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JNK and ERK1/2 pathways have a dual opposite effect on the expression of BACE1

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Abstract

The activity of β-secretase (BACE1), the endo-protease essential for the production of amyloid β (Aβ) peptides, is increased in brain of late-onset sporadic Alzheimer's disease (AD), and oxidative stress is the potential cause of this event. Oxidative stress up-regulates the expression and the activity of BACE1 in cellular and animal models, through a mechanism that involves the increase of γ-secretase cleavage on APP and the activation of c-jun N-terminal kinase/activator protein 1 (JNK/AP1) pathway. We further characterized the cellular pathways that control BACE1 expression under oxidative stress. We investigated the involvement of extracellular signal regulated MAP kinase (ERK1/2) pathway in the regulation of BACE1 expression, since it has been recently shown that ERK1/2 is an endogenous regulator of the γ-secretase activity. We found that ERK1/2 pathway negatively modulates BACE1 expression and activity. Moreover, we observed that conditions that abrogate the γ-secretase activity favor the activation of signalling pathways that promote cell survival, such as ERK1/2 and the serine/threonine kinase Akt/protein kinase B (Akt). These data suggest that the positive or negative cellular responses to oxidative stress parallel the activities of the β- and the γ-secretase. ERK1/2 and JNK pathways are involved in this bipartite response, which can lead to neurodegeneration or neuroprotection depending on the cellular and environmental conditions or cooperation with other signalling pathways such as Akt cascade.

Keywords: Alzheimer's disease; BACE1; γ-Secretase; Oxidative stress; ERK1/2; c-jun N-terminal kinase

1. Introduction

The amyloidogenic processing of amyloid precursor protein (APP) involves two sequential cleavages operated by β- and γ-secretase on APP, a transmembrane type 1 protein. The β-secretase (BACE1) cleaves the ectodomain of APP, producing an APP C-terminal fragment that is further cleaved, within the transmembrane domain, by the γ-secretase complex, resulting in the release of amyloid β (Aβ) peptides (Selkoe, 2001).

The altered activity of both γ-secretase and BACE1 are involved in the pathogenesis of Alzheimer's disease (AD). Presenilin 1 (PS1) gene mutations linked with early-onset familial AD increase the production of Aβ 42, which aggregates and accumulates faster than Aβ 40 (Lemere et al., 1996; Citron et al., 1997). The expression and the activity of BACE1 is elevated in the brain of late-onset sporadic AD patients (Fukumoto et al., 2002; Holsinger et al., 2002; Yang et al., 2003).

Oxidative stress may contribute to the pathogenesis of late-onset sporadic AD, being oxidative stress an age-dependent event (Nunomura et al., 2001; Cutler et al., 2004) and aging

Abbreviations: BACE1, β-site APP cleaving enzyme; APP, amyloid precursor protein; Aβ, β amyloid; PS, presenilin; ERK1/2, extracellular signal regulated MAP kinase; Akt, serine/threonine kinase Akt/protein kinase B; SEK, stress-activated protein kinase-ERK kinase; PAK, p21-activated kinase; MKK, mitogen activated kinase kinase; MKKK, mitogen activated kinase kinase kinase; Ask1, apopstosis signal-regulating kinase; JNK, c-jun N-terminal kinase.

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the major risk factor for AD. We and others have shown that the expression and activity of BACE1 is increased by oxidant agents 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 between BACE1 activity and oxidative markers in sporadic AD brain tissue (Borghi et al., 2007). Moreover, we recently found that the up-regulation of BACE1, induced by oxidative stress, is mediated by the γ-secretase activity on APP and requires the activation of the c-jun N-terminal kinase/activator protein 1 (JNK-AP1) pathway (Tamagno et al., 2008). Both JNK and extracellular signal regulated MAP kinase (ERK1/2) pathways are potentially involved in the pathogenesis of AD. Increased levels of activated ERK1/2 (Zhu et al., 2002; Pei et al., 2002; Canet-Aviles et al., 2002; Uemura et al., 2006) and JNK (Zhu et al., 2001, 2003a; Otth et al., 2003) have been found in brains of AD cases. The activation of JNK was associated with age-dependent amyloid plaque deposition, tau phosphorylation, and the loss of synaptophysin in a Tg2576/PS1 double transgenic mice (Puig et al., 2004; Pearson et al., 2006; Tatebayashi et al., 2006). ERK1/2 activation blocks Aβ-induced cell death (Savage et al., 2002; Guerra et al., 2004; Watson and Fan, 2005) as well as reduces Aβ generation (Mills et al., 1997; Desdouits-Magnen et al., 1998; Liu et al., 2003). Kim et al. (2005) recently found that ERK1/2 act as negative regulator of the γ-secretase activity directly interacting with Nicastrin, the γ-secretase component that binds both the N- terminal and the C-terminal of presenilins. Here, we have investigated the role of ERK1/2 pathway in the up-regulation of BACE1 induced by oxidative stress. We show that ERK1/2 is a constitutive suppressor of the expression and the activity of BACE1. We also investigated the behaviour of the cellular pathways in conditions that suppressed the γ-secretase activity. We found that, in these conditions, oxidative stress was able to activate pathways that are related to cell survival such as ERK1/2 and serine/threonine kinase Akt/protein kinase B (Akt) signalling.

2. Materials and methods

2.1. Cell culture and treatments

NT_2 undifferentiated cells were maintained in Dulbecco's modified Eagle's medium (DMEM/F12), supplemented with 5% fetal bovine serum and 1% antibiotic mixture comprising penicillin-streptomycin-amphotericin, in a humified atmosphere at 37 °C with 5% CO_2.

For differentiation, 2×10^6 cells were exposed to 10 μM retinoic acid for 5 weeks. Growth medium was changed three times a week. Cells were then replated and, 48 h later, mitotic inhibitors cytosine arabinoside (1 μM), fluorodeoxyuridine (10 μM) and uridine (10 μM) were added for 2 weeks to inhibit the division of non-neuronal cells. Experiments were performed 4–5 weeks after cessation of retinoic acid treatment (Tamagno et al., 2002).

SK-N-BE neuroblastoma cells were cultured as described previously (Tamagno et al., 2003a) and 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 μM or with H_2O_2 (Sigma Chemical Company, St. Louis, USA) at a concentration of 20 μM. The γ-secretase inhibitor L685,458 (Bachem, Weil am Rhein, Germany) was added (at a final concentration of 1 μM) to SK-N-BE cells, 8 h before treatments with pro-oxidants. The pharmacological MEK inhibitor PD 98059 (Calbiochem, Darmstadt, Germany), that acts by inhibiting the ERK1/2 phosphorylation, was added to NT_2 neuronal cells (at a final concentration of 30 μM) 30 min before treatment with pro-oxidants. Epidermal growth factor (EGF) (Sigma Chemical Company, St. Louis, USA) were used as ERK1/2 activators and added to SK-N-BE cells at the final concentration of 100 ng/ml up to 12 h.

2.2. Cell extracts

Preparation of cell lysates were performed as described previously (Tamagno et al., 2003b). Briefly, cells, treated with the appropriate experimental conditions, were quickly placed on ice in a buffer consisting in Tris–HCl buffer, pH 7.4, containing 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 0.05% aprotinin (w/v), and 1 mM dithiotreitol.

To determine the γ-secretase activity and the protein levels of full length N-cadherin, membrane fraction were isolated as described by Kim et al. (2005). Briefly, cells were lysed in a hypotonic buffer containing 10 mM Tris–HCl (pH 7.4), 1 mM EGTA and 1 mM 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, St. Louis, USA) at 4 °C for 30 min. The supernatants were collected after centrifugation.

2.3. Antibodies and immunoblot analysis

The following antibodies were used: monoclonal anti-BACE1 antibody (Chemicon); monoclonal anti-pERK and polyclonal anti-ERK, anti-pAkt, anti-Akt, anti-pAsk1 Thr 845, anti-Ask1, anti-pRAC1 Ser 71, and anti-RAC1 (Santa Cruz Biotechnology, Heidelberg, Germany); monoclonal anti-β-actin and polyclonal anti-N-cadherin antibodies (Sigma Chemical Company, St. Louis, USA); polyclonal anti-PS1, anti-PS2, anti-pJNK and anti-JNK (Cell Signalling Technology, Beverly, MA, USA). Lysates and membrane fraction extracts were subjected to 9.3% (pERK, ERK, pAkt, Akt, pRac1 Ser 71, Rac1, pJNK, JNK, PS1, PS2, BACE1, β-actin) or 7.5% (full length N-cadherin, pAsk1 Thr 845 and Ask1) acrylamide gels using the mini-PROTEAN II electrophoresis cell, according to Laemmli (1970). Proteins were transferred onto nitrocellulose membranes (Hybond-C

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extra Amersham Life Science, Arlington Heights, IL, USA). Unspecific binding was blocked with 50 g/l non-fat dry milk in 50 mM Tris–HCl, pH 7.4, containing 200 mM NaCl and 0.5 mM Tween-20 (TBS-Tween). The blots were incubated with different primary antibodies, followed by incubation with peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins in TBS-Tween containing 20 g/l non-fat dry milk. Reactions were developed with an enhanced chemiluminescence system according to manufacturer's protocol (Amersham-Pharmacia Biotech Italia, Cologno Monzese, Italy).

2.4. Enzyme-linked immunosorbent assay

The levels of Aβx-42 were measured by sandwich enzyme-linked immunosorbent assay (ELISA) method following the manufacturer's instructions (IBL, Gunma, Japan). Three 75 cm^2 flasks of differentiated NT_2 cells for each conditions were collected to prepare cell lysates. Media were concentrated 20 folds. Samples were then analyzed following the manufacturer's instructions. The Aβ concentration was detected using a Benchmark Microplate Reader and evaluated by "microplate manager" v. 5.1 software. ELISA analysis of all samples was performed in two different experiments.

2.5. Analysis of gene expression

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

- human BACE1: 5′-CATTGGAGGTATCGACCACTCGCT-3′ and 5′-CCACAGTCTTCCATGTCCAAGGTG-3′.
- human β actin: 5′-GGCACTCTTCCAGCCTTCCTTC-3′ and 5′-GCGGATGTCCACGTCACACTTCA-3′.

Quantitative PCR was performed on a real time iCycler sequence detector instrument (BioRad 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 done for 30 s at 60 °C for BACE1 and for β actin. Specificity of the produced amplification product was confirmed by examination of dissociation reaction plots. A distinct single peak indicated that a single DNA sequence was amplified during PCR. Each sample was tested in triplicate and threshold cycle (Ct) values were averaged from each reaction. 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 S.D.

2.6. RNA interference

RNA interference experiments to knockdown the ERK1/2 in SK-N-BE neuroblastoma cells were performed using a RNAi human/mouse control kit, including the small RNA duplex (Qiagen Italia, Milano, Italy). The siRNAs (2.5 μg) were transfected into cells using the proper RNAiFect reagent ratio (1:6) in a DMEM medium for 48 h.

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

2.7. 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. Cells were lysed in cold 1× Extraction Buffer (ready to use in the kit) to yield a final protein concentration of 1 mg/ml. The method is based on the secretase-dependent cleavage of a secretase-specific peptide substrate conjugated to the fluorescent reporter molecules EDAS and DABCYL, which results in the release of a fluorescent signal that can be detected on a fluorescence microplate reader (excitation wavelength of 355 nm and emission 510 nm). The level of secretase enzymatic activity is proportional to the fluorimetric reaction. Data were expressed as a percentage of the activity level of control cells.

2.8. Statistical analysis

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

3. Results

We have previously shown that oxidative stress up-regulates BACE1 expression and activity, as well as Aβ production in differentiated NT_2 cells, through the activation of JNK and $p38^{MAPK}$ pathways (Tamagno et al., 2005). Moreover, we ascertained that this induction is mediated by the γ-secretase activity on APP (Tamagno et al., 2008). In this study we investigated the role of ERK1/2 pathway, that has been shown to be an endogenous negative regulator of γ-secretase activity (Kim et al., 2005), on the up-regulation of BACE1 as mediated by oxidative stress. To study the role of ERK1/2 pathway we used two different approaches.

We pre-treated NT_2 neurons with 30 μM PD98059, a pharmacological inhibitor of MEK, the kinase that mediates the phosphorylation of ERK1/2, 30 min before treatment with HNE or H_2O_2. Pre-treatment of NT_2 neurons with PD98059 completely blocked ERK1/2 activation, as confirmed by the inhibition of its phosphorylation (Fig. 1A). Six hours treatment with both HNE or H_2O_2 was followed by 1.5–1.8-fold

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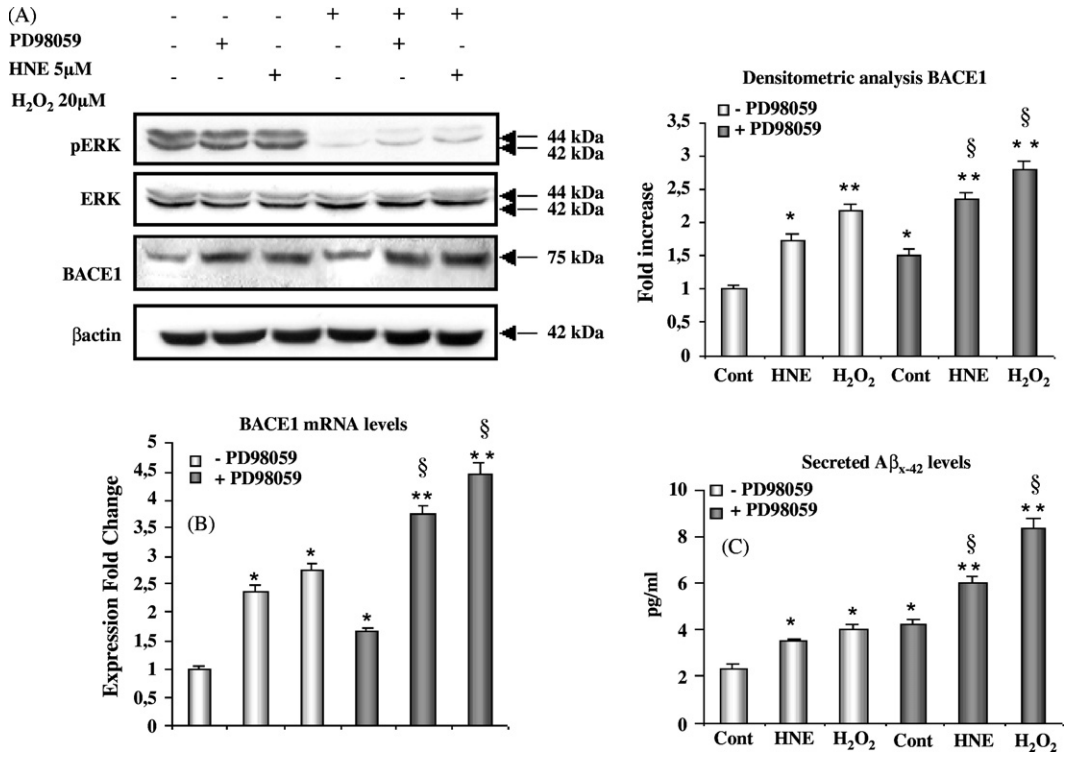

Fig. 1. Effect of pharmacological inhibition of ERK1/2 on protein levels, mRNA and activity of BACE1 induced by oxidative stress in NT_2 neurons. (A) Western blot showed the levels of phosphorylated ERK1/2 and BACE1 protein levels in NT_2 neurons after 6 h treatment with 5 μM HNE or 20 μM H_2O_2 with or without pre-treatment with the MEK inhibitor PD98059 (30 μM 30 min before pro-oxidant agents). PD98059 completely blocked the ERK1/2 activation, as confirmed by the inhibition of its phosphorylation; moreover, inhibition of ERK1/2 resulted in higher basal BACE1 protein levels and in its additional increase induced by pro-oxidant agents. (B) One hour treatment of neuronal cells with pro-oxidant agents significantly increased BACE1 mRNA; after pharmacological ERK1/2 inhibition, basal levels and oxidative stress induction of BACE1 resulted significantly higher. (C) Aβx-42 release in the medium of NT_2 neurons, both basal and after 6 h treatment with HNE or H_2O_2, was also higher after ERK1/2 inhibition. *$p < 0.05$ vs. control cells; **$p < 0.02$ vs. control cells; §$p < 0.05$ vs. HNE or H_2O_2 without ERK1/2 inhibition. Values are means ± S.D. of three experiments performed in duplicate.

increase in BACE1 protein levels, but, when ERK1/2 pathway was inhibited by PD98059, pro-oxidant agents induced a significant higher increase in BACE1 protein levels (2.5-fold and 2.7-fold after HNE and H_2O_2 treatments) (Fig. 1A). Of note, basal BACE1 protein levels were significantly higher (1.3-fold) after pre-treatment of neurons with MEK inhibitor (Fig. 1A).

Identical results were obtained silencing ERK1/2 (Fig. 2). After transfection of human SK-N-BE neuroblastoma cells with siRNA against ERK1/2, ERK1/2 levels markedly decreased without affecting β actin protein levels (Fig. 2A). The silencing of ERK1/2 was followed by a significant increase in basal BACE1 protein levels as compared to non-transfected cells (Fig. 2A). Again, pro-oxidants raised BACE1 protein levels significantly higher in silenced cells (3.8–4-fold versus 2.5-fold, respectively) (Fig. 2A).

Expression and activity of BACE1 paralleled the results obtained by immunoblot. Pharmacological inhibition of ERK1/2 determined 1.5-fold increase of BACE1 mRNA in basal conditions and 3.5- and 4-fold increase after treatment with HNE and H_2O_2. Likewise, ERK1/2 silencing induced 4.5–5.7-fold increase of BACE1 mRNA (Fig. 2B). Pre-treatment of cells with actinomycin D blocked the overexpression of BACE1 (data not shown). BACE1 activity was evaluated indirectly, measuring Aβx-42 release in the medium (Fig. 1C) in NT_2 neurons, and directly, using a fluorimetrically commercially available secretase kit (Fig. 2C) in SK-N-BE cells. As shown, both the examined parameters were significantly higher after pharmacological inhibition and silencing of ERK1/2 in basal conditions and after treatment with pro-oxidant agents (Figs. 1C and 2C).

Next, we examined the effect of the activation of ERK1/2 on BACE1 expression and activity. Treatment of SK-N-BE cells with EGF, an activator of ERK1/2 signalling pathway, produced a 2-fold increase of ERK1/2 phosphorylation after 1 and 3 h and a 3.2-fold increase after 6 h. ERK1/2 activation was followed by a significant decrease of BACE1 protein levels, mRNA, and activity. BACE1 protein levels were halved after 3 and 6 h of EGF treatment (Fig. 3A). The decrease of BACE1 protein levels was preceded by a 40% decrease in BACE1 mRNA after 1 and 3 h of EGF treatment and was simultaneous to the decrease of BACE1 activity (40–50% after 3 and 6 h) (Fig. 3B and C). Moreover, we showed that pre-treatment of cells with the pharmacological inhibitor of MEK, PD 98059, almost completely abolished EGF-induced inhibition of BACE1 expression and protein levels, thus con-

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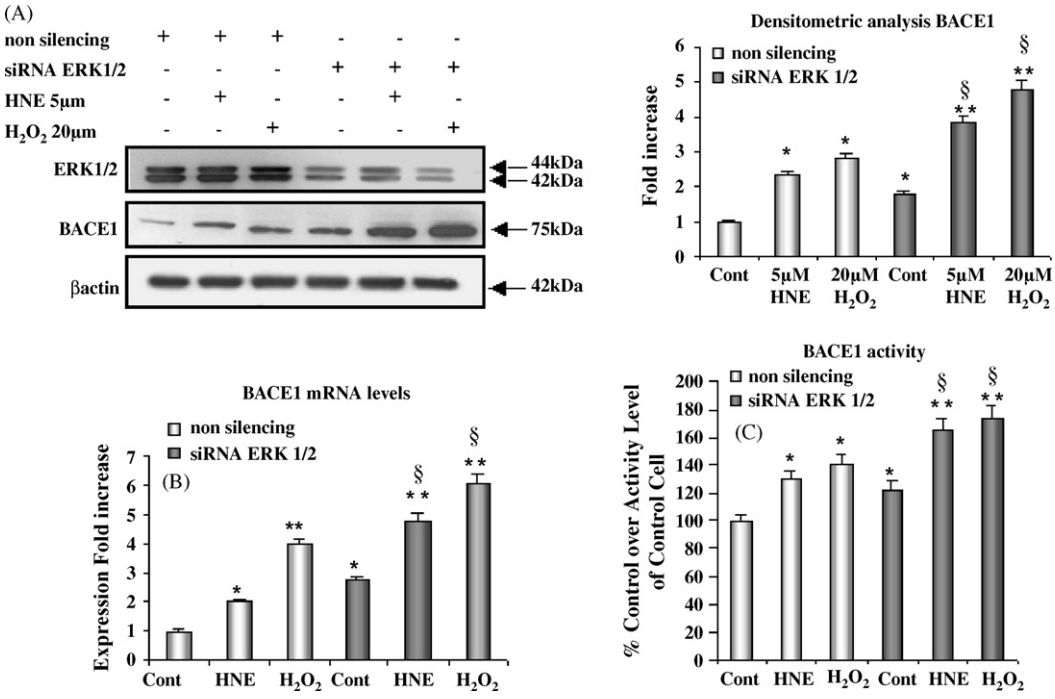

Fig. 2. ERK1/2 knockdown by RNA interference was followed by a significant higher increase in protein levels, mRNA and activity of BACE1 in SK-N-BE neuroblastoma cells. (A) Western blot showed the levels of ERK1/2 and BACE1 protein levels in SK-N-BE neuroblastoma cells after 6 h treatment with HNE or H_2O_2 and transfection with non-silencing or ERK1/2 RNA interference. ERK1/2 knock down significantly reduced the amount of ERK1/2 in these cells, as well as determined a significant higher increase in BACE1 protein levels induced by oxidative stress. (B) BACE1 mRNA, was higher after 1 h treatment with HNE or H_2O_2 in ERK1/2 silenced SK-N-BE cells. (C) BACE1 activity in SK-N-BE neuroblastoma was significantly higher after 6 h treatment with HNE or H_2O_2 in cell transfected with ERK1/2 siRNA. *$p < 0.05$ vs. control cells; **$p < 0.02$ vs. control cells; §$p < 0.05$ vs. HNE or H_2O_2 without ERK1/2 inhibition. Values are means ± S.D. of three experiments performed in duplicate.

firming the specific effect of ERK1/2 activation on BACE1 (Fig. 3D and E).

3.1. Inhibition of the γ-secretase activity activates ERK1/2 and Akt signalling pathways, and inhibits the JNK cascade

Our previous data showed that pre-treatment of SK-N-BE neuroblastoma cells with the transition analogue L685,458 was followed by a significant reduction of apoptosis induced by oxidative stress (unpublished observations). These findings have suggested that the inhibition of γ-secretase may shifts the equilibrium toward pathways that promote cell survival.

To investigate this hypothesis we studied the behaviour of ERK1/2 pathway in conditions that suppress the γ-secretase activity. The pharmacological inhibition of γ-secretase, as well as the silencing of both presenilins, was followed by a complete prevention of the HNE- or H_2O_2-induced increase in BACE1 protein levels, expression and activity (Figs. 4 and 5A–C), as described elsewhere (Tamagno et al., 2008). Inhibition of the γ-secretase activity was confirmed by the accumulation of full length and the decrease of truncated N-cadherin, which is a substrate of the γ-secretase (Marambaud et al., 2003; Uemura et al., 2006) (Figs. 4D and 5D). As shown in Figs. 4E and 5E, HNE and H_2O_2 were not able to increase the ERK1/2 phosphorylation in SK-N-BE cells in normal conditions. Instead, the block of the γ-secretase activity determined a 2.7–3.5-fold increase of ERK1/2 phosphorylation upon oxidative stress (Figs. 4E and 5E).

To further confirm the hypothesis that the inhibition of γ-secretase and, consequently, of BACE1, activates pathways that promote cell survival, we studied the response of Akt, another pathway that acts as mediator of anti-apoptotic activity (Yuan and Yankner, 2000; Levresse et al., 2000). Akt is known to be activated by oxidative stress (Chong and Maiese, 2004; Chong et al., 2004), and to be activated concomitantly to ERK1/2 (Kilic et al., 2006; Amadoro et al., 2007). In our cellular model, pro-oxidant agents did not increase Akt phosphorylation in basal conditions. When the γ-secretase activity was inhibited oxidative stress elicited a strong increase (2–2.5-fold increase) in the phosphorylation of Akt (Fig. 6A and B). We also observed that the inhibition of the γ-secretase activity blocked the activation of the JNK pathway (Fig. 6A and B). It has been suggested that Akt exerts its anti-apoptotic action through the inhibition of JNK pathway (Levresse et al., 2000). In our experimental model, the activation of Akt was followed by the inhibition of Rac1, through its phosphorylation in Ser 71, and by the inhibition of the phosphorylation of apoptosis signal-regulating kinase 1 (Ask1) in Ser 845 (Fig. 6A and B). Both these signalling events are related to the inhibition of JNK pathway (Kwon et al., 2000; Kim et al., 2001). Indeed, the phosphorylation of

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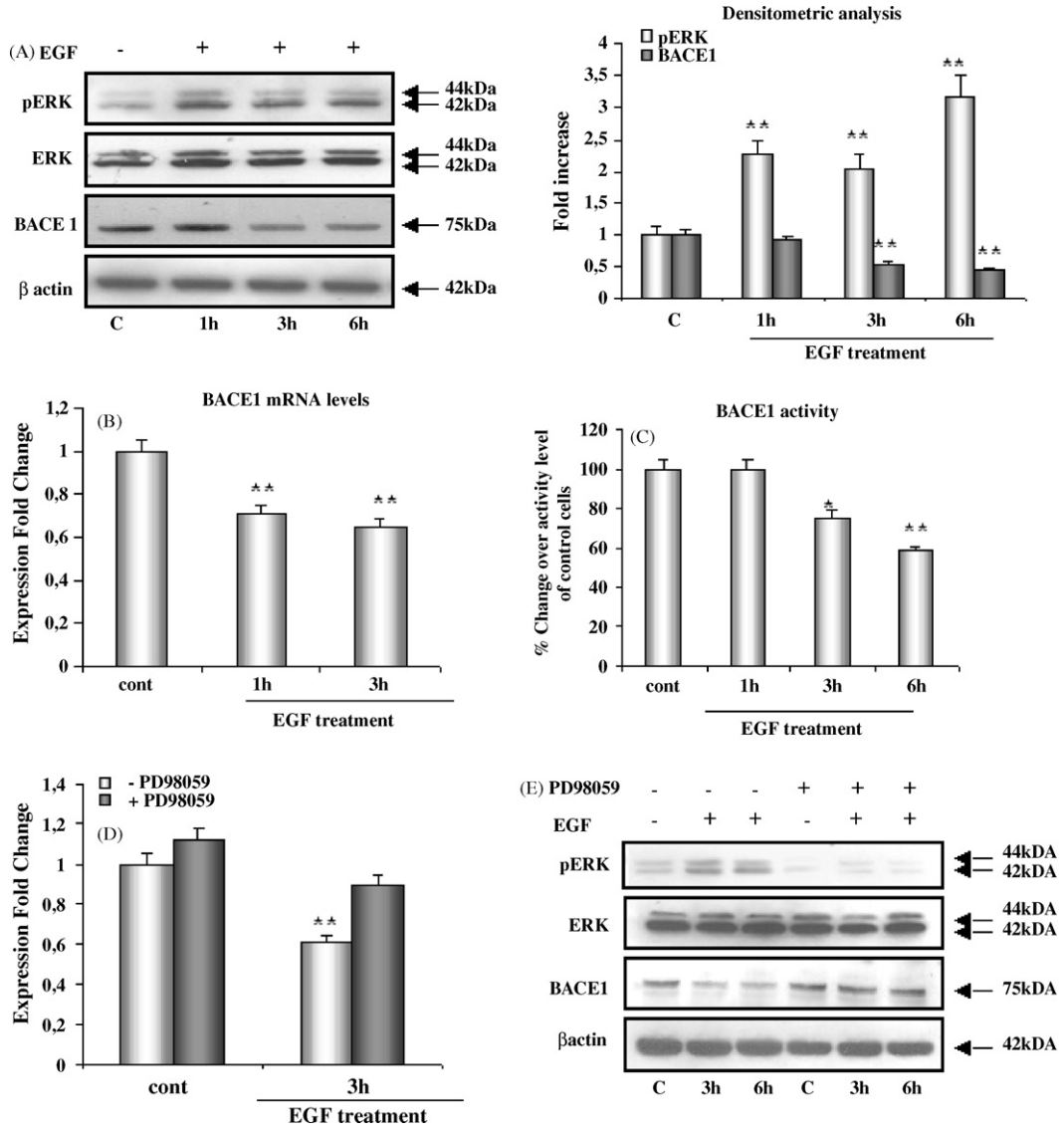

Fig. 3. Treatment of neuroblastoma cells with EGF, a negative regulator of γ-secretase activity, decreased protein levels, mRNA and activity of BACE1. (A) Western blot showed the levels of phosphorylated ERK1/2 and BACE1 protein levels in SK-N-BE neuroblastoma cells after 1, 3 and 6 h treatment with EGF. EGF significantly increased ERK1/2 phosphorylation and decreased BACE1 protein levels. (B) BACE1 mRNA levels were significantly decreased in SK-N-BE neuroblastoma cells after 1 and 3 h treatment with EGF. (C) BACE1 activity in SK-N-BE neuroblastoma cells was decreased after 3 and 6 h treatment with EGF. (D) Pre-treatment of cells with the pharmacological inhibitor of MEK, PD PD98059 (30 μM 30 min before EGF) abolishes the decrease the EGF-mediated BACE1 mRNA. (E) Pre-treatment of cells with the pharmacological inhibitor of MEK, PD PD98059 (30 μM 30 min before EGF) abolishes the decrease the EGF-mediated BACE1 protein levels. *$p < 0.05$ vs. control cells; **$p < 0.02$ vs. control cells. Values are means ± S.D. of three experiments performed in duplicate.

Rac1 by Akt inhibits Rac1-GTPbinding and in turn, blocks the phosphorylation of kinases upstream of JNK (Kwon et al., 2000). Moreover, the phosphorylation of Ask1 blocks a mitogen-activated protein kinase kinase kinase family member that acts upstream of JNK (Kim et al., 2001).

4. Discussion

We have previously shown that oxidative stress significantly increases the expression, protein levels and activity of BACE1 in NT_2 neurons and that this up-regulation is modulated by JNK and $p38^{MAPK}$, also known as stress activated protein kinases (SAPK) (Tamagno et al., 2005). More recently we found that the up-regulation of BACE1 induced by oxidative stress is mediated by the γ-secretase activity (Tamagno et al., 2008). These findings would provide a mechanistic explanation for the role of oxidative stress in sporadic late-onset AD, in which oxidative stress occurs as direct effect of aging (Nunomura et al., 2001; Cutler et al., 2004), or as consequence of the toxic effect of Aβ (Selkoe, 2001; Mills et al., 1997). We have also found that the activation of the positive feed-forward loop between β- and γ-secretase requires the JNK/AP1 signalling pathway (Tamagno et al., 2008),

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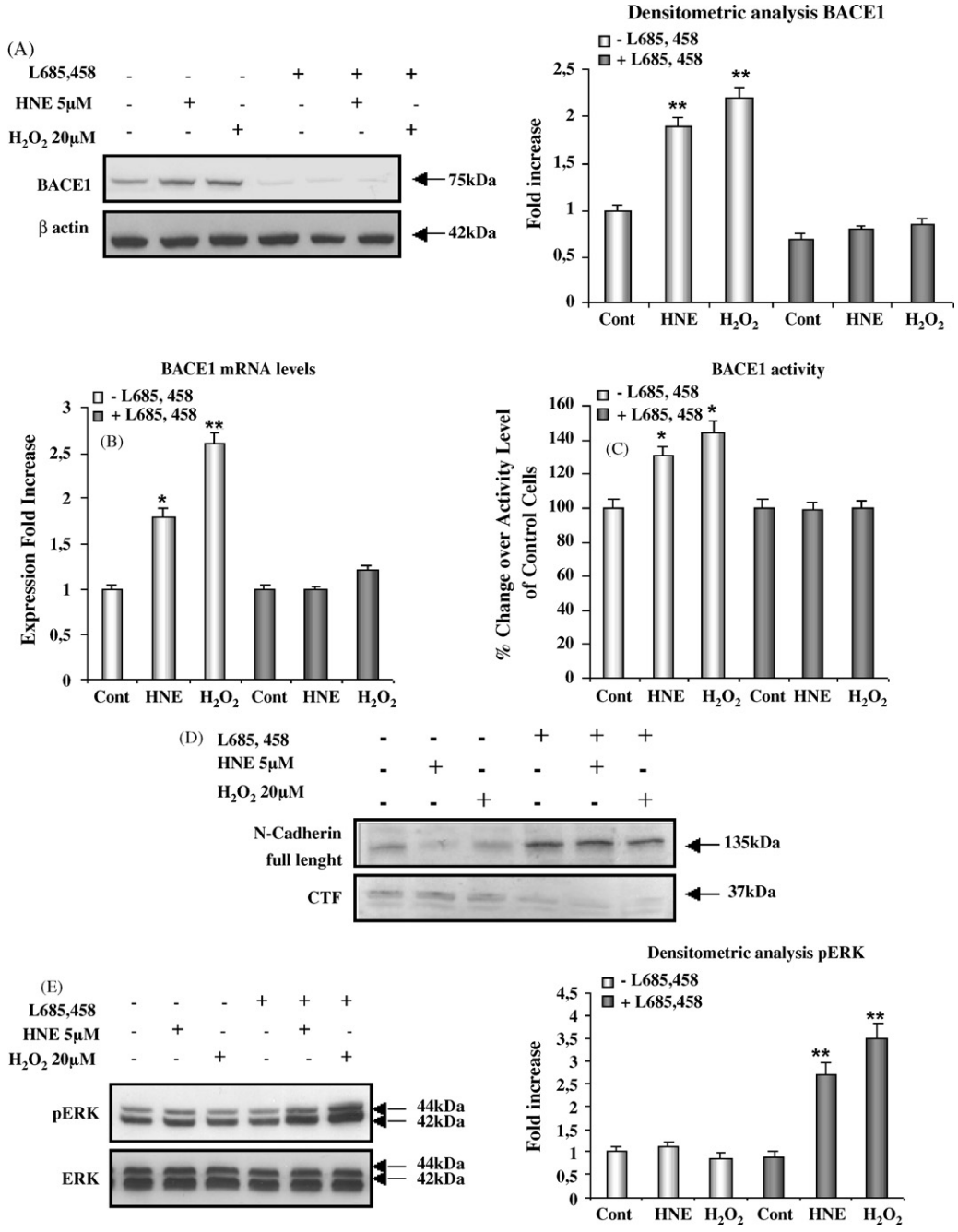

Fig. 4. Pharmacological inhibition of γ-secretase prevented the activation of BACE1 induced by oxidative stress while increased ERK1/2 phosphorylation. (A–C) Pre-treatment of cells with L685,458 prevented the increase of BACE1 protein levels, expression and activity in SK-N-BE neuroblastoma cells after treatment with HNE or H_2O_2. (D) Inhibition of γ-secretase activity was confirmed by the accumulation of full length N-cadherin, which is substrate of the γ-secretase, and by the decrease in its truncated fragment. Equal protein loading of membrane-enriched fractions was controlled by staining the membranes with Ponceau Red (data not shown). (E) Pre-treatment of neuroblastoma cells with L685,458 was accompanied by a strong activation of ERK1/2 pathway induced by oxidative stress. *$p < 0.05$ vs. control cells; **$p < 0.02$ vs. control cells. Values are means ± S.D. of three experiments performed in duplicate.

which is known to be activated in AD brain (Zhu et al., 2003b; Lagalwar et al., 2006; Thakur et al., 2007), to respond to cell stress, and to mediate apoptosis (Pugazhenthi et al., 2006; Kanzawa et al., 2006; Zhang et al., 2007).

Many author's found several evidence that the ERK1/2 pathway is pivotally involved in the pathophysiology of AD (Perry et al., 1999; Zhu et al., 2003c; Mei et al., 2006). Recent evidence suggests that ERK1/2, involved in anti-apoptotic signalling, is an endogenous regulator of the γ-secretase activity (Kim et al., 2005). The complex interplay among these opposite pathways and the proteases involved in APP processing might elucidate the pathologic production of Aβ.

Here we showed that the inhibition of ERK1/2, which is known to mediate the cell signalling toward survival,

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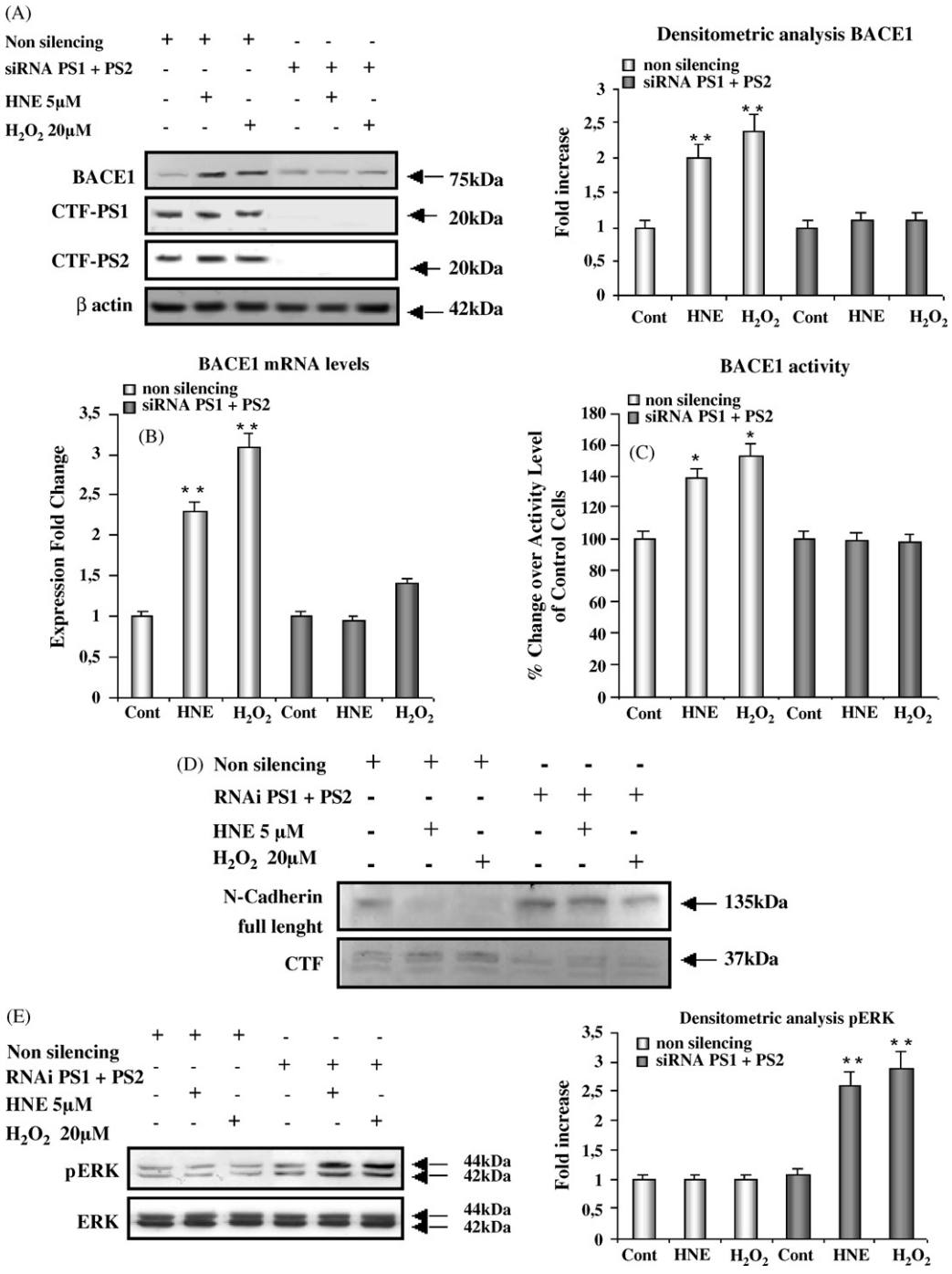

Fig. 5. Knock down of presenilins prevented the activation of BACE1 induced by oxidative stress while increased ERK1/2 phosphorylation. (A–C) PS silencing prevented the increase of BACE1 protein levels, mRNA and activity in SK-N-BE neuroblastoma cells, after 6 h of treatment with HNE or H_2O_2. (D) Inhibition of γ-secretase activity was confirmed by the accumulation of full length N-cadherin, which is substrate of the γ-secretase and by the decrease in its truncated fragment. Equal protein loading of membrane enriched fractions was controlled by staining the membranes with Ponceau Red (data not shown). (E) PS silencing was accompanied by a strong activation of ERK1/2 pathway induced by oxidative stress, and by a less significant increase of basal ERK1/2 phosphorylation. $^*p < 0.05$ vs. control cells; $^{**}p < 0.02$ vs. control cells. Values are means ± S.D. of three experiments performed in duplicate.

increases the expression and activity of BACE1, and finally results in an augmented production of Aβ 42. This is true in basal conditions, but is greatly enhanced under oxidative stress. The role of ERK1/2 is confirmed by its activation with EGF, that produces a down-regulation of BACE1. In our system, ERK1/2 acts as a restraint to the processing of APP and production of Aβ 42, which would eventually be detrimental to the cell. In conditions where, for any reason, the protec-

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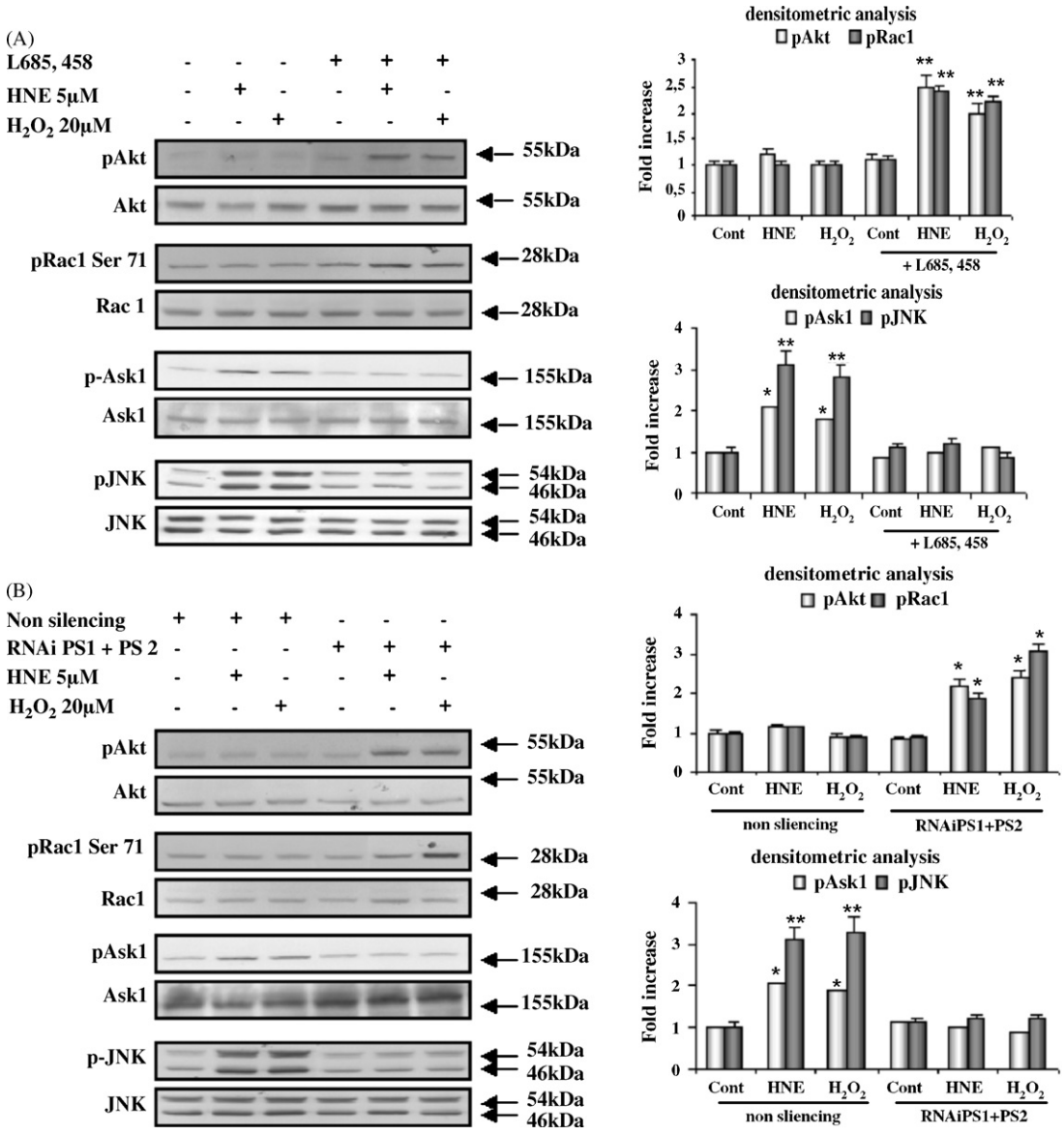

Fig. 6. Inhibition of the γ-secretase activity activated Akt signalling pathways, and inhibited the JNK cascade. (A and B) Inhibition of γ-secretase by pre-treatment of cells with L685,458 or by silencing of presenilins was followed by an increase of Akt phosphorylation as well as of Rac1 in serine 71, as induced by oxidative stress. As shown, in these conditions oxidative stress did not activate the Ask1 and the JNK kinases. *$p < 0.05$ vs. control cells; **$p < 0.02$ vs. control cells. Values are means ± S.D. of three experiments performed in duplicate.

tive effect of ERK1/2 fails, this mechanism contributes to the Aβ overproduction secondary to oxidative stress (Mills et al., 1997; Kim et al., 2005).

The focus of the second part of this study is to understand which are the conditions that impair the activation of ERK1/2. Recent data show that BACE1 over-expression reduces ERK1/2 phosphorylation (Nizzari et al., 2007) and that ERK1/2 is a negative regulator of the γ-secretase (Kim et al., 2005). Here we showed that the γ-secretase activity acts to negatively modulate the response of ERK1/2 to oxidative stress. Also, the upstream Akt signalling pathway, which targets several key proteins related to cell survival, including apoptotic regulators (Yuan and Yankner, 2000; Levresse et al., 2000) and transcription factors (Du and Montminy, 1998; Kane et al., 1999), seems to be regulated in a similar way. It has been suggested that the negative regulation of the JNK pathway by Akt contributes to the anti-apoptotic signalling in neuronal cells (Levresse et al., 2000).

In our work, we described two possible ways through which Akt may inhibit JNK activation under oxidative stress. We showed that the activation of Akt was followed by an increased Rac1 phosphorylation in serine 71. Rac1 binds to and phosphorylates PAK65 protein kinase. Activated PAK65 can stimulate MEKK1, which in turn phosphorylates and activates SEK/JNK kinase (Knaus and Bokoch, 1998; Hu et al., 1996). The active SEK/JNK kinase phosphorylates JNK. The phosphorylation of Rac1 by Akt in serine 71 inhibits the Rac1-GTP binding activity, blocking the phosphorylation

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cascade described (Kwon et al., 2000). Moreover, we showed that Ask1 is phosphorylated in Thr 845 when Akt was inactivated. Ask1 represents a mitogen-activated protein kinase kinase kinase (MKKK) family member that acts upstream of JNK. Ask1 phosphorylates and activates MKK4 o MKK7, which in turn induce JNK. It has been demonstrated that Ask1 is a substrate for phosphorylation by Akt and that this phosphorylation, in Ser 83, is associated with a decrease of Ask1 activity, including inhibition of JNK activation and in turn of apoptosis induction (Kim et al., 2001).

In conclusion, our data suggest that the cellular responses to oxidative stress are closely related to the positive feedback loop existing between γ-secretase and BACE1. The interruption of this loop allows the activation of ERK1/2 and Akt and the suppression of JNK pathway, thus protecting the cell from apoptosis. In the AD brain, any event that could impair the clearance of the oxidation products may trigger a cycle leading to a further activation of the amyloidogenic APP processing.

Disclosure statement

All authors disclose:

(a) There are not 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.
(b) In this study there are not data concerning human subjects and animals.

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