



# Interaction between 24-hydroxycholesterol, oxidative stress, and amyloid- $\beta$ in amplifying neuronal damage in Alzheimer's disease: three partners in crime

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## Summary

**All three cholesterol oxidation products implicated thus far in the pathogenesis of Alzheimer's disease, 7 $\beta$ -hydroxycholesterol, 24-hydroxycholesterol, and 27-hydroxycholesterol, markedly enhance the binding of amyloid-beta (A $\beta$ ) to human differentiated neuronal cell lines (SK-N-BE and NT-2) by up-regulating net expression and synthesis of CD36 and  $\beta$ 1-integrin receptors. However, only 24-hydroxycholesterol markedly potentiates the pro-apoptotic and pro-necrogenic effects of A $\beta$ <sub>1–42</sub> peptide on these cells: 7 $\beta$ -hydroxycholesterol and 27-hydroxycholesterol, like unoxidized cholesterol, show no potentiating effect. This peculiar behavior of 24-hydroxycholesterol at physiologic concentrations (1  $\mu$ M) depends on its strong enhancement of the intracellular generation of NADPH oxidase-dependent reactive oxygen species (ROS), mainly H<sub>2</sub>O<sub>2</sub>, and the consequent impairment of neuronal cell redox equilibrium, measured in terms of the GSSG/GSH ratio. Cell incubation with antioxidants quercetin or genistein prevents 24-hydroxycholesterol's pro-oxidant effect and potentiation of A $\beta$ -induced necrosis and apoptosis. Thus, the presence of 24-hydroxycholesterol in the close vicinity of amyloid plaques appears to enhance the adhesion of large amounts of A $\beta$  to the plasma membrane of neurons and then to amplify the neurotoxic action of A $\beta$**

**by locally increasing ROS steady-state levels. This report further supports a primary involvement of altered brain cholesterol metabolism in the complex pathogenesis of Alzheimer's disease.**

**Key words: cholesterol; Alzheimer's disease; oxysterols; amyloid- $\beta$ ; oxidative stress; neurotoxicity.**

## Introduction

Alzheimer's disease (AD), a protein misfolding disorder, is the commonest type of dementia in developed countries; it is characterized by progressive neuronal degeneration, gliosis, and the accumulation of intracellular inclusions (neurofibrillary tangles) and extracellular deposits of amyloid- $\beta$  (senile plaques) in the discrete regions of the basal forebrain, hippocampus, and association cortices (Selkoe, 1991; Hashimoto *et al.*, 2003). Amyloid- $\beta$  (A $\beta$ ) is also deposited in the cerebral vessels. Accumulation of A $\beta$  peptides is thought to be an early and causative event in AD pathogenesis and increases markedly during disease progression (Selkoe, 1994).

Several intertwined causative factors lead to the development of AD, including increased release and polymerization of A $\beta$ , hypertension, advanced glycation end-products, oxidative stress, inflammation, and hypercholesterolemia (Querfurth & LaFerla, 2010). At least three important lines of evidence have implicated cholesterol in AD: (i) hypercholesterolemia is unanimously recognized to be a risk factor for sporadic AD, a form that accounts for the great majority of cases (Puglielli *et al.*, 2003; Panza *et al.*, 2007); (ii) epidemiological studies have shown homozygosity for the APO-E4 allele (APO-E  $\epsilon$ 4 genotype) to be associated with an increased risk of AD (Corder *et al.*, 1993; Evans *et al.*, 2004); (iii) feeding cholesterol and copper to rabbits produces some of the pathological signs of AD including amyloid-like plaques (Sparks & Schreurs, 2003). In addition, early epidemiological studies indicated that cholesterol-lowering agents belonging to the family of statins reduce the prevalence of AD (Jick *et al.*, 2000), a conclusion not yet fully accepted, because of contradictory results of prospective clinical studies (Kandiah & Feldman, 2009).

The brain is the organ with the highest concentration of cholesterol, which is essential for its normal function; most of the cholesterol is present in the free form and derives from *de novo* synthesis by astrocytes, as plasma lipoproteins cannot cross the blood–brain barrier (Puglielli *et al.*, 2003). Cholesterol can only efflux to the plasma in normal conditions if it is transformed into oxysterols, cholesterol oxidation products that are thus impor-

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tant to balance the local synthesis of sterols (Lütjohann *et al.*, 1996).

The major oxysterol involved in this excretion mechanism appears to be 24-hydroxycholesterol (24-OH), also known as cerebrosterol, which is produced almost exclusively in the brain by cholesterol 24-hydroxylase (cytochrome P<sub>450</sub>-46A1) (Björkhem & Meaney, 2004; Björkhem, 2006). Another oxysterol, 27-hydroxycholesterol (27-OH), has been found to be produced *in situ* in the brain by the cytochrome P<sub>450</sub>-27A1, or even, unlike its parent compound, to flow from the circulation into the brain (Heverin *et al.*, 2005). A third compound, 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH), may derive in the brain from the oxidation of cholesterol by A $\beta$  and, to a lesser extent, by amyloid precursor protein (Nelson & Alkon, 2005).

Notably, an increased concentration of 24-OH has been detected in the cerebrospinal fluid of patients with AD (Schönknecht *et al.*, 2002), while decreased 24-OH and increased 27-OH are consistently found in autoptic brain samples from patients with AD (Heverin *et al.*, 2004). Further, an abnormal pattern of cholesterol hydroxylases has been observed in the AD brain, with a prominent expression of 24-hydroxylase in astrocytes and around amyloid plaques (Brown *et al.*, 2004). Hence, changes in the brain cholesterol/oxysterol balance, with pathological accumulation of cholesterol oxides in the Central Nervous System, may be the missing link between hypercholesterolemia and AD.

Oxysterols, which may either originate in the body or derive from the diet, are 27-carbon molecules produced by the oxidation of cholesterol. It is widely accepted that both enzymatic and nonenzymatic reactions are responsible for oxysterol generation in the body (Leonarduzzi *et al.*, 2002; Poli *et al.*, 2009). Enzymatic processes mainly involve the side chain of cholesterol yielding 24-OH and 27-OH. On the contrary, oxidation of the sterol nucleus, which primarily occurs at position 7 or at the 5,6 double bond, is commonly a non-enzymatic (autooxidative) reaction and generates, among others, 7 $\beta$ -OH of probable interest in the pathogenesis of neurodegenerative diseases. A typical nonenzymatic oxidation process of cholesterol is that mediated by inflammatory cells undergoing oxidative burst. Oxysterols have been shown to exert several *in vitro* and *in vivo* biochemical activities of both physiologic and pathologic relevance (Sottero *et al.*, 2009). Particularly relevant is the strong pro-inflammatory action shown by several components of this class of compounds (Vejud & Lizard, 2009). Thus, inflammation readily generates oxysterols that in turn up-regulate the flogistic process.

To determine how the oxysterols 24-OH, 27-OH, and 7 $\beta$ -OH could modulate and perhaps amplify the expression of AD, we investigated the potential interaction between these cholesterol oxides and A $\beta$ , whose extracellular accumulation in neuritic plaques is one of the hallmarks of AD. A possible manner of interaction could be by enhancing binding to A $\beta$  toxic peptides by vicinal neuronal cells. To date, this event has been demonstrated to occur for another cell type, namely microglia: human THP-1 monocytes and murine microglial cells have been shown to

readily bind to the fibrillar form of A $\beta$ , through a receptor complex involving CD36,  $\alpha$ 6 $\beta$ 1-integrin, and CD47 (Bamberger *et al.*, 2003).

On the basis of our recent demonstration that certain cholesterol oxidation products, unlike the parent compound, markedly up-regulate both the expression and the synthesis of CD36 in the cells of the macrophage lineage (Leonarduzzi *et al.*, 2008), we decided to investigate whether oxysterols also exert the same effect on neuronal cells.

All three cholesterol oxidation products considered strongly enhanced the binding of A $\beta$  to human differentiated neuronal cell lines (SK-N-BE and NT-2), by strongly up-regulating expression and synthesis not only of CD36 but also of  $\beta$ 1-integrin receptors. However, only the combination of 24-OH plus the A $\beta$ <sub>1-42</sub> peptide exerted net neurotoxicity. The reason for this selective behavior of 24-OH turned out to be dependent on its strong pro-oxidant action.

## Results

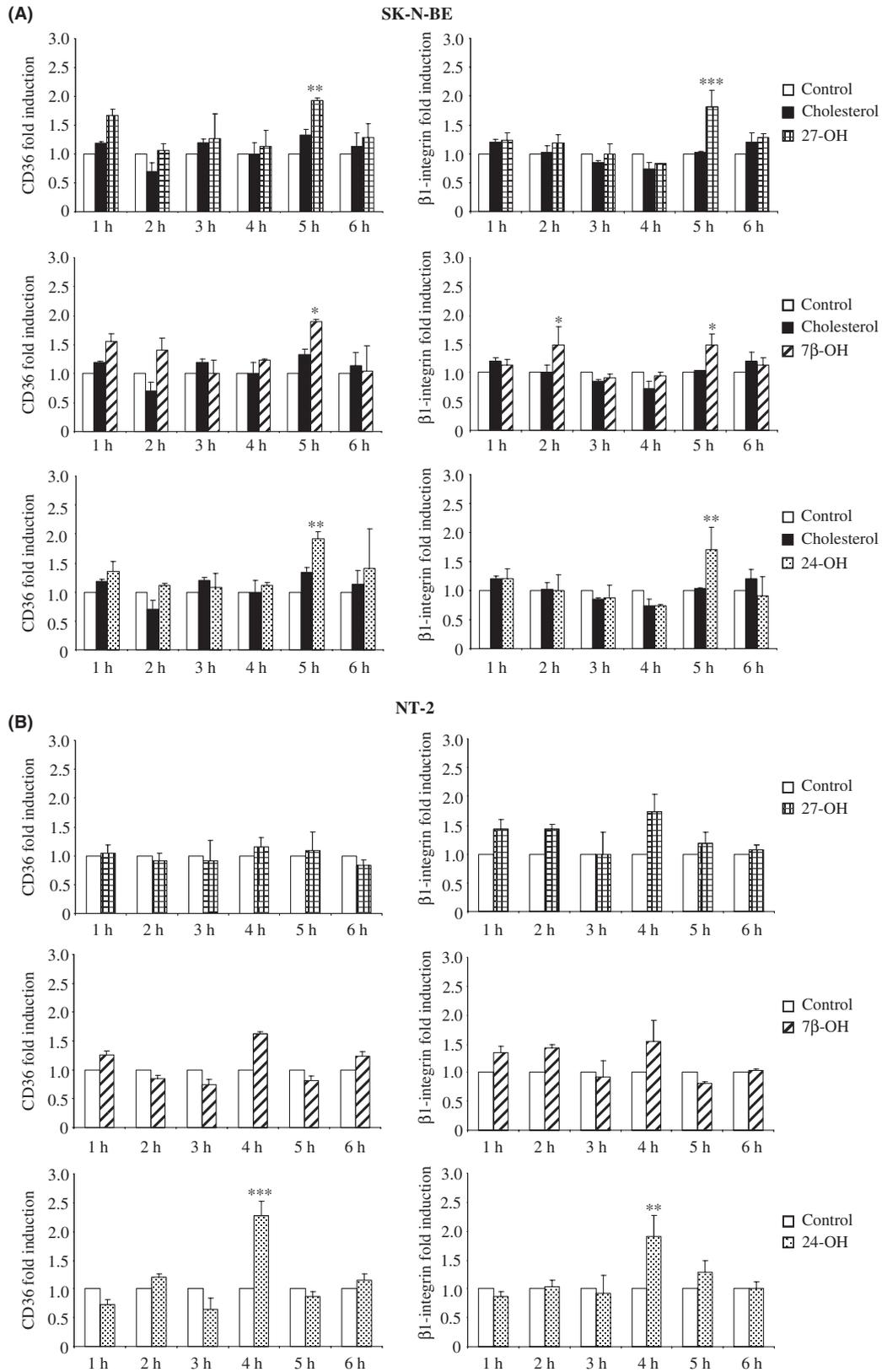
### 27-OH, 7 $\beta$ -OH, and 24-OH up-regulate the expression of the CD36 and $\beta$ 1-integrin genes, but not that of the CD47 gene, in SK-N-BE and NT-2 neuronal cells

The three oxysterols considered as being potentially implicated in the pathogenesis of Alzheimer's disease were first checked for their ability to modulate the CD36/ $\beta$ 1-integrin/CD47 receptor complex in two differentiated human neuronal cell lines, namely SK-N-BE and NT-2 cells.

In SK-N-BE cells, a statistically significant increase (about 1.5 to 2-fold induction,  $P < 0.001$ – $0.05$ ) in CD36 mRNA and  $\beta$ 1-integrin mRNA levels was evident after 5 h cell incubation with any of the three oxysterols in biologically compatible concentrations (1  $\mu$ M) compared with control. Of interest, this over-expression appeared to occur in a synchronous manner and was not inducible even minimally when oxysterols were replaced by an identical amount of unoxidized cholesterol (Fig. 1A). A trend to increased CD36 mRNA and  $\beta$ 1-integrin mRNA levels was consistently observed also in NT-2 neuronal cells after 4 h cell incubation with 7 $\beta$ -OH or 24-OH, even if statistically significant (about 2-fold induction compared with control,  $P < 0.001$  and  $P < 0.01$ ) only in the latter case. As regards 27-OH, it appeared to increase the expression of  $\beta$ 1-integrin and not that of CD36 (Fig. 1B). Expression of the CD47 gene was in no case modulated by any of the three oxysterols considered in this study, neither in SK-N-BE nor in NT-2 cells (Fig. 2).

### 27-OH, 7 $\beta$ -OH, and 24-OH increase CD36 and $\beta$ 1-integrin synthesis

A statistically significant increase ( $P < 0.001$  and  $P < 0.05$  vs. control) in the synthesis of the two receptors corresponded to the over-expression of CD36 and  $\beta$ 1-integrin genes, in both SK-N-BE and NT-2 neuronal cells, measured by Western blotting after 48 h treatment with 27-OH, 7 $\beta$ -OH, or 24-OH. Of note,



**Fig. 1** Effect of 27-hydroxycholesterol (27-OH), 7β-hydroxycholesterol (7β-OH), and 24-hydroxycholesterol (24-OH) on the expression of CD36 and β1-integrin receptors. Gene expression was quantified by real-time RT-PCR in SK-N-BE (A) and NT-2 (B) cells treated for times up to 6 h with 1 μM 27-OH, 7β-OH, or 24-OH or with 1 μM cholesterol (only SK-N-BE cells). Untreated cells were taken as control. Data, normalized to β2-microglobulin, are expressed as mean values ± standard deviation of three different experiments. \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001 vs. control group.

the effects of the three oxysterols on CD36 and  $\beta$ 1-integrin synthesis appeared quantitatively quite similar: about 150–190% (SK-N-BE) and 300% (NT-2) the increment of CD36 levels and 180–250% (SK-N-BE) and 250% (NT-2) the increment of  $\beta$ 1-integrin levels vs. control taken as 100% (Fig. 3).

### AD-relevant oxysterols strongly enhance the adhesion of neuronal cells to amyloid- $\beta$ ; the effect is mediated by up-regulation of CD36 and $\beta$ 1-integrin receptors

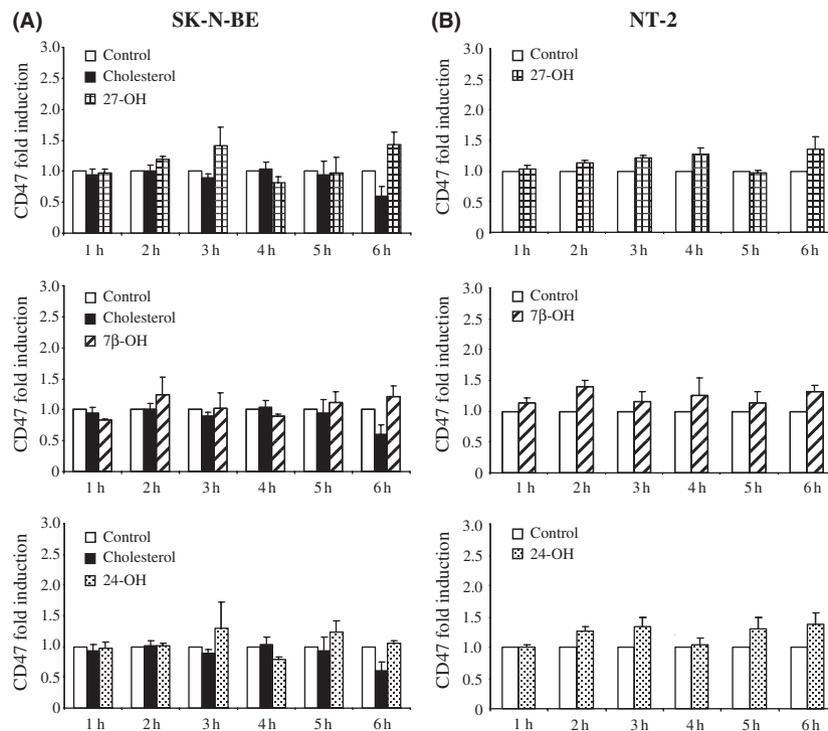
The consequence of increased availability of CD36 and  $\beta$ 1-integrin receptors, in terms of binding to A $\beta$ , was investigated on SK-N-BE neuronal cells incubated for 48 h in the presence of 27-OH, 7 $\beta$ -OH, or 24-OH and then challenged for 8 h with the A $\beta$ <sub>1–42</sub> peptide. The binding of A $\beta$ <sub>1–42</sub> (detected by confocal microscopy) to neuronal cells was greatly stimulated by cell pretreatment with any of the three oxysterols vs. that recovered in cells either untreated or simply incubated with unoxidized cholesterol (Fig. 4). This marked enhancement of A $\beta$ <sub>1–42</sub> peptide binding was fully prevented by the addition of either anti-CD36- or anti- $\beta$ 1-integrin-specific antibodies to the incubation medium before the challenge with the A $\beta$ <sub>1–42</sub> peptide (Fig. 4). At Congo red staining, SK-N-BE neuronal cells treated with the oxysterols and then with A $\beta$ <sub>1–42</sub> showed highly stimulated A $\beta$ <sub>1–42</sub> binding vs. internal controls (untreated and A $\beta$ <sub>1–42</sub>-treated cells) and vs. unoxidized cholesterol plus A $\beta$ <sub>1–42</sub>-treated cells; the amyloid

peptide that bound to the cells was mainly concentrated in clusters (Fig. 5).

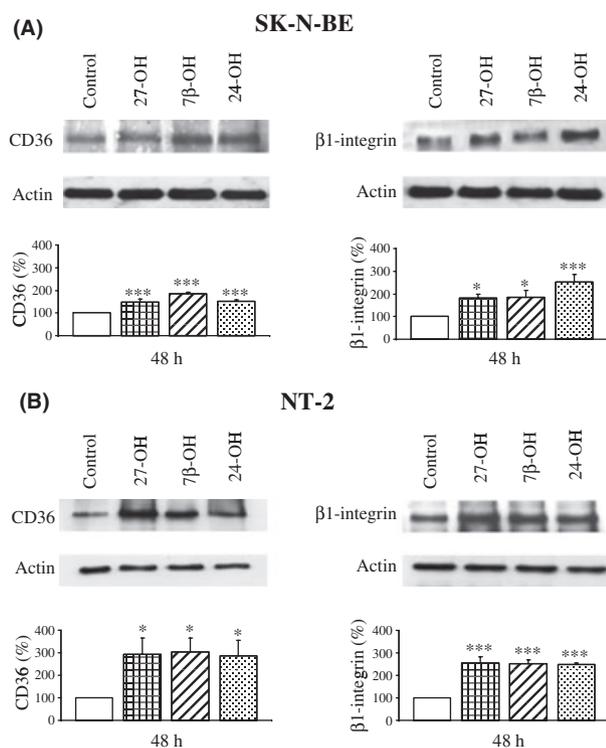
### Different effects of 27-OH, 7 $\beta$ -OH, and 24-OH in potentiating amyloid- $\beta$ 's neurogenic effect on neuronal cells

To determine whether increased binding of amyloid- $\beta$  to neuronal cells potentiates the peptide's toxic effects, SK-N-BE and NT-2 cells, differentiated with retinoic acid, were first incubated for 48 h with one of the three oxysterols and then challenged for 24 h with A $\beta$ <sub>1–42</sub> peptide. At the end of the experiment, necrosis was measured by the cell release of lactate dehydrogenase (LDH): 24-OH, not neurogenic *per se*, increased about 3- to 3.5-fold ( $P < 0.01$  vs. control) the LDH release induced by A $\beta$ <sub>1–42</sub>, in both SK-N-BE and NT-2 differentiated cells (Fig. 6A,C). On the contrary, neither 27-OH, nor 7 $\beta$ -OH, nor unoxidized cholesterol caused any significant modulation of the induced LDH release (Fig. 6A,C). As expected, cell treatment with the scramble peptide neither induced nor permitted any change in LDH release.

Importantly, 24-OH's marked potentiation of A $\beta$ <sub>1–42</sub>'s neurogenic effect was significantly quenched (30–40% reduction,  $P < 0.01$ ) compared with 24-OH-treated cells, in both neuronal cell lines, by addition of anti-CD36 antibodies, and was fully prevented ( $P < 0.05$ ) by addition of anti- $\beta$ 1-integrin-specific antibodies (Fig. 6B,D).



**Fig. 2** Effect of 27-hydroxycholesterol (27-OH), 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH), and 24-hydroxycholesterol (24-OH) on the expression of CD47 receptor. Gene expression was quantified by real-time RT-PCR in SK-N-BE (A) and NT-2 (B) cells treated up to 6 h with 1  $\mu$ M 27-OH, 7 $\beta$ -OH, or 24-OH or with 1  $\mu$ M cholesterol (only SK-N-BE cells). Untreated cells were taken as control. Data, normalized to  $\beta$ 2-microglobulin, are expressed as mean values  $\pm$  standard deviation of three different experiments.



**Fig. 3** Effect of 27-hydroxycholesterol (27-OH), 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH), and 24-hydroxycholesterol (24-OH) on CD36 and  $\beta$ 1-integrin synthesis. SK-N-BE (A) and NT-2 (B) cells were treated for 48 h with oxysterols at a final concentration of 1  $\mu$ M. Untreated cells were taken as control. CD36 and  $\beta$ 1-integrin levels were analyzed by Western blotting. Top: blot representative of three experiments. Bottom: histogram representing mean values  $\pm$  standard deviation of three experiments. CD36 and  $\beta$ 1-integrin densitometric measurements were normalized against the corresponding actin levels and expressed as percentage of control value. \* $P$  < 0.05 and \*\*\* $P$  < 0.001 vs. control.

### Pretreatment of neuronal cells with 24-OH, but not with 27-OH, 7 $\beta$ -OH, or unoxidized cholesterol, potentiates the A $\beta$ -dependent apoptotic effect

A similar set of data were obtained when AD-relevant oxysterols were examined for their potential enhancement of A $\beta$ 's pro-apoptotic effect: 4,4-diamidino-2-phenylindole dihydrochloride (DAPI) staining showed that the moderate but consistent apoptotic effect exerted on SK-N-BE cells by A $\beta$ <sub>1-42</sub> (15% DAPI-stained cells), but not by the scramble, was markedly increased when cells were incubated with 24-OH (50% DAPI-stained cells), as was said not toxic *per se*. On the contrary, neither 27-OH nor 7 $\beta$ -OH, exactly like an identical concentration of unoxidized cholesterol, altered the observed A $\beta$ <sub>1-42</sub> apoptotic effect (Fig. 7A). These findings were fully confirmed on NT-2 neuronal cells challenged with A $\beta$ <sub>1-42</sub> (Fig. 7C). As in the case of potentiation of A $\beta$ 's neurogenic effect on neuronal cells, both anti-CD36 and anti- $\beta$ 1-integrin competitive treatments entirely prevented the appearance of apoptotic bodies in SK-N-BE and NT-2 cultures incubated with 24-OH and then challenged with A $\beta$ <sub>1-42</sub> (Fig. 7B,D).

### Only treatment of neuronal cells with 24-OH induces an oxidative imbalance of the cellular redox equilibrium

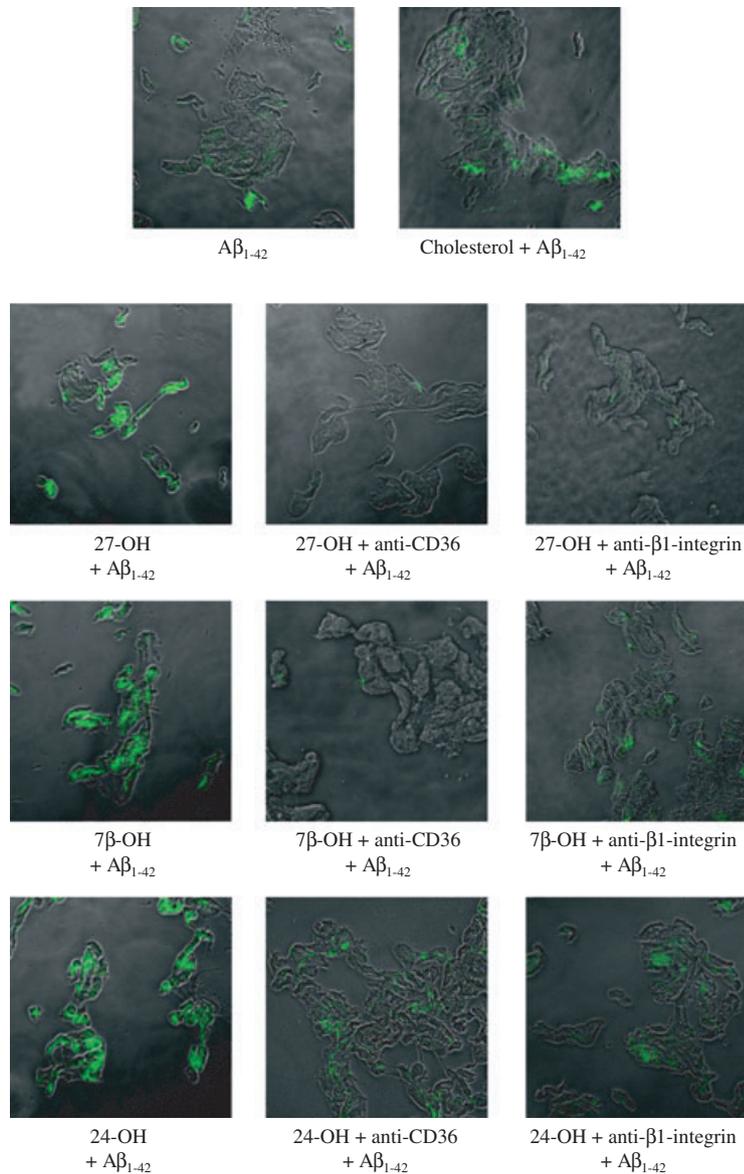
In an attempt to explore the remarkably different behaviors shown by the three oxysterols of interest in AD pathogenesis as regards A $\beta$ 's neurotoxicity, we decided to analyze their potential pro-oxidant effects employing the same cellular models and oxysterol concentrations employed in the rest of this study. Importantly, treatment of both neuronal cell lines with 24-OH produced a strong up-regulation of cell steady-state levels of reactive oxygen species (ROS) measured by both biochemical and fluorescence ( $P$  < 0.05 vs. control) methods (Figs 8B,D and 9A,B). As a consequence, the redox equilibrium of both SK-N-BE and NT-2 neuronal cells appeared deranged, as indicated by the net increase in the oxidized/reduced glutathione (GSSG/GSH) ratio during cell treatment ( $P$  < 0.05 vs. control) (Fig. 8A,C). Unlike what occurred with 24-OH, ROS steady-state levels and cellular redox equilibrium did not appear significantly affected by either 27-OH, 7 $\beta$ -OH, or unoxidized cholesterol (Figs 8 and 9).

The incubation of differentiated SK-N-BE neuronal cells with 27-OH, or 7 $\beta$ -OH, or 24-OH in the presence of A $\beta$ <sub>1-42</sub> did not significantly modulate GSSG/GSH ratio (Fig. 8A) and H<sub>2</sub>O<sub>2</sub> production (Fig. 8B) as well as ROS production (Fig. 9C) vs. oxysterol-treated SK-N-BE cells.

To confirm that NADPH oxidase is a primary source of the observed ROS over-production, SK-N-BE cells were pretreated with diphenyleneionium (DPI) (5  $\mu$ M) or with apocynin (APO) (300  $\mu$ M). Both NADPH oxidase inhibitors were able to block the 24-OH-induced enhancement of ROS production by neuronal cells (Fig. 9D).

### The potentiating effect of 24-OH on A $\beta$ <sub>1-42</sub>-dependent neurotoxicity appears chiefly dependent on this cholesterol oxidation product's pro-oxidant effect

To test the possible involvement of the pro-oxidant activity of 24-OH in its potentiating A $\beta$  neurotoxicity, suitable supplementation of the neuronal cell incubation medium was provided, with two flavonoids known to possess strong antioxidant properties, namely quercetin and genistein. Incubation of SK-N-BE and NT-2 differentiated cells, in the presence of either quercetin or genistein, fully prevented the oxidative stress condition generated in the neuronal cell cultures by incubation with 24-OH, in terms of ROS production ( $P$  < 0.01 vs. 24-OH-treated cells) (Figs 9A,B and 10B,D) as well as of the GSSG/GSH ratio ( $P$  < 0.01 vs. 24-OH-treated cells) (Fig. 10A,C). More importantly, this flavonoid-mediated inhibition of 24-OH's pro-oxidant effect completely prevented the potentiation exerted by this oxysterol on A $\beta$ <sub>1-42</sub>'s neurogenic ( $P$  < 0.01 vs. 24-OH+A $\beta$ <sub>1-42</sub>-treated cells) and apoptotic (1-3% DAPI-stained cells) effects, in both SK-N-BE and NT-2 cells (Fig. 11).



**Fig. 4** Oxysterols enhance the adhesion of neuronal cells to amyloid- $\beta$ . SK-N-BE cells were incubated for 48 h in the presence of 1  $\mu\text{M}$  of cholesterol, 27-OH, 7 $\beta$ -OH, or 24-OH and then treated for 8 h with 1  $\mu\text{M}$  A $\beta_{1-42}$ . Some cell aliquots were also pre-incubated for 1 h with anti-CD36 (2  $\mu\text{g mL}^{-1}$ ) or anti- $\beta$ 1-integrin (2  $\mu\text{g mL}^{-1}$ ) antibodies before adding A $\beta_{1-42}$ . Cells treated with 1  $\mu\text{M}$  A $\beta_{1-42}$  for 8 h were used as control. A $\beta_{1-42}$  peptide binding was detected by confocal laser microscopy using fluorescein isothiocyanate (FITC) fluorochrome (excitation from the 488 nm Ar laser line and emission passing through a longpass 505–550  $\mu\text{m}$  filter) and equipped with an inverted microscope with Plan-NEOFLUAR lenses (20  $\times$ /0.5, 2  $\times$ ). Images are from one representative experiment.

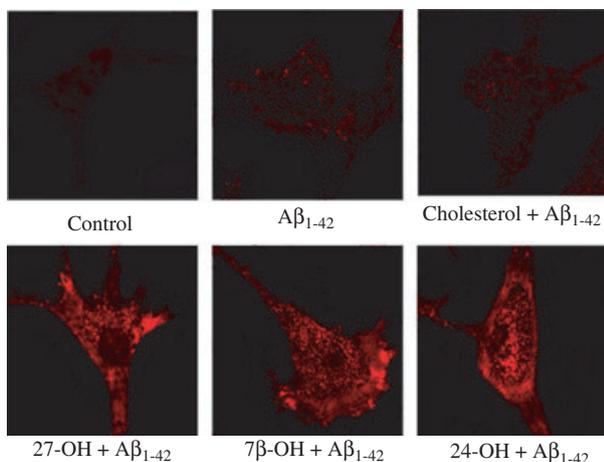
## Discussion

Altered cholesterol metabolism in the brain has repeatedly been suggested to be implicated in the pathogenesis of Alzheimer's disease, but the molecular mechanisms underlying such an involvement are still largely undefined. A net increase in free cholesterol has been clearly demonstrated in the cerebral cortical tissue of aging mice, in the primary cultures of rat hippocampal neurons exposed to A $\beta$ , and in the frontal cortex of autopsy-confirmed patients with AD (Cutler *et al.*, 2004). Interestingly, this cholesterol increase is accompanied by increased ceramide production and is consistently associated, in both experimental

models and human autolytic material, with a biochemical condition of oxidative stress, i.e., an imbalance of cell/tissue redox equilibrium toward oxidation (Cutler *et al.*, 2004).

Oxidative stress is increasingly considered to play a pivotal role in the initiation and promotion of the neurodegenerative events that characterize AD (Bonda *et al.*, 2010; Rothman & Mattson, 2010); it may be triggered within the brain by any environmental or age-related factor that can induce an oxidative burst in the microglia, by inflammatory molecules, and also by A $\beta$  (Zhu *et al.*, 2007a).

The association of oxidative stress with the accumulation of free cholesterol in AD brains should, in principle, facilitate the



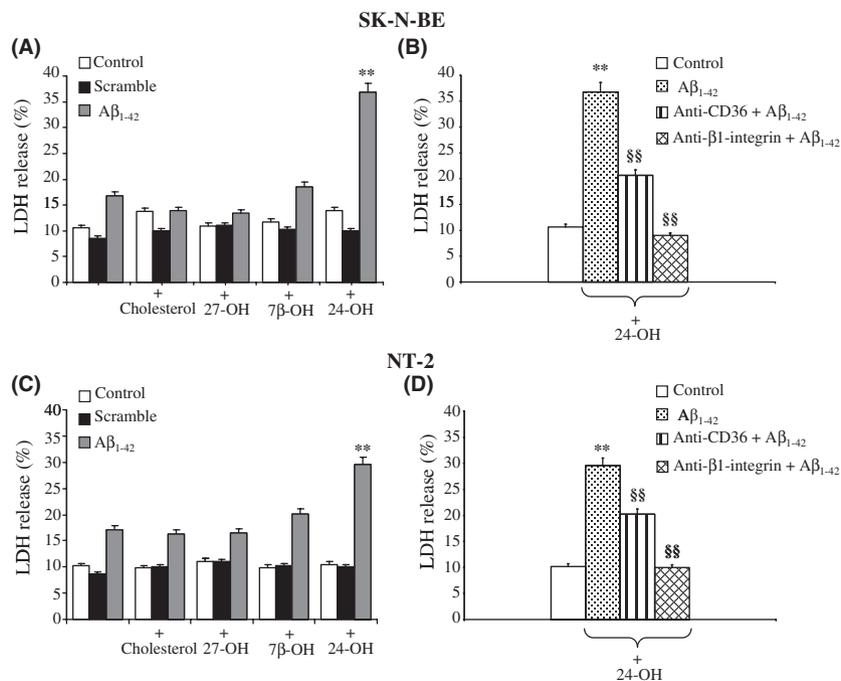
**Fig. 5** Congo red staining of amyloid- $\beta$  binding in SK-N-BE cells. SK-N-BE neuronal cells were treated with 27-OH (1  $\mu$ M), 7 $\beta$ -OH (1  $\mu$ M), 24-OH (1  $\mu$ M) or with cholesterol (1  $\mu$ M) for 48 h and then with  $A\beta_{1-42}$  (1  $\mu$ M) for 8 h. Other cells were treated with  $A\beta_{1-42}$  alone for 8 h. Untreated cells were taken as control.  $A\beta_{1-42}$  binding to cells was observed by confocal laser microscopy: excitation 488–543 nm and emission 560 nm; lens  $20\times/0.5$ ,  $3\times$ . Images are from one representative experiment of two performed.

nonenzymatic generation of cholesterol oxidation products, i.e., oxysterols, which, unlike the parent compound, often possess strong pro-inflammatory and toxic effects (Sottero et al., 2009; Vejux & Lizard, 2009). In addition to ROS-mediated oxidation of free cholesterol, dysregulated activity of cytochrome  $P_{450-46A1}$

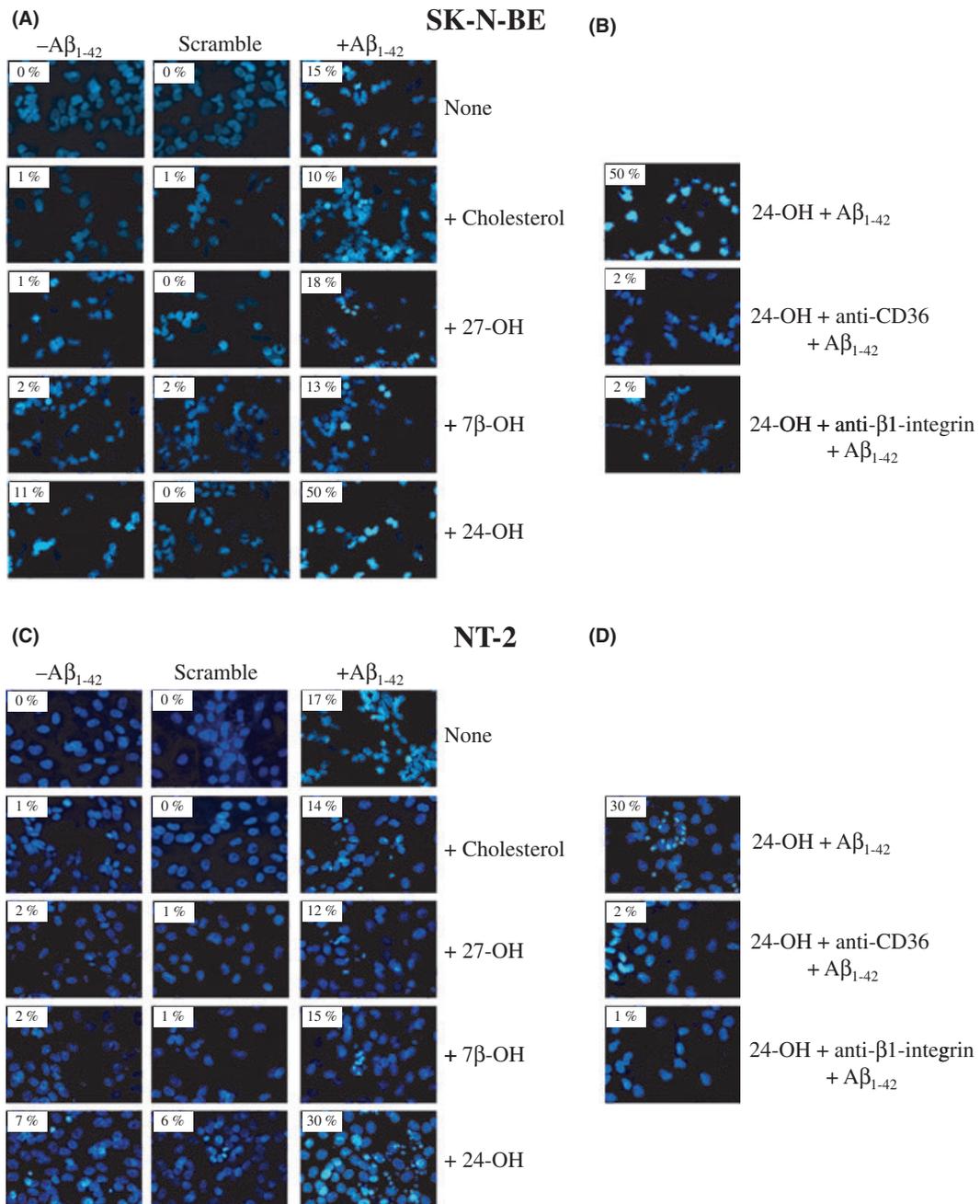
and cytochrome  $P_{450-27A1}$ , which respectively generate 24-OH and 27-OH, could contribute to an abnormal accumulation of oxysterols in certain brain areas.

The idea that oxysterols might be the missing link between altered cholesterol metabolism in the brain and AD was first proposed by Björkhem's group having developed a highly reliable method to measure cholesterol oxidation products, they showed that 24-OH may reach quite elevated concentrations in the brain, up to 30  $\mu$ M (Lütjohann et al., 1996). They also demonstrated that 24-OH, unlike cholesterol, can cross the blood-brain barrier from the brain into the circulation (Lütjohann et al., 1996) and that 27-OH, the major oxysterol found in human plasma (Brown & Jessup, 1999), readily flows from the circulation into the brain (Heverin et al., 2005). Further, as regards the potential neurotoxicity of oxysterols, the same team showed that 24-OH caused cell death when added at a 50  $\mu$ M concentration to undifferentiated SH-SY5Y neuronal cells (Kölsch et al., 1999).

In this connection, in brain cortex obtained *post mortem* from patients with AD, selective expression of cytochrome  $P_{450-46A1}$  (the enzyme that synthesizes 24-OH) was demonstrated in astroglial cells (Bogdanovic et al., 2001) and at the neuritic periphery of amyloid plaques (Brown et al., 2004). The latter finding could be very important despite the fact that the same immunocytochemical method showed a decrease in cortical neurons expressing cytochrome  $P_{450-46A1}$ , likely because of neuronal loss caused by the disease. The number of neurons



**Fig. 6** Effect of 27-hydroxycholesterol (27-OH), 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH), and 24-hydroxycholesterol (24-OH) on lactate dehydrogenase (LDH) release. SK-N-BE (A) or NT-2 (C) cells were treated with 27-OH, 7 $\beta$ -OH, or 24-OH at the final concentration of 1  $\mu$ M for 48 h and then for 24 h with scramble (1  $\mu$ M) or  $A\beta_{1-42}$  (1  $\mu$ M). Untreated cells were taken as control. Some SK-N-BE (B) or NT-2 (D) cell aliquots were also pre-incubated for 1 h with anti-CD36 (4  $\mu$ g  $mL^{-1}$ ) or anti- $\beta$ 1-integrin (4  $\mu$ g  $mL^{-1}$ ) antibodies after 24-OH incubation and before adding  $A\beta_{1-42}$ . Histograms represent the mean values  $\pm$  standard deviation of three experiments. \*\* $P < 0.01$  vs. control group; §§ $P < 0.01$  vs. 24-OH +  $A\beta_{1-42}$ .

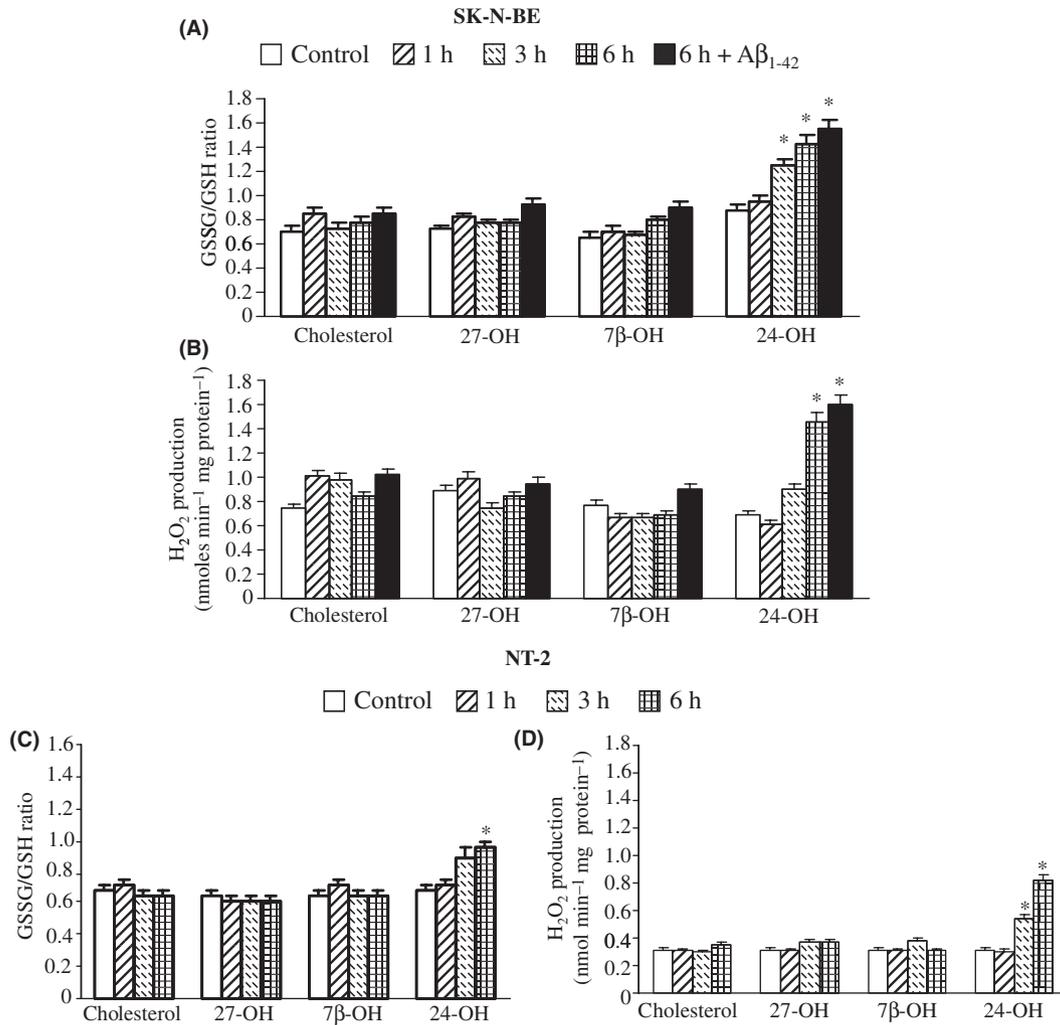


**Fig. 7** Pro-apoptotic effect of 27-hydroxycholesterol (27-OH), 7β-hydroxycholesterol (7β-OH), and 24-hydroxycholesterol (24-OH) on SK-N-BE or NT-2 cells. The formation of apoptotic nuclei was evaluated in terms of DAPI staining in SK-N-BE (A) or NT-2 (C) cells treated with 27-OH, 7β-OH, or 24-OH at a final concentration of 1 μM for 48 h and then for 24 h with scramble (1 μM) or Aβ<sub>1-42</sub> (1 μM). Some SK-N-BE (B) or NT-2 (D) cell aliquots were also pre-incubated for 1 h with anti-CD36 (4 μg mL<sup>-1</sup>) or anti-β1-integrin (4 μg mL<sup>-1</sup>) antibodies after 24-OH incubation and before adding Aβ<sub>1-42</sub>. The reported percentage of cells with chromatin condensation represents the means of three experiments.

expressing cytochrome P<sub>450</sub>-27A1 also appeared to be reduced in the cortical tissue from patients with AD but the enzyme was found to be prominently expressed in white matter oligodendrocytes (Brown *et al.*, 2004). This change in the distribution and net amount of these two enzymes in the brain of patients with AD was confirmed by mass-spectrometric measurement of 24-OH and 27-OH in the autaptic samples of AD subjects; the for-

mer oxysterol decreased, while the latter increased in all brain areas examined (Heverin *et al.*, 2004).

Although the actual concentration and distribution of 24-OH- and 27-OH-producing enzymes in AD brains is still debated, the abnormal increase in cytochrome P<sub>450</sub>-46A1 in the astrocytes of AD brains (Bogdanovic *et al.*, 2001) and the prominent localization of cytochrome P<sub>450</sub>-46A1 around the amyloid plaques



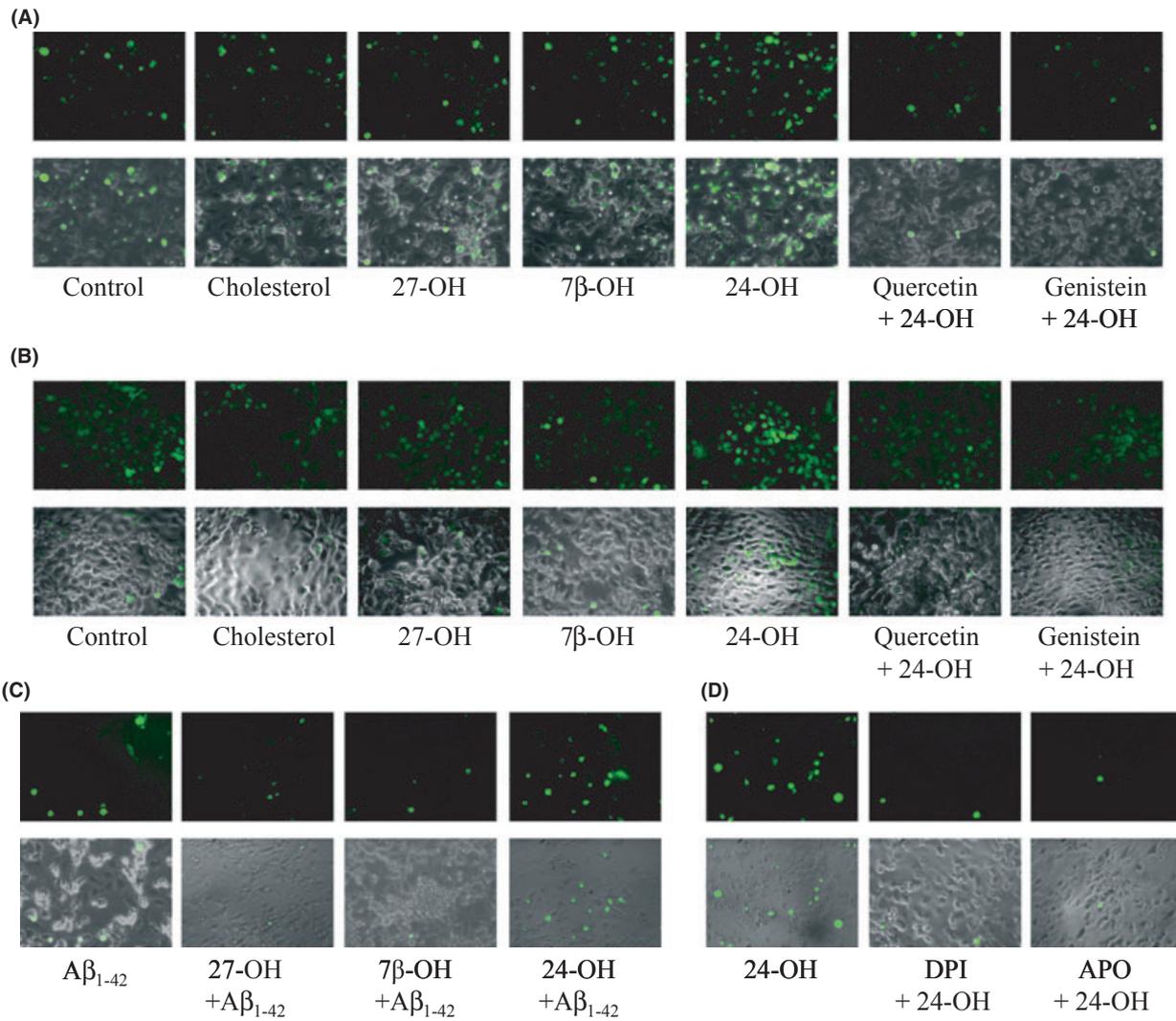
**Fig. 8** Effect of 27-hydroxycholesterol (27-OH), 7β-hydroxycholesterol (7β-OH), and 24-hydroxycholesterol (24-OH) on GSSG/GSH ratio and on H<sub>2</sub>O<sub>2</sub> production. GSSG/GSH ratio and H<sub>2</sub>O<sub>2</sub> production were measured in SK-N-BE (A and B) and NT-2 (C and D) cells after incubation with oxysterols or cholesterol, at a final concentration of 1 μM, for 1, 3, or 6 h. Untreated cells were taken as control. Other SK-N-BE cells were treated with the oxysterols for 3 h and then for three additional hours also with Aβ<sub>1-42</sub> (1 μM). Histograms represent mean values ± standard deviation of three experiments. \**P* < 0.05 vs. control.

(Brown *et al.*, 2004) clearly point to a potential interaction between 24-OH and Aβ peptides in bringing about neurotoxic effects.

Amyloid-β may bind to a number of biomolecules and receptors, the best characterized of which are those mediating the binding to glial cells, namely a multireceptor complex involving CD36, α6β1-integrin, and CD47 (Bamberger *et al.*, 2003; Verdier *et al.*, 2004). Of interest, binding of the peptide to cell membranes facilitates the formation of amyloid oligomers and fibrils, which are responsible for a series of structural and functional cell changes (Jiang *et al.*, 2009; Sakono & Zako, 2010). To our knowledge, the present study offers the first report of the enhancement of Aβ binding to neuronal cells exerted by AD-relevant oxysterols. All three of the oxysterols specifically implicated in brain pathophysiology, namely 27-OH, 7β-OH, and 24-OH, have been proved to strongly up-regulate not only

CD36 but also β1-integrin expression and synthesis (the specific integrin α subunits involved were not determined) in two different human differentiated neuronal cell lines, SK-N-BE and NT-2 (Figs 1 and 3). In a second part of this research, SK-N-BE neuronal cell pretreatment with either 27-OH, or 7β-OH, or 24-OH, followed by addition of synthetic Aβ<sub>1-42</sub>, strongly increased the amount of the peptide actually bound to cell plasma membranes, vs. oxysterol-untreated cells (Fig. 4). Congo red staining of oxysterol-treated SK-N-BE (Fig. 5) indirectly confirmed the key role of the CD36/β1-integrin/CD47 receptor complex in binding and concentrating the amyloid peptide on the cell surface.

The most interesting finding reported here, however, is the demonstration that even though all three oxysterols of potential relevance in AD pathogenesis may up-regulate the CD36/β1-integrin binding complex and stimulate Aβ binding

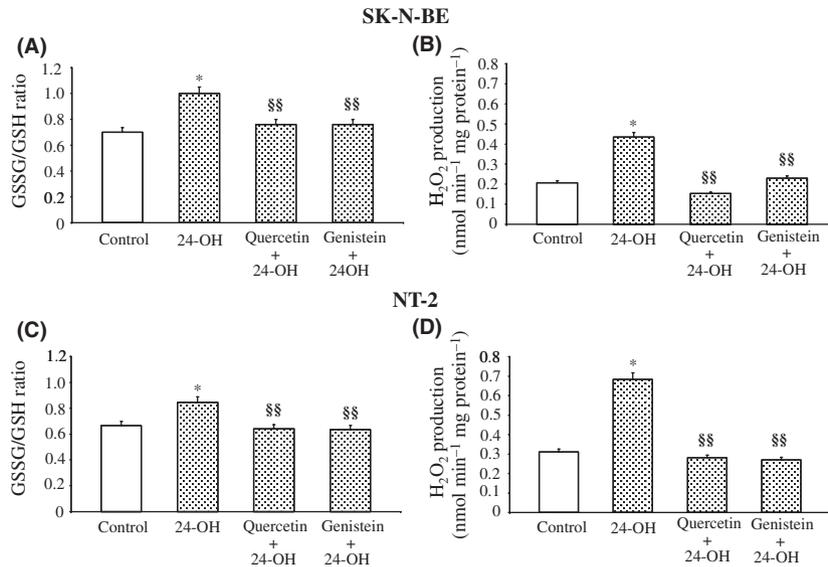


**Fig. 9** Pro-oxidant effect of 27-hydroxycholesterol (27-OH), 7β-hydroxycholesterol (7β-OH), and 24-hydroxycholesterol (24-OH). Intracellular generation of reactive oxygen species was run with 2'7'-dichlorodihydrofluorescein (DCFH-DA) in SK-N-BE (A, C, D) or NT-2 (B) cells. Cells were incubated with the oxysterols (all at 1 μM), cholesterol (1 μM), or Aβ<sub>1-42</sub> (1 μM) for 1 h. Defined cell aliquots were simultaneously treated with the oxysterols and with Aβ<sub>1-42</sub> (1 μM) for 1 h. Untreated cells were taken as control. Other cells were pre-incubated with quercetin (5 μM) or genistein (5 μM) for 1 h or pre-incubated with diphenyleneiodonium (DPI) (5 μM) or apocynin (APO) (300 μM) for 30 min and then treated with 24-OH (1 μM).

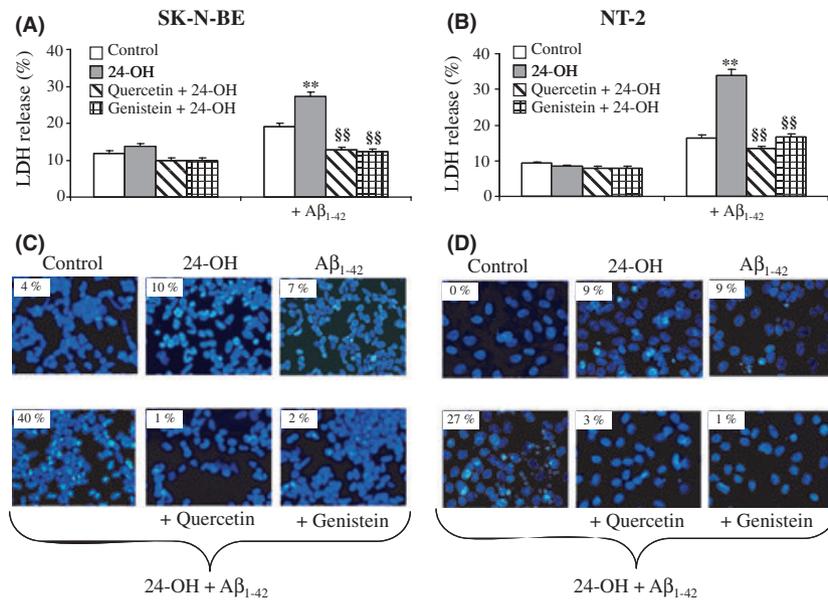
to human neuronal cells, only 24-OH significantly potentiates both the necrogenic and the apoptotic effects exerted by Aβ<sub>1-42</sub> peptide on these cells (Figs 6A,C and 7A,C). These effects were inhibited when 24-OH-treated neuronal cells were incubated with anti-CD36 and anti-β1-integrin antibodies before Aβ<sub>1-42</sub> addition because Aβ peptide binding to cell surface was prevented (Figs 6B,D and 7B,D). A study by Ferrera *et al.* (2008) showed that treatment of the human differentiated neuroblastoma cell line MSN with Aβ<sub>1-42</sub> peptide moderately impaired the mitochondrial reducing capacity, leading to about a 20% decrease in cell viability. Addition of a relatively high concentration (50 μM) of either cholesterol or 24-OH further increased, by about 10%, the toxic effect of the Aβ peptide. Data reported here are consistent with that study, but refer to the effect of 1 μM 24-

OH, and show much stronger potentiation of Aβ toxicity by the oxysterol, as well as demonstrating it in greater detail. In addition, the Ferrera study reported increased ROS generation induced in MSN cells by supplementation with 50 μM cholesterol (Ferrera *et al.*, 2008) and considered this finding to be the consequence of the already established generation of H<sub>2</sub>O<sub>2</sub> by amyloid-β in the presence of cholesterol as reducing agent (Opazo *et al.*, 2002). Notably, they did not directly check whether 24-OH exerted a pro-oxidant effect in their model system.

At least one significant reason for the selective neurotoxic behavior of 24-OH reported here appears to be the marked pro-oxidant action on neuronal cells that this compound, but not 27-OH nor 7β-OH, exerts at relatively low concentrations (1 μM) (Figs 8 and 9). The addition of Aβ<sub>1-42</sub> to oxysterol-treated SK-N-



**Fig. 10** Protection exerted by antioxidants quercetin and genistein on GSSG/GSH ratio and on H<sub>2</sub>O<sub>2</sub> production. GSSG/GSH ratio and H<sub>2</sub>O<sub>2</sub> production were measured in SK-N-BE (A and B) and NT-2 (C and D) cells after incubation with 1 μM 24-OH for 6 h. Untreated cells were taken as control. Other cells were pre-incubated with quercetin (5 μM) or genistein (5 μM) for 1 h and then treated with 24-OH (1 μM) for 6 h. Histograms represent the mean values ± standard deviation of three experiments. \**P* < 0.05 vs. control; \*\**P* < 0.01 vs. 24-OH alone.



**Fig. 11** Protection exerted by antioxidants quercetin or genistein on lactate dehydrogenase (LDH) release as well as on apoptotic nuclei formation. Lactate dehydrogenase release and the formation of apoptotic nuclei were analyzed in SK-N-BE (A and C) or NT-2 (B and D) cells. Cells were incubated with 1 μM 24-OH for 48 h and then with or without 1 μM Aβ<sub>1-42</sub> for 24 h. Untreated cells were taken as control. Other cells were incubated with Aβ<sub>1-42</sub> alone (1 μM) for 24 h and others were pre-incubated with quercetin (5 μM) or genistein (5 μM) for 1 h and then treated with 24-OH (1 μM) for 48 h and with Aβ<sub>1-42</sub> (1 μM) for 24 h. Histograms represent mean values ± standard deviation of three experiments. \*\**P* < 0.01 vs. control group; \*\**P* < 0.01 vs. 24-OH + Aβ<sub>1-42</sub>. The reported percentage of cells with chromatin condensation represents the means of three experiments.

BE neuronal cells did not modify the pro-oxidant effect of the three oxysterols (Figs 8A,B and 9C). 24-OH-dependent potentiation of Aβ neurotoxicity was completely inhibited by incubation of differentiated SK-N-BE or NT-2 cells with either the flavonol quercetin or the isoflavone genistein (Fig. 11), both of which

efficiently prevented ROS over-production and GSSG/GSH imbalance induced by 24-OH (Figs 9 and 10). Fully consistent with these data is the previous report of significant quenching of 24-OH's pro-apoptotic effect by physiologic concentrations of vitamin E in differentiated human neuronal cells SH-SY5Y (Kölsch *et al.*,

2001). However, antioxidant molecules such as quercetin and genistein appear by far more promising, because they can cross the blood–brain barrier thank to their polyphenolic hydroxyl groups (Sarkar & Das, 2006; Jakóbkiewicz-Banecka *et al.*, 2007).

In conclusion, the 24-OH produced by cytochrome P<sub>450</sub>-46A1 in the close vicinity of amyloid plaques appears to strongly enhance the adhesion of large amounts of A $\beta$  to the plasma membrane of neurons and then to amplify the neurotoxic action of the peptide by locally increasing ROS (mainly H<sub>2</sub>O<sub>2</sub>) steady-state levels.

Oxidative stress is now generally recognized as one of the earlier changes in AD, and it is considered to be a main driving force in its early promotion and progression (Odetti *et al.*, 1998; Perry *et al.*, 1998; Nunomura *et al.*, 2001; Cutler *et al.*, 2004; Zhu *et al.*, 2007b; Smith *et al.*, 2010). In relation to this, numerous studies carried out on animal models have demonstrated the ability of dietary docosahexaenoic acid supplementation to modulate amyloid pathology by preserving neuronal and brain functions: among the mechanisms involved, the anti-oxidant and anti-inflammatory properties of the n-3 fatty acid likely play a primary role (Oster & Pillot, 2010).

There are various sources of ROS in AD, including abnormal mitochondria, redox active iron and copper, activated glial cells, lipid peroxidation, and glycation end-products (Zhu *et al.*, 2007a). The here reported inhibition by NADPH oxidase inhibitors of 24-OH-induced neuronal ROS levels points to this enzyme as an additional and primary source of oxidant species (Fig. 9D). However, the mechanisms underlying the action of ROS in AD are still under investigation. The ROS-mediated neurotoxic interaction between 24-OH and A $\beta$  might be one such mechanism, and at least the aliquot of 24-OH localized at senile plaques should probably be added to the list of ROS sources in AD. Future studies should cast further light on the possible modulation of neurotoxicity because of the triad 24-OH-oxidative stress-A $\beta$ , by the simultaneous presence of 27-OH and other oxysterols, by astrocytes and microglial cells, by redox active metals, and, last but not least, by anti-inflammatory drugs and antioxidants that can cross the blood–brain barrier.

The obtained findings support the therapeutic use of statins in lowering the risk of dementia, not only because of their cholesterol-lowering, antioxidant, and anti-inflammatory effects but also because of their proved lowering effect on plasma oxysterols, including 24-OH (Locatelli *et al.*, 2002; Vega *et al.*, 2003).

## Experimental procedures

### Cell culture and differentiation

SK-N-BE neuroblastoma cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium containing 2 mM glutamine and supplemented with 10% fetal bovine serum, 1% antibiotic mixture (penicillin–streptomycin–amphotericin). NT-2 neuronal cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum and 1% antibiotic mixture, comprising penicillin–streptomycin–

amphotericin. Both cell lines were maintained in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>. For differentiation, 2 × 10<sup>6</sup> cells were plated in 75-cm<sup>2</sup> flasks (Costar, Lowell, MA, USA) and exposed to 10  $\mu$ M retinoic acid for 10 days in the case of SK-N-BE cells and for 5 weeks for NT-2 cells. Growth medium was changed three times a week for both cell lines. After 5 weeks of differentiation of NT-2 cells, the mitotic inhibitors cytosine arabinoside (1  $\mu$ M), fluorodeoxyuridine (10  $\mu$ M), and uridine (10  $\mu$ M) were added for 2 weeks to inhibit the division of non-neuronal cells.

### Cell treatments

Cells were treated with 1  $\mu$ M 27-hydroxycholesterol (27-OH), 1  $\mu$ M 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH), 1  $\mu$ M 24-hydroxycholesterol (24-OH), or 1  $\mu$ M unoxidized cholesterol (Steraloids, Newport, RI, USA), all dissolved in ethanol. Some oxysterol-treated cells were then treated with A $\beta$ <sub>1–42</sub> (1  $\mu$ M) (Bachem, Bubendorf, Switzerland) or with scramble A $\beta$  (1  $\mu$ M) (AnaSpec, Fremont, CA, USA). Fresh A $\beta$ <sub>1–42</sub> peptide stock solutions were prepared at 1 mg mL<sup>-1</sup> in hexafluoro-2-isopropanol (Sigma-Aldrich, Milan, Italy), quickly dried under nitrogen, and directly solubilized at the experimental concentration in the culture medium. In certain experiments, cells were pretreated with anti-CD36 antibody (2 or 4  $\mu$ g mL<sup>-1</sup>) (Clone FA6-152; HyCult Biotechnology b.v., Uden, the Netherlands) or with anti- $\beta$ 1-integrin antibody (2 or 4  $\mu$ g mL<sup>-1</sup>) (Clone 4B7R; Santa Cruz, Biotechnology Inc., Santa Cruz, CA, USA) and others were pretreated with quercetin (5  $\mu$ M) (Sigma-Aldrich) or genistein (5  $\mu$ M) (Alexis Biochemicals, Lausen, Switzerland) and with diphenyleneiodonium (5  $\mu$ M) or apocynin (300  $\mu$ M) (Sigma-Aldrich), two NADPH oxidase inhibitors. Incubation times for all experiments are reported in the Results section and Figure legends.

### RNA extraction

Total RNA was extracted from cells using TRIzol Reagent (Applied Biosystems, Monza, Italy) following the manufacturer's instructions after the treatment times indicated. RNA was dissolved in RNase-free water fortified with RNase inhibitors (RNase SUPERase-In; Ambion, Austin, TX, USA). The amount and purity (A260/A280 ratio) of the extracted RNA were assessed spectrophotometrically.

### cDNA preparation and real-time RT-PCR

cDNA was synthesized by reverse transcription from 2  $\mu$ g RNA with a commercial kit and random primers (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems) following the manufacturer's instructions. Singleplex real-time RT-PCR was performed on 30 ng of cDNA using TaqMan Gene Expression Assay kits prepared for human CD36,  $\beta$ 1-integrin, CD47, and  $\beta$ 2-microglobulin, TaqMan Fast Universal PCR Master Mix, and 7500 Fast Real-Time PCR System (Applied Biosystems). Negative controls did not include cDNA. The oligonucleotide sequences

are not revealed by the manufacturer because of proprietary interests. The cycling parameters were as follows: 20 s at 95°C for AmpErase UNG activation, 3 s at 95°C for AmpliTaq Gold DNA polymerase activation, 40 cycles of 3 s at 95°C (melting), and 30 s at 60°C (annealing/extension). The fractional cycle number (Ct) at which fluorescence passes the threshold in the amplification plot of fluorescence signal vs. cycle number was determined for each gene considered. The results were then normalized to the expression of  $\beta_2$ -microglobulin, as house-keeping gene. Relative quantification of target gene expression was achieved with a mathematical method proposed by Livak & Schmittgen (2001).

### Western blotting

Whole-cell extracts were prepared in ice-cold lysing buffer (1 mL of phosphate-buffered saline (PBS) was added with 10  $\mu$ L Triton X-100, 10  $\mu$ L SDS 10%, 5  $\mu$ L DTT 1 M, 6  $\mu$ L PMSF 0.1%, 10  $\mu$ L aprotinin) for 30 min and sonicated for 20 s. The lysates were then cleared by centrifugation at 17 860 *g* for 15 min. The protein concentration was measured following Bradford's method (Bradford, 1976).

To analyze the levels of CD36 and  $\beta$ 1-integrin, 100  $\mu$ g of total proteins were immunoprecipitated, respectively, with 8  $\mu$ L of anti-CD36 primary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or with 8  $\mu$ L of anti- $\beta$ 1-integrin primary antibody (Santa Cruz Biotechnology Inc.), purified on Protein A Sepharose resin (GE Healthcare Europe, Milan, Italy), boiled in Laemmli buffer for 5 min, separated by electrophoresis in 8% denaturing SDS/polyacrylamide gel, and then electroblotted onto Hybond ECL nitrocellulose membrane (GE Healthcare Europe). For CD36 and  $\beta$ 1-integrin level analysis, after saturation of nonspecific binding sites with 5% nonfat milk in Tris-buffered saline (TBS) 1  $\times$ -Tween 20 0.05%, membranes were immunoblotted overnight at 4°C with the appropriate primary antibody against CD36 (1:150) or  $\beta$ 1-integrin (1:150) (Santa Cruz Biotechnology Inc.), both diluted in 5% nonfat milk in TBS 1  $\times$ -Tween 20 0.05% and subsequently probed with an anti-mouse secondary antibody (1:1000) (Santa Cruz Biotechnology Inc.) for 3 h at room temperature.

The supernatants obtained after CD36 and  $\beta$ 1-integrin immunoprecipitation were again immunoprecipitated with 5  $\mu$ L of anti-actin primary antibody (Sigma-Aldrich). Proteins were separated by electrophoresis in 8% denaturing SDS/polyacrylamide gels, electroblotted onto nitrocellulose membranes, and incubated with anti-actin primary antibody (1:10 000) and then with anti-rabbit secondary antibodies (1:7500) (Santa Cruz Biotechnology Inc.), as described earlier. Proteins detected by the antibodies were visualized by enhanced chemiluminescence using the ECL-plus kit (GE Healthcare Europe) following the manufacturer's directions. The immunoreactive bands were scanned and subjected to densitometric analysis using 'Image Tool' software (Windows 3.00). The results were evaluated as relative units determined by normalization of the density of each band to that of the corresponding actin protein band.

### Analysis of A $\beta_{1-42}$ by confocal laser microscopy

Cells were grown on glass slides and, after the treatment times, specimens were fixed in 4% formalin for 15 min at room temperature. They were then washed (0.1 M PBS) and incubated with a 100 mM sodium cyanoborohydride reducing agent for 10 min at 37°C. To block nonspecific binding, cells were incubated with 3% BSA in 0.01 M PBS containing 5% goat serum and 0.3% Tween 20, for 30 min at room temperature. After blocking nonspecific binding, slides were incubated in the presence of antibodies to human A $\beta_{1-42}$  (1:500) (Bachem) and then with purified goat anti-mouse fluorescein isothiocyanate (FITC) fluorochrome-conjugated secondary antibodies (1:300) (Alexa Fluor 488; Molecular Probes-Invitrogen Srl, San Giuliano, Milanese, Italy). Slides mounted with glycerol/distilled water (1:1) plus 0.1% NaN<sub>3</sub> were observed through the LSM 510 confocal laser microscope (Carl Zeiss SpA, Arese, Milan, Italy) equipped with an inverted microscope with Plan-NEOFLUAR lenses (40  $\times$ /0.75).

### Congo red staining

Cells were grown on glass slides and, after the treatment times, specimens were fixed in 4% formalin for 15 min at room temperature. SK-N-BE neuroblastoma cells were washed with 0.1 M PBS and then dipped into Harris hematoxylin for 3 min. After washing with tap water, specimens were stained with a fresh solution of 0.5% filtered Congo red (Sigma-Aldrich) at room temperature for 3 min. After several washes with deionized water, specimens were dehydrated in increasing alcohol solutions (50%, 70%, 80%, 95%, and 100%) and cleared with xylene. Slides mounted in DPX (Sigma-Aldrich) were observed with a LSM 510 confocal laser microscopy system (Carl Zeiss SpA).

### Preparation of cell lysates and cytosolic fraction

Confluent differentiated cells were treated under the appropriate experimental conditions and placed immediately on ice-cold PBS. Cell lysates and cytosolic extracts were obtained by the method of Andrews & Faller (1991).

### Analysis of cell death

Lactate dehydrogenase (LDH) activity was determined in culture medium using a photometric assay based on the conversion of pyruvic acid to lactic acid by the enzyme, as described elsewhere (Tamagno *et al.*, 2000). Values for control and treated cells were expressed as percentages of the total LDH activity released by untreated cells, which were lysed with Triton X-100.

The rate of apoptosis was evaluated through 4',6'-diamidino-2-phenylindole (DAPI) staining. To identify apoptotic nuclei, cells were washed in PBS, fixed and permeabilized with 95% cold ethanol for 5 min, and then stained with DAPI solution for 30 min at 37°C. After rinsing in PBS, cells were observed and photographed under a Zeiss fluorescence microscope.

## Oxidative stress determinations

Intracellular generation of ROS was detected through the conversion of 2',7'-dichlorofluorescein diacetate (used at 5  $\mu$ M concentration), once taken up by cells and de-acetylated by esterase, into the corresponding fluorescent derivative (Rezvani *et al.*, 2007). Cells were observed and photographed under a Zeiss fluorescence microscope. The amount of ROS was expressed as percentage of fluorescent cells.

Antioxidant levels in the cytosolic fractions were evaluated in terms of the GSSG/GSH ratio, by the Owens & Belcher (1965) method. A mixture was directly prepared in a cuvette: 0.05 mM Na phosphate buffer, pH 7.0; 1 mM EDTA, pH 7.0; and 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid) plus an aliquot of the sample. Total thiol content was evaluated after 2 min at 412 nm and expressed as  $\mu$ g/mg protein. Suitable volumes of diluted GSH reductase and of NADPH were added to evaluate total GSH. The ratio between GSSG and GSH content is considered to be a measure of antioxidant status.

Generation of H<sub>2</sub>O<sub>2</sub> was monitored after adding horseradish peroxidase and acetylated ferrocyanide to cells. H<sub>2</sub>O<sub>2</sub> content was evaluated as the increase in acetylated ferrocyanide oxidation rate as described by Zoccarato *et al.* (1993).

## Statistical analysis

All values are expressed as means  $\pm$  standard deviation (SD). Statistical analysis of the data was assessed using one-way ANOVA with Bonferroni's post-test for multiple comparisons. Differences at  $P < 0.05$  were considered statistically significant. Statistical calculations were made with GRAPHPAD INSTAT3 software (GraphPad Software Inc., San Diego, CA, USA).

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