Chondroitin sulphate protects SH-SY5Y cells from oxidative stress by inducing Heme Oxygenase-1 via PI3K/Akt

Noelia Cañas, Teresa Valero, Mercedes Villarroya, Laia Montell L, Josep Vergés, Antonio G. García, Manuela G. López

Instituto Teófilo Hernando and Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid, 28029 Madrid, Spain. (NC, TV, MV, AGG, MGL) Servicio de Farmacología Clínica. Hospital Universitario la Princesa. Madrid. Spain (AGG) Instituto Universitario de Investigación Gerontológico y Metabólica, Hospital de la Princesa, 28006 Madrid, Spain (MGL, AGG) Bioibérica SA. Barcelona. Spain (LM, JV)

Running title: Neuroprotective effects of chondroitin sulphate

Corresponding author: Manuela G. López

Departamento de Farmacología, Facultad de Medicina,

Universidad Autónoma de Madrid

C/ Arzobispo Morcillo 4 E-28029 Madrid. Spain

E-mail: manuela.garcia@uam.es

Phone: +34-914975386 Fax: +34-914973120

Number text pages: 28

Number of tables: 0

Number of figures: 7

Number of references: 40

Number of words in Abstract: 218

Number of words in Introduction: 634

Number of words in Discussion: 1078

Abbreviations: CS: Chondroitin Sulphate, LDH: Lactate Dehidrogenase, ROS: Reactive Oxygen Species, DCFDA : 2´,7´-dichlorofluorescein diacetate, PKC: Protein Kinase C, PG: Proteoglicane, GAG: Glycosaminoglycane, BV: Biliverdin, BR: Bilirubin, HO-1: Heme Oxygenase 1: NGF: Neurotrophic Growth Factor, CSPG: Chondroitin Sulphate Proteoglicanes.

Section assignment: Neuropharmacology

ABSTRACT

INTRODUCTION

Proteoglycans (PGs) represent a diverse class of complex macromolecules that share a general molecular structure, comprising 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-acetylhexosamine), which are either or both sulphated (Hascall and Sajdera, 1970). The composition of the GAG chains in the CNS varies with development. For example, the four-carbon sulphated GAG is most abundantly expressed in the developing CNS (Kitagawa et al., 1997) while in the adult animal, the six-carbon sulphated GAG is upregulated after CNS injury (Lemons et al., 2003; Properzi et al., 2005).

PGs can be classified as heparan sulphate, dermatan sulphate, keratin sulphate or CS proteoglycans (CSPGs). The PG´s core proteins can be secreted into the extracellular matrix; CSPGs are soluble, and other PGs like heparan sulphate and keratan sulphate 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 sulphated 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 sulphatation, which confers different biological properties and functions to the different types of CSPGs (Sugahara et al., 2003).

CS has crucial functions in growth factor signalling, wound repair, morphogenesis, infection and cell division (Sugahara et al., 2003). Also, proteoglygans 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 excytotoxic concentrations of glutamate (Sugiura and Dow, 1994).

The neuroprotective effects of PGs have been described in several studies. For example, neurocan is overexpresed after ischemia in adult rat brain (Deguchi et al., 2005), heparan sulphate is released from synaptic vesicles upon the addition of glutamate, (Sugiura and Dow, 1994), and CS has shown to protect neurons against glutamate and excitatory amino acid induced injury (Okamoto et al., 1994a; Okamoto et al., 1994b). In the case of neurodegenerative diseases like Alzheimer´s, cortical areas highly rich in perineural nets are less severely affected by neurofibrillary degeneration, and the perineuronal net associated neurons are devoid of tangles (Bruckner 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) 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 prooxidant heme group and produces equimolecular quantities of carbon monoxide (CO), iron, and biliverdin (BV). BV is subsequently reduced to bilirubin (BR) 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 upregulated 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 oxidative stress injury (blockade of the mitochondrial respiratory chain) by inducing HO-1 via the
PI3K/Akt intracellular signalling pathway.

MATERIALS AND METHODS

Materials

Chondroitin sulphate (CS), composed of a chain of alternating sugars D-glucuronic acid beta(1-3)D –N- acetyl galactosamine beta (1-4) sulphated 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 2-(2-amino-3methoxyphenyl)-4*H*-1-benzopyran-4-one (PD98059) were purchased from Tocris (Biogen
Científica, Spain). Sn (IV) protoporphyrin IX dichloride (SnPP) was obtained from Frontier Scientific Europe (Lancashire, United Kingdom). Dulbecco´s Modified Eagle´s Medium (DMEM), foetal calf serum, and penicillin/streptomycin were purchased from
GIBCO (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 UAM/CSIC, Madrid, Spain. SH-SY5Y cells, at passages between 3 and 16 after de-freezing, were maintained in a DMEM supplemented with 10% foetal calf serum (FCS), 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. SH-SY5Y cells were seeded into flasks containing supplemented medium and maintained at 37ºC in 5% CO_2/humidified air. Stock cultures were passaged 1:3 twice weekly. For assays, SHSY5Y cells were sub-cultured in 48 well plates at a seeding density of 10^5 cells per well, or in 6 well plates at a seeding density

of $5x10^5$ cells per well. Cells were treated with the drugs before confluence in serum free DMEM.

Measurement of lactic dehydrogenase (LDH) 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 X100 in water (intracellular LDH). Extracellular and intracellular LDH activity was spectrophotometrically measured using a cytotoxicity cell death kit (RocheBoehringer Mannheim) according to the manufacturer's indications. 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 (Mosman, 1983). Following the experiments, MTT was added to each well at a final concentration of 0.5 mg/ml in Krebs-HEPES solution (in mM: NaCl, 144; KCl, 5.9; $MgCl_2$, 1.2; $CaCl_2$, 2; HEPES, 10; glucose, 11; pH 7.3), and incubation at 37ºC was continued for an additional 2 h period. Then, the insoluble formazan was dissolved with dimethylsulfoxide; 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) (Robinson, 1997). Cells were grown in 6-well plates until they reached 50% confluence (typically after 2448h 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 PBS by pipetting thoroughly to avoid cell clumping. The cell suspension was transferred to 4.5 ml 70% cold ethanol and kept in this fixative for a minimum of 2 h at 4ºC. Ethanol– fixed cells were centrifuged and washed once with 10 ml PBS. Finally, the cell pellet was suspended in 1 ml of PI/RNase staining buffer solution (BD Pharmigen) and incubated for

15 min at 37ºC. Samples were analyzed by flow cytometry (FACS-Calibur, Beckton Dickinson). 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 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 G0/G1 DNA content in the PI intensity-area histogram plot.

ROS measurement

To measure cellular reactive oxygen species (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 non-fluorescent form dichlorofluorescein (DCFH). DCFH reacts with intracellular H_2O_2 to form dichlorofluorescin (DCF), a green fluorescent dye. Fluorescence was measured in a fluorescence microplate reader (FLUOstar Galaxy, BMG, 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 ($5x10^5$) were washed once with cold phosphate-buffered saline and lysed in 100 µl 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 (Amersham Biosciences). Membranes were incubated with anti-total-AKT (1:1000), antiphospho-AKT (1:1000) (Santa Cruz Biotechnology); anti-HO-1 (1:1000) (Chemicon); anti-β-actin (1:100000) (Sigma). Appropriate peroxidase-conjugated secondary antibodies
(1:10000) 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. Immunoblots correspond to a representative experiment that was repeated four to eight times with similar results. Data are given as means ± s.e.m. Differences between groups were determined by applying a one-way ANOVA followed by Tuckey 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). On the other hand, pre-treatment of the cells with 10 or 60 µM of 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 sulphate 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 H_2O_2 (50 μM) increased basal cell death, measured as % 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).

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 rotenone (10 μM) plus oligomycin-A (1 μM) (Rot/oligo) respectively. As a result of mitochondrial disruption, the cell can not 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 panel A and cells treated with CS (60 μM) plus Rot/oligo, in panel C. 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 prior and during the toxic stimulus, afforded significant protection already at 0.3 μM. Maximum protection (48%) was 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 vitamine E) in this oxidative stress model. The results are represented in figure 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

In order 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

Since 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 DCFDA 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 DCFDA. Fluorescence increased in H_2O_2 (50 μM) treated cells (Fig 5B) and decreased when cells were pre-treated with CS, before adding H_2O_2 (Fig 5C).

Figure 5G represents pooled data of 6 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 pre-treated 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 DFCDA 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 pre-treated with CS 10 μM 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; pre-treatment of the cells with CS 24 h before exposing them to Rot/oligo, totally prevented the increased ROS production (Fig. 5H).

These results show that CS pre-treatment can reduce the formation of free radicals induced by H_2O_2 or the combination of Rot/oligo.

The protective effect of CS is PKC and PI3K-dependent but ERK independent

To analyze the signalling pathway that could participate in the neuroprotective mechanism of CS we performed experiments with PKC, PI3K, MEK1/2 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 chelerythrin and the PI3K inhibitor LY 294002, but not by the MEK1/2 antagonist PD 98059 (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 if 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 chelerythrin.

These results suggest that activation of Akt by CS is PKC dependent (Fig. 6B).

The protective effects of CS are related to the induction of hemo oxygenase-1

For these experiments, cells were incubated for 24 h in the presence of CS (10 μM) and cell lysates were resolved in SDS-PAGE and analysed by immunoblot with anti-HO-1 antibody (Fig. 7A); CS increased by almost two-fold the expression of HO-1, in comparison to control cells. Co-incubation of the cells with CS and the PI3K/Akt inhibitor LY 294002 reduced the over-expression of HO-1 to basal levels, indicating that PI3K/Akt was implicated in the over-expression of HO-1, induced by CS. Moreover, 24 h coincubation of the cells with CS, in the presence of chelerythrin, also reduced HO-1 expression to basal levels (Fig. 7B).

To further analyse the involvement of HO-1 in the protective effect of CS, we used Sn(IV) protoporphyrin IX (SnPP), a HO-1 inhibitor (Marinissen et al., 2006). Preincubation of CS (10 μM) during 24 h, followed by 24 h Rot/oligo, reduced cell death by 61% ($p<0.001$); such protection was lost when the cells were co-incubated 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 sulphate, 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 if CS could be protective. The first one consists of an exogenous source of ROS produced by incubating the

cells with H_2O_2 and the second 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 instance, protection against Rot/oligo was achieved at lower concentrations (0.3 to 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 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 like 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 like Cu^{2+} or Fe^{2+} that are, in turn, responsible of the initiation of Harber-Weiss and Fenton´s reaction. On the other hand, Campo and co-workers have shown that hyaluronic acid and C4S can reduce death by reducing protein oxidation, OH generation and lipid peroxidation by improving antioxidant defence as shown by an increase in SOD 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 great variety of stressinducing pathological conditions (Keyse and Tyrrell, 1987); moreover, studies in HO-1deficient 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 post-mortem brains of these patients, there was HO-1 induction in neurons of the cerebral cortex and the hippocampus, and HO-1 was co-localized 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 upregulate 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 signalling pathway responsible for phosphorylation of AKt is PKC. Upregulation 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 NGF against the parkinsonian neurotoxin 6hydroxydopamine where Salinas and co-workers (2003) reported NGF-induced upregulation 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 IL-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 (Dore 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 DCFDA; 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.

REFERENCES

Arai H, Kashiwagi S, Nagasaka Y, Uchida K, Hoshii Y and Nakamura K (1999) Oxidative modification of apolipoprotein E in human very-low-density lipoprotein and its inhibition by glycosaminoglycans. *Arch Biochem Biophys* **367**:1-8.

Arruda MA, Rossi AG, de Freitas MS, Barja-Fidalgo C and Graca-Souza AV (2004) Heme inhibits human neutrophil apoptosis: involvement of phosphoinositide 3-kinase, MAPK, and NF-kappaB. *J Immunol* **173**:2023-2030.

Bruckner G, Hausen D, Hartig W, Drlicek M, Arendt T and Brauer K (1999) Cortical areas abundant in extracellular matrix chondroitin sulphate proteoglycans are less affected by cytoskeletal changes in Alzheimer's disease. *Neuroscience* **92**:791-805.

Campo GM, Avenoso A, Campo S, D'Ascola A, Ferlazzo AM and Calatroni A (2004) The antioxidant and antifibrogenic effects of the glycosaminoglycans hyaluronic acid and chondroitin-4-sulphate in a subchronic rat model of carbon tetrachlorideinduced liver fibrogenesis. *Chem Biol Interact* **148**:125-138.

Chung SW, Chen YH and Perrella MA (2005) Role of Ets-2 in the regulation of heme oxygenase-1 by endotoxin. *J Biol Chem* **280**:4578-4584.

Deguchi K, Takaishi M, Hayashi T, Oohira A, Nagotani S, Li F, Jin G, Nagano I, Shoji M, Miyazaki M, Abe K and Huh NH (2005) Expression of neurocan after transient middle cerebral artery occlusion in adult rat brain. *Brain Res* **1037**:194-199.

Dore S, Sampei K, Goto S, Alkayed NJ, Guastella D, Blackshaw S, Gallagher M, Traystman RJ, Hurn PD, Koehler RC and Snyder SH (1999) Heme oxygenase-2 is neuroprotective in cerebral ischemia. *Mol Med* **5**:656-663.

Fox K and Caterson B (2002) Neuroscience. Freeing the bain from the perineuronal net. *Science* **298**:1187-1189.

Hascall VC and Sajdera SW (1970) Physical properties and polydispersity of proteoglycan from bovine nasal cartilage. *J Biol Chem* **245**:4920-4930.

Ivanov VN and Hei TK (2005) Combined treatment with EGFR inhibitors and arsenite upregulated apoptosis in human EGFR-positive melanomas: a role of suppression of the PI3K-AKT pathway. *Oncogene* **24**:616-626.

Keyse SM and Tyrrell RM (1987) Both near ultraviolet radiation and the oxidizing agent hydrogen peroxide induce a 32-kDa stress protein in normal human skin fibroblasts. *J Biol Chem* **262**:14821-14825.

Kim YS, Zhuang H, Koehler RC and Dore S (2005) Distinct protective mechanisms of HO-1 and HO-2 against hydroperoxide-induced cytotoxicity. *Free Radic Biol Med* **38**:85-92.

Kitagawa H, Tsutsumi K, Ujikawa M, Goto F, Tamura J, Neumann KW, Ogawa T and Sugahara K (1997) Regulation of chondroitin sulfate biosynthesis by specific sulfation: acceptor specificity of serum beta-GalNAc transferase revealed by structurally defined oligosaccharides. *Glycobiology* **7**:531-537.

Koh JY and Choi DW (1987) Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *J Neurosci Methods* **20**:83-90.

LeBel CP, Ischiropoulos H and Bondy SC (1992) Evaluation of the probe 2',7'dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. *Chem Res Toxicol* **5**:227-231.

Lemons ML, Sandy JD, Anderson DK and Howland DR (2003) Intact aggrecan and chondroitin sulfate-depleted aggrecan core glycoprotein inhibit axon growth in the adult rat spinal cord. *Exp Neurol* **184**:981-990.

Maines MD (2004) The heme oxygenase system: past, present, and future. *Antioxid Redox Signal* **6**:797-801.

Mariani E, Polidori MC, Cherubini A and Mecocci P (2005) Oxidative stress in brain aging, neurodegenerative and vascular diseases: an overview. *J Chromatogr B Analyt Technol Biomed Life Sci* **827**:65-75.

Marinissen MJ, Tanos T, Bolos M, de Sagarra MR, Coso OA and Cuadrado A (2006) Inhibition of heme oxygenase-1 interferes with the transforming activity of the Kaposi sarcoma herpesvirus-encoded G protein-coupled receptor. *J Biol Chem* **281**:11332-11346.

Martin D, Rojo AI, Salinas M, Diaz R, Gallardo G, Alam J, De Galarreta CM and Cuadrado A (2004) Regulation of heme oxygenase-1 expression through the phosphatidylinositol 3-kinase/Akt pathway and the Nrf2 transcription factor in response to the antioxidant phytochemical carnosol. *J Biol Chem* **279**:8919-8929.

Mosman, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Meth.* **65**, 55-63

Morawski M, Bruckner MK, Riederer P, Bruckner G and Arendt T (2004) Perineuronal nets potentially protect against oxidative stress. *Exp Neurol* **188**:309-315.

Nimura T, Weinstein PR, Massa SM, Panter S and Sharp FR (1996) Heme oxygenase-1 (HO-1) protein induction in rat brain following focal ischemia. *Brain Res Mol Brain Res* **37**:201-208.

Okamoto M, Mori S and Endo H (1994a) A protective action of chondroitin sulfate proteoglycans against neuronal cell death induced by glutamate. *Brain Res* **637**:57-67.

Okamoto M, Mori S, Ichimura M and Endo H (1994b) Chondroitin sulfate proteoglycans protect cultured rat's cortical and hippocampal neurons from delayed cell death induced by excitatory amino acids. *Neurosci Lett* **172**:51-54.

Parfenova H, Basuroy S, Bhattacharya S, Tcheranova D, Qu Y, Regan RF and Leffler CW (2006) Glutamate induces oxidative stress and apoptosis in cerebral vascular endothelial

cells: contributions of HO-1 and HO-2 to cytoprotection. *Am J Physiol Cell Physiol* **290**:C1399-1410.

Poss KD and Tonegawa S (1997) Reduced stress defense in heme oxygenase 1-deficient cells. *Proc Natl Acad Sci U S A* **94**:10925-10930.

Properzi F, Carulli D, Asher RA, Muir E, Camargo LM, van Kuppevelt TH, ten Dam GB, Furukawa Y, Mikami T, Sugahara K, Toida T, Geller HM and Fawcett JW (2005) Chondroitin 6-sulphate synthesis is up-regulated in injured CNS, induced by injuryrelated cytokines and enhanced in axon-growth inhibitory glia. *Eur J Neurosci* **21**:378-390.

Ricchetti GA, Williams LM and Foxwell BM (2004) Heme oxygenase 1 expression induced by IL-10 requires STAT-3 and phosphoinositol-3 kinase and is inhibited by lipopolysaccharide. *J Leukoc Biol* **76**:719-726.

Robinson JP, Darzynkiewicz, Z., Dean, P.N., Orfao, A., Rabinovitch, P.S., Stewart, C.C., Tanke, H.J. and Weeless, L.L., (1997) *Current Protocols in Cytometry*. John Wiley & Sons, Inc, New York.

Saito A, Maier CM, Narasimhan P, Nishi T, Song YS, Yu F, Liu J, Lee YS, Nito C, Kamada H, Dodd RL, Hsieh LB, Hassid B, Kim EE, Gonzalez M and Chan PH (2005) Oxidative stress and neuronal death/survival signaling in cerebral ischemia. *Mol Neurobiol* **31**:105-116.

Salinas M, Diaz R, Abraham NG, Ruiz de Galarreta CM and Cuadrado A (2003) Nerve growth factor protects against 6-hydroxydopamine-induced oxidative stress by increasing expression of heme oxygenase-1 in a phosphatidylinositol 3-kinasedependent manner. *J Biol Chem* **278**:13898-13904.

Schipper HM (2004) Heme oxygenase expression in human central nervous system disorders. *Free Radic Biol Med* **37**:1995-2011.

Schipper HM, Cisse S and Stopa EG (1995) Expression of heme oxygenase-1 in the senescent and Alzheimer-diseased brain. *Ann Neurol* **37**:758-768.

Seidenbecher CI, Richter K, Rauch U, Fassler R, Garner CC and Gundelfinger ED (1995) Brevican, a chondroitin sulfate proteoglycan of rat brain, occurs as secreted and cell surface glycosylphosphatidylinositol-anchored isoforms. *J Biol Chem* **270**:2720627212.

Sugahara K, Mikami T, Uyama T, Mizuguchi S, Nomura K and Kitagawa H (2003) Recent advances in the structural biology of chondroitin sulfate and dermatan sulfate. *Curr Opin Struct Biol* **13**:612-620.

Sugiura M and Dow KE (1994) Activity-dependent regulation of neuronal synthesis and release of neurite-promoting heparan sulfate proteoglycans. *Dev Biol* **164**:102-110.

Tacchini L, De Ponti C, Matteucci E, Follis R and Desiderio MA (2004) Hepatocyte growth factor-activated NF-kappaB regulates HIF-1 activity and ODC expression, implicated in survival, differently in different carcinoma cell lines. *Carcinogenesis* **25**:2089-2100.

Vitali SH, Mitsialis SA, Christou H, Fernandez-Gonzalez A, Liu X and Kourembanas S (2005) Mechanisms of heme oxygenase-1-mediated cardiac and pulmonary vascular protection in chronic hypoxia: roles of carbon monoxide and bilirubin.

Chest **128**:578S-579S.

Wang JY, Shum AY, Ho YJ and Wang JY (2003) Oxidative neurotoxicity in rat cerebral cortex neurons: synergistic effects of H2O2 and NO on apoptosis involving activation of p38 mitogen-activated protein kinase and caspase-3. *J Neurosci Res*

72:508-519.

FOOTNOTES

We acknowledge the financial support from Bioiberica SA (Barcelona, Spain), the Ministry of Science and Technology Ref. SAF2006-085540 to MGL and the Spanish Ministry of Health (Instituto de Salud Carlos III) RETICS-RD06/0026 and Comunidad Autonoma de Madrid SAL2006/0275 to AGG, and to Mutua Madrileña grants. We also thank the Fundación Teófilo Hernando for continued support. N. C. has a Mutua Madrileña predoctoral fellowship.

This work was previously presented:

- in a poster at the 10th International Conference of Alzheimer's Disease. 2006, Madrid Spain. Cañas C, Valero T, Montell L, Vergés J, García AG, López MG. "Chondroitin Sulphate promotes neuroprotection against free radicals-induced damage in vitro".

Reprint requests:

Dr. Manuela G. López

Departamento de Farmacología, Facultad de Medicina, Universidad Autónoma de Madrid

C/ Arzobispo Morcillo, 4

28029 Madrid, Spain

Fax: +34-914973120; Phone: +34-914975386

E-mail: **manuela.garcia@uam.es**

LEGENDS FOR FIGURES

Figure 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 extracelular medium was measured as an index of cell death. Data correspond to the mean and S.E.M of triplicates of 4 different batches of cells; $p<0.05$ with respect to basal untreated cells. (B) Analysis of the % of cells under the different cell cycle phase, after incubating the cells for 48h in the presence or absence of CS 10 or 60 μM. Data correspond to the mean and S.E.M of 4 experiments. (C) The 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 4 different batches of cells

Figure 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 20X of control cells (A), cells treated for 24 h with H_2O_2 50 μM (B) and cells pre-treated 24 h with 600 μM CS before being exposed for another 24 h with H_2O_2 50 μM, still in the presence of CS (C). Panel D represents the H_2O_2 damaging effects, measured as LDH release in the absence and the presence of increasing concentrations of CS (60-1000 μM). The cells were pre-treated 24 h before and during exposure to the toxic stimuli. Panel E shows the 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 4-6 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.

Figure 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 20X of control cells (A), cells treated for 24 h with 10 μM rotenone plus 1 μM oligomycin-A (Rot/oligo) (B) and cells pre-treated 24 hours with 60 μM CS before being exposed for another 24 h to Rot/Oligo, still in the presence of Rot/oligo (C). Panel D represents the damaging effects, measured as LDH release, elicited by Rot/oligo in the absence and presence of increasing concentrations of CS (0.3-100 μM). Panel E shows 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 5 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).

Figure 4: CS does not afford significant protection against calcium overload toxicity. The cells were pre-treated 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 6 different experiments; ###p<0.001 with respect to basal conditions.

Figure 5: CS reduced ROS production elicited by H_2O_2 and Rot/oligo. On the top part of the figure, DCFDA fluorescence images, taken at 20X, of SH-5YSY under basal conditions (A), after exposure to 50 μM H_2O_2 (B) or after 24h pre-treatment with CS 600 μM and exposed thereafter to 50 μM H_2O_2 (C). D, E and F images correspond to a similar experiment as 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 pre-treated 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 7 different cell batches. *p<0.05, **p<0.01 and *** p<0.001 in comparison to the ROS donor. # p<0.05, ## p<0.01 ### p<0.001 in comparison to basal.

Figure 6: Implication of PKC and the PI3/AKt signalling pathway in the protective effects of CS. (A) The 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 MAPK inhibitor PD98059 (10 μM) did not revert the protection afforded by CS. All the antagonists were co-incubated 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 7 different experiments; ###p<0.001 with respect to Rot/oligo induced toxicity; **p<0.01 and ***p<0.001 respect to CS protection. (B) The top part of the figure shows 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). The bottom part of the figure represents the densitometric quantification of P-Akt levels in comparison to total Akt. Values are means ± S.E.M. of at least 5 different cell batches. *p<0.05 **p<0.01 in comparison to control cells and ## p<0.01 in comparison to CS-
treated cells

Figure 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 ± S.E.M. of 6 different cell batches. **p<0.01 in comparison to control cells and, ###p<0.001 in comparison to CS-treated cells. (C) Cells were pre-incubated 24 h either with CS alone or with CS in the presence of 10 and 30 μM of SnPP, a HO-1 blocker. After this pre-incubation period, cells were incubated 24 h with Rot/oligo. Values are expressed as means ± S.E.M. of 4 different cell batches. ### p<0.001 in comparison to control cells, **p<0.01 in comparison to Rot/oligo-lesioned cells in the absence or presence of CS. $ p<0.05 in comparison to cells in presence or absence of

SnPP.

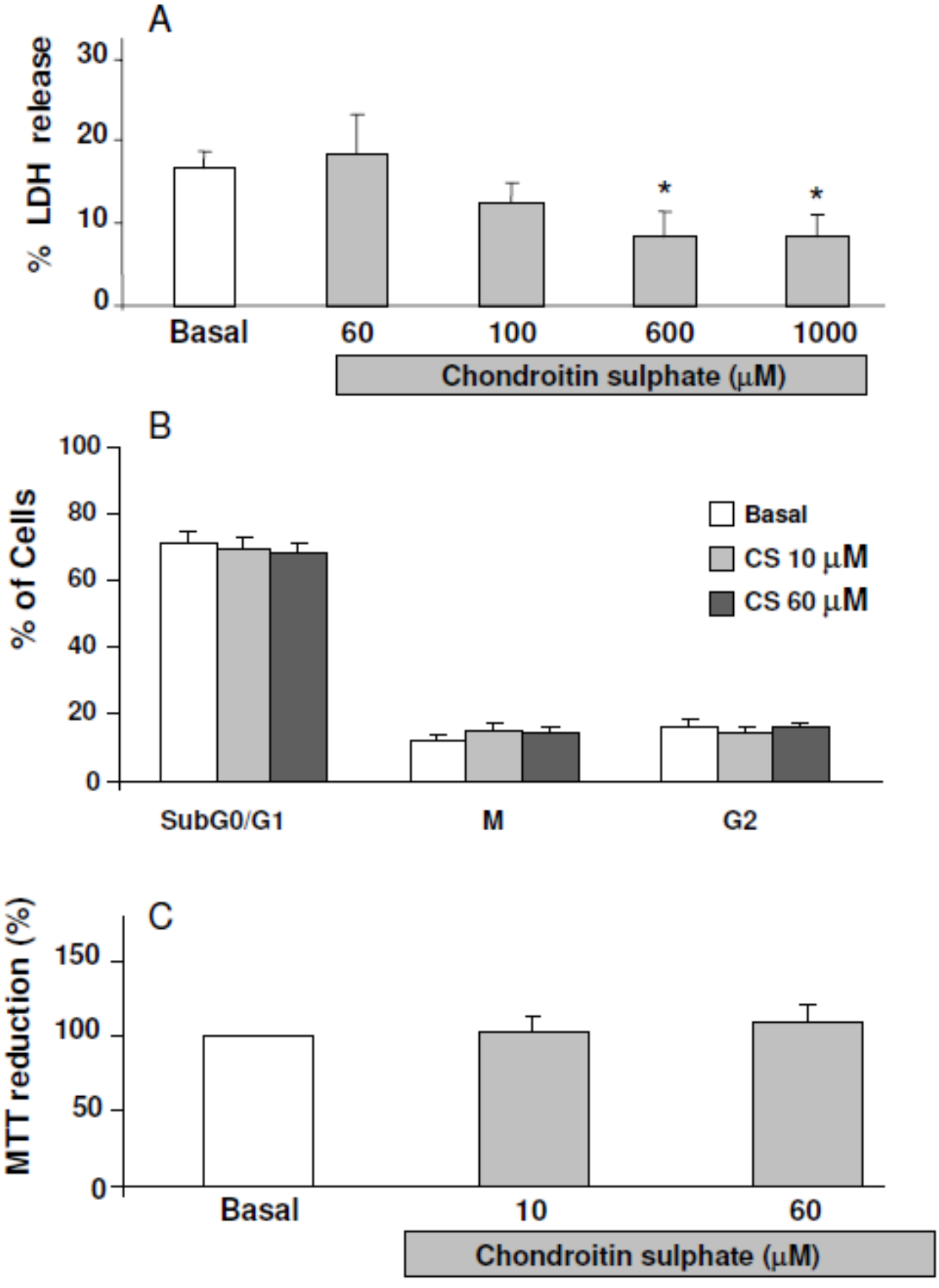
A
% LDH release
30
20
10
0
*
*
Basal
60
100
600
1000
Chondroitin sulphate (μM)
B
% of Cells
100
80
60
40
20
0
Basal
CS 10 μM
CS 60 μM
SubG0/G1
M
G2
C
MTT reduction (%)
150
100
50
0
Basal
10
60
Chondroitin sulphate (μM)

Figure 1

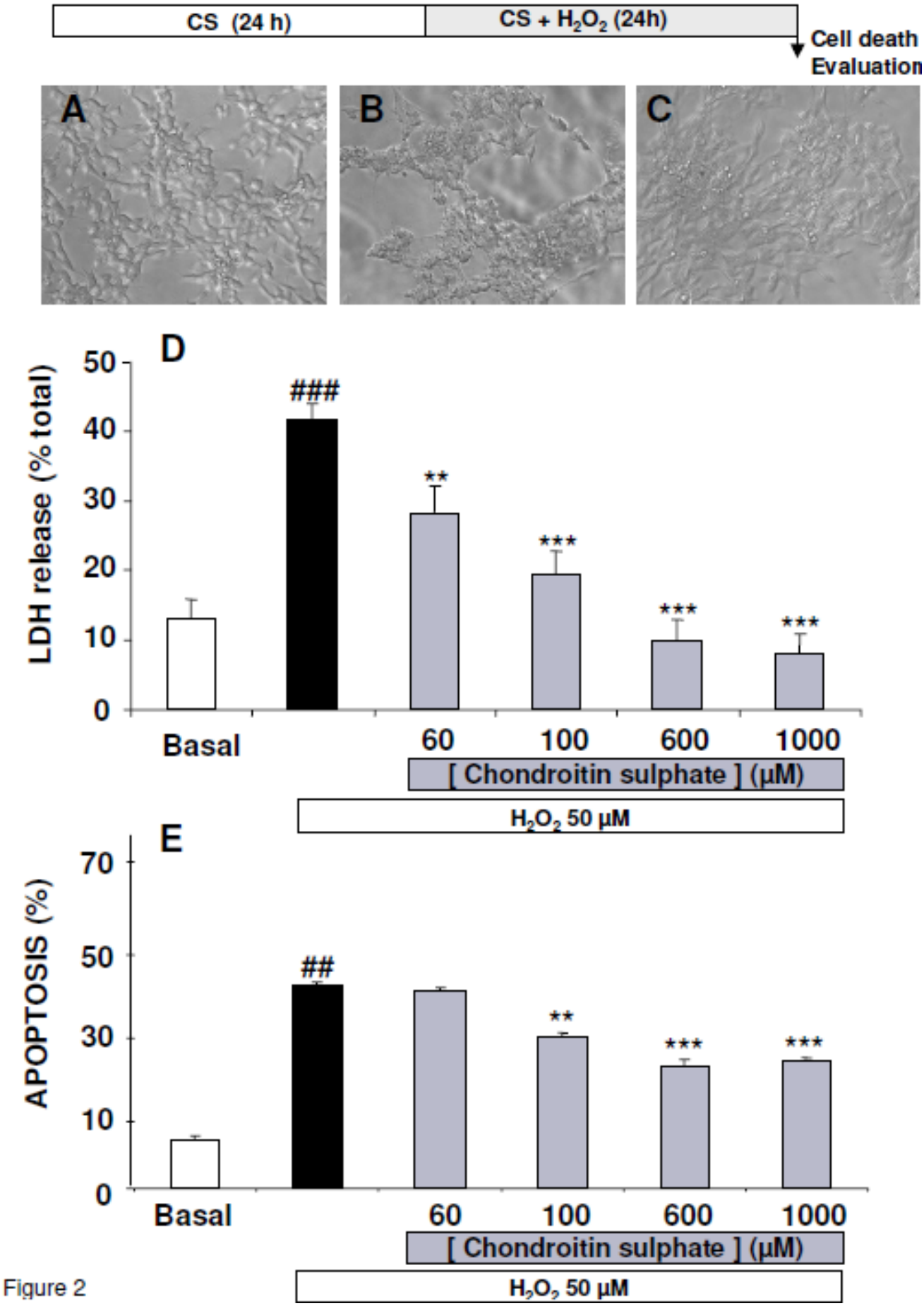
CS (24 h)
CS + H2O2 (24h)
Cell death Evaluation
A
B
C
D
LDH release (% total)
50
40
30
20
10
0
###
**
***
***
***
Basal
60
100
600
1000
[Chondroitin sulphate] (µM)
H2O2 50 µM
E
APOPTOSIS (%)
70
50
30
10
0
##
**
***
***
Basal
60
100
600
1000
[Chondroitin sulphate] (µM)
H2O2 50 µM

Figure 2

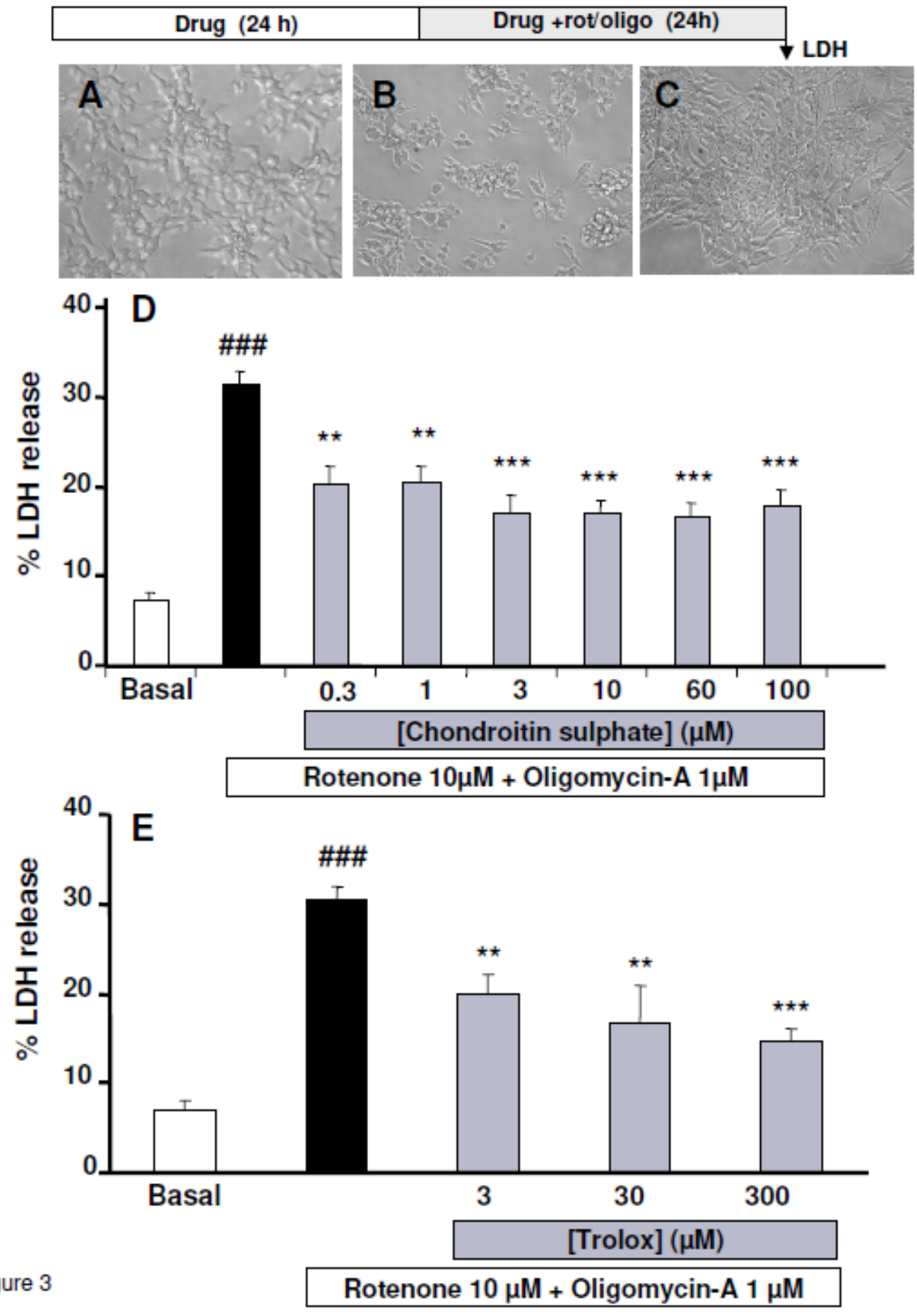
Drug (24 h)
Drug +rot/oligo (24h)
LDH
A
B
C
D
% LDH release
###
**
**
***
***
***
***
Basal
0.3
1
3
10
60
100
[Chondroitin sulphate] (µM)
Rotenone 10µM + Oligomycin-A 1µM
E
% LDH release
###
**
**
***
Basal
3
30
300
[Trolox] (µM)
Rotenone 10 µM + Oligomycin-A 1 µM

Figure 3

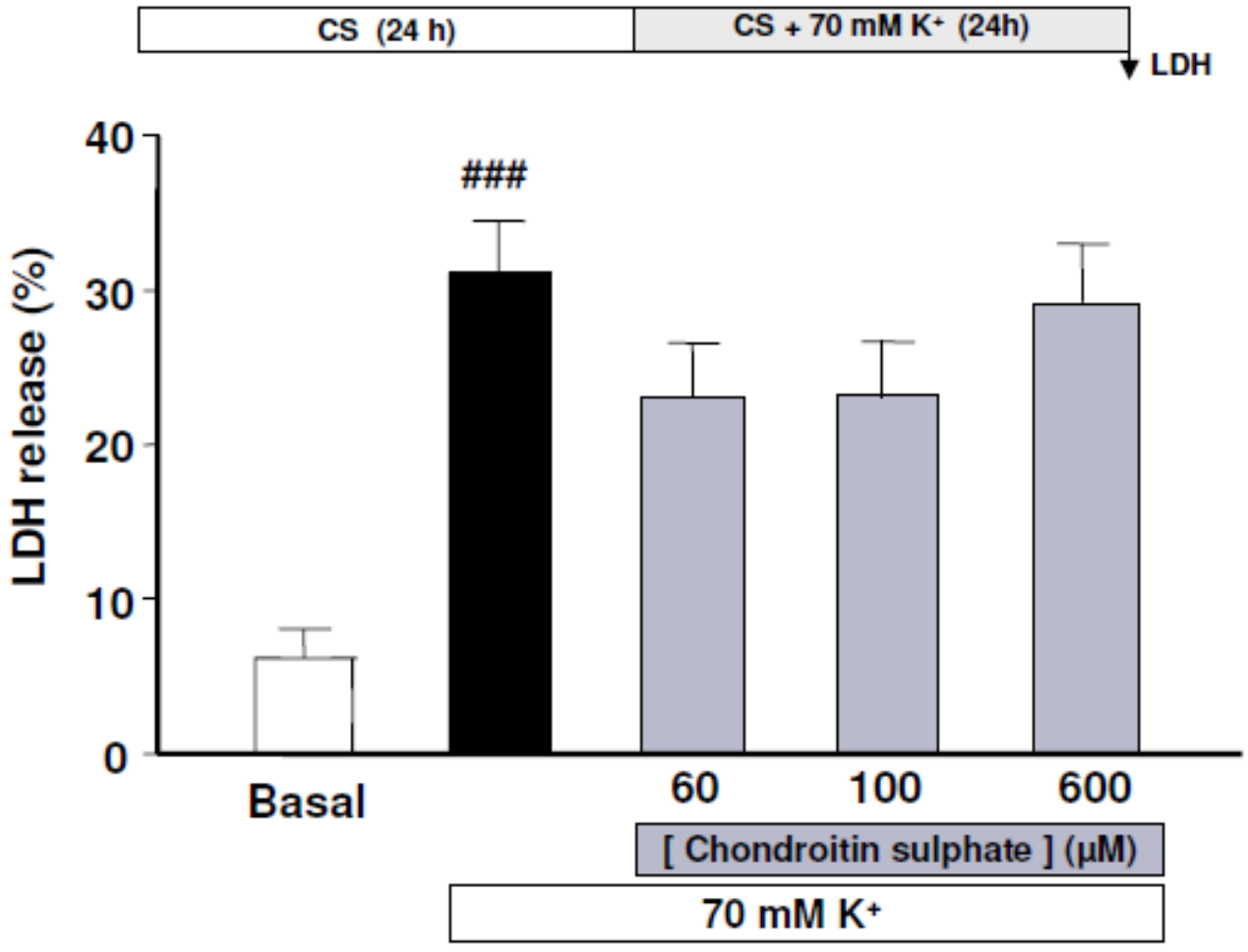
CS (24 h)
CS + 70 mM K+ (24h)
LDH
LDH release (%)
40
30
20
10
0
###
Basal
60
100
600
[Chondroitin sulphate] (µM)
70 mM K+

Figure 4

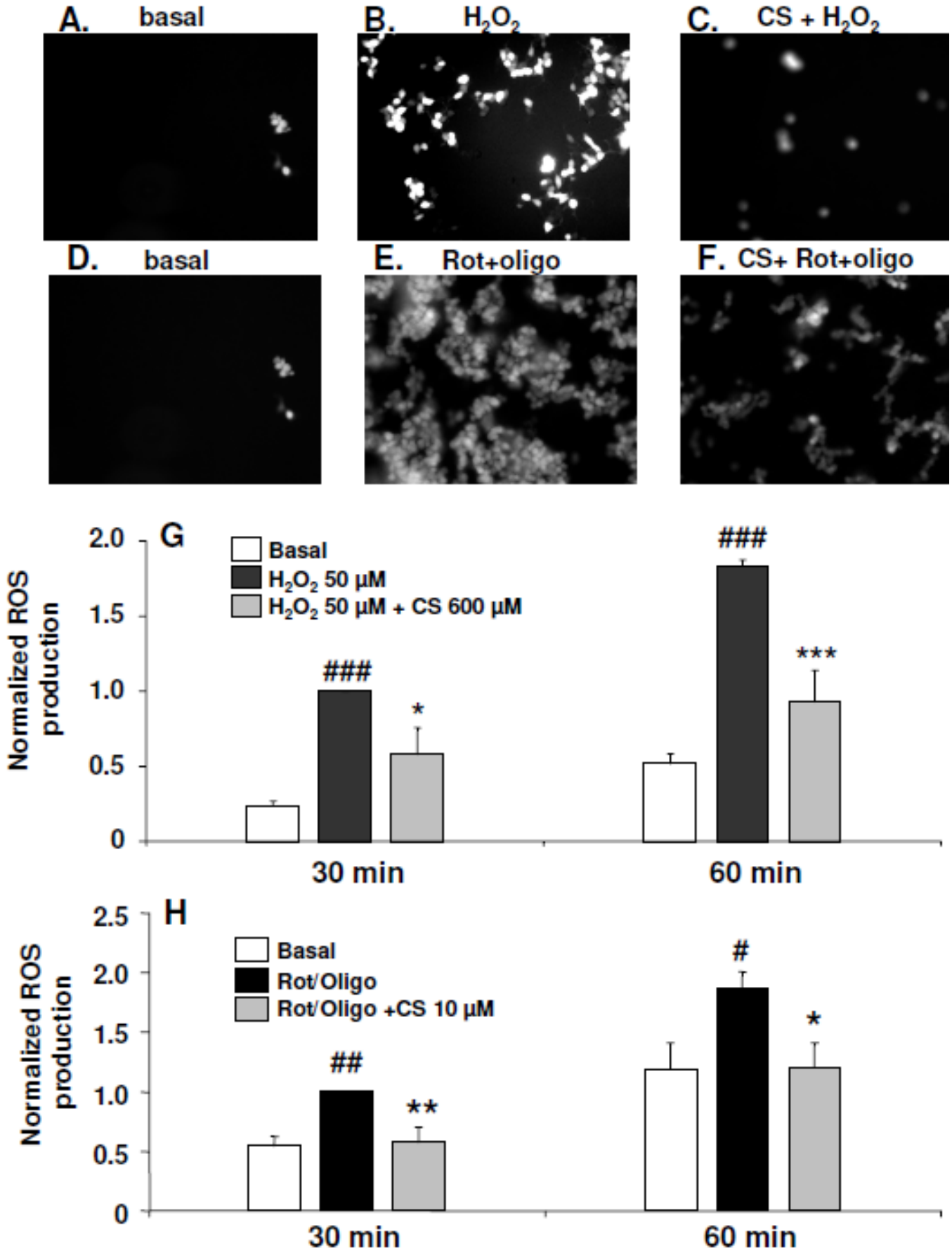
A. basal
B. H_2O_2
C. CS + H_2O_2
D. basal
E. Rot+oligo
F. CS+ Rot+oligo
G
Basal
H_2O_2 50 µM
H_2O_2 50 µM + CS 600 µM
Normalized ROS production
2.0
1.5
1.0
0.5
0
###
*
###
***
30 min
60 min
H
Basal
Rot/Oligo
Rot/Oligo +CS 10 µM
Normalized ROS production
2.5
2.0
1.5
1.0
0.5
0
##
**
#
*
30 min
60 min

Figure 5

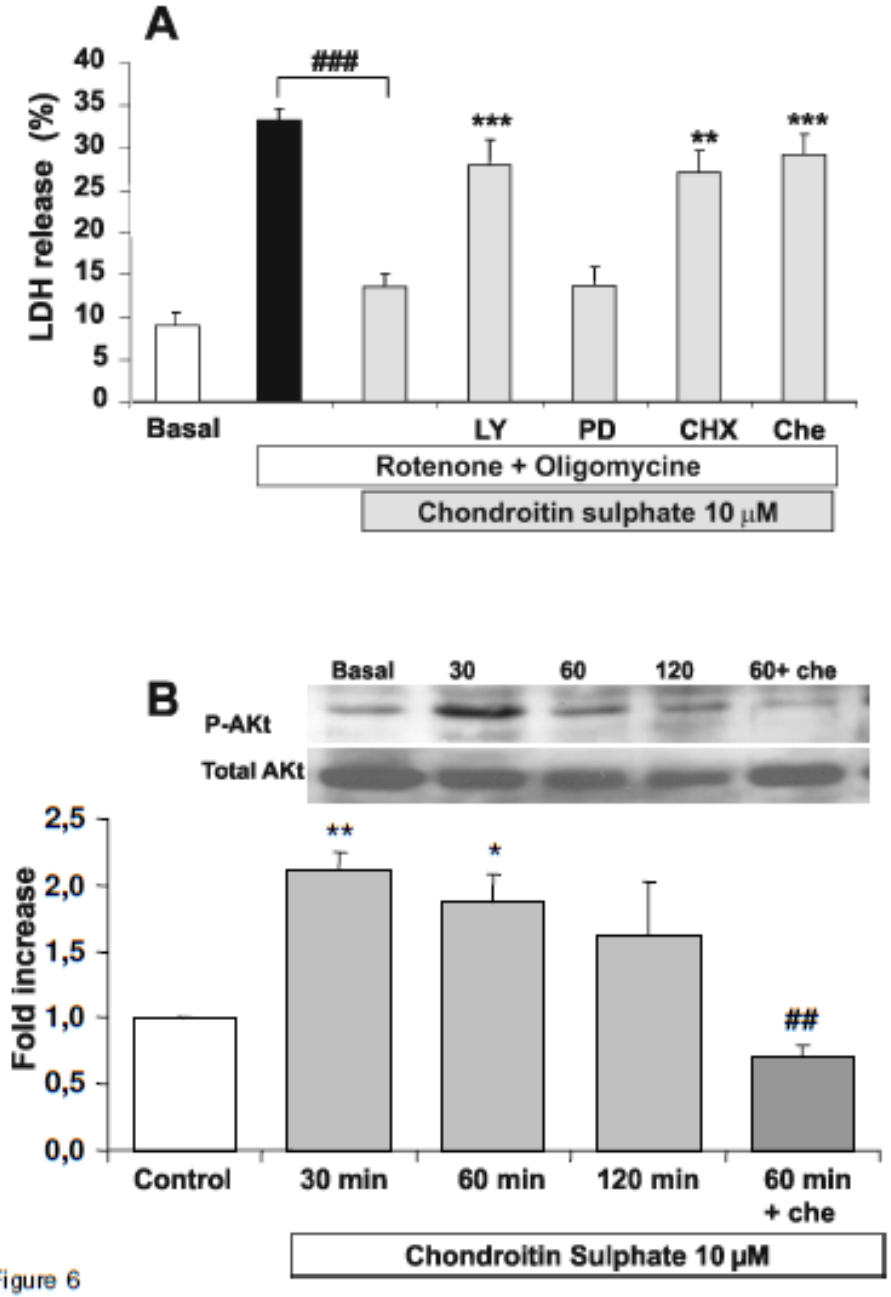
A
LDH release (%)
40
35
30
25
20
15
10
5
0
###
***
**
***
Basal
LY
PD
CHX
Che
Rotenone + Oligomycine
Chondroitin sulphate 10 μM
B
Basal
30
60
120
60+ che
P-AKt
Total AKt
Fold increase
2,5
2,0
1,5
1,0
0,5
0,0
**
*
##
Control
30 min
60 min
120 min
60 min
+ che
Chondroitin Sulphate 10 μM

Figure 6

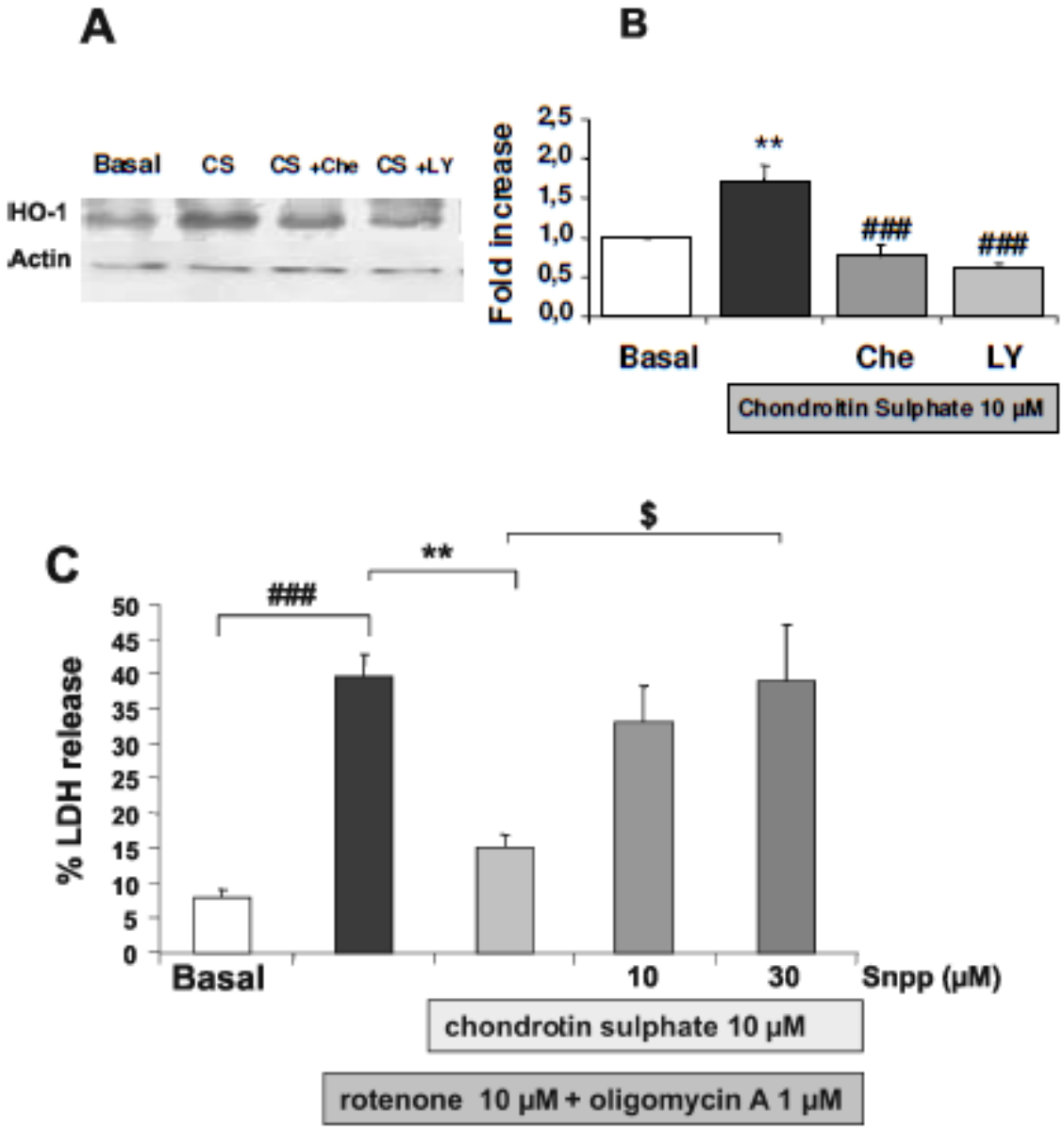
A
Basal
CS
CS +Che
CS +LY
HO-1
Actin
B
Fold increase
2,5
2,0
1,5
1,0
0,5
0,0
**
###
###
Basal
Che
LY
Chondroitin Sulphate 10 µM
C
$
**
###
% LDH release
50
45
40
35
30
25
20
15
10
5
0
Basal
10
30
Snpp (µM)
chondrotin sulphate 10 µM
rotenone 10 µM + oligomycin A 1 µM

Fig.7