

Blockade of 12-lipoxygenase expression protects cortical neurons from apoptosis induced by β -amyloid peptide

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Abstract

The cyclo-oxygenase (COX) and lipoxygenase (LOX) pathways belong to the eicosanoid synthesis pathway, a major component of the chronic inflammatory process occurring in Alzheimer's disease (AD). Clinical studies reported beneficial effects of COX inhibitors, but little is known about the involvement of LOXs in AD pathogenesis. β -amyloid peptide ($A\beta$) accumulation contributes to neurodegeneration in AD, but mechanisms underlying $A\beta$ toxicity have not been fully elucidated yet. Here, using an antisense oligonucleotide-based strategy, we show that blockade of 12-LOX expression prevents both $A\beta$ -induced apoptosis and overexpression of c-Jun, a factor required for the apoptotic process, in cortical neurons. Conversely, the 12-LOX metabolite, 12(S)-HETE (12(S)-hydroxy-(5Z, 8Z, 10E, 14Z)-eicosatetraenoic acid), promoted c-Jun-dependent apoptosis. Specificity of the 12-LOX involvement was further supported by the observed lack of contribution of 5-LOX in this process. These data indicate that blockade of 12-LOX expression disrupts a c-Jun-dependent apoptosis pathway, and suggest that 12-LOX may represent a new target for the treatment of AD.

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Abbreviations: $A\beta$, β -amyloid peptide; AD, Alzheimer's disease; BSO, buthionine sulfoximine; COX, cyclo-oxygenase; DAPI, 4',6-diamidino-2-phenylindole; LOX, lipoxygenase; NSAIDs, nonsteroidal anti-inflammatory drugs; 5(S)-HETE, 5(S)-hydroxy-(6E, 8Z, 11Z, 14Z)-eicosatetraenoic acid; 12(S)-HETE, 12(S)-hydroxy-(5Z, 8Z, 10E, 14Z)-eicosatetraenoic acid

Introduction

Alzheimer's disease (AD) involves a chronic CNS inflammatory process that contributes to the neuronal loss and all the

pathological features of the disease.¹ A major component of this process is the eicosanoid synthesis pathway, which involves two major routes: the cyclo-oxygenase (COX) and the lipoxygenase (LOX) pathways.² Clinical studies showing that chronic treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) was associated with a delay of AD onset focused attention on the beneficial effect of COX inhibitors.³ In contrast, very few studies evaluated the potential interest of LOX inhibitors in the treatment of AD.

One of the main features of AD is the presence of senile plaques in the brain that contain deposits of the 40–42 amino-acid β -amyloid peptide ($A\beta$). The central role of $A\beta$ in AD pathogenesis has been well documented.^{4,5} In particular, the rate of accumulation of this peptide correlated with the extent of neurodegeneration.⁶ Furthermore, injection of $A\beta$ aggregates in the rat brain caused neuronal death and behavioral alterations.^{7,8} $A\beta$ generated apoptosis in several *in vitro* models, associated with caspase activation,^{9–14} both of which were also documented in AD brains.^{15,16} Recent data further evidenced a major role of the transcription factor c-Jun in mediating $A\beta$ -induced apoptosis.^{17–19} Overexpression of this factor was detected in neurons surrounding senile plaques.²⁰ However, the exact signaling pathways underlying $A\beta$ -induced apoptosis have not been fully elucidated yet.

LOXs incorporate oxygen into specific sites of polyunsaturated fatty acids and are generally classified as 5-, 12- or 15-LOXs depending on the site of incorporation.²¹ Both 12- and 5-LOX are expressed in the brain and especially in neurons.^{22,23} LOXs represent important regulators of cell proliferation and death in different cell types.^{24–26} Moreover, a few studies suggested the involvement of 5- or 12-LOX in pathways leading to neuronal death, such as kainic acid excitotoxicity,²⁷ apoptosis induced by prion peptide²⁸ and oxidative glutamate toxicity.²³

In this work, we show that blockade of 12-LOX expression protects cortical neurons from $A\beta$ -induced apoptosis, through disruption of a c-Jun-dependent apoptosis pathway. These findings suggest that 12-LOX might be considered as a new target for the treatment of AD.

Results

Blockade of 12-LOX expression suppresses $A\beta$ -induced apoptosis and reduces $A\beta$ -induced caspase activation

12-LOX was previously shown to mediate both oxidative glutamate toxicity and apoptosis induced by buthionine sulfoximine (BSO) in primary cultures of rat cortical neurons, two processes involving glutathione depletion.²³ Alteration of glutathione metabolism is one of the events triggered by $A\beta$ ²⁹ and was reported to occur in AD,³⁰ which

suggested that this LOX might also participate in $A\beta$ -induced apoptosis.

Indeed, previous studies using broad-spectrum LOX inhibitors such as nordihydroguaiaretic acid on hippocampal cultures led to equivocal results, reporting either a lack of protection against $A\beta$ -induced apoptosis³¹ or a protective action at high concentrations.³² Using more selective inhibitors, we recently suggested a participation of 12-LOX in apoptosis triggered by $A\beta$ in cortical neurons.³³ However, inhibitors can only provide circumstantial evidence about the role of LOXs in $A\beta$ -induced toxicity, since most of these compounds also possess other characteristics such as pro- or antioxidant properties,^{25,26,34–36} which might lead to LOX-independent effects. In order to assess the protective potential of 12-LOX blockade towards $A\beta$ -induced apoptosis, we used here an antisense oligonucleotide-based strategy.

When extracts of cortical cells were probed with an antibody raised against murine leukocyte 12-LOX, two bands (75–78 kDa) were detected in Western blot experiments (Figure 1a). A similar doublet pattern was already observed in previous studies on these cultures.²³ As shown in Figure 1a, pretreatment of cortical cells for 48 h with the antisense 12-LOX oligonucleotide induced an important decrease in the expression of 12-LOX protein. When normalized to corresponding actin levels, the effect of the oligonucleotide represented an 85% decrease in 12-LOX protein levels.

As illustrated in Figure 1b, a 24-h treatment of cortical cells with 25 μ M $A\beta_{25-35}$ increased the percentage of apoptotic cells, as assessed by 4',6-diamidino-2-phenylindole (DAPI) staining. This proapoptotic effect of $A\beta$ was totally suppressed by a 48-h pretreatment of the cells with the 12-LOX antisense oligonucleotide, whereas the sense or the scramble 12-LOX oligonucleotide did not show any protective action (Figure 1b). Similar results were obtained when apoptosis was assessed by TUNEL experiments (Figure 1c).

Numerous studies indicated that $A\beta$ -induced cell death was associated with the activation of the caspase cascade.^{10–14} We therefore investigated the effect of 12-LOX downregulation on $A\beta$ -induced caspase activation. As shown in Figure 2, treatment of cortical cells with 25 μ M $A\beta$ led to the activation of caspase-2, -3 and -8. Pretreatment with the 12-LOX antisense oligonucleotide strongly inhibited $A\beta$ -induced activation of all three caspases (Figures 2a–c). The oligonucleotide effect represented 67 ± 4 , 68 ± 4 and $78 \pm 2\%$ ($n = 3$ independent experiments) decreases in the $A\beta$ -induced activation of caspase-2, -3 and -8, respectively. No alteration of the $A\beta$ -induced caspase activation was observed upon treatment with the 12-LOX sense oligonucleotide (Figures 2d–f).

The protection against $A\beta$ -induced apoptosis afforded by 12-LOX blockade further suggested a proapoptotic involvement of 12-LOX in cortical neurons. Previous studies^{23,37} indicated that the 12-LOX isoform present in primary cultures of rat cortical neurons was the 12-LOX cloned from rat brain,³⁸ belonging to the family of leukocyte-type 12-LOXs. Like other members of this family, rat brain 12-LOX presented high similarity with 15-LOX. However, a striking characteristic of this isoform was that it generated predominantly 12(S)-

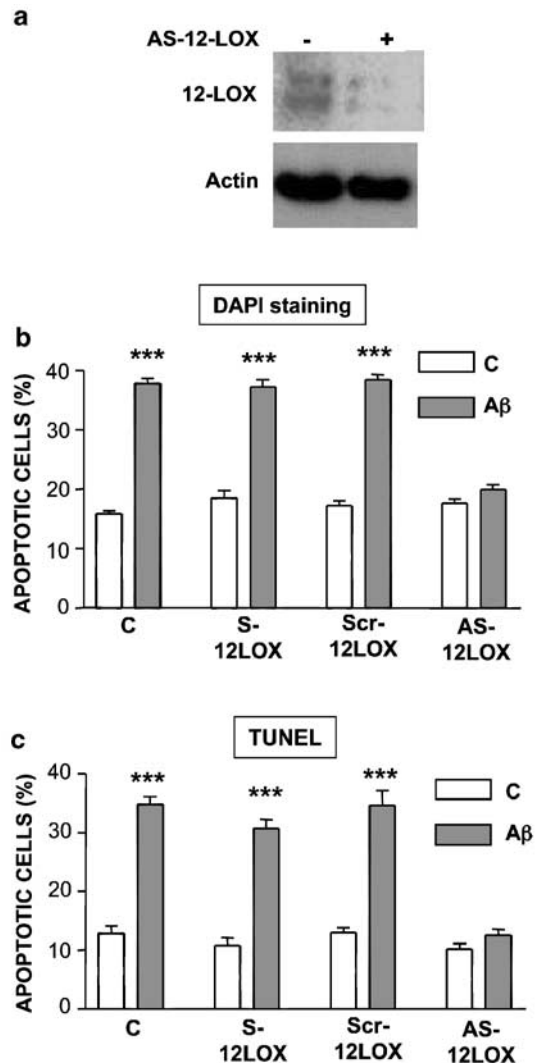


Figure 1 Downregulation of 12-LOX expression by antisense oligonucleotide prevents $A\beta$ -induced apoptosis in cortical neurons. (a) Cell lysates (25 μ g protein/lane) from untreated cortical cultures or cultures treated for 48 h with 12-LOX antisense oligonucleotide (AS-12-LOX, 4 μ M) were resolved in 4–20% SDS-polyacrylamide gel gradients and transferred to PVDF membranes. Membranes were probed with anti-12-LOX antibody, stripped and reprobed with anti-actin antibody as described in Materials and methods. (b and c) Cortical neurons in primary culture were treated for 48 h with 12-LOX antisense oligonucleotide (AS-12-LOX, 4 μ M), sense oligonucleotide (S-12-LOX, 4 μ M) or scramble oligonucleotide (Scr-12-LOX, 4 μ M) and then with (black bars) or without (white bars) 25 μ M $A\beta_{25-35}$ for an additional 24-h period. The percentage of apoptotic cells was evaluated by DAPI staining (b) or TUNEL labeling (c) as described in Materials and methods. Results represent means \pm S.E.M. ($n = 6$ –12 culture wells) obtained in one typical experiment. *** $P < 0.001$ versus corresponding control without $A\beta_{25-35}$ (one-way ANOVA followed by Newman-Keuls test)

hydroxy-(5Z, 8Z, 10E, 14Z)-eicosatetraenoic acid (12(S)-HETE), with only small amounts of 15(S)-HETE.^{38,39}

We therefore evaluated whether exogenous 12(S)-HETE could induce apoptosis in cortical cells. As illustrated in Figure 3, a concentration-dependent increase in the percentage of apoptotic cells was observed following a 24-h treatment of the cultures with 12(S)-HETE.

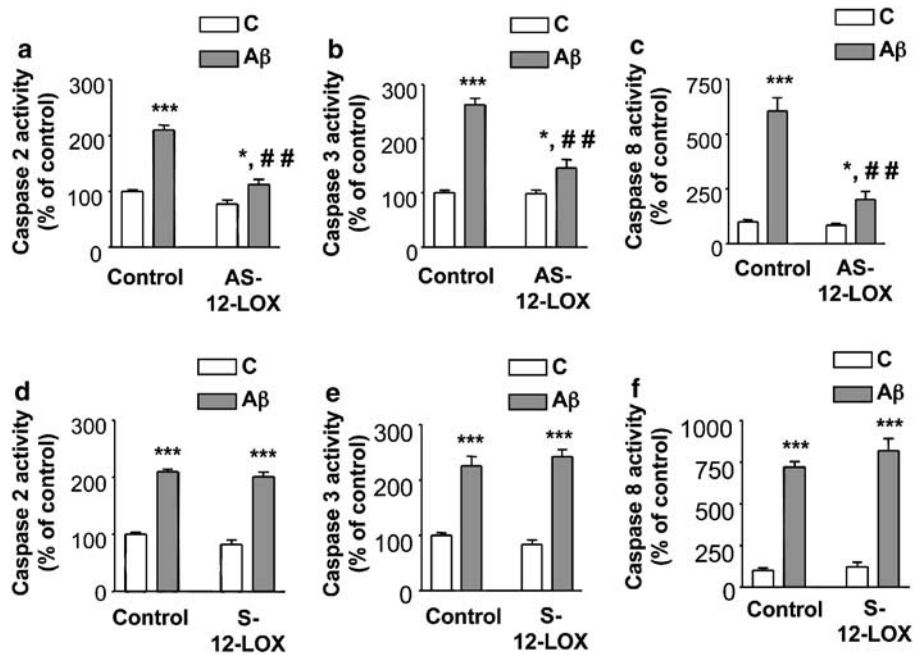


Figure 2 Downregulation of 12-LOX expression by antisense oligonucleotide decreases A β -induced caspase activation in cortical neurons. Cortical neurons in primary culture were treated for 48 h with 12-LOX antisense oligonucleotide (a–c: AS-12-LOX, 4 μ M) or sense oligonucleotide (d–f: S-12-LOX, 4 μ M) and then with (black bars) or without (white bars) 25 μ M A β_{25-35} for an additional 24-h period. Activity of caspase-2 (a, d), -3 (b, e) and -8 (c, f) were assessed by a fluorimetric assay using the fluorogenic substrates Ac-VDVAD-AMC, Ac-DEVD-AMC and Ac-IETD-AMC, respectively. Results represent means \pm S.E.M. ($n=6-12$ culture wells) obtained in one typical experiment. *** $P < 0.001$ versus corresponding control without A β_{25-35} ; * $P < 0.05$ versus corresponding control without A β_{25-35} ; # $P < 0.01$ versus A β_{25-35} alone (one-way ANOVA followed by Newman–Keuls test)

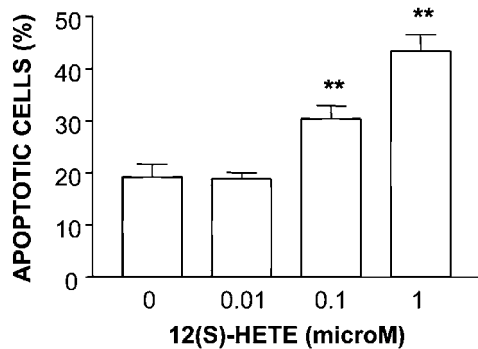


Figure 3 12-LOX metabolite, 12(S)-HETE, induces apoptosis in cortical neurons. Cortical neurons in primary culture were treated for 24 h with increasing concentrations of 12(S)-HETE. The percentage of apoptotic cells was evaluated by DAPI staining as described in Materials and methods. Results represent means \pm S.E.M. ($n=6$ culture wells) obtained in one typical experiment. ** $P < 0.01$ versus control without 12(S)-HETE (one-way ANOVA followed by Dunnett's test)

Blockade of 5-LOX expression does not alter A β -induced apoptosis

Previous studies indicated that 5-LOX could play a critical role in neuronal cell death, mediating, for instance, kainic acid-induced excitotoxicity²⁷ and apoptosis generated by prion peptide.²⁸ We recently reported that the 5-LOX inhibitor, caffeic acid, did not counteract apoptosis induced by A β in cortical neurons, suggesting that 5-LOX was not involved in this degeneration process.³³ In the present study, we further

evaluated this hypothesis through the same strategy as that used for 12-LOX.

As shown in Figure 4a, pretreatment of cortical cells for 48 h with the antisense 5-LOX oligonucleotide induced an important decrease in the expression of the 5-LOX protein. When normalized to corresponding actin levels, the effect of the oligonucleotide represented a 75% decrease in 5-LOX protein levels.

However, at variance with what was observed above for 12-LOX, such a pretreatment with the 5-LOX antisense oligonucleotide did not counteract the proapoptotic effect of A β (Figure 4b). Similarly, no effect was observed with the corresponding sense oligonucleotide (Figure 4b).

We also evaluated whether the 5-LOX metabolite, 5(S)-hydroxy-(6E, 8Z, 11Z, 14Z)-eicosatetraenoic acid (5(S)-HETE), could induce apoptosis in cortical cells. Contrasting with what was obtained above with 12(S)-HETE, no increase in the percentage of apoptotic cells was observed following a 24-h treatment of the cultures with 5(S)-HETE concentrations up to 1 μ M (Figure 4c).

Blockade of 12-LOX expression disrupts a c-Jun-dependent A β -induced apoptotic pathway

Several studies revealed that the transcription factor c-Jun represented a common mediator in neuronal apoptosis triggered by several stimuli, such as growth factor deprivation, oxidative stress or excitotoxicity.^{40–42} Moreover, both c-Jun expression and activation were found to be increased following exposure of neurons to A β , and recent data suggested a

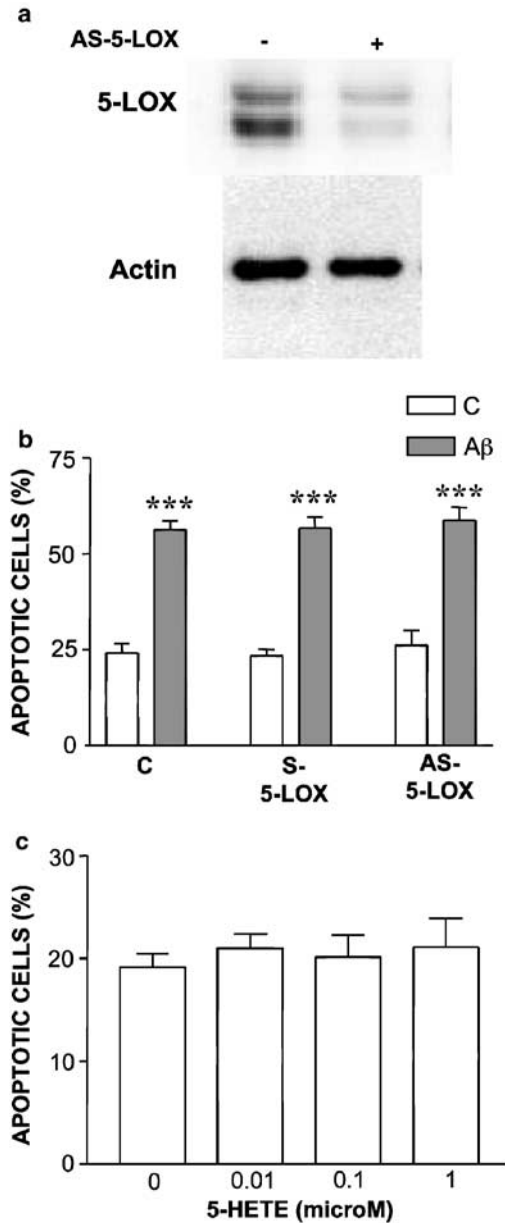


Figure 4 Downregulation of 5-LOX expression does not counteract A β -induced apoptosis, and 5(S)-HETE does not induce apoptosis in cortical neurons. **(a)** Cell lysates (25 μ g protein/lane) from untreated cortical cultures or cultures treated for 48 h with 5-LOX antisense oligonucleotide (AS-5-LOX, 4 μ M) were resolved in 4–20% SDS-polyacrylamide gel gradients and transferred to PVDF membranes. Membranes were probed with anti-5-LOX antibody, stripped and reprobed with anti-actin antibody as described in Materials and methods. **(b)** Cortical neurons in primary culture were treated for 48 h with 5-LOX antisense oligonucleotide (AS-5-LOX, 4 μ M) or sense oligonucleotide (S-5-LOX, 4 μ M) and then with (black bars) or without (white bars) 25 μ M A β_{25-35} for an additional 24-h period. The percentage of apoptotic cells was evaluated by DAPI staining as described in Materials and methods. Results represent means \pm S.E.M. ($n = 6-12$ culture wells) obtained in one typical experiment. *** $P < 0.001$ versus corresponding control without A β_{25-35} (one-way ANOVA followed by Newman-Keuls test). **(c)** Cortical neurons in primary culture were treated for 24 h with increasing concentrations of 5(S)-HETE. The percentage of apoptotic cells was evaluated by DAPI staining as described in Materials and methods. Results represent means \pm S.E.M. ($n = 6$ culture wells) obtained in one typical experiment

critical role of this transcription factor in A β -induced apoptosis.^{17–19,43,44} We therefore investigated whether 12-LOX blockade could interfere with such an A β -induced c-Jun-dependent apoptotic pathway in cortical cells.

We first examined the effect of 12-LOX antisense oligonucleotide on A β -induced increase in c-Jun expression. In agreement with our previous observations,³³ treatment of the cultures with 25 μ M A β induced an increase in the percentage of c-Jun-positive neurons (Figure 5). A 48-h pretreatment with the 12-LOX antisense oligonucleotide suppressed this effect, suggesting that 12-LOX mediated the increase in c-Jun expression induced by A β (Figure 5). No alteration of the A β -induced increase in the percentage of c-Jun-positive neurons was observed upon treatment with the sense 12-LOX oligonucleotide (Figure 5).

We then determined whether c-Jun was involved in A β -induced apoptosis under our experimental conditions. Pretreatment with a c-Jun antisense oligonucleotide decreased the expression of c-Jun protein as observed in Western blot experiments (Figure 6a). When normalized to corresponding actin levels, the oligonucleotide effect represented a 60% decrease in the amount of c-Jun protein. As illustrated in Figure 6b, pretreatment of cortical cells with the c-Jun antisense oligonucleotide, but not with the sense oligonucleotide, inhibited apoptosis induced by A β . The effect of the c-Jun antisense oligonucleotide represented a 75% decrease in A β -induced apoptosis. Similar results were obtained when apoptosis was assessed by TUNEL experiments (Figure 6c). Consistent with previous data showing a protective action of dominant-negative mutants of c-Jun in this experimental system,¹⁸ these results indicated that c-Jun was involved in the death process triggered by A β in cortical cells.

Recent data suggested that the downstream components of the c-Jun-dependent apoptosis pathway induced by A β included induction of Fas ligand expression and caspase-8

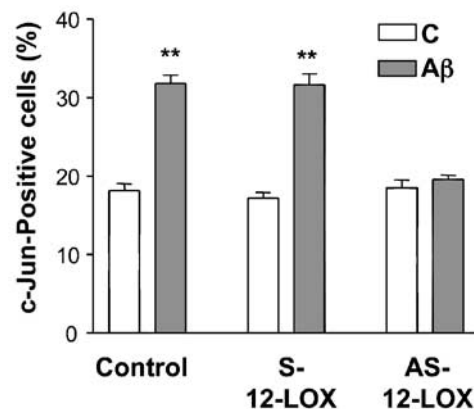


Figure 5 12-LOX antisense oligonucleotide suppresses the increase in the percentage of c-Jun-positive cells induced by A β in cortical neurons. Cortical neurons in primary culture were treated for 48 h with 12-LOX antisense oligonucleotide (AS-12-LOX, 4 μ M) or sense oligonucleotide (S-12-LOX, 4 μ M) and then with (black bars) or without (white bars) 25 μ M A β_{25-35} for an additional 24-h period. The percentage of c-Jun-positive cells was evaluated by immunocytochemistry as described in Materials and methods. Results represent means \pm S.E.M. ($n = 6$ culture wells) obtained in one typical experiment. ** $P < 0.01$ versus corresponding control without A β_{25-35} (one-way ANOVA followed by Newman-Keuls test)

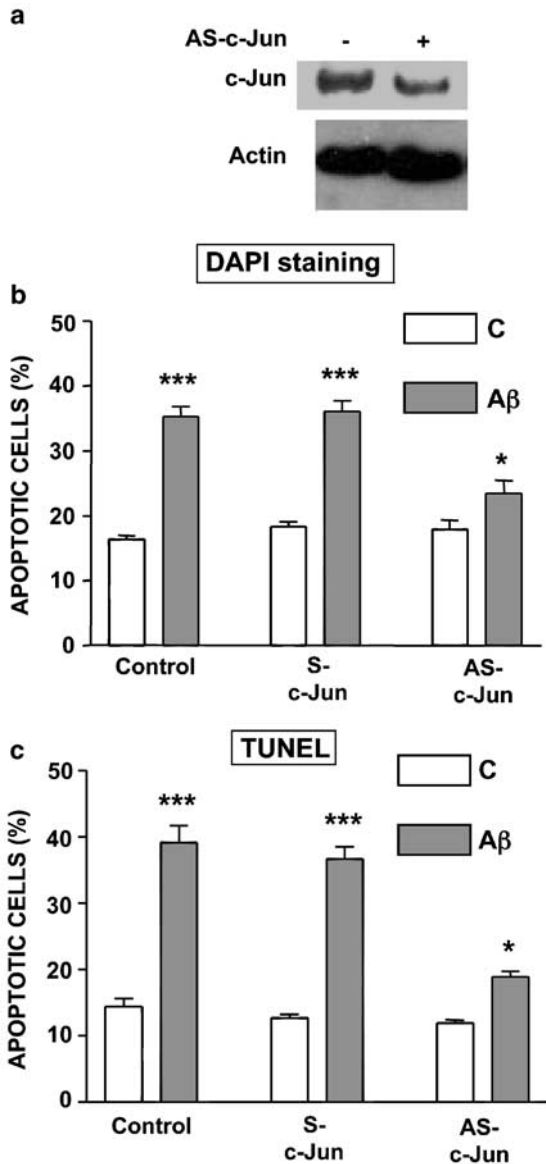


Figure 6 Downregulation of c-Jun expression by c-Jun antisense oligonucleotide prevents A β -induced apoptosis in cortical neurons. (a) Cell lysates (25 μ g protein/lane) from untreated cortical cultures or cultures treated for 48 h with c-Jun antisense oligonucleotide (AS-c-Jun, 4 μ M) were resolved in 4–20% SDS-polyacrylamide gel gradients and transferred to PVDF membranes. Membranes were probed with anti-c-Jun antibody, stripped and re-probed with anti-actin antibody as described in Materials and methods. (b and c) Cortical neurons in primary culture were treated for 48 h with c-Jun antisense oligonucleotide (AS-c-Jun, 4 μ M) or sense oligonucleotide (S-c-Jun, 4 μ M) and then with (black bars) or without (white bars) 25 μ M A β_{25-35} for an additional 24-h period. The percentage of apoptotic cells was evaluated by DAPI staining (b) or TUNEL labeling (c) as described in Materials and methods. Results represent means \pm S.E.M. ($n = 6-12$ culture wells) obtained in one typical experiment. *** $P < 0.01$ versus corresponding control without A β_{25-35} ; * $P < 0.05$ versus corresponding control without A β_{25-35} (one-way ANOVA followed by Newman-Keuls test)

activation.¹⁸ However, nothing had been reported yet on the consequences of c-Jun downregulation on A β -induced caspase activation. We therefore investigated the effect of c-Jun antisense oligonucleotide on caspase activities triggered by A β in our experimental model. As illustrated in

Figure 7c, pretreatment of cortical cultures with c-Jun antisense oligonucleotide decreased the A β -induced activation of caspase 8. In contrast, no alteration of the A β -induced activation of caspase-2 and -3 was observed (Figures 7a and b). Treatment with the c-Jun sense oligonucleotide did not alter A β -induced activation of caspase-2, -3 and -8 (Figures 7d–f). These data indicated an involvement of c-Jun in the activation of caspase-8 triggered by A β .

Taken together, our findings indicated both an involvement of 12-LOX in the A β -mediated induction of c-Jun and a participation of this transcription factor in the apoptotic process, suggesting that 12-LOX lay upstream of c-Jun in the A β -induced apoptotic pathway. The ability of 12-LOX to trigger a c-Jun-dependent apoptotic pathway was further investigated by evaluating the role of c-Jun in apoptosis induced by 12(S)-HETE.

As presented in Figure 8a, a 24-h treatment of cortical cells with 12(S)-HETE induced a concentration-dependent increase in the percentage of c-Jun-positive neurons, which strikingly paralleled the proapoptotic action of this compound (Figure 3). Furthermore, pretreatment with the c-Jun antisense oligonucleotide totally protected cortical cells from apoptosis induced by 12(S)-HETE, whereas no protection was observed following treatment with the corresponding sense oligonucleotide (Figure 8b). These data indicated that the 12-LOX metabolite, 12(S)-HETE, induced a c-Jun-dependent apoptotic pathway in cortical neurons.

Discussion

In the present report, we demonstrated that downregulation of 12-LOX suppressed apoptosis, strongly decreased the activation of caspase-2, -3 and -8 and prevented c-Jun overexpression induced by A β in rat cortical neurons. The requirement of c-Jun for the death process induced by A β in these cells was further indicated by the prevention of both A β -induced apoptosis and caspase-8 activation following pretreatment with a c-Jun antisense oligonucleotide. Moreover, we showed that the 12-LOX metabolite 12(S)-HETE induced apoptosis, associated with an increase in c-Jun expression and suppressed by a c-Jun antisense oligonucleotide. These data suggest that blockade of 12-LOX expression protects cortical neurons from A β -induced apoptosis, through disruption of a c-Jun-dependent apoptosis pathway. The specificity of involvement of 12-LOX in this process was further supported by both the lack of protection observed following downregulation of 5-LOX expression and the lack of toxicity of the 5-LOX metabolite, 5(S)-HETE, in this experimental model.

It has been reported that LOXs play an important role in neuronal metabolism and function.⁴⁵ Indeed, LOX metabolites were found to be synthesized in both neurons and glial cells.^{21,46–48} Some of them, such as 5- and 12-hydroxyeicosatetraenoic acids, have been reported to exert various actions on neurons, including modulation of Na–K ATPase activity,⁴⁹ neurotransmitter release⁵⁰ or alteration of membrane potential.^{51,52} LOXs also act as regulators of cell proliferation and death, and have been implied in the mediation of apoptosis induced by various physical or chemical agents such as UV light, oxidative stress or receptor ligands

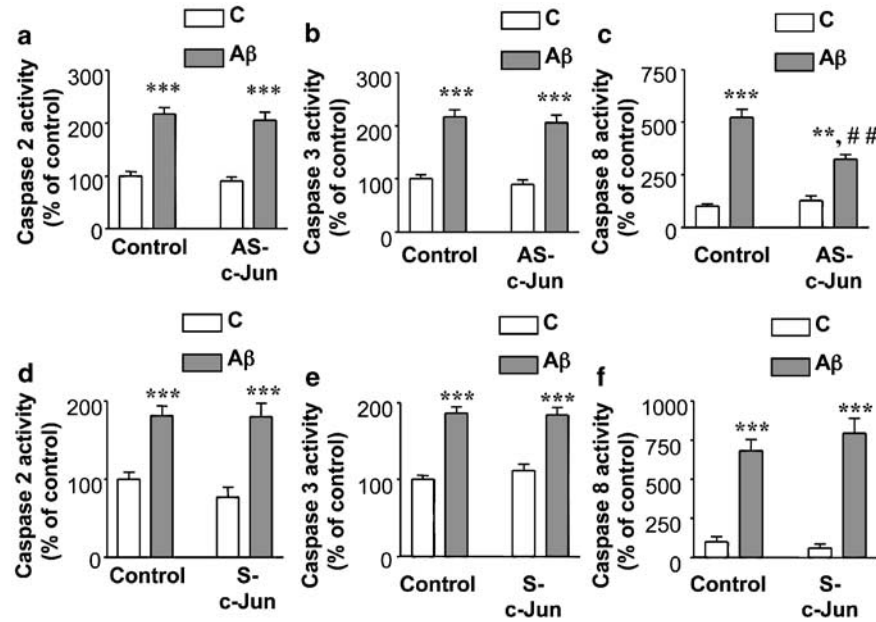


Figure 7 c-Jun antisense oligonucleotide decreases caspase-8 activation in cortical neurons. Cortical neurons in primary culture were treated for 48 h with c-Jun antisense oligonucleotide (a–c: AS-c-Jun, 4 μ M) or sense oligonucleotide (d–f: S-c-Jun, 4 μ M) and then with (black bars) or without (white bars) 25 μ M A β_{25-35} for an additional 24-h period. Activity of caspase-2 (a, d), -3 (b, e) and -8 (c, f) were assessed by a fluorimetric assay using the fluorogenic substrates Ac-VDVAD-AMC, Ac-DEVD-AMC and Ac-IEPD-AMC, respectively. Results represent means \pm S.E.M. ($n = 6-12$ culture wells) obtained in one typical experiment. *** or ** $P < 0.001$ or < 0.01 versus corresponding control without A β_{25-35} ; ## $P < 0.01$ versus A β_{25-35} alone (one-way ANOVA followed by Newman-Keuls test)

such as TNF α or Fas ligand, in different types of cells of peripheral origin.^{25,26}

In contrast, only a small number of studies investigated the involvement of LOXs in the various signaling pathways leading to neurodegeneration. However, data obtained thus far suggest a crucial participation of these enzymes in several of these pathways. For instance, 5-LOX was shown to mediate kainic acid-induced excitotoxicity²⁷ and apoptosis generated by prion peptide.²⁸ With regard to 12-LOX, this enzyme was previously shown to mediate both oxidative glutamate toxicity and BSO-induced apoptosis.²³ In a recent study,³³ we showed that baicalein, a 12-LOX inhibitor, protected cortical neurons from A β -induced apoptosis, suggesting a role for 12-LOX in the apoptotic process triggered by A β in these cells.

The present results indicate that downregulation of 12-LOX expression by an antisense oligonucleotide leads to the suppression of A β -induced apoptosis. When combining these data to those previously obtained with the 12-LOX inhibitor, it thus appears that protection against neuronal death induced by A β could be achieved upon blockade of 12-LOX through either inhibition of the enzyme or downregulation of its expression. Moreover, it is now well documented that caspases participate in A β -induced apoptosis,¹⁰⁻¹³ and that their activation correlates with apoptotic features in AD brains.^{53,54} In particular, caspase-2, -3 and -8 have been reported to be activated in neurons following A β treatment.^{10-12,14,18} We show here that the protective action of 12-LOX antisense oligonucleotide against A β -mediated apoptosis is associated with decreases in the activation of these caspases. Taken together, these data indicate that 12-LOX blockade counteracts the apoptotic process triggered by A β in cortical cells.

Both pro- and antiapoptotic roles of LOXs have been reported in various cell types.^{25,26} However, the protection afforded by 12-LOX blockade against A β -induced apoptosis as well as the induction of apoptosis by 12(S)-HETE observed in the present work favored a proapoptotic involvement of this enzyme in cortical cells. These findings add to previous studies showing that 12-LOX mediated apoptosis induced by other stimuli, such as glutamate or BSO,²³ and suggest that this LOX might represent a common intermediate in neurodegenerative processes triggered by various insults.

In the present study, we also demonstrated both a lack of protection of cortical neurons by pretreatment with a 5-LOX antisense oligonucleotide and a lack of toxicity of the 5-LOX metabolite, 5(S)-HETE. These findings add to our previous observations showing that the 5-LOX inhibitor, caffeic acid, did not counteract A β -induced apoptosis in this experimental system.³³ Taken together, these data suggest that 5-LOX does not participate in the apoptotic process induced by A β in cortical neurons, representing an important support to the specificity of the role of 12-LOX pathway in this process.

It should be stressed, however, that these results do not rule out the possibility of a participation of 5-LOX in the pathology of AD, since this pathology most probably encompasses several other parameters, which are not present in this *in vitro* model. In this respect, it is interesting to note that a relationship between 5-LOX promoter polymorphism and AD onset was recently reported.⁵⁵

One of the typical feature found in post-mortem studies on brains from AD patients is an overexpression of c-Jun,⁵⁶ which is also a hallmark of A β -induced apoptosis in neuronal cultures.^{17,44} This transcription factor was found to play a major role in A β -induced neurotoxicity.^{17,18} In the present

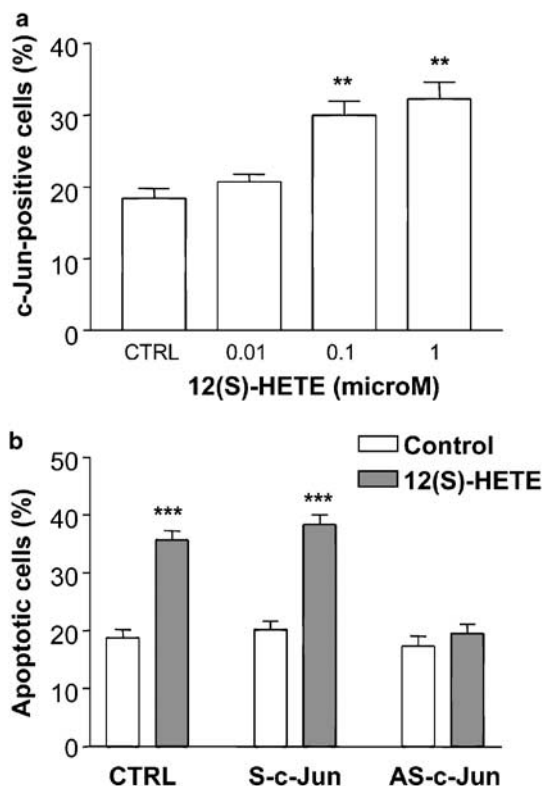


Figure 8 12-LOX metabolite, 12(S)-HETE, induces a c-Jun-dependent apoptosis pathway in cortical neurons. (a) Cortical neurons in primary culture were treated for 24 h with 12(S)-HETE. The percentage of c-Jun-positive cells was evaluated by immunocytochemistry as described in Materials and methods. Results represent means \pm S.E.M. ($n=6$ culture wells) obtained in one typical experiment. ** $P<0.01$ versus control without 12(S)-HETE (one-way ANOVA followed by Dunnett's test). (b) Cortical neurons in primary culture were treated for 48 h with c-Jun antisense oligonucleotide (AS-c-Jun, 4 μ M) or sense oligonucleotide (S-c-Jun, 4 μ M) and then with (black bars) or without (white bars) 1 μ M 12(S)-HETE for an additional 24-h period. The percentage of apoptotic cells was evaluated by DAPI staining as described in Materials and Methods. Results represent means \pm S.E.M. ($n=6-12$ culture wells) obtained in one typical experiment. ** $P<0.01$ versus corresponding control without 12(S)-HETE (one-way ANOVA followed by Newman-Keuls test)

study, we showed that the protective action of 12-LOX antisense oligonucleotide against $A\beta$ -mediated toxicity was associated with the suppression of c-Jun overexpression. Furthermore, pretreatment with a c-Jun antisense oligonucleotide protected cortical neurons from $A\beta$ -induced apoptosis and decreased the activation of caspase-8, indicating that this transcription factor was involved in the death process induced by $A\beta$ under our experimental conditions. The data obtained with the c-Jun antisense oligonucleotide corroborate previous studies performed on cortical cultures, showing that dominant-negative mutants of c-Jun afforded protection against $A\beta$ -induced apoptosis.¹⁸

These findings suggested that 12-LOX was positioned upstream of c-Jun in the apoptotic pathway induced by $A\beta$. They also suggested that this enzyme could represent a critical step in the triggering of a c-Jun-dependent apoptosis pathway. This hypothesis was further supported by our observation that the 12-LOX metabolite, 12(S)-HETE, induced a c-Jun-dependent death process. Interestingly, although such

an involvement of 12-LOX in the induction of c-Jun-dependent apoptosis had not been previously evidenced, it lies in accordance with some data from literature since BSO-induced apoptosis, shown to be mediated by 12-LOX,²³ was also reported to be c-Jun dependent.⁵⁷

The relationship between c-Jun and $A\beta$ -induced activation of caspases has not been fully elucidated yet. The activation of caspase-8 upon $A\beta$ treatment of cortical neurons was recently suggested to occur through a c-Jun-mediated induction of Fas ligand.¹⁸ Our observation that $A\beta$ -induced caspase-8 activation is prevented by a c-Jun antisense oligonucleotide provides evidence for an involvement of this factor in the activation of caspase-8. Interestingly, the lack of effect of the c-Jun antisense oligonucleotide on caspase-2 and -3 activations lies, in contrast, with the previously reported inhibition of these caspases upon treatment with CEP-1347, an inhibitor of the c-Jun N-terminal kinase (JNK).^{19,58} It could thus be postulated that the effect of CEP-1347 on these two caspases reflected disruption of the JNK action on a target different from c-Jun, such as activating transcription factor 2 or Elk1.⁵⁹

It should also be noticed that, despite its lack of effect on the activation of caspase-2 and -3, the c-Jun antisense oligonucleotide afforded protection of cortical neurons against $A\beta$ -induced apoptosis. Whereas the involvement of caspase-3 in $A\beta$ -induced neuronal apoptosis still remains controversial,^{10,13,60} caspase-8 activation¹¹ and caspase-2 activation^{12,61} were separately identified as necessary steps for $A\beta$ -induced neuronal death. Our results support the hypothesis that the induction of these two caspases is needed for completion of the apoptotic process.

The involvement of c-Jun in the $A\beta$ -induced activation of caspase-8 further suggests the existence of a causal link between the suppression of c-Jun induction and the decrease in caspase-8 activation observed following 12-LOX downregulation. However, the decreased activation of caspase-2 and -3, also obtained under those experimental conditions, cannot be explained by the sole disruption of c-Jun induction and would thus imply additional mechanisms. For instance, as mentioned above for the action of CEP-1347, the effect of 12-LOX downregulation on these two caspases could involve decreased activation of other targets of the JNK signaling pathway.

The beneficial effects of NSAIDs in AD focused attention on the therapeutic interest of COX inhibitors.³ However, the present study suggests that the 12-LOX pathway could also play an important role in this pathology. Indeed, the 12-LOX inhibitor baicalein is the main component of an herbal medicine (Sho-Saiko-To) commonly administered in Asian countries to outpatients with chronic hepatitis.⁶² Our findings suggest that such compounds may also offer new potential strategies in the pharmacological treatment of AD.

Materials and Methods

Primary neuronal culture

Rat cortical cultures were prepared from 17-day-old embryonic Wistar rats as described previously.^{33,63} The culture medium consisted of minimum

essential medium with Earle's salts (Gibco-BRL, Life Technologies, Rockville, MD, USA) supplemented with 5% fetal calf serum, 2 mM L-glutamine, 5 $\mu\text{g/ml}$ insulin, 2×10^{-8} M progesterone, 100 $\mu\text{g/ml}$ human transferrin 10^{-4} M putrescine, 3×10^{-8} M sodium selenite and 10 $\mu\text{g/ml}$ bovine serum albumin. Cells were seeded in 24-well Costar plates previously coated with poly-D-lysine at a density of 3×10^5 cells per well and grown in a humidified 5% $\text{CO}_2/95\%$ air atmosphere at 37°C .

Treatment of cultures with antisense or sense oligonucleotides

Oligonucleotides (Invitrogen, Life Technologies, Rockville, MD, USA) used for antisense experiments corresponded to the sequences previously reported to downregulate selectively expression of rat 12-LOX⁶⁴ (antisense: 5'-CTC-AGG-AGG-GTG-TAA-ACA-3'; sense: TGT-TTA-CAC-CCT-CCT-GAG; scramble: AAG-ATT-GCG-GCG-CGA-CGA-TGA), 5-LOX⁶⁵ (antisense: TCT-GGG-AAG-TGA-GCG-CTG; sense: CAG-CGC-TCA-CTT-CCC-AGA) and c-Jun⁶⁶ (antisense: CGT-TTC-CAT-CTT-TGC-AGT-CAT; sense: ATG-ACT-GCA-AAG-ATG-GAA-ACG). After 5 days, cultures were treated with the different antisense and sense oligonucleotides at a final concentration of 4 μM during 48 h, and then exposed to the β -amyloid peptide fragment $A\beta_{25-35}$ (25 μM , Alexis Biochemicals, San Diego, CA, USA) for an additional 24 h.

Western blot analysis

Cells from six wells were washed with phosphate-buffered saline (PBS), and total proteins were extracted by scraping the cells in 100 μl of protein extracting buffer (10 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40) containing 0.5 mM EDTA, 1 mM EGTA and phosphatase and protease inhibitors (0.5 mM sodium orthovanadate, 1 μM okadaic acid, 1 $\mu\text{g/ml}$ *N*-tosyl-L-phenylalanine chloromethyl ketone, 1 $\mu\text{g/ml}$ *N* α -*p*-tosyl-L-lysine chloromethyl ketone, 20 mM sodium fluoride, 1 μM phenylmethylsulfonyl fluoride, 10 $\mu\text{g/ml}$ leupeptin, 10 $\mu\text{g/ml}$ aprotinin and 5 $\mu\text{g/ml}$ pepstatin). Lysates were then sonicated and centrifuged, the resulting supernatants were collected and protein concentrations were determined by BCA assay (Pierce, CA, USA). Samples were mixed with $2 \times$ Laemmli sample buffer and heated at 90°C for 5 min. Proteins (25 $\mu\text{g/lane}$) were separated by electrophoresis on 4–20% SDS-polyacrylamide gel gradients, transferred to PVDF membranes (Millipore, USA) and probed with either polyclonal anti-12-LOX antibody (1:2000; Cayman Biochemical, Ann Arbor, MI, USA), monoclonal anti-5-LOX antibody (1:250; Transduction Laboratories, Lexington, KI, USA) or polyclonal anti-c-Jun antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (DAKO, Denmark), immunoblots were developed using enhanced chemiluminescence (Amersham Bioscience, Orsay, France), stripped and reblotted with an anti-actin antibody (1:4000; Sigma, St. Louis, MO, USA). The densitometric analysis of immunoblot was carried out using ImageQuant software (Molecular Dynamics, Amersham Bioscience, Orsay, France). The 12-LOX 5-LOX and c-Jun immunoreactivities were normalized to actin immunoreactivity.

Treatment of cultures with 12(S)-HETE or 5(S)-HETE

12(S)- and 5(S)-HETE were purchased from Cayman Biochemical, Ann Arbor, MI, USA. Rat cortical cells grown for 5 days were treated with increasing concentrations (10 nM–1 μM) of 12(S)- or 5(S)-HETE

for 24 h and then subjected to DAPI staining for quantification of apoptotic cells.

Quantification of apoptosis

To detect morphological evidence of apoptosis, cell nuclei were visualized following DNA staining with the fluorescent dye DAPI (Sigma, St. Louis, MO, USA). Cells were rinsed with PBS, fixed for 20 min in formalin (Sigma, St. Louis, MO, USA), rinsed with PBS and then incubated for 10 min with DAPI (1 $\mu\text{g/ml}$ PBS). After washing with PBS, cultures were examined using fluorescence microscopy (Microphot FX, Nikon France, Champigny-sur-Marne, France), and the percentage of apoptotic nuclei with condensed or fragmented chromatin was evaluated.

Detection of apoptosis was also evaluated through TUNEL labeling using the *in situ* Death Detection Kit POD (Roche Applied Science, Mannheim, Germany). Following DAPI staining, cells were subjected to TUNEL labeling according to the instructions of the manufacturer.

c-Jun immunocytochemistry

After fixation, cultures were rinsed with PBS and sequentially incubated with 3% H_2O_2 in PBS for 10 min to inhibit endogenous peroxidase, 10% horse serum (HS) for 30 min to saturate aspecific sites, anti-c-Jun primary antibody (Sigma, St. Louis, MO, USA) 1/300 in 1% HS for 1 h at 37°C , biotinylated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) for 1 h, ABC solution (Vector Laboratories, Burlingame, CA, USA) in 1% HS for 1 h, rinsed rapidly in 0.1 M Tris-HCl buffer (pH 7.4) and incubated with 0.05% diaminobenzidine (Sigma, St. Louis, MO, USA) and 0.01% H_2O_2 in 0.1 M Tris-HCl for 15 min. The percentage of c-Jun-positive neurons was evaluated after nuclear staining with DAPI.

Caspase assay

Caspase activities were assayed using AMC (7-amino-4-methylcoumarin)-related fluorogenic substrates (Alexis Biochemicals, San Diego, CA, USA): Ac-VDVAD-AMC for caspase-2, Ac-DEVD-AMC for caspase-3 and Ac-IEPD-AMC for caspase-8. Cortical neurons were harvested following a 24-h treatment with 25 μM $A\beta_{25-35}$. To measure caspase activity, cells were washed once with ice-cold PBS, and 250 μl ice-cold extraction buffer was added directly to the cultures. The extraction buffer consisted of 50 mM HEPES, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% CHAPS, 0.1% Triton X-100, 0.5 mM PMSF and 1 $\mu\text{g/ml}$ pepstatin and aprotinin. After 15 min incubation on ice, the extraction buffer was removed, sonicated, centrifuged for 5 min at $17\,000 \times g$ at 4°C and 100 μl of supernatant was added to each well of a 96-well plate. Then, 100 μl of 20 μM fluorogenic substrate were added to each well to give a final substrate concentration of 10 μM . Plates were incubated at 37°C and cleavage of the fluorogenic substrate was measured over 2 h with a Spectrafluor fluorometer (TECAN France, Trappes, France) (excitation wavelength: 365 nm; emission wavelength: 460 nm). Protein concentration was determined by the Biorad Protein Assay (Bio-Rad Laboratories, München, Germany).

Statistical analysis

All experiments were performed at least three times. All values are presented as means \pm S.E.M. Statistical analysis of data was carried out using ANOVA variance analysis followed by Dunnett's or Newman-Keuls tests, using Instat software (GraphPad, San Diego, CA, USA). Differences at $P < 0.05$ were considered as significant.

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