,458, an inhibitor of the γ-secretase, completely prevented the HNE- or $H_2O_2$-induced increase of BACE1 mRNA, protein and activity levels (Fig. 2a–c). To complement this result, we silenced PS1 and PS2 expression using a double-stranded RNA-mediated interference in SK-N-BE cells. Accordingly, we observed that the inhibition of the production of PS prevented the increase of BACE1 protein levels induced by $H_2O_2$ or HNE (Fig. 2d).

To confirm that the γ-secretase cleavage is required to induce the up-regulation of BACE1 we used MEFs from PS1/PS2 double knock-out (PSdko) mice (Herreman *et al.* 1999, 2003). Treatment with HNE and $H_2O_2$ induced a 100–150% increase of BACE1 mRNA, protein and activity levels in wild-type MEFs, as expected (Fig. 3a–c). Instead, HNE or $H_2O_2$ failed to induce any changes of BACE1 mRNA, protein or activity levels in PSdko-MEFs, when the same experimental conditions were applied (Fig. 3a–c). To confirm that the activation of BACE1 requires the PS, we re-introduced PS1/PS2 into PSdko-MEFs by transfection of PSdko-MEFs with PS1 and PS2 constructs. The re-expression of PS1/PS2 in PSdko-MEFs restored the increase of BACE1 protein levels induced by HNE and $H_2O_2$ to the same level of that observed in the wild-type MEFs (Fig. 3d).

## The γ-secretase cleavage regulates the expression of BACE1 in the brain of mice subjected to cerebral ischaemia/reperfusion

To confirm that γ-secretase functions as a positive regulator of BACE1 activity in response to OS *in vivo*, we used a well-established animal model, in which OS is induced in mice by transient MCAO followed by reperfusion (Huang *et al.* 1994; Cho *et al.* 2007).

Mice that had undergone ischaemic injury, followed by 1 or 3 h of reperfusion, exhibited a 30% or 90% increase in ROS in the brain, respectively, as shown in Fig. 4a. The increased production of ROS was accompanied by a significant depletion of reduced GSH content, as indicated by the increased ratio of GSSG/GSH (Fig. 4b).

We next measured BACE1 mRNA, protein and activity levels. The protein levels of BACE1 were significantly increased during reperfusion, ranging from a 50% increase compared with sham-operated animals after 1 h of reperfusion to 150% after 3 h (Fig. 4c). At 1 and 3 h of reperfusion a 2- to 2.5-fold increase of BACE1 mRNA was observed (Fig. 4d) and BACE1 activity was found to be significantly increased after 3 and 6 h of reperfusion, when compared with sham-operated animals (Fig. 4e). The use of a specific inhibitor of BACE1 halved the basal activity of BACE1 and completely blocked the increase induced by oxidation (Fig. 4e).

The γ-secretase activity was increased in the brain in response to ischaemia/reperfusion. After 1–3 h of reperfusion, brain tissue showed a 100% increase in the PS1 and PEN-2 mRNA (Fig. 4f). The activity of the γ-secretase cleavage of APP was tested using a specific peptide cleavage assay (Kim *et al.* 2005), as well as using a measurement of the levels of full-length *N*-cadherin and of full-length APP, as substrates of the γ-secretase (Marambaud *et al.* 2003;Uemura *et al.* 2006). At 1–3 h of reperfusion there was a significant increase in the fluorescence intensity read-out (40–50%), which is directly proportional to the γ-secretase activity (Fig. 4g), while a strong decrease in the amounts of full-length *N*-cadherin and APP were observed (Fig. 4h). Equal protein loading of membrane enriched fractions was ensured by monitoring the staining of the membranes with Ponceau Red (data not shown).

### The JNK/c-*jun* pathway mediates the OS-dependent up-regulation of γ-secretase and BACE1

The signalling pathways potentially involved in the OS-dependent up-regulation of BACE1 mediated by the γ-secretase cleavage were next investigated. The c-*jun* N-terminal kinase (JNK) signalling pathway is a potential candidate, as it can be activated by both Aβ and OS (Wang *et al.* 2004; Yao *et al.* 2005). First, we tested if the JNK pathway was activated in the OS models used in this study. HNE or $H_2O_2$ induced a robust activation of JNK and c-*jun* in SK-N-BE neuroblastoma cells, as shown by increased levels of phospho-JNK and phospho-c-*jun* (Fig. 5a). Similarly, brains of ischaemic/reperfused mice showed a significant increase of JNK and c-*jun* phosphorylation, the levels increasing by 100% after 1 h of reperfusion, to 120% after 3 h (Fig. 5a). To confirm that there was an activation of the JNK pathway in our experimental models we evaluated the activation of the transcription factor AP-1. It is composed of a mixture of heterodimeric complexes of proteins derived from the *fos* and *jun* families. Phosphorylation of AP-1 family members by kinases is required for transactivation activity. In the case of c-*jun*, the activation domain is regulated to a large extent by the JNK (Karin 1995). Three hours incubation of SK-N-BE cells with HNE or $H_2O_2$ and 1–3 h of reperfusion of ischaemic brains resulted in a significant activation of AP-1 (Fig. 5b). To ascertain the role of the JNK/AP-1 pathway in the OS-dependent up-regulation of the γ-secretase and BACE1, we used MEFs deficient for JNK (JNKko-MEFs). Treatment of cells with HNE or $H_2O_2$ resulted in a 90–100% increase in BACE1 mRNA in WT-MEFs over 1–6 h incubation periods. By contrast, there was no BACE1 mRNA increase induced by HNE or $H_2O_2$ in JNKko–MEFs (Fig. 5c). PS1 mRNA was also increased in WT-MEFs, but not in JNKko-MEFs, upon treatment with HNE or $H_2O_2$ (Fig. 5d). The same result was observed for PEN-2 (data not shown).

To check the activation of the JNK/AP-1 pathway in the transient MCAO-reperfusion model, a cell permeable selective JIP (JIP-1-HIV-TAT peptide), able to abrogate the activation of JNK, without affecting its phosphorylation level (Borsello *et al.* 2003), was injected intraperitoneally 30 min prior to the MCAO.

Accordingly, the 2- to 2.5-fold increase of BACE1 mRNA after 1–3 h of reperfusion was also completely prevented by pre-treatment with the JNK inhibitor peptide (Fig. 5e). Similarly, the levels of PS1 mRNA also showed a 1.5- to twofold increase after 1–3 h of reperfusion and again the increase was prevented by pre-treatment with the JNK inhibitor peptide (Fig. 5f). The inhibitor peptide also prevented the increase of PEN-2 (data not shown).

### Role of the γ-secretase cleavage-derived APP derivatives on the up-regulation of BACE1

As APP is the substrate of the γ-secretase, we next investigated whether the presence of APP was essential for obtaining the up-regulation of BACE1. For this experiment we used MEFs lacking $APP_{695}$ (APPko) (Heber *et al.* 2000; Leissring *et al.* 2002). Neither HNE nor $H_2O_2$ induced any change in BACE1 mRNA and protein levels in APPko–MEFs, in contrast to the result obtained with wild-type MEFs (Fig. 6a). The re-introduction of APP into APPko–MEFs by transfection with the $APP_{695}$ construct restored the increase of BACE1 protein levels to the same levels observed in the WT-MEFs (Fig. 6a).

The activation of BACE1 depends on both the activity of the γ-secretase and the presence of APP, we therefore explored whether the APPko phenotype can be rescued by transfecting the cells with APP–Carboxy terminal fragments, or by treating them with Aβ peptides. We first transfected APPko–MEFs with APP-C99, the APP derivative resulting from the β-cleavage, both in normal conditions or under OS (Fig. 6b). An increase of BACE1 mRNA by 100–150% and of protein levels by 100–200%, were observed upon transfection with APP-C99 (Fig. 6b). We then looked at the APP intracellular domain (AID/AICD) as a potential player of the positive feedback on APP processing. To accomplish this APPko–MEFs were transfected with

an AID/AICD construct (AID/AICD59) which expresses the APP C-terminal fragment of 59 amino acid residues, the latter resulting from the γ-secretase cleavage at position 40 of the Aβ domain. The expression of AID/AICD59 did not modify BACE1 mRNA and protein levels (Fig. 6c). This result is consistent with the Aβ peptides, resulting from γ-secretase cleavage of APP derivatives, being the signalling molecules in the loop. We incubated APPko–MEFs with Aβ 1–40 and Aβ 1–42 synthetic peptides, at a concentration of 1 μmol/L. The state of the Aβ preparations was examined by electron microscopy and no fibrillar or globular structures were detected (data not shown). APPko–MEFs showed a 3.5-fold increase of BACE1 mRNA after 1 h treatment with Aβ 1–42, whereas incubation with Aβ 1–40 produced a twofold increase in BACE1 expression (Fig. 6d).

## Discussion

We found that both in cultured cells and *in vivo*, the up-regulation of BACE1 induced by OS is mediated by γ-secretase activity, and also that it requires the activation of the JNK/c-*jun* pathway. The results have three implications for the pathogenesis of sporadic AD. First, they suggest that OS, as effect of ageing, can increase the expression of both PS1/PEN2 and BACE1, thereby enhancing Aβ production. The activity of the γ-secretase is modified by PS1 mutations, as well as by molecules that interact directly with PS1, generally inhibiting the function of the γ-secretase complex (Beher *et al.* 2004; Matsuda *et al.* 2005; Cai *et al.* 2006; Heneka and O'banion 2007). OS is the only known factor able to augment the γ-secretase cleavage by increasing the expression of PS1, the catalytic subunit of the endoprotease. Secondly, our data reveal the existence of a positive feedback loop in which increased γ-secretase activity results in up-regulation of BACE1 expression, which is mediated in part by the release of Aβ peptides that act as signalling molecules (Tamagno *et al.*, in preparation). Moreover, the effect of the up-regulation of BACE1 is not only the increased production of total Aβ, but also an augmented production of N terminally truncated Aβ species (Lee *et al.* 2003), which exert higher rates of aggregation and toxicity than the full-length peptides (Piccini *et al.* 2005). Thirdly, we showed that the activation of the positive feed-forward loop between the β- and the γ-secretase requires the JNK/c-*jun* signalling pathway. The JNK/c-*jun* signalling cascade, which is activated in the AD brain (Zhu *et al.* 2003a,b Lagalwar *et al.* 2006), responds to cell stress and mediates apoptosis (Kanzawa *et al.* 2006; Pugazhenthi *et al.* 2006; Zhang *et al.* 2007). The extracellular regulated kinases, that promote cell survival, are also activated in AD brain. Of note, extracellular regulated kinases inhibit the γ-secretase activity (Kim *et al.* 2005) and decrease the expression of BACE1 (Tamagno *et al.*, submitted). Thus, the positive or negative cellular responses to OS parallel the activities of the β- and the γ-secretases that generate APP.

The sources and nature of OS in brain regions vulnerable in AD have not been established, but are likely to include processes involved in normal ageing. During normal ageing, and to a greater extent in AD, levels of protein oxidation and lipid peroxidation are increased (Butterfield and Kanski 2001; Cutler *et al.* 2004). Hydroxyl radicals, a ROS generated from $H_2O_2$, are potent inducers of membrane lipid peroxidation and the generation of HNE, a neurotoxic lipid peroxidation product, is associated with Aβ pathology in AD (Mark *et al.* 1997; Sayre *et al.* 1997). OS and HNE have been shown to induce activation of JNK/c-*jun* in neurons, which at high enough levels can trigger apoptosis (Camandola *et al.* 2000; Tamagno *et al.* 2003). Our findings suggest that the activation of JNK/c-*jun* mediates the increased expression of PS1 and BACE1 in response to OS and HNE. The latter mechanism is consistent with previous findings suggesting that c-*jun* can induce the expression of PS1 (Pastorcic and Das 2002) and BACE1 (Sambamurti *et al.* 2004).

Oxidative stress produces several effects that may contribute to synaptic function and cell death in AD (Mattson 2004; Bayer *et al.* 2006; Nunomura *et al.* 2006). We and other groups observed that OS induces *in vitro* an increased production of Aβ (Paola *et al.* 2000; Tamagno *et al.*

2005; Tong *et al.* 2005), and in this report we show a possible mechanism of this effect. OS correlates with ageing, the major risk factor of sporadic AD. This study presents evidence that OS contributes to Aβ accumulation. Aβ, in turn, induces OS and HNE production resulting in JNK/c-*jun* activation and increased levels of β- and γ-secretases, which further enhances Aβ production.

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## Abbreviations used

**AD**
: Alzheimer's disease

**AID/AICD**
: amyloid precursor protein intracellular domain

**AP-1**
: activator protein 1

**APP**
: amyloid precursor protein

**Aβ**
: β amyloid

**BACE1**
: β-site amyloid precursor protein cleaving enzyme 1

**HNE**
: 4-hydroxynonenal

**JIP**
: c-*jun* N-terminal kinase-inhibitory peptide

**JNK**
: c-*jun* N-terminal kinase

**MCAO**
middle cerebral artery occlusion

**MEFs**
mouse embryonic fibroblasts

**OS**
oxidative stress

**PEN-2**
presenilin enhancer-2

**PS**
presenilin

**PSdko**
PS1/PS2 double knock-out

**ROS**
reactive oxygen species

**shRNA**
short hairpin RNA

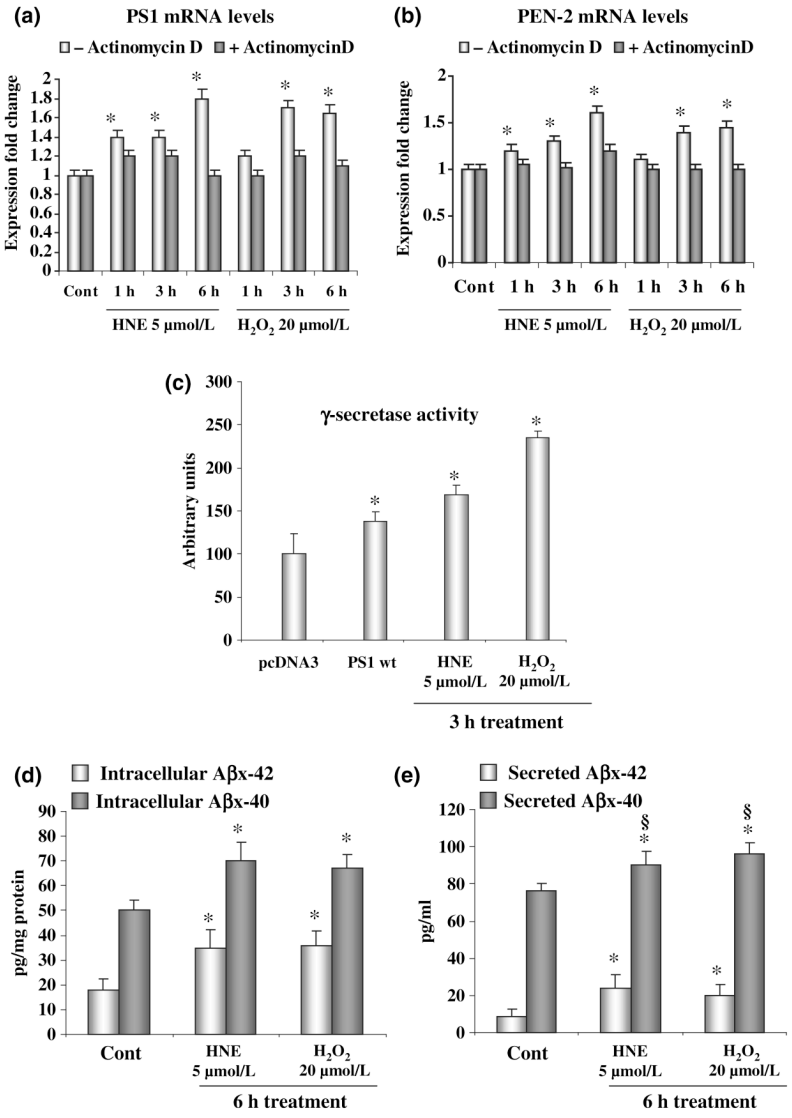


**Fig. 1.**

Oxidative stress increases presenilin (PS) 1/PEN2 expression and the γ-secretase activity. (a) PS1 and (b) PEN-2 mRNA levels were significantly increased, as revealed by real-time PCR, after treatment of SK-N-BE neuroblastoma cells with 4-hydroxynonenal (HNE) or $H_2O_2$. Pre-treatment of cells with 1 µmol/L actinomycin D was able to completely rescue the increase of PS1 and PEN2 expression. (c) A significant increase of γ-secretase activity on the amyloid precursor protein (APP)-Gal4 fusion protein, as shown by the luciferase reporter assay, occurs after treatment of SK-N-BE neuroblastoma cells with oxidative agents. Values are expressed as arbitrary units of luminescence. (d) and (e) Incubation of SK-N-BE cells with HNE and $H_2O_2$ resulted in a significant increase of intracellular and secreted Aβx-40 and Aβx-42. Experiments were performed in triplicate; $*p < 0.05$ versus control.

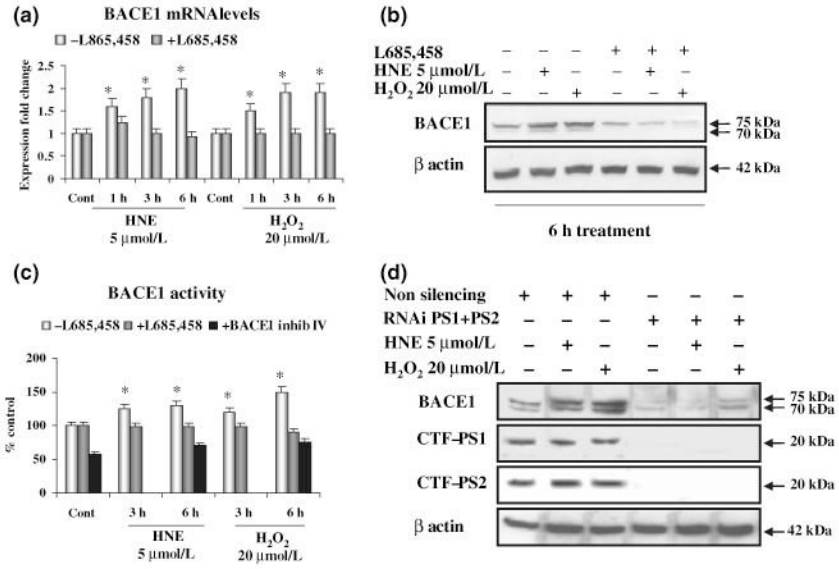


**Fig. 2.**

Inhibition of γ-secretase decreases β-site amyloid precursor protein cleaving enzyme 1 (BACE1) mRNA and protein levels, and its protease activity. (a–c) In SK-N-BE cells, inhibition of γ-secretase with the transition analogue L685,458 prevented the increase of BACE1 mRNA level (a), protein level (b) and enzyme activity (c) of BACE1, that are induced by 4-hydroxynonenal (HNE) and $H_2O_2$ in control cells. Addition of BACE1 inhibitor IV halved the basal activity of BACE1 and completely blocked its significant increase induced by oxidative stress (OS) (c). (d) In SK-N-BE cells, simultaneous knock-down of PS1/2 by RNA interference reduced the basal level of BACE1 protein levels and prevented the increase of BACE1 by HNE and $H_2O_2$. Non-silencing shRNA-treated cells exhibited BACE1 levels similar to those of wild-type cells. Experiments were performed in triplicate; $*p < 0.05$.

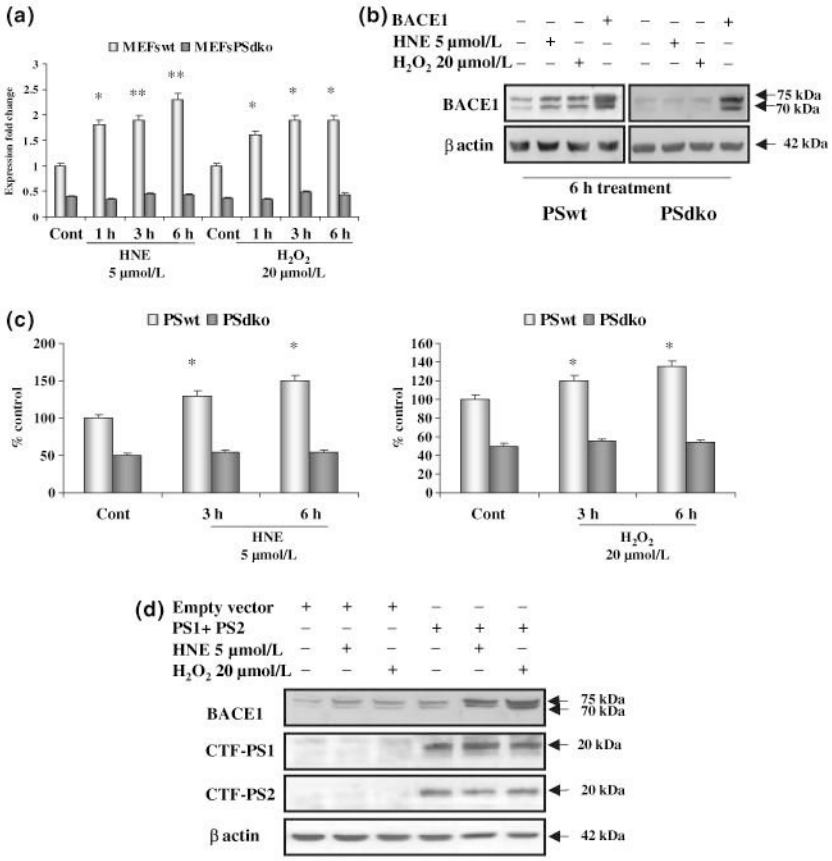


**Fig. 3.**

Presenilins are needed for oxidative stress dependent up-regulation of β-site amyloid precursor protein cleaving enzyme 1 (BACE1) expression and activity. (a–c) Pro-oxidative agents increased BACE1 transcription (a), protein levels (b) and activity (c) in WT-MEFs (PSwt), but not in MEFs lacking PS1 and PS2 (PSdko). (d) Transfection of PSdko-MEFs with PS1 and PS2 increased the basal level of BACE1 and restored the capacity of pro-oxidative agents to induce BACE1, as seen by western blot analysis. Experiments were performed in triplicate; $*p < 0.05$.

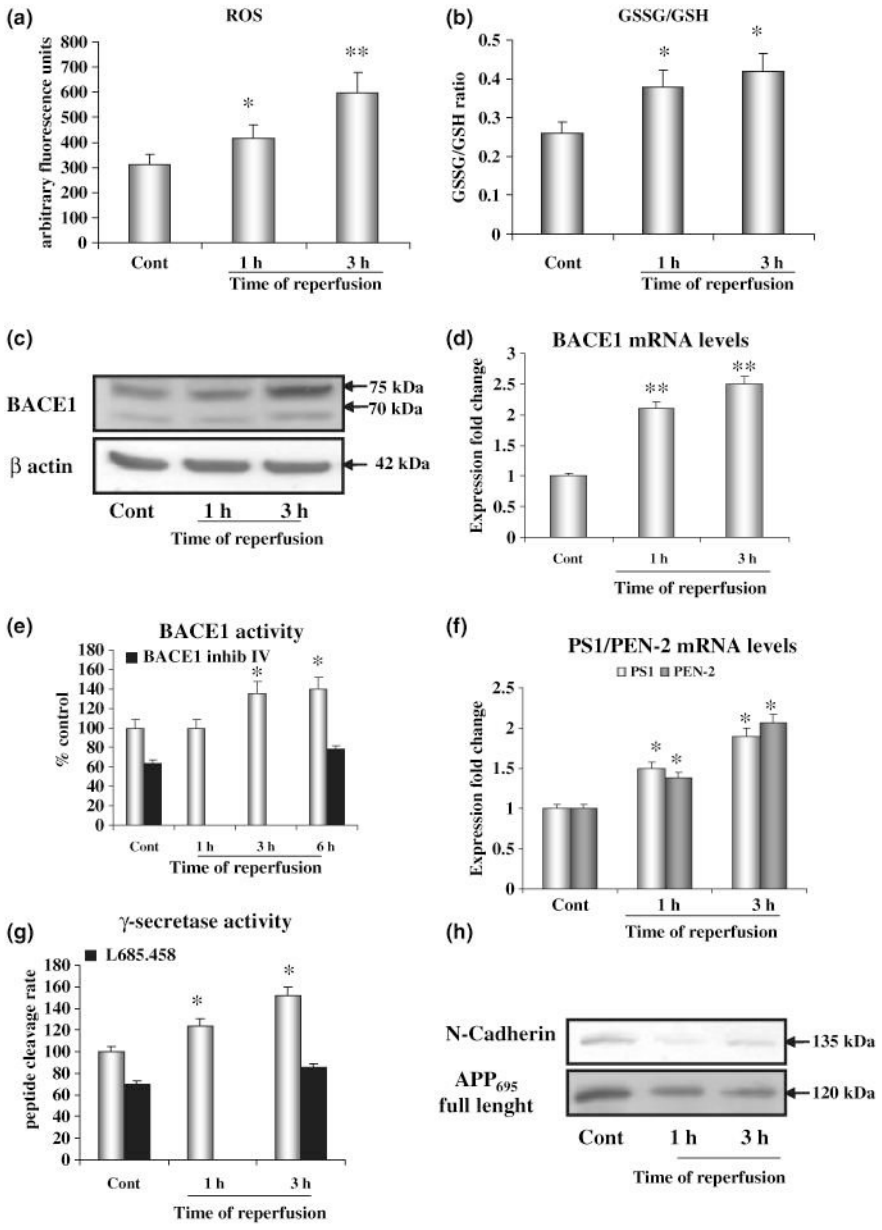


**Fig. 4.**

Cerebral ischaemia/reperfusion induces oxidative stress and β-site amyloid precursor protein cleaving enzyme 1 (BACE1) expression and γ-secretase activity in mice brain. (a and b) Mice subjected to ischaemia and 1 or 3 h of reperfusion exhibit a significant increase in the production of reactive oxygen species (a) as well as a significant depletion in GSH content evaluated as a significant increase in the GSSG/GSH ratio (b). (c–e) Compared with sham-operated animals, BACE1 protein levels (c), mRNA levels (d) and enzyme activity (e) are significantly increased in brains of mice subjected to a stroke. Addition of BACE1 inhibitor IV halved the basal activity of BACE1 and completely blocked the increase induced by oxidative stress (OS) (e). (f) PS1 and PEN-2 transcription is increased in the ischaemic cortex after 1 and 3 h of reperfusion. (g) Increase in the activity of γ-secretase is seen in ischaemic-reperfused mice, as shown by the *in vitro* peptide cleavage assay, within 1 h and up to 3 h of reperfusion. Addition of L685,458 halved the basal activity of γ-secretase and completely blocked the increase induced by OS

(g). (h) At 1 and 3 h of reperfusion, a significant decrease in protein levels of full-length *N*-cadherin and amyloid precursor protein (APP), substrates of γ-secretase, was also observed. Equal protein loading of membrane enriched fractions was controlled by staining the membrane with Ponceau Red (data not shown). There were six mice per group: sham operated (cont), or ischaemia followed by reperfusion for 1, 3 or 6 h; $*p < 0.05$; $**p < 0.02$.

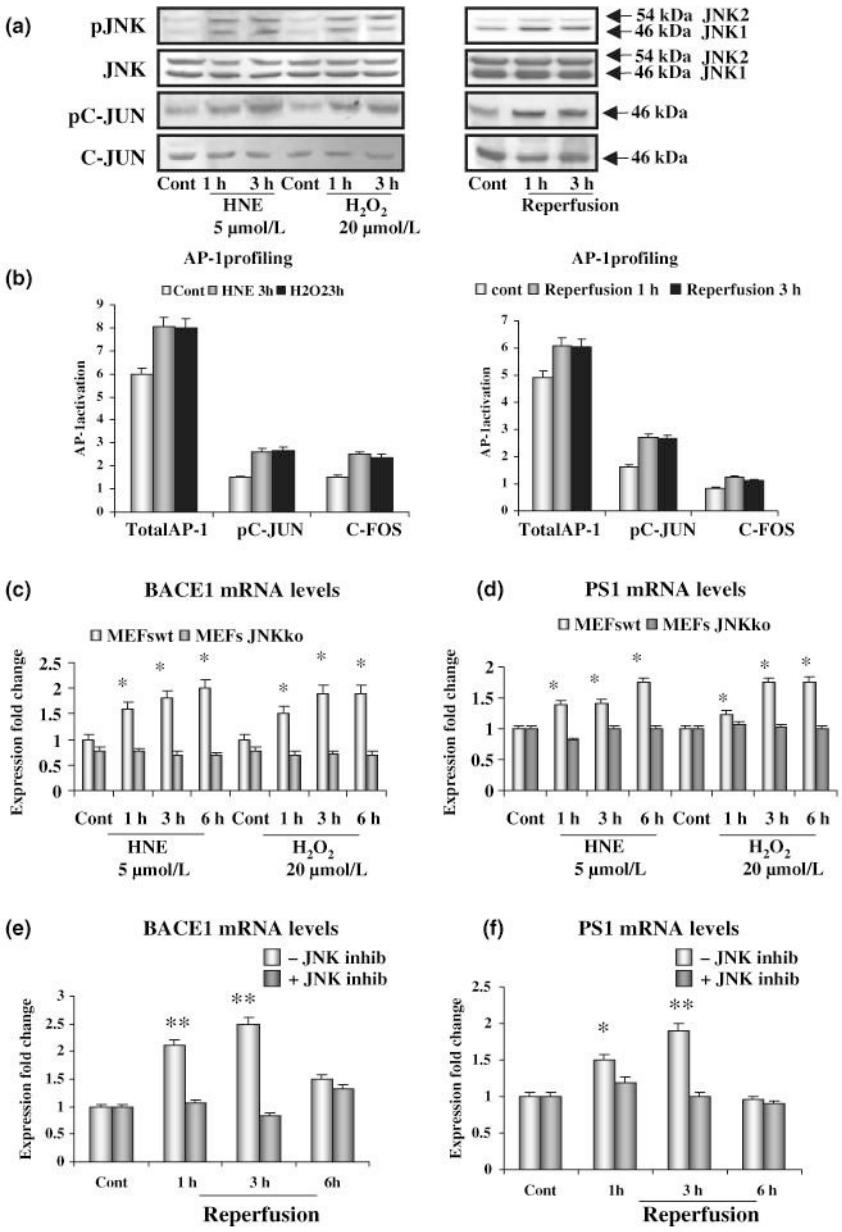


**Fig. 5.**

The c-*jun* N-terminal kinase (JNK)/activator protein 1 (AP-1) signalling pathway mediates oxidative stress (OS)-induced increases in β-site amyloid precursor protein cleaving enzyme 1 (BACE1) and PS1 expression. (a) Significant increase in phosphorylation of JNK and c-*jun* after treatment of SK-N-BE neuroblastoma cells with HNE or $H_20_2$, and after 1 and 3 h of reperfusion, in brains of ischaemic mice. (b) Activation of AP-1 transcription factor, together with pc-*jun* and c-*fos*, follows OS SK-N-BE cells and mouse models of OS. (c) BACE1 transcription, measured by real-time PCR, is not affected by oxidative stimuli in JNKko–MEFs. (d) Levels of PS1 mRNA are not changed by oxidative stimuli in JNKko–MEFs. (e and f) Pre-treatment of mice with the JNK inhibitory peptide prevents the increase of BACE1 and PS1 transcription observed in ischaemic/reperfused mice; $*p < 0.05$, $**p < 0.02$.

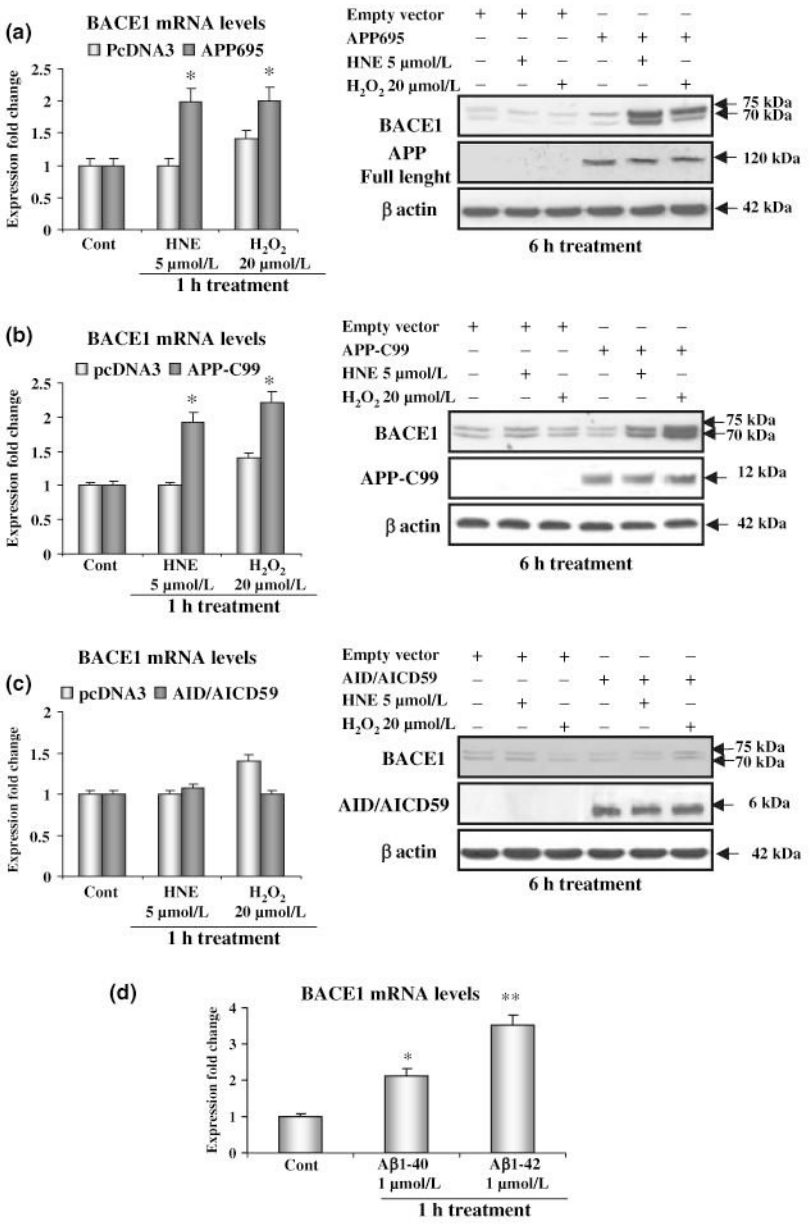


**Fig. 6.**

Role of amyloid precursor protein (APP) derivatives on the up-regulation of β-site amyloid precursor protein cleaving enzyme 1 (BACE1). (a) Transfection with $APP_{695}$, significantly increases BACE1 expression and protein levels in APPko–mouse embryonic fibroblasts (MEFs). (b) Transfection with APP-C99, expressing the APP C-terminal domain resulting from the β-secretase cleavage restores the up-regulation of BACE1 in APPko-MEFs. (c) Transfection with APP intracellular domain (AID/AICD) 59, the APP derivative released by the γ-secretase cleavage at position 40 of the Aβ domain, has no effect on BACE1 expression and protein levels in APPko-MEFs. (d) Incubation with freshly prepared Aβ 1–42 induced a 3.5-fold increase in BACE1 mRNA in APPko–MEFs. Incubation with freshly prepared Aβ 1-40 induced a twofold increase in BACE1 mRNA in APPko–MEFs; $*p < 0.05$, $**p < 0.02$.