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Chondroitin Sulfate Protects SH-SY5Y Cells from Oxidative Stress by Inducing Heme Oxygenase-1 via Phosphatidylinositol 3-Kinase/Akt

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ABSTRACT

We investigated the mechanism of the neuroprotective properties of chondroitin sulfate (CS), an endogenous perineuronal net glycosaminoglycan, in human neuroblastoma SH-SY5Y cells subjected to oxidative stress. Preincubation with CS for 24 h afforded concentration-dependent protection against H_2O_2-induced toxicity (50 μM for 24 h) measured as lactic dehydrogenase released to the incubation media; cell death was prevented at the concentrations of 600 and 1000 μM. Cell death caused by a combination of 10 μM rotenone plus 1 μM oligomycin-A (Rot/oligo) was also reduced by CS at concentrations ranging from 0.3 to 100 μM; in this toxicity model, maximum protection was achieved at 3 μM (48%). No significant protection was observed in a cell death model of Ca^{2+} overload (70 mM K^+, for 24 h). H_2O_2 and Rot/oligo generated reactive oxygen species (ROS) measured as an increase in the fluorescence of dichlorofluorescein diacetate-loaded cells. CS drastically reduced ROS generation induced by both H_2O_2 (extracellular ROS) and Rot/oligo (intracellular ROS). CS also increased the expression of phosphorylated Akt and heme oxygenase-1 by 2-fold. The protective effects of CS were prevented by chelerythrine, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002), cycloheximide, and Sn(IV)-protoporphyrin IX. Taken together, these results show that CS can protect SH-SY5Y cells under oxidative stress conditions by activating protein kinase C, which phosphorylates Akt that, via the phosphatidylinositol 3-kinase/Akt pathway, induces the synthesis of the antioxidant protein heme oxygenase-1.

Proteoglycans (PGs) represent a diverse class of complex macromolecules that share a general molecular structure that includes a central core protein with a number of covalently attached carbohydrate chains, the glucosaminoglycans (GAGs). Each GAG is made up of repeating disaccharide units (hexuronic acid and *N*-acetyl hexosamine), which are either or both sulfated (Hascall and Sajdera, 1970). The composition of the GAG chains in the CNS varies with development. For example, the four-carbon sulfated GAG is most abundantly expressed in the developing CNS (Kitagawa et al., 1997), whereas in the adult animal, the six-carbon sulfated GAG is up-regulated after CNS injury (Lemons et al., 2003; Properzi et al., 2005).

PGs can be classified as heparan sulfate, dermatan sulfate, keratin sulfate, or chondroitin sulfate (CS) proteoglycans (CSPGs). The core proteins of the PGs can be secreted into the extracellular matrix; CSPGs are soluble, and other PGs

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ABBREVIATIONS: PG, proteoglycan; GAG, glycosaminoglycan; CNS, central nervous system; CS, chondroitin sulfate; CSPG, chondroitin sulfate proteoglycans; ROS, reactive oxygen species; HO-1, heme oxygenase 1; BV, biliverdin; PI3K, phosphatidylinositol 3-kinase; PD98059, 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one; SnPP, Sn(IV)-protoporphyrin IX; DMEM, Dulbecco's modified Eagle's medium; DCFDA, 2′,7′-dichlorofluorescein diacetate; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PI, propidium iodide; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; Rot/oligo, rotenone/oligomycin-A; ERK, extracellular signal-regulated kinase; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; DCFH, dichlorofluoroscein.

such as heparan sulfate and keratan sulfate are bound to the membrane (Seidenbecher et al., 1995). CSPGs are the most abundant type of PGs expressed in the mammalian central nervous system, and CS chains are the major sulfated carbohydrate chains attached to PGs present in the extracellular matrix. In the central nervous tissue, the extracellular matrix forms a special matrix called perineuronal net. This net contains several types of CSPGs (Fox and Caterson, 2002) with different patterns of sulfation, which confers different biological properties and functions to the different types of CSPGs (Sugahara et al., 2003).

CS has crucial functions in growth factor signaling, wound repair, morphogenesis, infection, and cell division (Sugahara et al., 2003). Furthermore, proteoglycans have shown to protect the brain in a physiological manner (Deguchi et al., 2005), and they are released from neurons that have been exposed to excitotoxic concentrations of glutamate (Sugiura and Dow, 1994). The neuroprotective effects of PGs have been described in several studies. For example, neurocan is overexpressed after ischemia in adult rat brain (Deguchi et al., 2005), heparan sulfate is released from synaptic vesicles upon the addition of glutamate (Sugiura and Dow, 1994), and CS has been shown to protect neurons against glutamate and excitatory amino acid-induced injury (Okamoto et al., 1994a,b). In the case of neurodegenerative diseases like Alzheimer's disease, cortical areas highly rich in perineural nets are less severely affected by neurofibrillary degeneration, and the perineuronal net-associated neurons are devoid of tangles (Brückner et al., 1999). Another study has shown that the perineural net per se can protect the brain tissue against oxidative stress in Alzheimer's disease (Morawski et al., 2004).

Oxidative stress is a leading mechanism of cell death in distinct cytotoxic models such as glutamate (Parfenova et al., 2006), or H_2O_2-induced cytotoxicity (Kim et al., 2005) and in neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Mariani et al., 2005), and stroke (Saito et al., 2005). Overproduction of reactive oxygen species (ROS) such as superoxide free radicals or hydrogen peroxide leads to damage of both neuronal and vascular cells by cell membrane lipid destruction and DNA cleavage (Wang et al., 2003).

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme that degrades the pro-oxidant heme group and produces equimolecular quantities of CO, iron, and biliverdin. Biliverdin is subsequently reduced to bilirubin by biliverdin reductase. These three by-products have been related to cell protection against oxidative stress in distinct cellular models (Kim et al., 2005; Vitali et al., 2005).

Because oxidative stress has been implicated in several CNS diseases and CS is up-regulated during CNS injury, the objective of this investigation was to determine the mechanism by which this GAG can protect neurons under oxidative stress conditions. The results of this study provide evidence that CS, an endogenous GAG of the perineuronal net, can exert protective actions against extracellular (H_2O_2) or intracellular (blockade of the mitochondrial respiratory chain) oxidative stress injury by inducing HO-1 via the PI3K/Akt intracellular signaling pathway.

Materials and Methods

Materials. CS, composed of a chain of alternating sugars D-glucuronic acid β(1-3) D-*N*-acetyl galactosamine β(1-4) sulfated at position 4 of *N*-acetyl galactosamine, was provided by Bioibérica (Barcelona, Spain). Rotenone and oligomycin-A were obtained from Sigma (Madrid, Spain). Chelerythrine and PD98059 were purchased from Tocris (Biogen Científica, Spain). Sn(IV)-protoporphyrin IX (SnPP) dichloride was obtained from Frontier Scientific Europe (Lancashire, UK). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and penicillin/streptomycin were purchased from Gibco (Invitrogen, Madrid, Spain). Cytotoxicity cell death kit was obtained from Roche-Boehringer Mannheim (Madrid, Spain). 2′,7′-Dichlorofluorescein diacetate (H_2DCFDA) was obtained from Molecular Probes (Invitrogen, Madrid, Spain).

Culture of SH-SY5Y Cells. The neuroblastoma cell line SH-SY5Y was a kind gift from the Centro de Biología Molecular Universidad Autonoma de Madrid/Consejo Superior de Investigaciones Cientificas (Madrid, Spain). SH-SY5Y cells, at passages between 3 and 16 after defreezing, were maintained in a DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. SH-SY5Y cells were seeded into flasks containing supplemented medium, and they were maintained at 37°C in 5% CO_2, humidified air. Stock cultures were passaged 1:3 twice weekly; i.e., one plate was divided (subcultured or split) into three plates. This procedure was performed twice a week. For assays, SH-SY5Y cells were subcultured in 48-well plates at a seeding density of 10^5 cells per well, or in six-well plates at a seeding density of 5×10^5 cells per well. Cells were treated with the drugs before confluence in serum-free DMEM.

Measurement of Lactic Dehydrogenase Activity. Samples of incubation media were collected at the end of the 24-h incubation period with the toxic stimuli to estimate extracellular LDH as an indication of cell death (Koh and Choi, 1987). LDH activity was also measured in the cells after treatment with 1% Triton X-100 in water (intracellular LDH). Extracellular and intracellular LDH activity was spectrophotometrically measured using a cytotoxicity cell death kit (Roche-Boehringer Mannheim) according to the manufacturer's instructions. Total amount of LDH (intracellular plus extracellular) was normalized to 100%; then, the amount of LDH released to the extracellular medium was expressed as percentage of this total. This value was considered as cell death.

Measurement of MTT. Cell viability was measured using the MTT reduction assay as described previously (Mosmann, 1983). After the experiments, MTT was added to each well at a final concentration of 0.5 mg/ml in Krebs-HEPES solution (144 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 11 mM glucose; pH 7.3), and incubation at 37°C was continued for an additional 2-h period. Then, the insoluble formazan was dissolved with dimethyl sulfoxide; colorimetric determination of MTT reduction was measured at 500 nm. Control cells treated with vehicle (DMEM) were taken as 100% viability.

Measurement of Apoptosis by Flow Cytometry. Cellular cycle and apoptosis was determined by flow cytometry analysis of the cell cycle after DNA staining with propidium iodide (PI; Molecular Probes-Invitrogen) (Robinson et al., 1997). Cells were grown in six-well plates until they reached 50% confluence (typically after 24–48 h in culture). After treatment, cells that remained attached were harvested in PBS/EDTA (5 mM EDTA in PBS) and collected together with those floating (detached) cells. Cells were then centrifuged, the supernatant discarded, and the cell pellet suspended in 0.5 ml of PBS by pipetting thoroughly to avoid cell clumping. The cell suspension was transferred to 4.5 ml of 70% ice-cold ethanol and kept in this fixative for a minimum of 2 h at 4°C. Ethanol-fixed cells were centrifuged and washed once with 10 ml of PBS. Finally, the cell pellet was suspended in 1 ml of PI/RNase staining buffer solution (BD Biosciences PharMingen, Palo Alto, CA) and incubated for 15 min at 37°C. Samples were analyzed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA). The analysis of the samples included a first selection (gate 1) in which events with appropriate size (forward scatter) and complexity (side scatter) were selected. Then, selected events were analyzed to discard doublets by using a PI intensity-

width versus PI intensity-area dot plot (gate 2). Finally, events (cells) that were contained in gates 1 and 2 were plotted in a histogram representing the number of events (cells) containing a specific PI intensity-area (e.g., specific amount of DNA). Apoptosis was measured as the percentage of cells with a sub-G_0/G_1 DNA content in the PI intensity-area histogram plot.

ROS Measurement. To measure cellular ROS, we have used the molecular probe H_2DCFDA (LeBel et al., 1992). SH-SY5Y cells were loaded with 10 μM H_2DCFDA, which diffuses through the cell membrane and is hydrolyzed by intracellular esterases to the nonfluorescent form dichlorofluorescein. Dichlorofluorescein (DCFH) reacts with intracellular H_2O_2 to form dichlorofluorescin, a green fluorescent dye. Fluorescence was measured in a fluorescence microplate reader (FLUOstar Galaxy; BMG Labtech GmbH, Offenburg, Germany). Wavelengths of excitation and emission were 485 and 520 nm, respectively. The fluorescence caused after 30-min exposure to the ROS generator (H_2O_2 or Rot+oligo) was normalized to 1; the remaining variables were expressed in relation to this value.

Immunoblotting. SH-SY5Y cells (5×10^5) were washed once with cold phosphate-buffered saline and lysed in 100 μl of ice-cold lysis buffer (1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, and 1 mM Na_3VO_4). Proteins (30 μg) from this cell lysates were resolved by SDS-PAGE and transferred to Amersham membranes (GE Healthcare, Chalfont St. Giles, UK). Membranes were incubated with anti-total-AKT (1:1000), anti-phospho-AKT (1:1000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-HO-1 (1:1000) (Chemicon International. Temecula, CA); anti-β-actin (1:100,000) (Sigma). Appropriate peroxidase-conjugated secondary antibodies (1:10,000) were used to detect proteins by enhanced chemiluminescence.

Data Analysis and Statistics. Different band intensities corresponding to immunoblot detection of protein samples were quantified using the Scion Image program (Scion Corporation, Frederick, MD). Immunoblots correspond to a representative experiment that was repeated four to eight times with similar results. Data are given as means $\pm$ S.E.M. Differences between groups were determined by applying a one-way analysis of variance followed by Tukey test. Differences were considered to be statistically significant when $p \leq 0.05$.

Results

Effect of CS per se on SH-SY5Y Cells. Incubation of SH-SY5Y cells for 24 h with CS, at concentrations ranging from 0.06 to 1 mM, did not significantly increase cell death. In fact, a significant reduction of basal cell death was observed at the concentrations of 0.6 and 1 mM ($p < 0.01$; $n = 4$) (Fig. 1A). In contrast, pretreatment of the cells with 10 or 60 μM CS for 48 h did not modify the proliferative rate when analyzing the cellular cycle by flow cytometry in propidium iodide-stained cells (Fig. 1B) or by using the MTT test (Fig. 1C).

Chondroitin Sulfate Protects against H_2O_2-Induced Toxicity. H_2O_2 is commonly used to induce cell death secondary to free radicals; it is therefore considered a good model of oxidative stress. SH-SY5Y cells treated for 24 h with 50 μM H_2O_2 increased basal cell death, measured as percentage of LDH released to the extracellular medium, from 12 to 45%. Under these experimental conditions, pretreatment of the cells with CS, 24 h before and during the noxious stimulus, afforded concentration-dependent protection; total protection was achieved at the concentrations of 0.6 and 1 mM (Fig. 2D). In fact, LDH release during this period was below basal, suggesting a cytoprotective effect of CS against the spontaneous cell death resulting from culture aging. CS at concentrations ranging from 100 to 1000 μM also reduced apoptotic cell death induced by H_2O_2 (Fig. 2E).

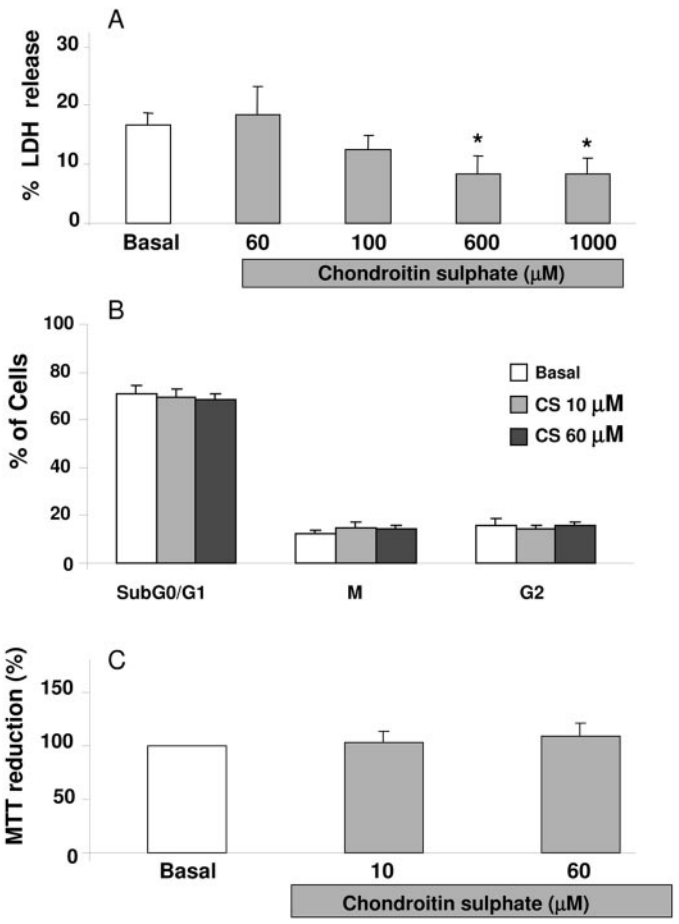

Fig. 1. Effects of CS per se on SH-SY5Y cells. A, cells were incubated for 48 h with increasing concentration of CS (60–100 μM), and thereafter, LDH released to the extracellular medium was measured as an index of cell death. Data correspond to the mean and S.E.M of triplicates of four different batches of cells; $p < 0.05$ with respect to basal untreated cells. B, analysis of the percentage of cells under the different cell cycle phase, after incubating the cells for 48 h in the presence or absence of 10 or 60 μM CS. Data correspond to the mean and S.E.M of four experiments. C, results of a similar experiment as in B, but measuring MTT reduction as an index of cell proliferation. Data correspond to the mean and S.E.M of triplicates of four different batches of cells.

CS Protects against the Toxicity Induced by the Combination of Rotenone and Oligomycin-A. The other oxidative stress model used in this study consists in causing mitochondrial disruption by blocking mitochondrial complexes I and V with the combination of 10 μM rotenone plus 1 μM oligomycin-A (Rot/oligo), respectively. As a result of mitochondrial disruption, the cell cannot further synthesize ATP, and free radicals are generated beyond the cell's capacity to buffer them, and, ultimately, the cell dies. Exposure of SH-SY5Y cells to Rot/oligo for 24 h increased cell death above basal, measured as LDH released to the extracellular medium, from 8 to 35%. See the cell damage in Fig. 3B, where the number of cells decreased and their outlines were irregular; note the difference with control cells in Fig. 3A and cells treated with CS (60 μM) plus Rot/oligo, in Fig. 3C. In the presence of CS, the cells recovered their initial density and exhibited a healthier appearance. This is better seen in the histogram of Fig. 3D. Pretreatment of the cells with CS, 24 h before and during the toxic stimulus, afforded significant protection already at 0.3 μM. Maximum protection (48%) was

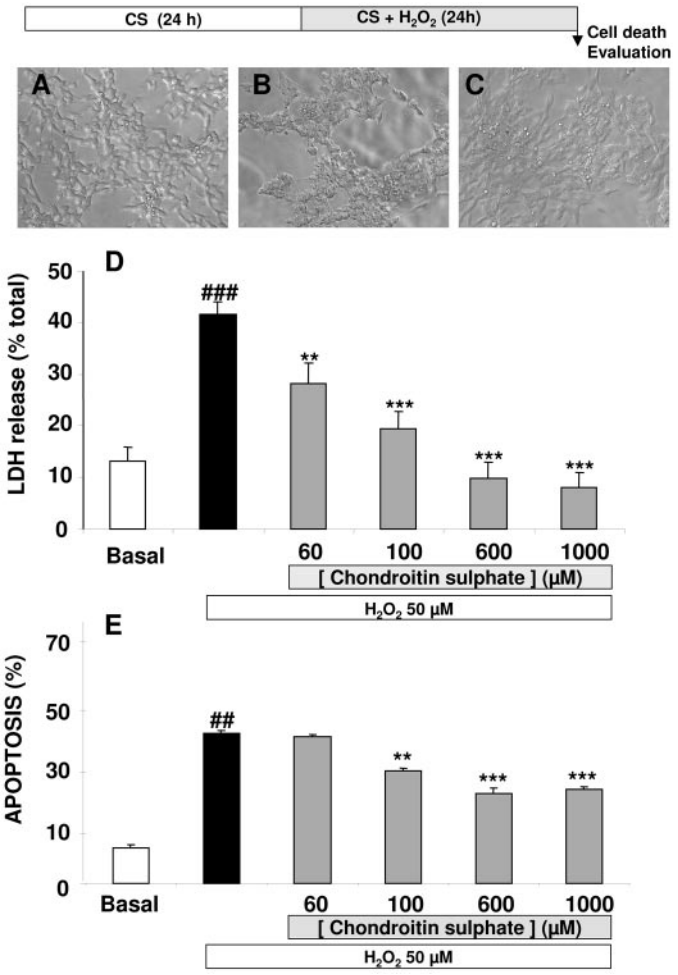

Fig. 2. CS causes concentration-dependent protection of SH-SY5H cells against H_2O_2-induced cell death. See experimental protocol on top of the figure. The microphotographs show phase-contrast images taken at 20× of control cells (A), cells treated for 24 h with 50 μM H_2O_2 (B), and cells pretreated 24 h with 600 μM CS before being exposed for another 24 h with 50 μM H_2O_2, still in the presence of CS (C). D, H_2O_2 damaging effects, measured as LDH release in the absence and the presence of increasing concentrations of 60 to 1000 μM CS. The cells were pretreated 24 h before and during exposure to the toxic stimuli. E, antiapoptotic effects of increasing concentrations of CS, measured by flow cytometry in propidium iodide-stained cells. Data correspond to the mean and S.E.M. of four to six different experiments; **, $p < 0.01$ and ***, $p < 0.001$ respect to H_2O_2-induced toxicity in the absence of CS (black column) and ###, $p < 0.001$ with respect to basal conditions.

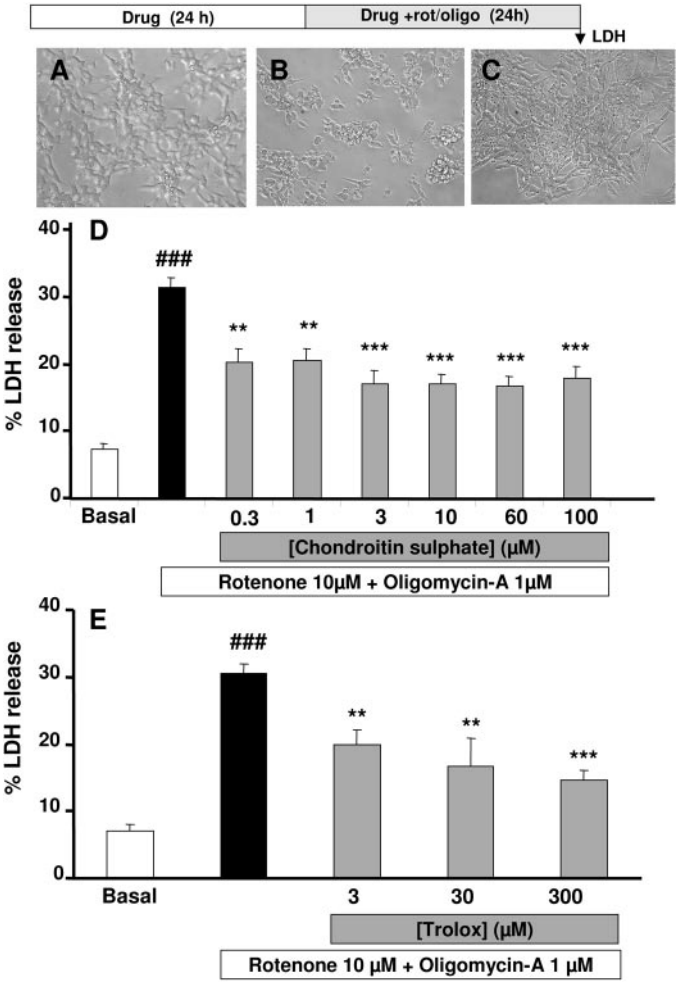

Fig. 3. CS protects SH-SY5H cells against rotenone + oligomycin-A-induced cell death. The experimental protocol used is represented on the top part of the figure. The microphotographs show phase-contrast images taken at 20× of control cells (A), cells treated for 24 h with 10 μM rotenone plus 1 μM oligomycin-A (Rot/oligo) (B), and cells pretreated 24 h with 60 μM CS before being exposed for another 24 h to Rot/oligo, still in the presence of Rot/oligo (C). D, damaging effects, measured as LDH release, elicited by Rot/oligo in the absence and presence of increasing concentrations of CS (0.3–100 μM). E, cell damage (LDH release; ordinate) caused by Rot/oligo in the absence and presence of increasing concentrations of Trolox. Data correspond to the mean and S.E.M. of five different experiments; **, $p < 0.01$ and ***, $p < 0.001$ respect Rot/oligo in the absence of Trolox (black column), and ###, $p < 0.001$ with respect to basal conditions (white column).

achieved at 3 μM; this protection was maintained up to 100 μM, the maximum concentration tested (Fig. 3D).

For comparative purposes, we evaluated the effects of Trolox (the active part of vitamin E) in this oxidative stress model. The results are represented in Fig. 3E; Trolox reduced cell death induced by Rot/oligo, although the concentrations required to obtain such protection were 10 times higher than those needed for CS. A similar pattern was observed when Trolox was used in the H_2O_2 model (data not shown).

Effect of CS on the Calcium Overload-Induced Toxicity. To gain information on the specificity of CS in protecting cells against oxidative stress, we decided to use another toxicity model. We treated the cells for 24 h with 70 mM K^+; this stimulus causes cell depolarization, opening of voltage-dependent calcium channels, and toxic calcium overloading. In this toxicity model, CS did not afford significant protection (Fig. 4). For comparative purposes, we also tested the calcium antagonist nimodipine that blocks L-type calcium channels in this model, that did afford significant protection (34.93%; $p < 0.001$, $n = 4$; data not shown). Therefore, CS was rather selective in protecting against death elicited by oxidative stress, but not against calcium overload-elicited cell death

CS Reduces ROS Production. Because CS was an effective neuroprotectant in different oxidative stress models, we studied how this drug could modify ROS production induced by H_2O_2 or Rot/oligo. For this purpose, we used the fluorescent dye H_2DCFDA that increases its fluorescence when free radicals are generated within the cell.

The microphotograph shown in Fig. 5A shows the basal fluorescence of SH-SY5Y cells loaded with the fluorescent dye H_2DCFDA. Fluorescence increased in H_2O_2 (50 μM)-treated cells (Fig. 5B), and it decreased when cells were pretreated with

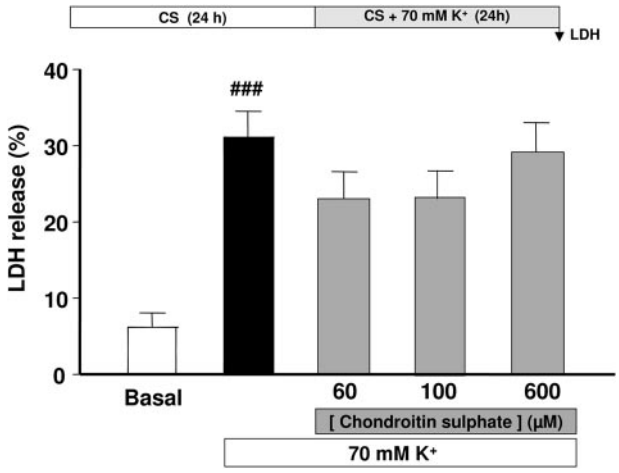

Fig. 4. CS does not afford significant protection against calcium overload toxicity. The cells were pretreated 24 h before and during exposure to the toxic stimuli as represented on the top part of the figure. The graph shows the damaging effects, measured as LDH release, caused by 24-h cell incubation with 70 mM K^+ in the absence and presence of increasing concentrations of CS (60–1000 μM). Data correspond to the mean and S.E.M. of six different experiments; ###, $p < 0.001$ with respect to basal conditions.

CS, before adding H_2O_2 (Fig. 5C). Figure 5G represents pooled data of six different cell cultures; addition of H_2O_2 to SH-SY5Y cells gave significant ROS production after 30- and 60-min incubation. When the cells were pretreated for 24 h with a neuroprotective concentration of CS against H_2O_2 toxicity, a significant reduction of fluorescence was observed.

A similar pattern emerged when ROS were generated by mitochondrial disruption with Rot/oligo. Microphotograph 5D hardly shows DCFH-positive cells in Rot/oligo untreated cells. However, when the cells were exposed to Rot/oligo, the number of fluorescent cells dramatically increased (Fig. 5E); when SH-SY5Y cells were pretreated with 10 μM CS for 24 h and then exposed to Rot/oligo for 30 min, the number of fluorescent cells decreased (Fig. 5F). Figure 5H represents pooled data of ROS production. The combination of Rot/oligo gave a significant increase of fluorescence after 30 and 60 min; pretreatment of the cells with CS 24 h before exposing them to Rot/oligo prevented the increased ROS production (Fig. 5H). These results show that CS pretreatment can reduce the formation of free radicals induced by H_2O_2 or the combination of Rot/oligo.

Protective Effect of CS Is PKC- and PI3K-Dependent but ERK-Independent. To analyze the signaling pathway that could participate in the neuroprotective mechanism of CS, we performed experiments with PKC, PI3K, mitogen-activated protein kinase kinase 1/2, and protein synthesis inhibitors. The protective effects of CS against Rot/oligo-induced toxicity were reversed by the protein synthesis inhibitor cycloheximide, the PKC inhibitor chelerythrine, and the PI3K inhibitor LY294002, but not by the mitogen-activated protein kinase kinase 1/2 antagonist PD98059 (Fig. 6A). These results show that PKC and PI3K/Akt, together with the synthesis of proteins, but not the ERK1/2 pathway are implicated in the protective effects of CS.

To determine whether CS was activating Akt, we treated SH-SY5Y cells with 10 μM CS for 30, 60, and 120 min, and cell lysates were resolved in SDS-PAGE and analyzed by immunoblot with Akt antibodies. CS caused maximum phosphorylation of Akt at 30 min; phosphorylation of Akt induced by CS was inhibited by the PKC inhibitor chelerythrine. These results suggest that activation of Akt by CS is PKC-dependent (Fig. 6B).

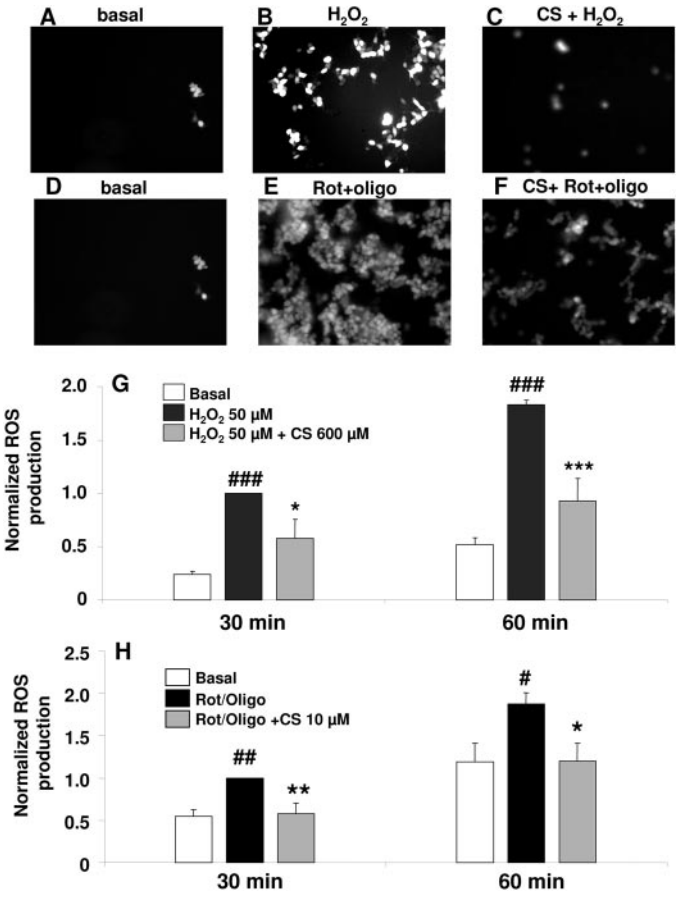

Fig. 5. CS reduced ROS production elicited by H_2O_2 and Rot/oligo. On the top part of the figure, DCFH fluorescence images, taken at 20× of SH-5YSY under basal conditions (A), after exposure to 50 μM H_2O_2 (B), or after 24-h pretreatment with 600 μM CS and exposed thereafter to 50 μM H_2O_2 (C). D to F, images correspond to a similar experiment as described above, but ROS were generated with the combination of Rot/oligo. G represents pooled data of ROS production, at 30- and 60-min incubation with H_2O_2, under basal conditions and after the addition of H_2O_2 in cells pretreated or not for 24 h with CS. H shows similar results as in G but in cells exposed to the combination of Rot/oligo. Values are expressed as means ± S.E.M. of seven different cell batches. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ compared with ROS donor. #, $p < 0.05$; ##, $p < 0.01$; and ###, $p < 0.001$ compared with basal.

Protective Effects of CS Are Related to Induction of Hemo Oxygenase-1. For these experiments, cells were incubated for 24 h in the presence of 10 μM CS, and cell lysates were resolved in SDS-PAGE and analyzed by immunoblot with anti-HO-1 antibody (Fig. 7A). CS increased by almost 2-fold the expression of HO-1, compared with control cells. Coincubation of the cells with CS and the PI3K/Akt inhibitor LY294002 reduced the overexpression of HO-1 to basal levels, indicating that PI3K/Akt was implicated in the overexpression of HO-1, induced by CS. Moreover, 24 h coincubation of the cells with CS, in the presence of chelerythrine, also reduced HO-1 expression to basal levels (Fig. 7B).

To further analyze the involvement of HO-1 in the protective effect of CS, we used SnPP, an HO-1 inhibitor (Marinissen et al., 2006). Preincubation of 10 μM CS during 24 h, followed by 24-h Rot/oligo, reduced cell death by 61% ($p <$

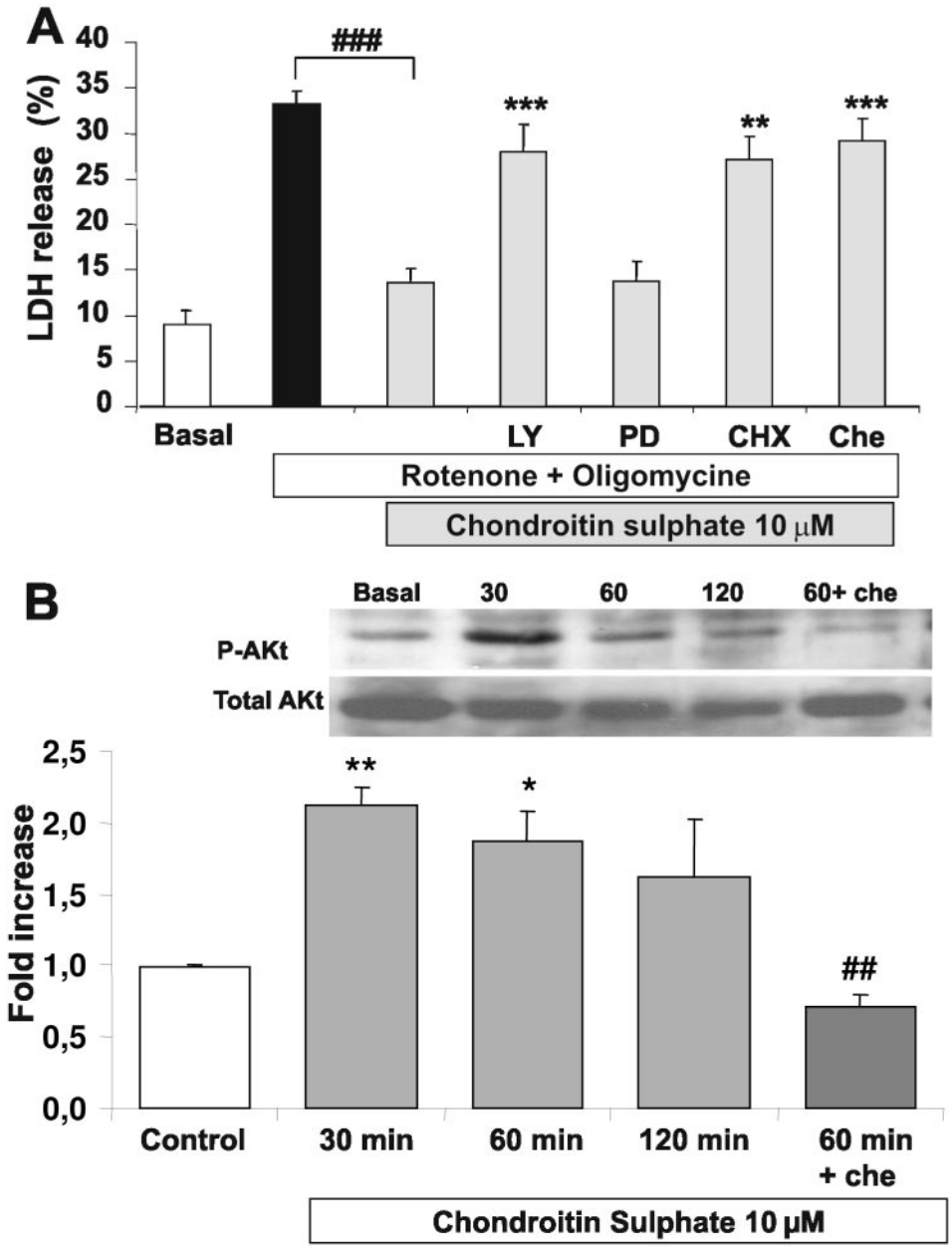

Fig. 6. Implication of PKC and the PI3K/AKt signaling pathway in the protective effects of CS. A, protective effects of CS were reverted by the PI3K/Akt antagonist LY294002 (10 μM), the protein synthesis blocker cycloheximide (1 μM), and the PKC inhibitor chelerythrine (0.1 μM). However, the mitogen-activate protein kinase inhibitor PD98059 (10 μM) did not revert the protection afforded by CS. All the antagonists were coincubated with CS 24 h before adding the toxic stimuli, and they were present during the 24-h incubation with the toxic compound. Data correspond to the mean and S.E.M. of seven different experiments; ###, $p < 0.001$ with respect to Rot/oligo-induced toxicity; **, $p < 0.01$ and ***, $p < 0.001$ respect to CS protection. B, top, immunoblots of phosphorylated-Akt and total Akt in SH-SY5Y cells treated for 30, 60, and 120 min with 10 μM CS and cells treated for 60 min in the presence of CS and the PKC blocker chelerythrine (0.1 μM). Bottom, densitometric quantification of phosphorylated-Akt levels compared with total Akt. Values are means ± S.E.M. of at least five different cell batches. *, $p < 0.05$ and **, $p < 0.01$ compared with control cells; and ##, $p < 0.01$ compared with CS-treated cells.

0.001); such protection was lost when the cells were coincubated with CS in the presence of 30 μM SnPP ($p < 0.01$). These results indicate that HO-1 is participating in the protective effect afforded by CS.

Discussion

In this study, we have shown that an endogenous component of the perineuronal net, the GAG chondroitin sulfate, can protect SH-SY5Y against oxidative stress via PI3K/Akt and induction of the antioxidant enzyme HO-1.

We have used two models of free radical-induced toxicity to evaluate whether CS could be protective. The first model consists of an exogenous source of ROS produced by incubating the cells with H_2O_2, and the second model consists in the interruption of the respiratory chain at complexes I (rotenone) and V (oligomycin-A); in this case, mitochondria are depolarized, ROS production is augmented, and a vicious circle leads to cell death. CS was able to protect neuroblastoma cells against both stimuli, although some differences were observed. For example, protection against Rot/oligo was achieved at lower concentrations (0.3–3 μM) of CS than those required to protect against H_2O_2 (60–1000 μM). However, in terms of protection, CS was more efficient against exogenous than endogenous ROS production, i.e., it completely prevented cell death induced by H_2O_2 but reduced partially (around 50%) that induced by Rot/oligo. A possible explanation for this result could be that the high concentrations of CS used to protect against H_2O_2 could be exerting a direct free radical scavenging effect of extracellular H_2O_2. For example, the GAG chains of the perineuronal net have been described to provide highly charged structures in the microenvironment of neurons that could potentially act as a buffering system for physiologically relevant ions such as calcium, potassium, and sodium (Morawski et al., 2004). Through

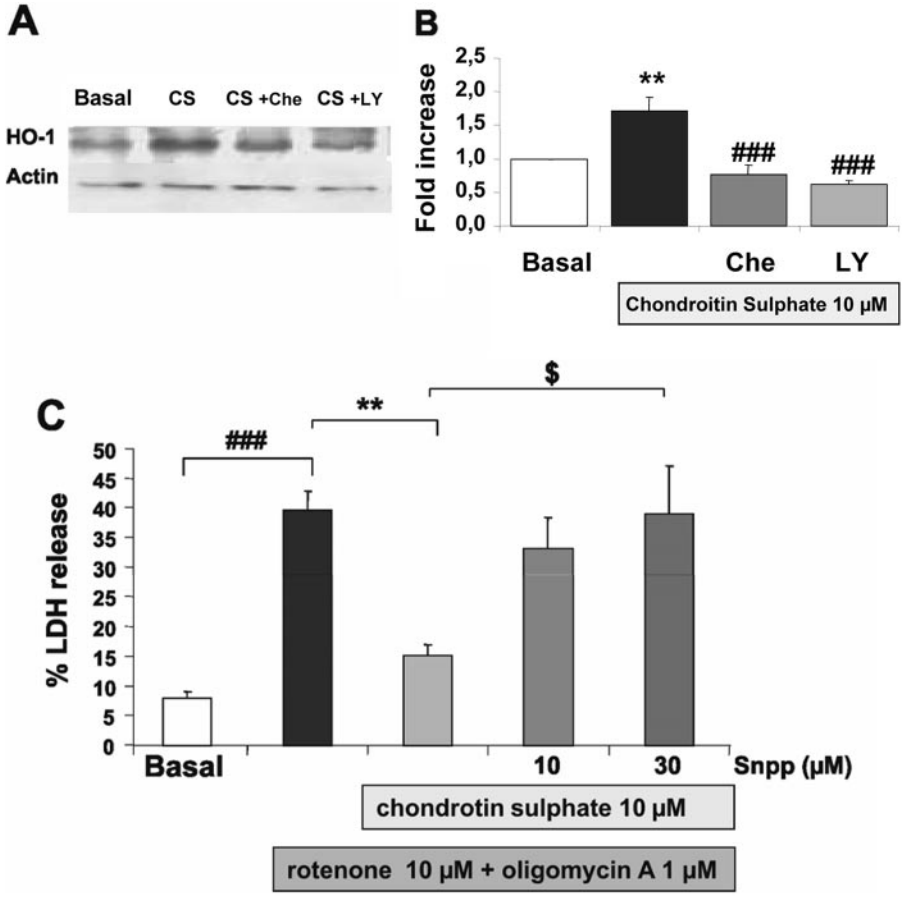

Fig. 7. CS induces HO-1 synthesis and its protective effect is related to this antioxidant enzyme. A, immunoblot showing HO-1 induction in SH-SY5Y cells incubated for 24 h with CS in the absence or in the presence of 10 μM LY294002 and 0.1 μM chelerythrine. B, densitometric quantification of HO-1 protein levels using β-actin for normalization. Values are means $\pm$ S.E.M. of six different cell batches. **, $p < 0.01$ compared with control cells; and ###, $p < 0.001$ compared with CS-treated cells. C, cells were preincubated 24 h either with CS alone or with CS in the presence of 10 and 30 μM SnPP, a HO-1 blocker. After this preincubation period, cells were incubated 24 h with Rot/oligo. Values are expressed as means $\pm$ S.E.M. of four different cell batches. ###, $p < 0.001$ compared with control cells; **, $p < 0.01$ compared with Rot/oligo-lesioned cells in the absence or presence of CS; and \$, $p < 0.05$ compared with cells in presence or absence of SnPP.

scavenging and binding of redox active ions, perineuronal nets might be able to neutralize or reduce potentially deleterious local oxidative potential in the neuronal microenvironment, thereby protecting neurons unsheathed by the perineuronal net against sequelae of oxidative stress.

The protective effects of GAGs in oxidative stress models have been previously reported by other groups, although the described mechanisms differ to the one described in this study. For example, GAGs such as hyaluronic acid and CS have been described to have antioxidant activity in "in vitro" and "in vivo" experimental models (Arai et al., 1999; Campo et al., 2004) by a mechanism related to their capacity to chelate transition metals such as Cu^{2+} or Fe^{2+} that are, in turn, responsible of the initiation of Harber-Weiss and Fenton's reaction. In contrast, Campo and coworkers have shown that hyaluronic acid and C4S can reduce death by reducing protein oxidation, OH generation, and lipid peroxidation by improving antioxidant defense as shown by an increase in superoxide dismutase and catalase activity (Campo et al., 2004). However, the results of our study support an additional mechanism that is related to the induction of the antioxidant enzyme HO-1.

HO is an antioxidant enzyme with two isoforms: an inducible isoform HO-1 and a constitutive isozyme HO-2. HO-1 is induced in response to a variety of stress-inducing pathological conditions (Keyse and Tyrrell, 1987); moreover, studies in HO-1-deficient mice have confirmed that the HO system is indispensable for cell protection against oxidative stress (Poss and Tonegawa, 1997). In neurons, there is a low expression of HO-1 (Maines, 2004), and its induction is related with protection against H_2O_2 (Kim et al., 2005), focal ischemia (Nimura et al., 1996), and glutamate excitotoxicity (Parfenova et al., 2006). Furthermore, in neurodegenerative diseases such as Alzheimer's disease, it has been shown that in the postmortem brains of these patients, there was HO-1 induction in neurons of the cerebral cortex and the hippocampus, and HO-1 was colocalized with neurofibrillary tangles (Schipper et al., 1995). Therefore, it is generally accepted that HO-1 represents a physiological protective mechanism against oxidative stress. In this study, CS was able to up-regulate HO-1 and this effect was related to the protective effects of CS because when the activity of HO-1 was blocked with SnPP, the protective effects of CS were lost.

The fact that CS-induced phosphorylation of Akt was prevented by the PKC inhibitor chelerythrine indicates that the upstream signaling pathway responsible for phosphorylation of Akt is PKC. Up-regulation of HO-1 was also prevented by chelerythrine and LY294002, indicating that PKC and the PI3K/AKt pathway are participating in the induction of this antioxidant enzyme. Our results are consistent with those found for the cytoprotective effects of neurotrophic growth factor against the parkinsonian neurotoxin 6-hydroxydopamine, where Salinas et al. (2003) reported neurotrophic growth factor-induced up-regulation of HO-1 expression by a PI3K-dependent mechanism. The participation of the survival pathway PI3K/AKt in the regulation of HO-1 has also been described in other cellular contexts, including the response to interleukin-10, hepatocyte growth factor (Ricchetti et al., 2004; Tacchini et al., 2004), endotoxin, arsenite, hemin, and carnosol (Arruda et al., 2004; Martin et al., 2004; Chung et al., 2005; Ivanov and Hei, 2005).

An interesting observation of this study was that the induction of HO-1 by CS was not very high, suggesting a physiological role for neuroprotection. High induction of HO-1 activity as that observed in senile plaques of Alzheimer's disease patients, and Lewy bodies of Parkinson's disease or following induction with hemin, may not be compatible with cell viability because it compromises the availability of heme for hemoproteins function and because the release of high levels of free iron may in turn result in free radical production through Fenton reaction (Schipper, 2004). A modest increase in HO-1 activity results in a modest increase in biliverdin/bilirubin, and it has been reported that nanomolar amounts of bilirubin reduce micromolar amounts of H_2O_2 (Doré et al., 1999). Therefore, a modest increase in HO-1 activity would provide efficient antioxidant protection without compromising cell viability. Considering that neurons are subjected to high levels of ROS, the induction of HO-1 by CS could attenuate oxidative stress and cell death induced by H_2O_2 or mitochondrial disruption by blockade of complexes I and V of the respiratory mitochondrial chain.

The induction of the antioxidant enzyme HO-1 correlated to the limitation of ROS production as shown in the experiments with the fluorescent dye H_2DCFDA; CS was able to reduce both exogenous ROS production induced by H_2O_2 treatment and endogenous ROS secondary to mitochondrial disruption.

In summary, this study shows that CS can afford protection of SH-SY5Y neuroblastoma cells under oxidative stress conditions by induction of the antioxidant enzyme HO-1 via Akt/PI3K pathway. This finding may have therapeutic implications because CS, or its smaller active fragments, could have neuroprotective properties that might be useful in the acute treatment of stroke, to prevent neuronal damage and the ensuing neurological sequelae. Experiments in animal models of cerebral ischemia may be useful to test this hypothesis.

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