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ORIGINAL PAPER

β-Amyloid-evoked Apoptotic Cell Death is Mediated Through MKK6–p66shc Pathway

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Abstract We have previously shown the involvement of p66shc in mediating apoptosis. Here, we demonstrate the novel mechanism of β-Amyloid-induced toxicity in the mammalian cells. β-Amyloid leads to the phosphorylation of p66shc at the serine 36 residue and activates MKK6, by mediating the phosphorylation at serine 207 residue. Treatment of cells with antioxidants blocks β-Amyloid-induced serine phosphorylation of MKK6, reactive oxygen species (ROS) generation, and hence protected cells against β-Amyloid-induced cell death. Our results indicate that serine phosphorylation of p66shc is carried out by active MKK6. MKK6 knock-down resulted in decreased serine 36 phosphorylation of p66shc. Co-immunoprecipitation results demonstrate a direct physical association between p66shc and WT MKK6, but not with its mutants. Increase in β-Amyloid-induced ROS production was observed in the presence of MKK6 and p66shc, when compared to triple mutant of MKK6 (inactive) and S36 mutant of p66shc. ROS scavengers and knock-down against p66shc, and MKK6 significantly decreased the endogenous level of active p66shc, ROS production, and cell death. Finally, we show that the MKK6–p66shc complex mediates β-Amyloid-evoked apoptotic cell death.

Keywords β-Amyloid · P66shc · MKK6 · Reactive oxygen species (ROS) · Phosphorylation · Cell death

Rafia A. Baba and Hina F. Bhat have contributed equally to the work.

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Introduction

Alzheimer's disease(AD) has been identified as a protein misfolding disease, or proteopathy, due to the accumulation of abnormally folded Amyloid beta (Aβ) or β-Amyloid proteins in the brains of AD patients (Hashimoto et al. 2003). Aβ or Abeta is a peptide of 39–43 amino acids that appear to be the main constituent of Amyloid plaques, in the brain of AD patients (Glenner et al. 1984; Selkoe 1994). The β-Amyloid precursor protein (APP) is a ubiquitously expressed cell membrane protein that is sequentially cleaved by β-secretase and γ-secretase to release the extracellular peptides, including Amyloid beta peptides and the APP intracellular domain (Strooper and Annaert 2000). The γ-secretase that produces the C-terminal end of the β-Amyloid peptide cleaves within the transmembrane region of APP and can generate a number of isoforms of 39–43 amino acid residues in length. The most common isoforms are Aβ40 and Aβ42; the shorter form is typically produced by cleavage that occurs in the endoplasmic reticulum, while the longer form is produced by cleavage in the trans-Golgi network (Hartmann et al. 1997). The biological function of APP and the factor(s) that trigger the APP proteolytic cascade remains unclear. The β-Amyloid so produced is toxic to neurons in vitro, and the high concentration of β-Amyloid in vivo causes loss of synapses and neurons in the brain of animal models (Smith et al. 2006). Although β-Amyloid is involved in mediating many cell-damaging events (Mattson et al. 1993; Iversen et al. 1995; Pagani and Eckert 2010; Spuch et al. 2012), the common transducer emerging from these events is reactive oxygen species (ROS) and one of the major consequences of the ROS stress is the activation of p66shc.

P66shc is an oxidative stress-induced protein that regulates the apoptotic response to oxidative stress (Migliaccio et al.

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1999; Purdom and Chen 2003). It belongs to the shc family of adaptor proteins. The three mammalian shcA isoforms (p46, p52, and p66) share structural features, including a C-terminal Src homology 2 (SH2) domain, a proline- and glycine-rich region, a collagen-homologous region 1 (CH1), and an N-terminal phosphotyrosine-binding (PTB) domain (Luzi et al. 2000; Pelicci et al. 1992). In addition, there is a unique collagen-homologous region 2 (CH2) domain at the N terminus of the p66Shc isoform containing a serine phosphorylation site (S36). The pro-apoptotic activity of p66Shc is predominantly dependent on phosphorylation of S36 in the CH2 domain present at its N-terminal. S36 phosphorylation is observed in response to many stimuli, including H_2O_2, UV (Migliaccio et al. 1999), and Fas ligation (Pacini et al. 2004). Depending on the cellular context and on the identity of the stimulus, Erk, JNK, or p38 MAPkinase (MAPKs) is responsible for S36 phosphorylation (Le et al. 2001; Okada et al. 1997), though alternate mechanisms do exist (Khanday et al. 2006a).

Stress-activated protein kinases (SAPK), such as c-jun N-terminal kinase (JNK) and p38MAP kinase, are ectopically activated and participate in the pathogenesis of AD (Hensley et al. 1999; Zhu et al. 2003; Tare et al. 2011; Corrêa and Eales 2012). However, the involvement of these SAPKs in β-Amyloid-mediated events is only just being unraveled. It has been previously shown that β-Amyloid is involved in the activation of p66shc in neuroblastoma cell lines through the activation of JNK (Smith et al. 2005). Although p38MAP kinase can phosphorylate p66shc at S36 in vitro (Le et al. 2001), the role of its upstream activator MKK6 in the β-Amyloid-mediated activation of p66shc pathway is yet to be elucidated. MKK6 is an upstream activator of the p38 mitogen-activated protein kinase, a redox-regulated signaling factor activated by many environmental stimuli and proinflammatory cytokines (Derijard et al. 1995; Stein et al. 1996; Das et al. 2004; Nebreda and Porras 2000). The role of MKK6 in the activation of p66shc has not been explored so far. Since MKK6 is an apoptotic kinase and the main activator of p38MAPK, in response to several stimuli both in vitro and in vivo, we decided to explore the role of MKK6 in the β-Amyloid-induced p66shc activation. Here, we show that β-Amyloid stimulation of C6 and U87MG cells induces MKK6 and p66shc phosphorylation, resulting in the formation of MKK6/p66shc complex that ultimately leads to ROS production and apoptotic cell death.

Materials and Methods

Cell Lines, Plasmids, Antibodies, and Reagents

Rat C6 glioma and U87MG glioblastoma cell lines were obtained from NCCS (National Centre for Cell science) and were grown in DMEM (Dulbecco's modified Eagle's medium), with 10 % FBS (fetal bovine serum) and 1× antibiotic (100 U/ml penicillin, 100 µg/ml streptomycin) at 37 °C in 5 % CO_2/95 % air. Xpress-tagged p66shc WT CDNA in PCDNA 3.1/His A, PCDNA 3.1, and Flag-tagged MKK6 in PcDNA3.1 were a kind gift from Dr. Shaida Andrabi. PCDNA 3.1 was obtained from Addgene.Org. P66shc S36A mutant, MKK6 double mutant (S207A and Thr 211A), and MKK6 triple mutant (TM) (S207A, Thr 211A, and Y219F) were made by site-directed mutagenesis (Quick Change, Stratagene) and confirmed by DNA sequencing. Antibody to Shc (Bio-Rad Inc.), MKK6 (Cell Signalling Technologies), phospho-S36-p66shc, phospho-S207-MKK6 (Abcam), anti-vinculin (Upstate Biotechnology Inc.), and Horseradish peroxidase-conjugated secondary anti-mouse and anti-rabbit (Sigma-Aldrich) were purchased from the indicated sources. All the reagents were purchased from Sigma-Aldrich or otherwise mentioned. β-Amyloid 35–25 and β-Amyloid 25–35 (Gen Script) were dissolved in sterile double-distilled water and incubated at 37 °C for 4 days before use and stored at −20 °C until use. SiRNA against MKK6 was purchased from Santa Cruz Inc., USA. ShRNA against p66shc was kindly provided by Kaikobad Irani, UPMC, USA. Ascorbic acid and N-acetylcysteine were purchased from Sigma-Aldrich, USA. Both the chemicals were dissolved in culture media.

Cell Culture and Transfections

C6 and U87MG cells were maintained in DMEM containing 10 % heat-inactivated FBS, 2 mM glutamine, and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) at 37 °C in 5 % CO_2. For transfection, C6 cells were plated in 10-cm dishes (obtained from Corning and Greiner) at a density of 3×10^6. The following day, cells were incubated with a total of 20 µg of DNA and 50 µl of lipofectamine (Invitrogen) in a final volume of 5 ml OPTI-MEM (Gibco Life Technologies). After 5 h, the transfection mix was aspirated and cells were replaced in fresh medium containing 0.5 % FBS and incubated further for 36 h. β-Amyloid was added to these serum-starved cells for specific time periods, depending on the purpose of the experiment. For knock-down experiments, cells were grown in antibiotic-free media overnight, and the experiment was performed as per manufacturer's recommendations (SiRNA against MKK6) as previously described for ShRNA against p66shc (Khanday et al. 2006b).

Cell Lysis and Immunoblotting

Cells were washed with phosphate-buffered saline (PBS) and lysed in magnesium lysis buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 % NP-40,

25 mM $MgCl_2$, 20 % glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM NaF, protease inhibitor cocktail, and 10 µl/1 ml of lysis buffer (1000 × stock). The lysates were then put on ice for 45 min. This was followed by centrifugation at 12,000 rpm for 10 min at 4 °C. Protein concentration of the supernatant was determined spectrophotometrically (Shimadzu, Japan) at 595 nm using the Bio-Rad assay kit, as suggested by the manufacturer. Then, 2× Laemmli sample buffer was added to the cell lysates, and samples were resolved by 10 % SDS-PAGE. Gels were transferred to PVDF membranes (Milipore) at 100 V for 1 h. After blocking with nonfat dry milk (5 %), proteins were probed overnight using their respective antibodies at appropriate dilution. Anti-Shc (1:3,000), anti-MKK6 (1:1,000), anti-phospho-S36-p66shc (1:1,000), anti-phospho-S207-MKK6 (1:500), and anti-vinculin (1:5,000) were used. The excess antibody was removed by sequential washing of the membranes in Tween-TBS; 1:7,000 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody was added for 1 h at room temperature, followed by sequential washing of the membranes in T-TBS. All the steps of western blotting were followed as done previously (Bashir et al. 2010). Signal was detected by chemiluminescence using the ECL system (Cell Biosciences). Blots were stripped and reprobed as required.

Co-immunoprecipitation

Co-immunoprecipitation was typically performed by incubating 3 µg of antibody and 35 µl of slurry of protein A–Sepharose beads (Invitrogen) at 4 °C overnight. Antibody–Sepharose complex was washed three times with PBS and incubated with 1.0 mg of cell lysate in lysis buffer for 4 h at 4 °C. Sepharose beads were collected, washed by repeated centrifugation and then boiled. Supernatant from a final centrifugation was analyzed by SDS-PAGE/western blotting. An equivalent amount of nonimmune IgG was used as a control for immunoprecipitations.

Apoptotic Assay

Cell death detection ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN) was used to detect apoptosis after β-Amyloid treatment of cells. Assay is based on a quantitative sandwich using antibodies directed against DNA and histones to detect mono- and oligonucleosomes in the cytoplasm of cells undergoing apoptosis. Assay was carried out according to the manufacturer's protocol. Measured OD_{405} was normalized for cell number/protein content (Khanday et al. 2006a).

Measurement of ROS Generation

Reactive oxygen species were measured by using the Amplex Ultra Red assay (Molecular Probes, Invitrogen) as per the manufacturer's protocol. Transfected cells were treated with β-Amyloid (20 µM concentration) for 15 min. This was followed by addition of 10 mM Amplex Ultra Red mixture. The cells along with the mixture were protected from light and incubated at 37 °C for 15–20 min. Fluorescence signal was then detected using spectrofluorophotometer (Shimadzu, RF-5301) with excitation at 568 nm and emission at 581 nm. The wells containing assay mixture and the cells untreated with β-Amyloid were used as the control.

Statistical Analysis

In this study, representative experiments from three independent experiments are shown. Results for each experiment are given as mean of triplicates ± SD. Statistically significant differences between sample groups were determined using *t* tests (Excel, Microsoft). A p value of <0.01 was considered significant.

Results

β-Amyloid Induces Significant Cellular Toxicity

We first investigated the role of β-Amyloid in the toxicity of C6 cell lines. Cells were grown overnight to near confluency in serum-free media, followed by treatment with β-Amyloid for 24 h, at concentrations of 10, 20, 30, and 50 µM. Progressive increase in apoptotic death was observed with β-Amyloid 25–35 treatment, whereas the control peptide 35–25 showed no effect (Fig. 1a, b). We carried out time-dependent ROS production in C6 cells when treated with 20 µM of β-Amyloid. Results indicate a progressive increase in ROS production up to 15-min time point. However, beyond 15-min time point, ROS production decreased but the decrease was not too significant as compared to untreated cells, although it stayed significantly higher than untreated cells till 24 h (Fig. 1c).

β-Amyloid Activates p66shc

Since β-Amyloid neurotoxicity leads to the production of ROS, we looked at the serine phosphorylation of p66shc upon treatment with β-Amyloid. Lysates from cells treated with β-Amyloid peptides at various time points and concentrations were subjected to immunoprecipitation to pull down the total Shc. It was followed by the western blot analysis, using S36-p66shc phospho-specific antibody, to

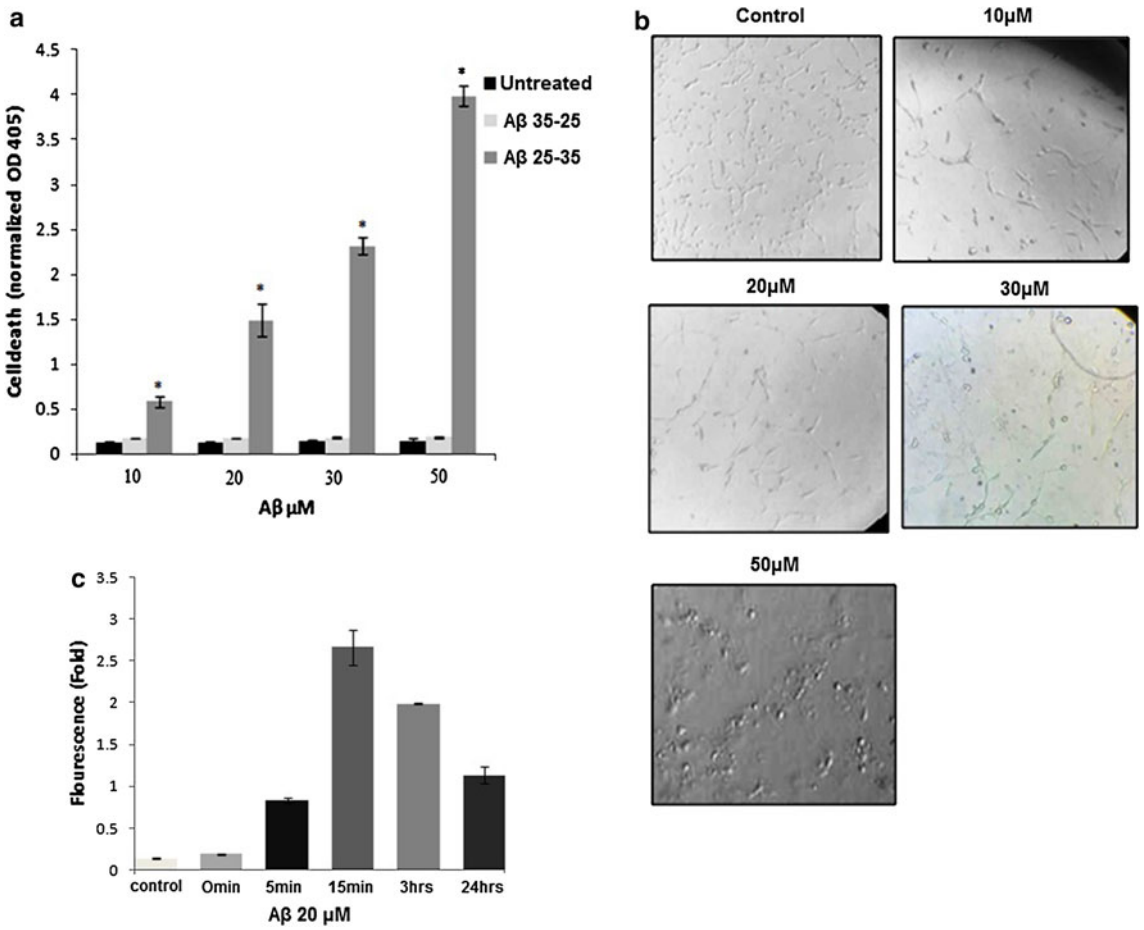

Fig. 1 β-Amyloid induces dose-dependent apoptotic cell death in C6 cells. **a** β-Amyloid causes apoptosis starting at 10 μM concentration and increases with an increase in the concentration and reaches a maximum at 50 μM with predominantly necrosis taking place. C6 cells were treated with increasing concentration of Aβ 25–35 and 35–25, respectively, for 24 h in serum-free media. Data are means of ±SD for three separate experiments, $p < 0.05$ versus untreated cells. **b** Representative photographs were taken to illustrate the changes in cell morphology. **c** Time-dependent ROS production in C6 cells at 20 μM β-Amyloid concentration

detect the serine phosphorylation of p66shc. Results indicate that the serine phosphorylation of p66shc remains elevated for 90 min in the presence of 10 and 20 μM, with the maximum serine phosphorylation being observed at 15 min and at 20 μM β-Amyloid concentration (Fig. 2a, b). Blots were stripped and probed with Shc antibody to look for the effect of β-Amyloid on total Shc expression. Expression of all the three isoforms, i.e., p46shc, p52shc, and p66shc, was unaltered (Fig. 2a, b, middle panel). Anti-vinculin antibody was used as loading control (Fig. 2a, b, lower panel). While comparing the phosphorylation of p66shc at 10 μM and 20 μM Aβ 25–35 peptide, respectively, a twofold increase in phosphorylation of S36 residue of p66shc was observed at 20 μM Aβ 25–35 (Fig. 2c). Similar experiments were performed in human glioblastoma cell line U87MG. Time-dependent treatment of U87MG cells with 20 μM β-Amyloid showed maximum p66shc serine phosphorylation at 15-min time point (Fig. 2d). Exposure at 20 μM β-Amyloid concentration caused cell death in both cell lines, whereas the control peptide showed no effect (Fig. 2e).

β-Amyloid Leads to Serine Phosphorylation of MKK6 Protein

Next, we examined the effect of β-Amyloid on the expression and activation of MKK6, in time- and concentration-dependent manner. Treatment of C6 cells with β-Amyloid activates endogenous MKK6, in a time-dependent manner at 20 μM concentration (Fig. 3a, upper panel). As observed for p66shc activation (Fig. 2a, d), maximum activation of MKK6 was observed at 15 min and 20 μM concentration (Fig. 3a, upper panel). Blot was stripped and probed with total MKK6, which showed the same level of total MKK6 expression (Fig. 3a, middle panel). Anti-vinculin was used as a loading control (Fig. 3a, lower panel). These results were reconfirmed in human glioblastoma U87MG cell line (Fig. 3b).

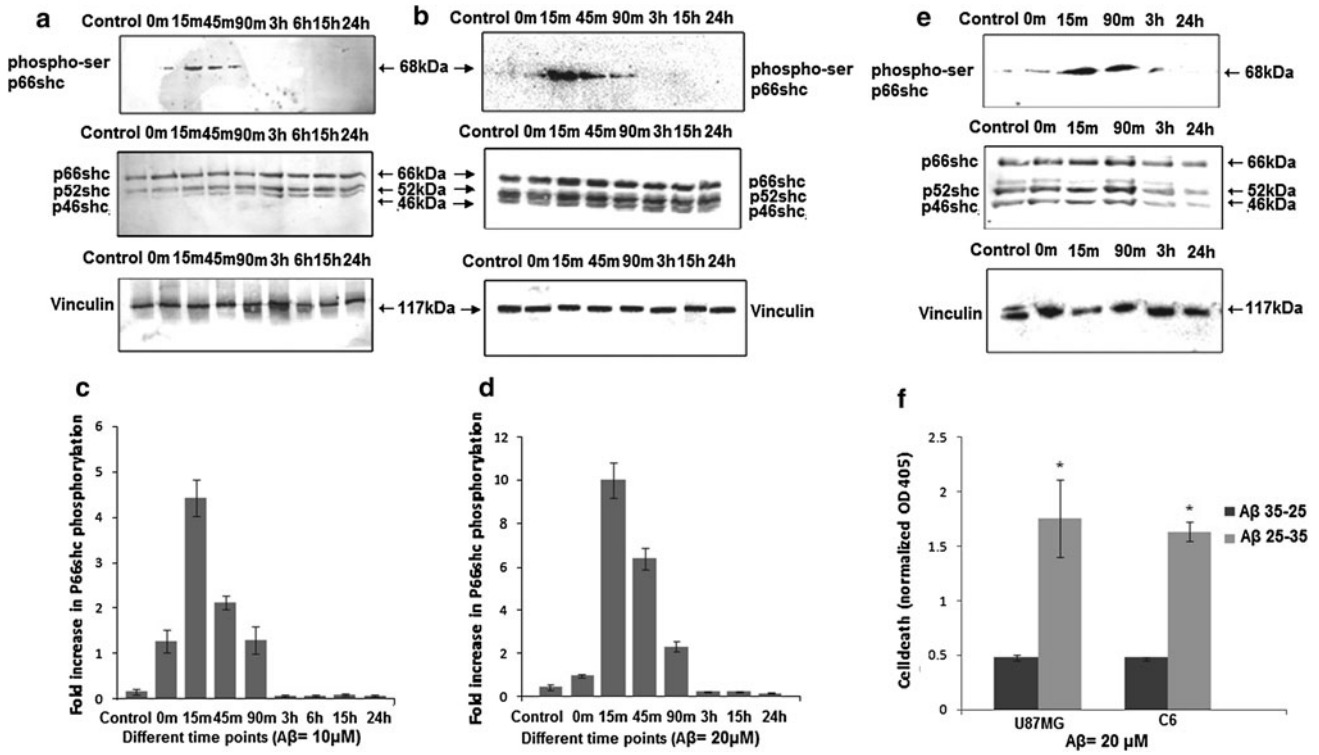

Fig. 2 β-Amyloid triggers serine 36 phosphorylation of p66shc in C6 cells. **a** Western blot analysis showing phosphorylation and total Shc expression in C6 cells treated with 10 μM β-Amyloid for the indicated time points after an overnight serum starvation period, by using a specific anti-phospho-p66shc (S36)antibody. Maximum phosphorylation was observed at 15-min time point (*Bar graph*, *lower panel*). **b** S36 phosphorylation of p66shc at 20 μM concentration. C6 cells treated with 20 μM β-Amyloid for indicated time points after an overnight serum starvation period. **c** Extent of phosphorylation was almost twofold as compared to 10 μM concentration (*Bar graph*, *lower panel*). The results are the mean ±SD of three different experiments. **d** Time-dependent serine phosphorylation of 36th residue of p66shc in U87MG cells at 20 μM β-Amyloid concentration. **e** Comparison of apoptotic cell death between U87MG and C6 cells using 20 μM β-Amyloid concentration

Serine Phosphorylation of p66shc is MKK6 Dependent

To identify whether MKK6 is responsible for p66shc phosphorylation on serine 36 residue, we first tested the serine phosphorylation of exogenous MKK6 and its mutants. It is well documented that oxidative stress plays a role in the activation of MKK6 in AD brains at S207/211 (Zhu et al., 2003), and indeed, T219 is the site of phosphorylation after H_2O_2 treatment (Harraz et al. 2007). Cells expressing vector control, exogenous MKK6, MKK6 double mutant (S207A and T211A), and MKK6 TM (S207A, T211A, Y219F) were treated with 20 μM β-Amyloid for 15 min. Lysates of β-Amyloid-treated cells were analyzed by western blotting with antibody recognizing phospho-MKK6 (S207). MKK6 transfected cells show a threefold to fourfold increase in phosphorylation (S207) as compared to vector and mutant constructs (Fig. 3c, upper panel). Blots were striped and reprobed with anti-Shc (Fig. 3c, middle panel) and anti-MKK6 (Fig. 3c, middle panel) antibodies. Results reveal the same level of total Shc and MKK6 expression in transfected cells. Anti-vinculin was used as a loading control (Fig. 3B, lower panel). Densitometric analysis indicates a twofold to threefold increase in MKK6 phosphorylation as compared to vector control and MKK6 mutants (bar graph) (Fig. 3d). Overall, results indicate that β-Amyloid triggers phosphorylation of MKK6 at S207, T211, and Y219 residues. Alternately, we reconfirmed the results by using ROS scavengers, i.e., ascorbic acid (100 μM for 4 h) and N-acetylcysteine (500 μM for 4 h) in β-Amyloid- and mock-treated cells. Treatment of C6 cells with ascorbic acid and N-acetylcysteine significantly decreased β-Amyloid-induced serine phosphorylation of MKK6 (Fig. 3e) and ROS generation (Fig. 3f). These findings suggest that β-Amyloid-induced ROS generation is responsible for MKK6 activation and ROS scavengers block β-Amyloid-induced cell death by inhibiting the phosphorylation of MKK6.

Since transfection with MKK6 did not show any effect on the expression levels of Shc, we studied whether β-Amyloid-mediated activation of MKK6 affects p66shc activity (phosphorylation at serine 36 residue of p66shc). As indicated in Fig. 4a, b, cells were transfected with vector control, p66shc, MKK6 or their mutant constructs.

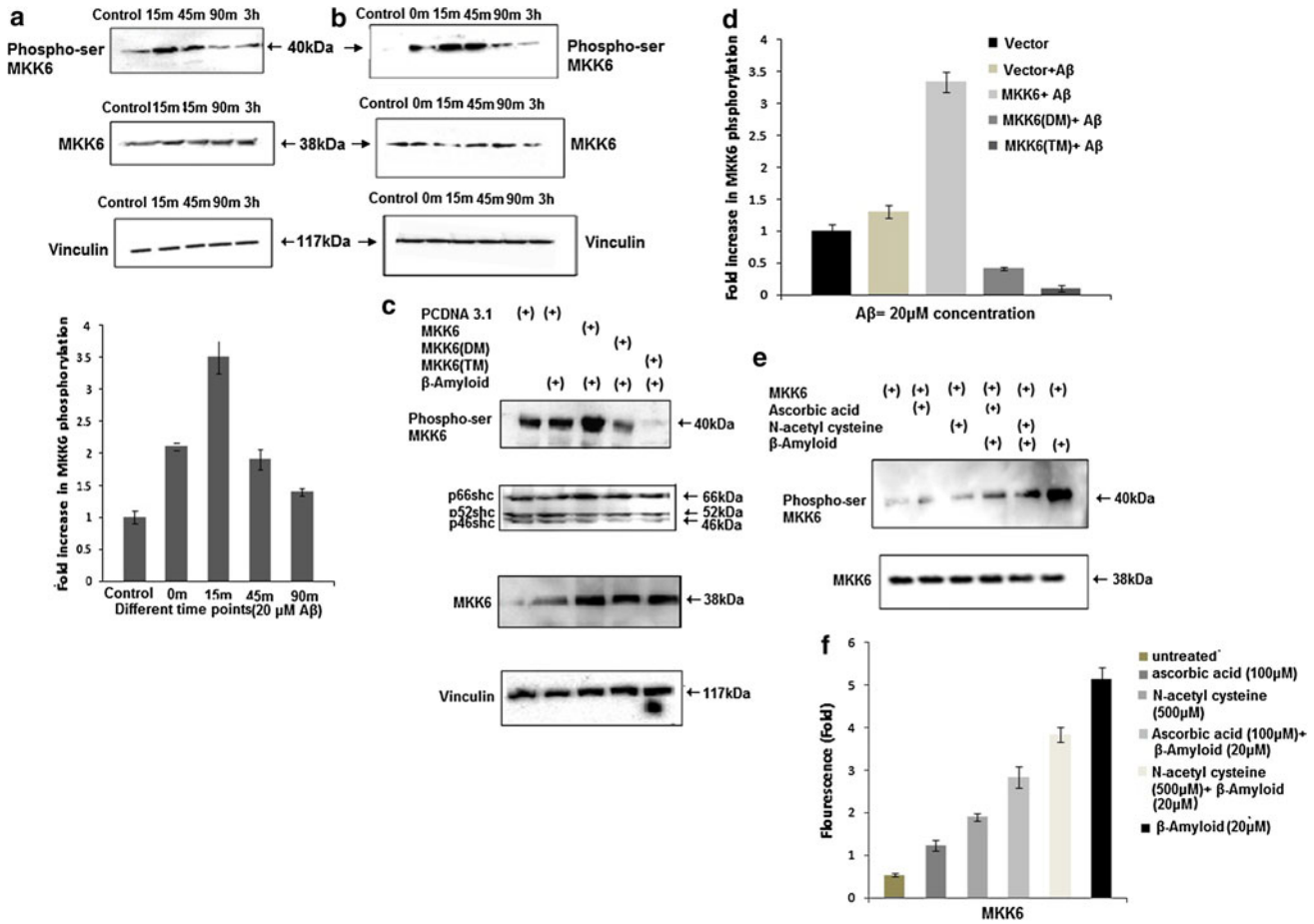

Fig. 3 β-Amyloid mediates serine phosphorylation of MKK6. **a** Western blot analysis showing serine phosphorylation and total MKK6 expression in C6 cells treated with 20 μM β-Amyloid for the indicated time points after an overnight serum starvation period by using a specific anti-phospho-MKK6 (S207)antibody. β-Amyloid-mediated serine phosphorylation of MKK6 was maximally observed at 15-min time point. **b** Time-dependent serine phosphorylation of MKK6 in the presence of 20 μM β-Amyloid concentration in U87MG cell lines. Total MKK6 (middle panel) and vinculin as loading control (*lower panel*) are also shown. **c** C6 cells were transfected with vector, MKK6, MKK6 DM, MKK6 TM. After an overnight serum starvation period, C6 cells were treated with 20 μM 25–35 β-Amyloid for 15 min. Ectopically expressed MKK6 was serine phosphorylated, while DM and TM showed least phosphorylation upon β-Amyloid treatment. **d** Densitometric analysis indicates twofold to threefold increase in MKK6 phosphorylation as compared to vector control and MKK6 mutants (*bar graph*). The results are the mean ±SD of three different experiments. **e** Serine phosphorylation of MKK6 in the presence of ascorbic acid (100 μM, for 4 h) and N-acetylcysteine (500 μM for 4 h) versus β-Amyloid (20 μM for 15 min). **f** ROS production in the presence of ascorbic acid and N-acetylcysteine treated with β-Amyloid or mock-treated cells

Cells were treated with β-Amyloid for 15 min and at 20 μM concentration. Co-immunoprecipitation results indicate a significant serine phosphorylation in p66shc–MKK6 co-transfected cells as compared to either p66shc-expressing cells or cells co-transfected with mutant constructs (Fig. 4a, upper panel). Blots were stripped and reprobed with anti-Shc (Fig. 4a, middle panel) and anti-MKK6 (Fig. 4a, middle panel) antibodies, respectively. Total protein level after transfection was observed to be the same. Vinculin expression was used as loading control (Fig. 4a, lower panel). We also studied the serine phosphorylation of MKK6 in the presence of exogenous p66shc and its mutant (S36A). However, no effect on serine phosphorylation (S207) of MKK6 was observed (Fig. 4c). Results conclude that MKK6 phosphorylates p66shc on Ser36 in response to β-Amyloid treatment and thus acts as an upstream target of p66shc.

MKK6 Knock-down Decreases Activation of p66shc

We treated MKK6– p66shc double transfectant cells with SiRNA against MKK6 and looked for the phosphorylation of serine 36 of p66Shc in the presence of β-Amyloid. Results indicate a significant decrease in the serine 36 phosphorylation of p66Shc (Fig. 5a). We observed a decrease in ROS production (Fig. 5b) and decrease in cell death (Fig. 5c), in the presence of knock-down of MKK6. The results validate the observation that MKK6 acts as an

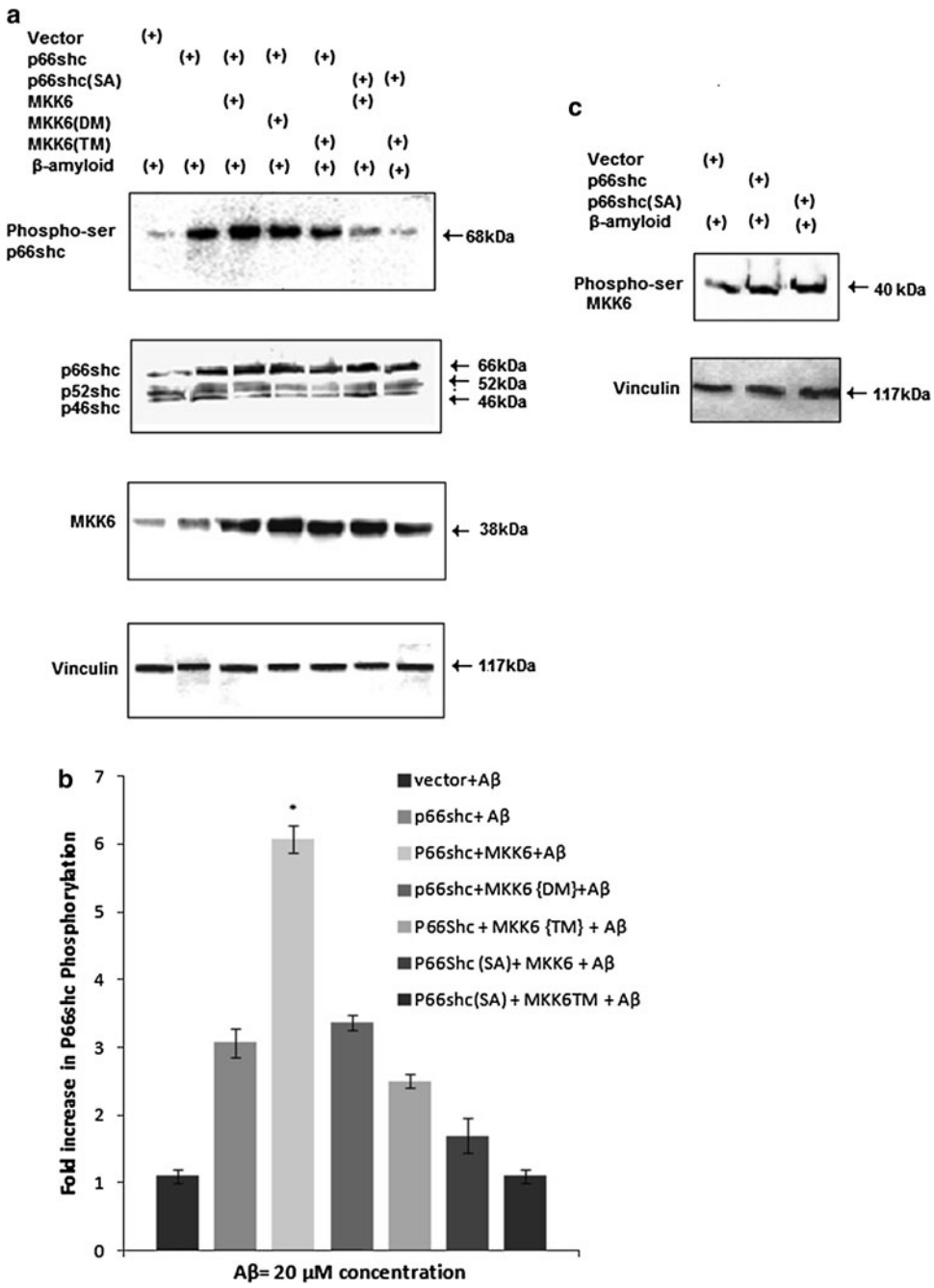

Fig. 4 MKK6 is an upstream target of p66shc. **a** P66shc phosphorylation at S36 in C6 cells expressing vector control, p66shc alone, MKK6–p66shc, MKK6 mutants, and p66shc mutant after addition of 20 μM β-Amyloid for 15 min sequentially using anti-phospho-p66shc (S36) and anti-shc antibody. Cell lysates were incubated with anti-MKK6 antibodies conjugated with agarose beads overnight, washed with lysis buffer, and analyzed by western blot using, anti-phospho-p66shc (S36), anti-p66shc, and anti-MKK6 antibodies. S36 phosphorylation of p66shc was maximum in cells co-transfected with MKK6–p66shc as compared to vector control, p66shc alone, MKK6 mutants, and p66shc mutant. **b** *Bar chart* showing the extent of phosphorylation from three independent experiments. Results are expressed as mean ±SD. **c** MKK6 was not phosphorylated in the presence of ectopically expressed p66shc or its mutant construct

upstream target of p66shc. We used ShRNA against p66shc to assess ROS production and apoptotic cell death. Results indicate a significant decrease in serine phosphorylation of p66shc (Fig. 5d), ROS production (Fig. 5e), and apoptotic cell death (Fig. 5f). MKK6 knock-down led to decrease in endogenous levels of MKK6, thereby decreasing the levels of active p66shc (S36 phosphorylation), ROS production, and cell death (Fig. 6a–c). P66shc knock-down significantly decreased the endogenous levels of active p66shc, in the presence of β-Amyloid leading to decreased ROS production and prevented apoptotic cell death (Fig. 6a–c). These results suggest the involvement of both MKK6 and p66shc in β-Amyloid-induced apoptosis.

P66shc and MKK6 Interact to Mediate β-Amyloid-triggered ROS Production and Cell Death

MKK6-mediated phosphorylation of p66shc at S36 requires these proteins to associate under in vivo conditions. Ectopically expressed p66shc was co-immunoprecipitated with anti-MKK6 antibody (Fig. 7a, upper panel). Alternatively, we used anti-p66shc antibody to pull down MKK6

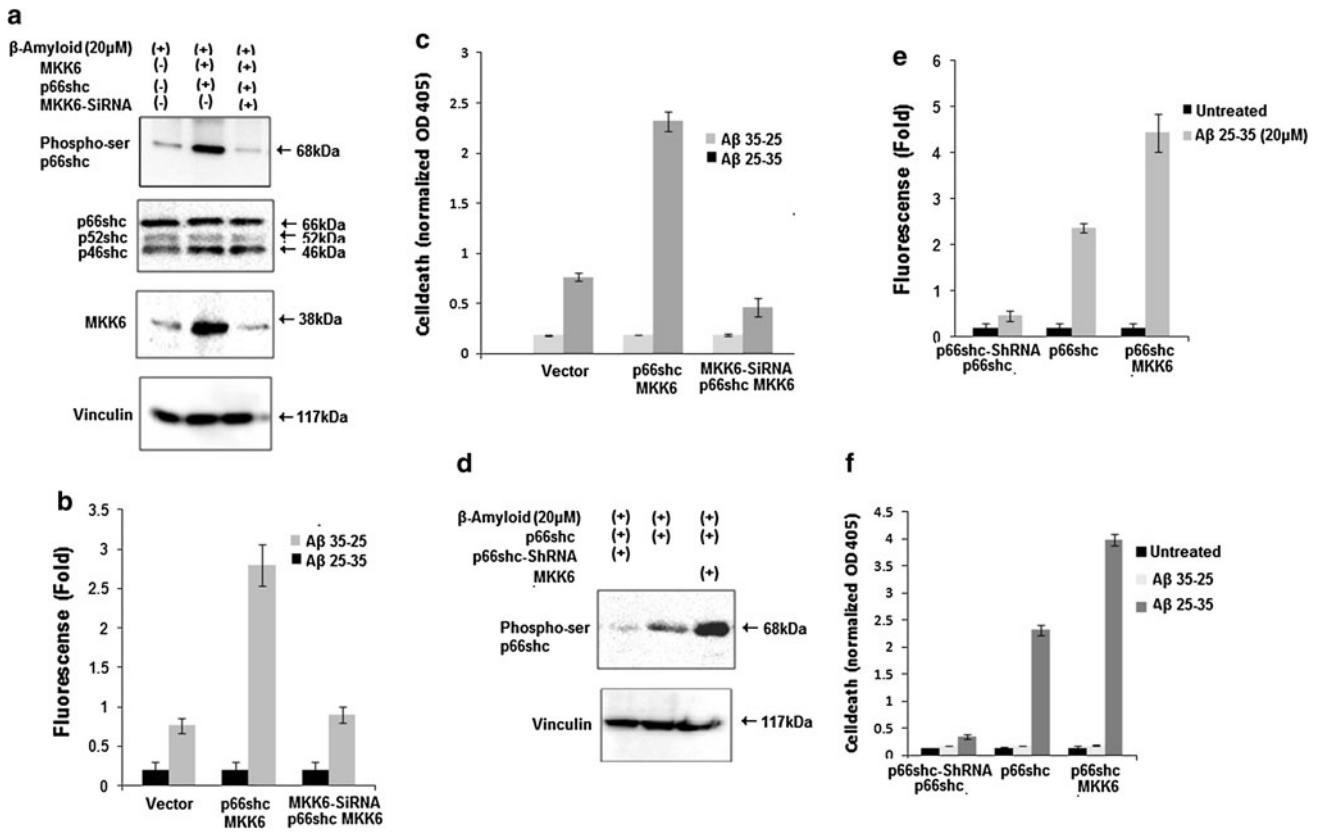

Fig. 5 Effect of knock-down on activation of MKK6 and p66shc. **a** C6 cells were treated with SiRNA against MKK6. Significant decrease in serine phosphorylation of p66shc was observed (*upper panel*). Total shc, MKK6, and vinculin expressions are represented in 2nd, 3rd, and 4th panel of the figure. **b** ROS production decreases on treatment of cells with SiRNA of MKK6 as compared to MKK6–p66shc co-transfectants. **c** Fourfold to fivefold decrease in apoptotic cell death was observed in the presence of SiRNA of MKK6. **d** Representative blot showing the decrease in serine phosphorylation of p66shc in the presence of ShRNA against p66shc. **e** ROS production in the presence of ShRNA of p66shc decrease compared to p66shc alone or p66shc–MKK6 co-transfected cells. **f** Apoptotic cell death decrease in the presence of ShRNA of p66shc as compared to p66shc alone or p66shc–MKK6 co-transfected cells

(Fig. 7a, lower panel). Results indicate MKK6 pull down in cells transfected with WT p66shc, but not in the control. Co-immunoprecipitation results suggest that MKK6 (TM) was not able to interact with WT p66shc (Fig. 7a).

ROS analysis indicates a threefold to fourfold increase in ROS production in β-Amyloid-treated cells, co-transfected with p66shc and WT MKK6 as compared to the control (Fig. 7b). We further carried out cell death analysis on cells co-transfected with MKK6/p66shc and p66shc(S36A)/MKK6 (TM), respectively. Results indicate a twofold increase in death of the cells, co-transfected with p66shc and WT MKK6 when compared to p66shc (SA) and MKK6 (TM) (Fig. 7c). Furthermore, C6 cells co-transfected with control, p66shc + MKK6, or p66Shc (SA) + MKK6(TM), and treated with β-Amyloid all exhibited a marked decrease in cell viability compared to cells transfected without β-Amyloid treatment. On treatment with ascorbic acid (100 μM) and N-acetylcysteine (500 μM), p66shc–MKK6-transfected cells showed a marked decrease in the serine phosphorylation of p66shc (Fig. 7d), and apoptotic cell death (Fig. 7e).

Discussion

A novel and important conclusion of our study is that MKK6–p66shc forms an important signaling cascade in the β-Amyloid-mediated cell toxicity. We demonstrate that the β-Amyloid causes apoptotic cell death through p66shc-serine 36 phosphorylation, with the phosphorylation being carried out by an apoptotic kinase MKK6. Aβ is the most likely candidate of neurodegeneration in AD. It is a small soluble aggregate that appears to insert into the lipid membrane and generate ROS (Canevari et al. 2004). However, this neurotoxic property of β-Amyloid resides in the amino acids, found at positions 25–35 of the β-Amyloid structure. β-Amyloid 25–35 fragment has been shown to a have toxic effect on neurons (Pike et al. 1995), produce free radicals in aqueous solutions (Hensley et al. 1999) as well as ROS generation in C6 astroglioma cell lines (Lee et al. 2000). Thus, we have used β-Amyloid 25–35 peptide for carrying out our experiments in C6 cell lines. C6 cells have been shown to be most sensitive and prime targets of

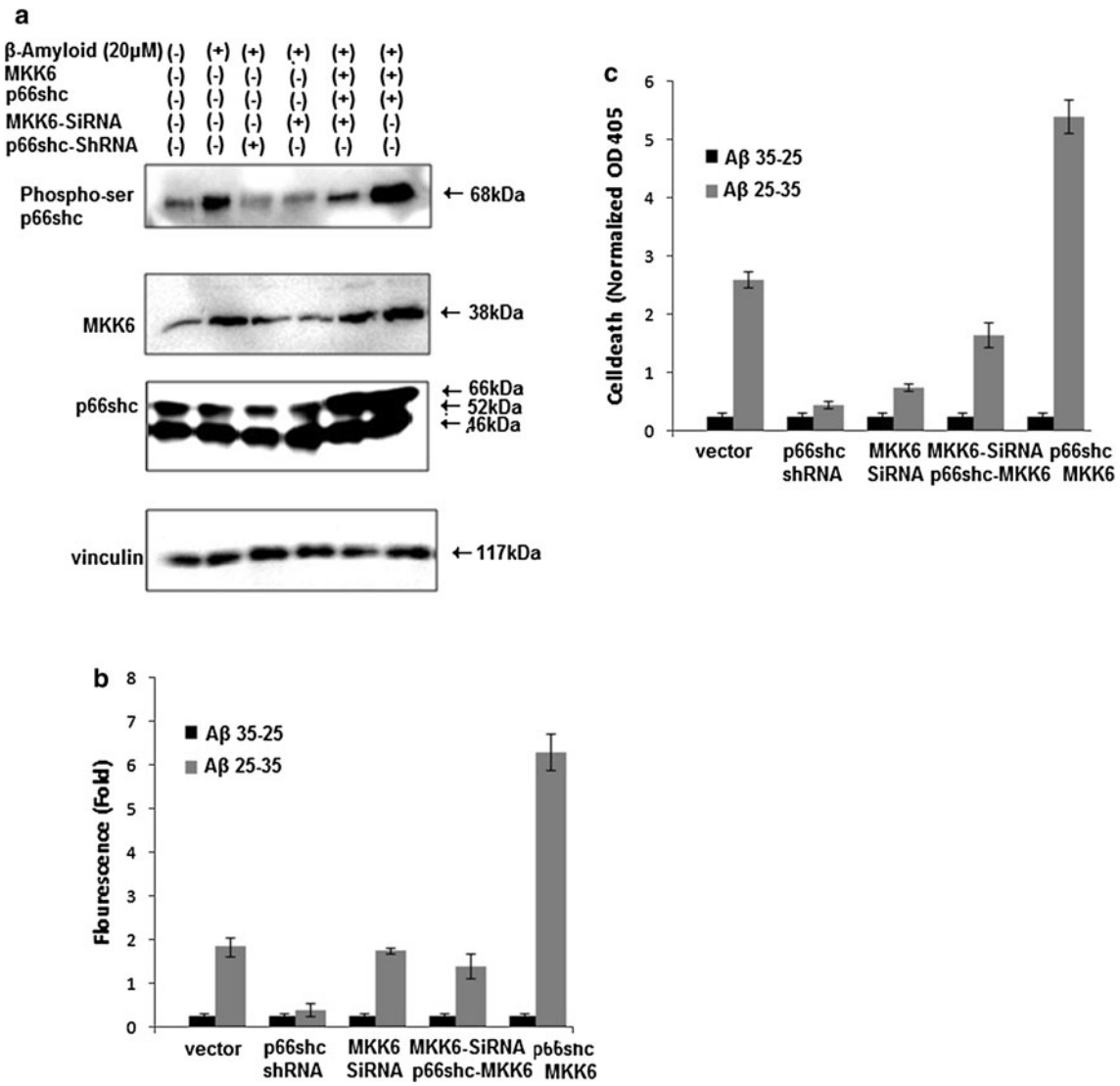

Fig. 6 Effect of knock-down of MKK6 and p66shc on activation of p66shc, ROS production, and apoptotic cell death. **a** Cells were treated with SiRNA of MKK6 and ShRNA of p66shc in the presence and absence of β-Amyloid. Significant decrease in serine 36 phosphorylation of p66shc was observed at endogenous levels when compared to the control (without β-Amyloid) or p66shc–MKK6 co-transfectants. Total MKK6, shc, and vinculin (loading control) are represented in 2nd, 3rd, and 4th panel of the figure. **b** ROS production decreases on treatment of cells with SiRNA of MKK6 and ShRNA of p66shc as compared to the control (without β-Amyloid) or p66shc–MKK6 co-transfectants. **c** Significant decrease in apoptotic cell death was observed in the presence of SiRNA of MKK6 and ShRNA of p66shc when compared to control (without β-Amyloid) or p66shc–MKK6 co-transfectants

the β-Amyloid-induced pathogenesis as compared to other neuronal cells as they become reactive in the presence of β-Amyloid (Kalaria 1999; Rodriguez et al. 2010). Cultured rat astrocytes can also respond to cytotoxic changes leading to cell death in the presence of β-Amyloid peptides (Brera et al. 2000). Furthermore, C6 astroglioma cell lines have been used earlier to study the pathway of Rac1 GTpase in the presence of β-Amyloid causing generation of ROS (Lee et al. 2000). Thus, we propose C6 cell line as a matter of choice for studying the mechanistic pathway of Aβ, though key observations have been repeated for confirmation in human glioblastoma U87MG cells (Figs. 2e, f and 3b).

We have shown that β-Amyloid causes a progressive increase in apoptotic cell death in C6 astroglioma cell lines. However, increasing the concentration of Aβ above 30 μM leads to an increase in apoptotic cell death predominantly necrosis taking place (Fig. 1a, b). Previous studies have also established that higher concentration of Aβ (Yankner et al. 1989; Cotman et al. 1994; Selkoe 1999) results in neuronal cell death and secondary apoptosis. Thus, we used 20 μM β-Amyloid concentration in this study that alters cell signaling pathway, without having lethal impact on cell viability (Fig. 1). Similar concentration of β-Amyloid has been previously used in the cell culture experiments

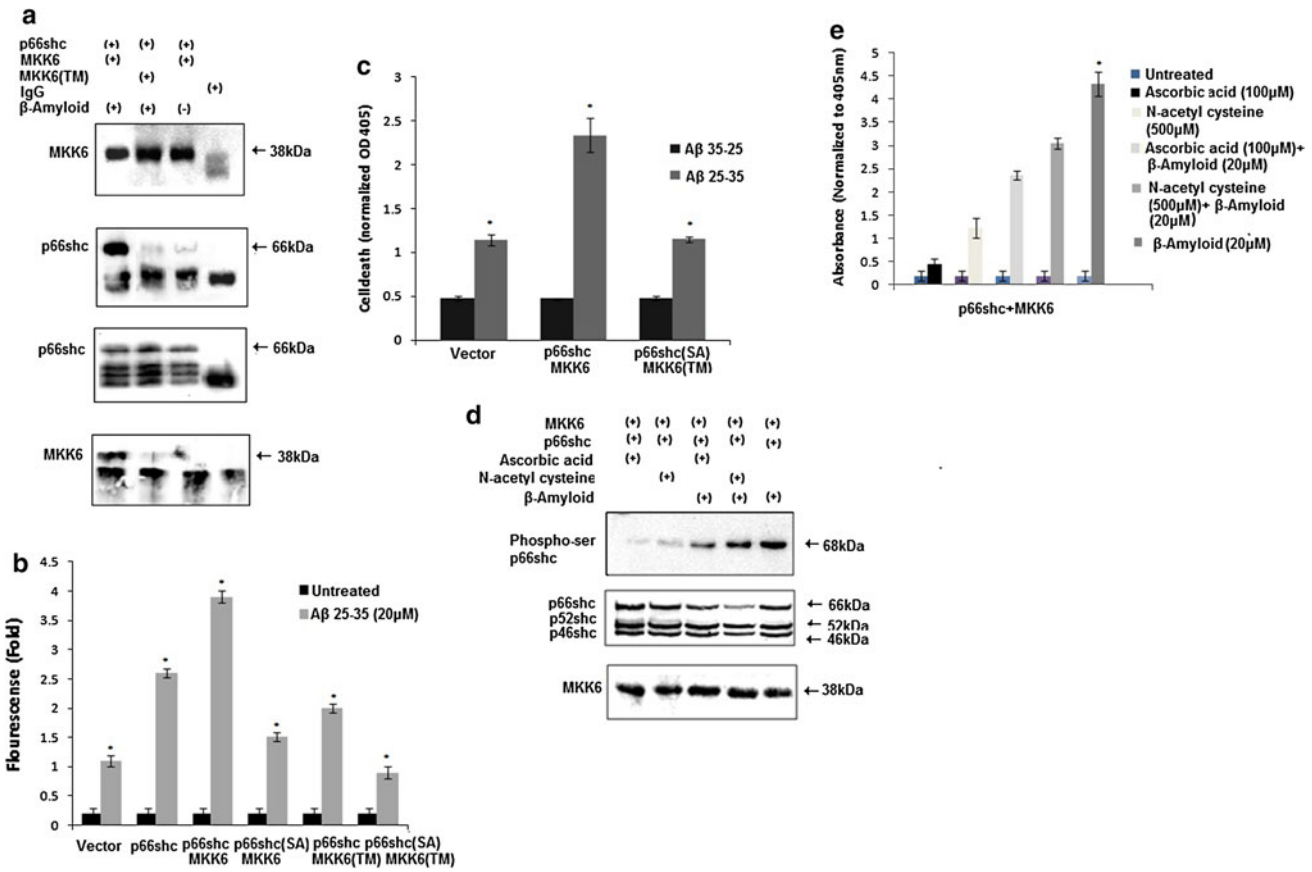

Fig. 7 P66shc and MKK6 interact to mediate β-Amyloid-triggered ROS production and cell death. **a** C6 cells were co-transfected with vector, p66shc–MKK6 and p66Shc (S36A)–MKK6(TM) 24 h in 0.5 % FBS DME media and then incubated in serum-free media and treated with 20 μM β-Amyloid for 15 min followed by Co-IP experiments. MKK6 interacts with p66shc in presence, but not in the absence of 20 μM β-Amyloid. TM of MKK6 did not interact with p66shc in the presence of β-Amyloid. **b** Threefold to fivefold increase in the production of ROS in the presence of MKK6–p66shc transfects as compared to MKK6(TM)–p66Shc(SA) and vector control. The results are the mean ±SD of three different experiments and probability <0.01. **c** *Bar graph* showing programmed cell death assay. A twofold increase in apoptotic death was observed in cells transfected with p66shc and MKK6 as compared to p66shc(SA) and TM of MKK6. The results are the mean ±SD of three different experiments and probability <0.01. **d** Treatment of cells with ascorbic acid (100 μM for 4 h) and N-acetylcysteine (500 μM for 4 h) decreases the serine phosphorylation of p66shc in the presence of β-Amyloid. **e** Treatment of cells with ascorbic acid (100 μM for 4 h) and N-acetylcysteine (500 μM for 4 h) decreases apoptotic cell death by eightfold and sixfold, respectively

(Tong et al. 2001). We also performed a time-dependent ROS generation assay in C6 cells that showed maximum ROS production at 15-min time point followed by decrease at beyond 15-min time point, which could be due to the decrease in serine phosphorylation of p66shc protein (Fig. 1c). ROS production will sustain for a longer period of time as a result of other prooxidant proteins like RhoGTPase rac1 being activated in positive feedback manner (Khanday et al. 2006a, b). The sustained increase in ROS will lead to eventual cell death.

Several studies have been conducted, suggesting the role of p66shc serine phosphorylation in signaling events leading to apoptosis, in response to oxidative damage (Migliaccio et al. 1999; Le et al. 2001; Nemoto and Finkel 2002). Since β-Amyloid leads to oxidative apoptotic death, we hypothesize this may act as a trigger for the serine phosphorylation of p66shc. Here, we have shown that β-Amyloid induces p66shc phosphorylation at S36 in C6 and U87MG cell lines (Fig. 2). Our results are in consonance with the earlier studies showing phosphorylation of p66shc in neuroblastoma cell lines, in response to β-Amyloid (Smith et al. 2005), but through a different mechanism. Here, we report novel findings of MKK6 phosphorylation when cells are treated with β-Amyloid (Fig. 3). We also confirmed our results using ascorbic acid and N-acetyl-cysteine, the common ROS scavengers. Treatment of cells with these antioxidants resulted in marked decrease in serine phosphorylation of MKK6 and ROS generation (Fig. 3e, f). Several studies have been conducted to point out the role of β-Amyloid in the activation of MKK6 protein. β-Amyloid 1–40 has been observed to induce an increase in phosphorylated MKK3/MKK6 and p38 MAPK

expressions in hippocampal tissue (Jin et al. 2006). It also induces MKK6 activation through ROS-induced Apoptosis signal-regulating kinase 1 (ASK1) pathway (Kadowaki et al. 2005). Activation of PP2A–ASK1–MKK3/6–p38MAPK apoptotic signaling cascade requires β-Amyloid-induced oxidative stress (Hsu et al. 2007). In response to oxidative stress, ASK1 activates the cell death-associated p38 MAPK pathway, by phosphorylating MKK6 after H_2O_2 treatment (Sturchler et al. 2010). Furthermore, we show that phosphorylated MKK6 acts as an upstream target for p66shc phosphorylation (Fig. 4). We used SiRNA against MKK6 and ShRNA against p66shc, to confirm the involvement of MKK6 and p66shc in β-Amyloid-induced apoptosis. Results suggest a significant decrease in the serine 36 phosphorylation of p66shc, ROS production, and apoptotic cell death in the presence of SiRNA of MKK6 both at exogenous (Fig. 5a, c) and endogenous levels (Fig. 6). Using ShRNA against p66shc led to its decreased activation, decrease in ROS production, and cell death both at exogenous (Fig. 5d–f) and endogenous levels (Fig. 6).

Although phosphorylation of p66shc at S36 residue by p38MAP kinase in vitro in response to UV radiation has been demonstrated previously (Le et al. 2001), the role of MKK6 in serine phosphorylation of p66shc cannot be ruled out. We were able to detect complex formation of p66shc–MKK6, when cells were treated with β-Amyloid (Fig. 7a). However, we could not detect the complex formation between MKK6 and p66shc at endogenous level, possibly due to low expression levels. We suggest that this complex facilitates p66shc phosphorylation (Fig. 7d), ROS production (Fig. 7b), and hence cell death (Fig. 7c). Treatment of MKK6 and p66shc co-transfected cells with antioxidants abrogated serine phosphorylation of p66shc (Fig. 4a) and thus inhibited cell death induced by β-Amyloid (Fig. 7e). The fact that β-Amyloid was involved in the association of MKK6–p66Shc complex could be due to the reason that β-Amyloid generates ROS (Su et al. 2008), more specifically H_2O_2, which is responsible for the tyrosine phosphorylation of MKK6 at Y219 residue (Harraz et al. 2007). Our results suggest this phosphorylated residue (Y219) to be responsible for the interaction of MKK6 with p66shc. We suggest this tyrosine phosphorylated residue to be involved in interaction with PTB domain of p66shc. Based on our findings, we propose that β-Amyloid generates ROS that ultimately leads to cell death (Fig. 8). However, we do not rule out the possibility of additional and alternative pathways apart from ROS generation that supports the role of β-Amyloid in neurodegeneration. Through separate signaling events, β-Amyloid is involved in disrupting Ca^{2+} homeostasis leading to cell death and phosphorylating tau and APP, thereby contributing to the formation of plaques and neurofibrillary tangles (Holscher 1998; Kawahara and Kuroda 2000; Mattson et al. 1993; Mattson 2002; Mattson and Chan 2003).

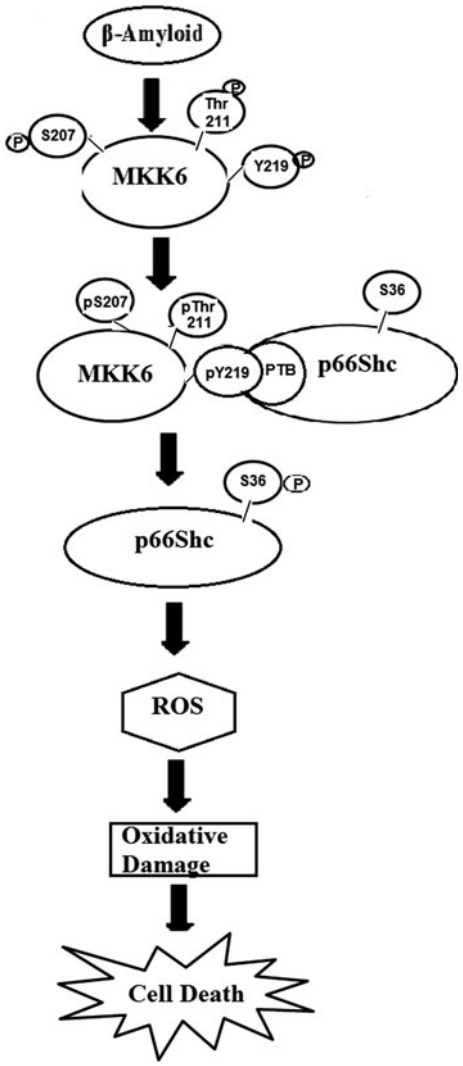

Fig. 8 Schematic diagram showing the interaction/activation of MKK6–p66shc proteins in the presence of Aβ leading to ROS production and cell death in C6 cells. Aβ causes phosphorylation of MKK6 at ser 207, Thr 211, and tyr 219, respectively. The phosphorylated MKK6 then interacts with p66shc resulting in the formation of MKK6–p66shc complex between the phosphorylated tyrosine of MKK6 and PTB domain of p66shc. P66shc gets phosphorylated at S36 residue, leading to the generation of ROS and cell death

β-Amyloid also binds to nAChR with high affinity (Wang et al. 2000), causing accumulation of Aβ1-42 (Nagele et al. 2003). Aβ1-42 triggers sustained increase in presynaptic Ca^{2+} and irreversibly block nAChR-dependent Ca^{2+} and acetylcholine release. These events critically affect memory and cognitive functions and contribute to the AD pathogenesis (Wang et al. 2000; Li and Buccafusco 2003; Lee and Wang 2003).

A number of mutations have been mapped to APP and a component of the γ-secretase presenilin 1 (PS1), which apparently favor the generation of β-Amyloid (Duyckaerts et al. 2008) and thereby AD. Emerging studies suggest that various intracellular signaling pathways are deregulated in AD brains; e.g., glycogen synthase kinase 3 has also been implicated in β-Amyloid-induced neurotoxicity (Ryder et al. 2004). Deregulation of such distinct signaling pathways lead to aberrant phosphorylation of cellular proteins and has a profound effect on the progression of AD (Ferrer et al. 2005; Hooper et al. 2008).

In conclusion, MKK6–p66shc interaction has a role in promoting cell toxicity in the presence of β-Amyloid and might have implications for understanding the initial stages of AD and for the design of therapies targeting β-Amyloid/MKK6/p66shc signaling cascade.

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Conflict of interest The authors declare no conflict of interest.

References

Bashir, M., Kirmani, D., Bhat, H. F., Baba, R. A., Hamza, R., Naqash, S., et al. (2010). P66shc and its associate targets are upregulated in esophageal cancers. *Cell Communication and Signaling, 8*, 13.

Brera, B., Serrano, A., & De Ceballos, M. L. (2000). β-amyloid peptides are cytotoxic to astrocytes in culture: A role for oxidative stress. *Neurobiology of Disease, 7*, 395–405.

Canevari, L., Abramov, A. Y., & Duchen, M. R. (2004). Toxicity of amyloid peptide: Tales of calcium, mitochondria, and oxidative stress. *Neurochemistry and Research, 29*, 637–650.

Corrêa, S. A. L., & Eales, K. L. (2012). The role of p38 MAPK and its substrates in neuronal plasticity and neurodegenerative diseases. *Journal of Signal transduction, 2012*, 1–12.

Cotman, C. W., Whittemore, E. R., Watt, J. A., Anderson, A. J., & Loo, D. T. (1994). Possible role of apoptosis in Alzheimer's disease. *Annuals of New York Acadamy Sciences, 747*, 36–49.

Das, D. K., Maulik, N., & Engelman, R. M. (2004). Redox regulation of angiotensin II signaling in the heart. *Journal of Cellular and Molecular Medicine, 8*, 144–152.

Derijard, B., Raingeaud, J., Barrett, T., Wu, I. H., Han, J., Ulevitch, R. J., et al. (1995). Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. *Science, 267*, 682–685.

Duyckaerts, C., Potier, M. C., & Delatour, B. (2008). Alzheimer disease models and human neuropathology: Similarities and differences. *Acta Neuropathologica, 115*, 5–38.

Ferrer, I., Gomez-Isla, T., Puig, B., Freixes, M., Ribe, E., Dalfo, E., et al. (2005). Current advances on different kinases involved in tau phosphorylation, and implications in Alzheimer's disease and tauopathies. *Current Alzheimer Research, 2*, 3–18.

Glenner, G. G., Wong, C. W., Quaranta, V., & Eanes, E. D. (1984). The amyloid deposits in Alzheimer's disease: Their nature and pathogenesis. *Applied Pathology, 2*, 357–369.

Hashimoto, Y., Tsuji, O., Niikura, T., Yamagishi, Y., Ishizaka, M., Kawasumi, M., et al. (2003). Involvement of c-Jun N-terminal kinase in amyloid precursor protein-mediated neuronal cell death. *Journal of Neurochemistry, 84*, 864–877.

Harraz, M. M., Park, A., Abbott, D., Zhou, W., Zhang, Y., & Engelhardt, J. F. (2007). MKK6 phosphorylation regulates production of superoxide by enhancing Rac GTPase activity. *Antioxidants & Redox Signaling, 9*, 1803–1813.

Hartmann, T., Bieger, S. C., Brühl, B., Tienari, P. J., Ida, N., Allsop, D., et al. (1997). Distinct sites of intracellular production for Alzheimer's disease Aβ40/42 amyloid peptides. *Nature Medicine, 3*, 1016–1020.

Hensley, K., Floyd, R. A., Zheng, N. Y., Nael, R., Robinson, K. A., Nguyen, X., et al. (1999). p38 kinase is activated in the Alzheimer's disease brain. *Journal of Neurochemistry, 72*, 2053–2058.

Holscher, C. (1998). Possible causes of Alzheimer's disease: Amyloid fragments, free radicals, and calcium homeostasis. *Neurobiology Disease, 5*, 129–141.

Hooper, C., Killick, R., & Lovestone, S. (2008). The GSK3 hypothesis of Alzheimer's disease. *Journal of Neurochemistry, 104*, 1433–1439.

Hsu, M. J., Hsu, C. Y., Chen, B. C., Chen, M. C., Ou, G., & Lin, C. H. (2007). Apoptosis signal-regulating kinase 1 in amyloid β peptide-induced cerebral endothelial cell apoptosis. *Journal of Neuroscience, 27*(21), 5719–5729.

Iversen, L. L., Mortishire-Smith, R. J., Pollack, S., & Shearman, M. S. (1995). The toxicity in vitro of β-amyloid protein. *Biochemical Journal, 311*, 1–16.

Jin, Y., Fan, Y., Yan, E. Z., Liu, Z., Zong, Z. H., & Qi, Z. M. (2006). Effects of sodium ferulate on amyloid-beta-induced MKK3/MKK6-p38 MAPK-Hsp27 signal pathway and apoptosis in rat hippocampus. *Acta Pharmacologica Sinica, 27*, 1309–1316.

Kalaria, R. N. (1999). Microglia and Alzheimer's disease. *Current Opinion in Hematology, 6*, 15–24.

Kadowaki, H., Nishitoh, H., Urano, F., Sadamitsu, C., Matsuzawa, A., Takeda, K., et al. (2005). Amyloid beta induces neuronal cell death through ROS-mediated ASK1 activation. *Cell Death and Differentaition, 12*, 19–24.

Kawahara, M., & Kuroda, Y. (2000). Molecular mechanism of neurodegeneration induced by Alzheimer's β-Amyloid protein: Channel formation and disruption of calcium homeostasis. *Brain Research Bulletin, 53*, 389–397.

Khanday, F. A., Santhanam, L., Kasuno, K., Yamamori, T., Naqvi, A., Dericio, J., et al. (2006a). SOS-mediated activation of Rac1 by p66shc. *Journal of Cell Biology, 172*, 817–822.

Khanday, F. A., Yamamori, T., Singh, I. M., Zhang, Z., Bugayenko, A., Naqvi, A., et al. (2006b). Rac1 Leads to Phosphorylation-dependent increase in stability of the p66shc adaptor protein: Role in rac1-induced oxidative stress. *Molecular Biolpgy of Cell, 17*, 122–129.

Le, S., Connors, T. J., & Maroney, A. C. (2001). c-Jun N-terminal kinase specifically phosphorylates p66ShcA at serine 36 in response to ultraviolet irradiation. *Journal of Biological Chemistry, 276*, 48332–48336.

Lee, D. H., & Wang, H. Y. (2003). Differential physiologic responses of alpha7 nicotinic acetylcholine receptors to β-amyloid1–40 and β-amyloid1–42. *Journal of Neurobiology, 55*, 25–30.

Lee, M., You, H. J., Cho, S. H., Woo, C. H., Yoo, M. H., & Joe, E. H. (2000). Implication of the small GTPase Rac1 in the generation of reactive oxygen species in response to β-amyloid in C6 astroglioma cells. *Biochemistry Journal, 366*, 937–943.

Li, X. D., & Buccafusco, J. J. (2003). Effect of β-Amyloid peptide 1-42 on the cytoprotective action mediated by alpha7 nicotinic acetylcholine receptors in growth factor-deprived differentiated PC-12 cells. *Journal of Pharmacological Experimental Therapy, 307*, 670–675.

Luzi, L., Confalonieri, S., Di Fiore, P. P., & Pelicci, P. G. (2000). Evolution of Shc functions from nematode to human. *Current Opinion in Genetics & Development, 10*, 668–674.

Mattson, M. P. (2002). Oxidative stress, perturbed calcium homeostasis, and immune dysfunction in Alzheimer's disease. *Journal of Neurovirology, 8*, 539–550.

Mattson, M. P., Barger, S. W., Cheng, B., Lieberburg, I., Smith-Swintosky, V. L., & Rydel, R. E. (1993). B-Amyloid precursor protein metabolites and loss of Ca homeostasis in Alzheimer's disease. *Trends in Neuroscience, 16*, 409–414.

Mattson, M. P., & Chan, S. L. (2003). Neuronal and glial calcium signaling in Alzheimer's disease. *Cell Calcium, 34*, 385–397.

Migliaccio, E., Giorgio, M., Mele, S., Pelicci, G., Reboldi, P., Pandolfi, P. P., et al. (1999). The p66shc adaptor protein controls oxidative stress response and life span in mammals. *Nature, 402*, 309–313.

Nagele, R. G., D'Andrea, M. R., Lee, H., Venkataraman, V., & Wang, H. Y. (2003). Astrocytes accumulate Aβ42 and give rise to astrocytic amyloid plaques in Alzheimer disease brains. *Brain Reearch, 971*, 197–209.

Nebreda, A. R., & Porras, A. (2000). p38 MAP kinases: Beyond the stress response. *Trends in Biochemical Sciences, 25*, 257–260.

Nemoto, S., & Finkel, T. (2002). Redox regulation of forkhead proteins through a p66shc dependent signaling pathway. *Science, 291*, 2450–2452.

Okada, S., Kao, A. W., Ceresa, B. P., Blaikie, P., Margolis, B., & Pessin, J. E. (1997). The 66-kDa Shc isoform is a negative regulator of the epidermal growth factor-stimulated mitogen activated protein kinase pathway. *Journal of Biological Chemistry, 272*, 28042–28049.

Pacini, S., Pellegrini, M., Migliaccio, E., Patrussi, L., Ulivieri, C., Ventura, A., et al. (2004). SHC promotes apoptosis and antagonizes mitogenic signaling in T cells. *Molecular and Cellular Biology, 24*, 1747–1757.

Pagani, L., & Eckert, A. (2010). Amyloid-Beta Interaction with Mitochondria. *International Journal of Alzheimer's Disease, 2011*, 1–12.

Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., et al. (1992). A novel transforming protein (SHC) within SH2 domain is implicated in mitogenic signal transduction. *Cell, 70*, 93–104.

Pike, C. J., Overman, M. J., & Cotman, C. W. (1995). Amino-terminal deletions enhance aggregation of β-amyloid peptides in vitro. *Journal of Biological Chemistry, 270*, 23895–23898.

Purdom, S., & Chen, Q. M. (2003). P66(Shc): At the crossroad of oxidative stress and the genetics of aging. *Trends in Molecular Medicine, 9*, 206–210.

Rodriguez, J. J., Witton, J., Olabarria, M., Noristani, H. N., & Verkhratsky, A. (2010). Increase in the density of resting microglia precedes neuritic plaque formation and microglial activation in a transgenic model of Alzheimer's disease. *Cell Death and Disease, 1*, 1–6.

Ryder, J., Su, Y., & Ni, B. (2004). Akt/GSK3β serine/threonine kinases: Evidence for a signalling pathway mediated by familial Alzheimer's disease mutations. *Cellular Signaling, 16*, 187–200.

Selkoe, D. J. (1994). Alzheimer's disease: A central role for amyloid. *Journal of Neuropathology and Experimental Neurology, 53*, 438–447.

Selkoe, D. J. (1999). Translating cell biology into therapeutic advances in Alzheimer's disease. *Nature, 399*(Supp), A23–A31.

Smith, W. W., Norton, D. D., Gorospe, M., Jiang, H., Nemoto, S., Holbrook, N. J., et al. (2005). Phosphorylation of p66Shc and forkhead proteins mediates Aβ Toxicity. *Journal of Cell Biology, 169*, 331–338.

Smith, W. W., Gorospe, M., & Kusiak, J. W. (2006). Signaling Mechanisms Underlying A beta Toxicity: Potential Therapeutic Targets for Alzheimer's Disease. *CNS Neurological Disordors-Drug Targets, 5*, 355–361.

Spuch, C., Ortolano, S., & Navarro, C. (2012). New insights in the amyloid-beta interaction with mitochondria. *Journal of Aging and Research, 2012*, 324968.

Stein, B., Brady, H., Yang, M. X., Young, D. B., & Barbosa, M. S. (1996). Cloning and characterization of MEK6 a novel member of the mitogen activated protein kinase kinase cascade. *Journal of Biological Chemistry, 271*, 11427–11433.

Strooper, B. D., & Annaert, W. (2000). Proteolytic processing and cell biological functions of the amyloid precursor protein. *Journal of Cell Sciences, 113*, 1857–1870.

Sturchler, E., Feurstein, D., McDonald, P., & Duckett, D. (2010). Mechanism of oxidative stress-induced ASK1-catalyzed MKK6 phosphorylation. *Biochemistry, 49*, 4094–4102.

Su, B., Wang, X., Nunomura, A., Moreira, P. I., Lee, H., Perry, G., et al. (2008). Oxidative Stress Signaling in Alzheimer's Disease. *Current Alzheimer Research, 5*, 525–532.

Tare, M., Modi, R. M., Nainaparampil, J. J., Puli, O. R., Bedi, S., Fernandez-Funez, P., et al. (2011). Activation of JNK signaling mediates amyloid-ss-dependent cell death. *PLoS One, 6*, e24361.

Tong, L., Thornton, P. L., Balazs, R., & Cotman, C. W. (2001). β-amyloid-(1–42) impairs activity-dependent cAMP-response element-binding protein signaling in neurons at concentrations in which cell survival is not compromised. *Journal of Biological Chemistry, 276*, 17301–17306.

Wang, H. Y., Lee, D. H., D'Andrea, M. R., Peterson, P. A., Shank, R. P., & Reitz, A. B. (2000). β-Amyloid(1–42) binds to alpha7 nicotinic acetylcholine receptor with high affinity. Implications for Alzheimer's disease pathology. *Journal Biological Chemistry, 275*, 5626–5632.

Yankner, B. A., Dawes, L. R., Fisher, S., Villa-Komaroff, L., Oster-Granite, M. L., & Neve, R. L. (1989). Neurotoxicity of a fragment of the amyloid precursor associated with Alzheimer's disease. *Science, 245*, 417–420.

Zhu, X., Ogawa, O., Wang, Y., Perry, G., & Smith, M. A. (2003). JKK1, an upstream activator of JNK/SAPK, is activated in Alzheimer's disease. *Journal of Neurochemistry, 85*, 87–93.