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Guanosine protects human neuroblastoma SH-SY5Y cells against mitochondrial oxidative stress by inducing heme oxigenase-1 via PI3K/Akt/GSK-3β pathway

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ABSTRACT

Mitochondrial perturbation and oxidative stress are key factors in neuronal vulnerability in several neurodegenerative diseases or during brain ischemia. Here we have investigated the protective mechanism of action of guanosine, the guanine nucleoside, in a human neuroblastoma cell line, SH-SY5Y, subjected to mitochondrial oxidative stress. Blockade of mitochondrial complexes I and V with rotenone plus oligomycin (Rot/oligo) caused a significant decrease in cell viability and an increase in ROS production. Guanosine that the protective effect of guanosine incubated concomitantly with Rot/oligo abolished Rot/oligo-induced cell death and ROS production in a concentration dependent manner; maximum protection was achieved at the concentration of 1 mM. The cytoprotective effect afforded by guanosine was abolished by adenosine A_1 or A_{2A} receptor antagonists (DPCPX or ZM241385, respectively), or by a large (big) conductance Ca^{2+}-activated K^+ channel (BK) blocker (charybdotoxin). Evaluation of signaling pathways showed that the protective effect of guanosine was not abolished by a MEK inhibitor (PD98059), by a $p38^{MAPK}$ inhibitor (SB203580), or by a PKC inhibitor (cheleritrine). However, when blocking the PI3K/Akt pathway with LY294002, the neuroprotective effect of guanosine was abolished. Guanosine increased Akt and p-Ser-9-GSK-3β phosphorylation confirming this pathway plays a key role in guanosine's neuroprotective effect. Guanosine induced the antioxidant enzyme heme oxygenase-1 (HO-1) expression. The protective effects of guanosine were prevented by heme oxygenase-1 inhibitor, SnPP. Moreover, bilirubin, an antioxidant and physiologic product of HO-1, is protective against mitochondrial oxidative stress. In conclusion, our results show that guanosine can afford protection against mitochondrial oxidative stress by a signaling pathway that implicates PI3K/Akt/GSK-3β proteins and induction of the antioxidant enzyme HO-1.

1. Introduction

In the central nervous system (CNS) guanosine, the endogenous guanine nucleoside, is available extracellularly through release from glial cells. In astrocytic cell cultures it has been reported that guanine nucleotides and guanosine can be released under basal or toxic conditions (Ciccarelli et al., 1999, 2001). Alternatively, nucleotides such as GTP, GDP and GMP can be metabolized by ecto-nucleotidases to produce extracellular guanosine (Caciagli et al., 2000; Ciccarelli et al., 2001). Guanosine and guanine nucleotides have been implicated in neuroprotection by exerting trophic effects (Ciccarelli et al., 2001; Decker et al., 2007), as well as by counteracting glutamate excitotoxicity *in vitro* (Molz et al., 2005, 2008; Oleskovicz et al., 2008) and *in vivo* (Schmidt et al., 2000, 2005, 2007). Guanosine also protects cultured rat astrocytes from staurosporine-induced apoptosis (Di Iorio et al., 2004) and SH-SY5Y cells from β-amyloid-induced apoptosis (Pettifer et al., 2004). In both cases, the anti-apoptotic effect of guanosine was mediated by stimulation of phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) and mitogen-activated protein kinase (MAPK) cell survival pathways.

Abbreviations: BK, large (big) conductance Ca^{2+}-activated K^+ channels; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; GUO, guanosine; GSK-3β, glycogen synthase kinase 3β; H_2DCFDA, 2′,7′-dichlorofluorescein diacetate; HO-1, heme oxygenase-1; LY294002, 2-(4-morpholinyl)-8phenyl-1(4*H*)-benzopyran-4-4hydrochloride; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PD98059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; PKC, protein kinase C; PI3K, phosphatidilinositol-3kinase; ROS, reactive oxygen species; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole; SnPP, Sn(IV) protoporphyrin IX dichloride; ZM241385, 4-(2-[7-amino-2-{2-furyl} {1,2,4} triazolo {2,3a}{1,3,5}triazin-5-ylamino]ethylphenol.

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Despite several evidences on the protective effect of the exact extracellular site of interaction and mechanisms of action for this nucleoside have not yet been fully characterized. Some studies suggest that guanosine participates in cell proliferation, neurite outgrowth and cellular protection by a mechanism that involves activation of adenosine receptors (Ciccarelli et al., 2000; D'Alimonte et al., 2007; Thauerer et al., 2010). However, we have recently demonstrated that guanosine-induced protection in hippocampal slices subject to oxygen/glucose deprivation depends on large conductance Ca^{2+}-activated K^+ (BK) channels activation (Dal-Cim et al., 2011).

Oxidative stress is a common mechanism of cell death in distinct cytotoxic models such as glutamate (Parfenova et al., 2006), β-amyloid (Tamagno et al., 2006), MPP^+ (Nicotra and Pavrez, 2000), or hydrogen peroxide-induced cytotoxicity (Kim et al., 2005). Oxidative stress has also been related to neurodegenerative diseases like Alzheimer and Parkinson's diseases (Mattson and Magnus, 2006) or stroke (Saito et al., 2005). Overproduction of reactive oxygen species (ROS) leads to damage of both neurons and astrocytes (Lin and Beal, 2006). In this study, we used an oxidative stress model evoked by mitochondrial activity disruption induced by blockade of mitochondrial complexes I and V, by using the combination of rotenone plus oligomycin-A (Rot/oligo) (Egea et al., 2007).

As mentioned above, the detrimental accumulation of ROS plays an important role in multiple pathologies; therefore, cells have developed an antioxidant armamentarium that includes a group of antixenobiotic genes termed phase II detoxification genes (Itoh et al., 1999) to maintain redox homeostasis. Among these genes is heme oxygenase-1 (HO-1), which is the rate-limiting enzyme that degrades the pro-oxidant heme group and produces equimolecular quantities of carbon monoxide (CO), iron, and biliverdin (BV). 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). HO-1 is induced in response to a great variety of stress-inducing pathological conditions (Keyse and Tyrrell, 1987; Nimura et al., 1996). Moreover, studies in HO-1-deficient mice have confirmed that HO system is indispensable for cell protection against oxidative stress (Poss and Tonegawa, 1997). Furthermore, it has been shown that in the post-mortem brains of Alzheimer's disease patients, there was a HO-1 induction in neurons of the cerebral cortex and hippocampus and HO-1 was co-localized with neurofibrillar tangles (Schipper et al., 1995). Therefore, it is generally accepted that HO-1 represents a physiological protective mechanism against oxidative stress.

The purpose of this study was to evaluate how guanosine, a guanine nucleoside that can be secreted under physiological or pathological conditions, could protect cells against oxidative stress caused by disruption of the mitochondrial respiratory chain. Herein we show that guanosine-induced protective effect depends on activation of adenosine receptors and BK channels. Guanosine can afford cytoprotection under circumstances of cell vulnerability caused by mitochondrial disruption through an intracellular biochemical pathway that implicates the activation of PI3K/Akt leading to inactivation of glycogen synthase kinase-3β (GSK-3β) and induction of the antioxidant enzyme HO-1.

2. Materials and methods

2.1. Materials

Charybdotoxin, DPCPX (1,3-dipropyl-8-cyclopentylxanthine), F-12 nutrient mixture, Eagle's minimum essential medium (MEM), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), oligomycin A, rotenone, SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole] and ZM241385 [4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3a}{1,3,5}triazin-5-ylamino]ethylphenol] were obtained from Sigma (Madrid, Spain). Chelerythrine and PD98059 [2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one] and LY294002 [2-(4-morpholinyl)-8phenyl-1(4*H*)-benzopyran-4-4hydrochloride] were purchased from Tocris (Biogen Científica, Spain). Sn(IV) protoporphyrin-IX dichloride (SnPP) was obtained from Frontier Scientific Europe (Lancashire, UK). Penicillin/streptomycin was purchased from GIBCO (Madrid, Spain). 2′,7′-dichlorofluorescein diacetate (H_2DCFDA) was obtained from Molecular Probes (Invitrogen, Madrid, Spain). Pyruvate and heat-inactivated fetal bovine serum (FBS) were purchase from Invitrogen. Bilirubin was purchase from Analisa Gold.

2.2. Culture and maintenance 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 were maintained in a 1:1 mixture of F-12 nutrient mixture (Ham 12) and Eagle's MEM supplemented with 15 nonessential amino acids, 1 mM sodium pyruvate, 10% heat-inactivated FBS, 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Madrid, Spain). 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 96-well plates at a seeding density of 1×10^5 cells per well (Cañas et al., 2007). Cells were treated with the drugs in MEM supplemented with 1% fetal calf serum. Cells were treated with the drugs before confluence in MEM/F-12 with 1% FBS. Cells were used at a passage below 13.

2.3. Induction of cell toxicity and evaluation of cytoprotection induced by guanosine

Cell death was induced by adding a combination of rotenone (30 μM) plus oligomycin-A (10 μM) (Rot/oligo) for 24 h. When present, guanosine was added to SH-SY5Y at the same time as Rot/oligo and remained in the culture medium for the duration of experiments (24 h). Bilirubin (50 nM) was added in medium culture as the same time as Rot/Oligo and remained in culture medium for the duration of experiments (24 h). In experiments where enzyme inhibitors, adenosinergic receptors antagonists or potassium channel blocker were tested, SH-SY5Y cells were pretreated with these agents for 30 min prior to the addition of guanosine, which remained in the incubation medium throughout the duration of the experiment. These treatments included: the potent and selective inhibitor of the PI3K (LY294002, 10 μM); the selective inhibitor of the MAP kinase kinase (MEK) (PD98059, 10 μM); the inhibitor of PKC (cheleritrine, 0,1 μM); the inhibitor of $p38^{MAPK}$ (SB203580, 10 μM); HO-1 inhibitor, Sn(IV) protoporphyrin IX dichloride (SnPP, 3 μM). Adenosinergic antagonists: A_1 receptor antagonist (DPCPX, 100 nM); A_{2A} receptor antagonist (ZM 241385, 50 nM); BK channel blocker (charybdotoxin, 100 nM). LY294002, PD98059 and cheleritrine were dissolved in and added to the culture medium at a final concentration of 0.01% dimethyl sulfoxide (DMSO).

2.4. Evaluation of cell viability by MTT reduction

SH-SY5Y cell viability was evaluated 24 h after Rot/oligo or Rot/oligo plus guanosine exposure. At the end of each experiment, 3-(4,5-dimethylthiazol-2-yl-diphenyltetrazolium bromide (MTT Sigma Aldrich) was added to each well to a final concentration of

0.5 mg/ml (Mosmann, 1983) and the plates were incubated for 2 h at 37 °C. Then the insoluble formazan was solubilized by adding DMSO, resulting in a colored compound which optical density was measured in an ELISA reader (550 nm). The evaluation of MTT reduction was performed in triplicates of four to five independent experiments.

2.5. Measurement of ROS production

To measure cellular ROS, we have used the molecular probe H_2DCFDA (Ha et al., 1997). 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 (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). Wavelengths of excitation and emission were 485 and 520 nm, respectively (Egea et al., 2007). Production of ROS was evaluated in triplicates of four independent experiments.

2.6. Immunoblotting

SH-SY5Y cells 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 mmol/L NaCl, 20 mmol/L Tris–HCl, pH 7.5, 1 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L NaF, 1 mmol/L sodium pyrophosphate, and 1 mmol/L Na_3VO_4). Protein (30 μg) from the cell lysates was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to PVDF membranes (GE Healthcare). Membranes were incubated with the following antibodies: Anti-Akt at 1:1000, Anti-phospho-Akt at 1:1000, Anti-phospho-Ser-9-GSK3-β at 1:1000, Anti-GSK-3β at 1:1000 (Cell Signaling, Izasa SA, Barcelona, Spain); anti HO-1 at 1:1000 (Chemicon, Temecula, CA, USA) anti-β-actin at 1:100,000 (Sigma, Madrid, Spain). Appropriate peroxidase-conjugated secondary antibodies at 1:10,000 were used to detect proteins by enhanced chemiluminescence. Immunodetection was performed in triplicates of four to six independent experiments.

2.7. Statistical analysis

Comparison among groups were performed by one-way analysis of variance (ANOVA) followed by Duncan's test if necessary, with $p < 0.05$ considered to be statistically significant.

3. Results

3.1. Guanosine protects SH-SY5Y neuroblastoma cells against Rot/oligo-induced cell death

Treatment of SH-SY5Y neuroblastoma cells for 24 h with 30 μM rotenone plus 10 μM oligomycin A (Rot/oligo) decreased by 70% the cellular viability, measured as MTT reduction, when compared to cultures incubated only with culture medium (basal). Guanosine *per se* (0.03–1 mM) did not alter cellular viability at any of the concentrations tested (Fig. 1A).

As illustrated in the microphotographs of Fig. 1B, Rot/oligo decreased the number of cells when compared to cultures in basal conditions and transformed healthy cells, isolated or grouped in clusters, into rounded shaped cells, with a granular morphology containing debris. SH-SY5Y cells co-incubated with 1 mM guanosine and Rot/oligo greatly recovered their initial density and exhibited a healthier appearance.

Increasing concentrations of guanosine (0.3–1 mM) added to the incubation medium at the same time as Rot/oligo significantly reduced Rot/oligo-induced cell death in SH-SY5Y cells in a concentration dependent fashion (Fig. 1C); maximum protection was achieved at the concentration of 1 mM (40%, $p < 0.01$). Thus, this was the concentration selected to study guanosine's protective mechanism of action in the following experiments.

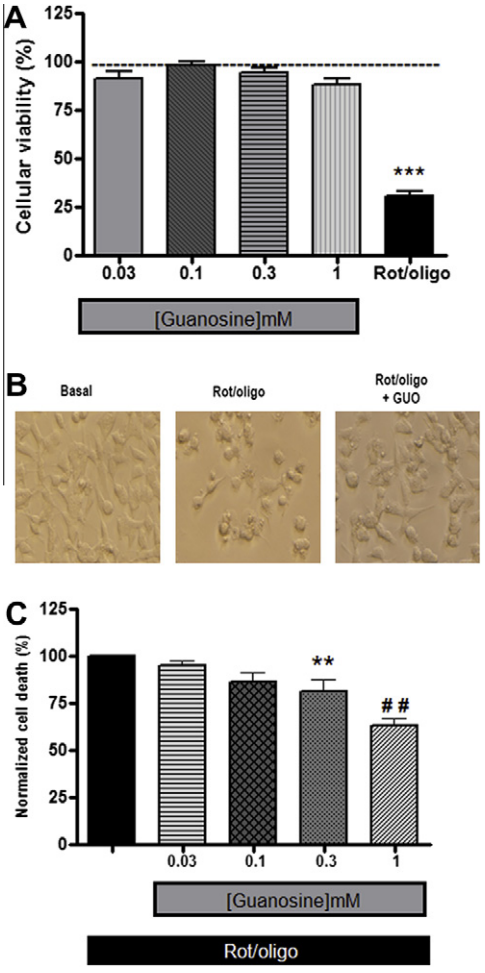

Fig. 1. Guanosine protects against cell death induced by Rot/oligo in SH-SY5Y neuroblastoma cells. (A) SH-SY5Y neuroblastoma cells were incubated for 24 h with culture medium (basal), with increasing concentrations of GUO (0.03–1 mM) or with 30 μM rotenone plus 10 μM oligomycin. Cellular viability was evaluated by MTT reduction assay and was expressed as percentage of control cells representing cells incubated for 24 h in culture medium (100% cellular viability). (B) Photomicrographs of control SH-SY5Y cells (Basal); cells exposed 24 h to Rot/oligo; cells co-incubated 24 h with 1 mM guanosine (Rot/oligo + GUO).Image magnification (40X). (C) Concentration–response curve of GUO co-incubated for 24 h with Rot/oligo. Cell death was normalized in each individual experiment, as percentage of the maximum cell death (Rot/oligo) that was considered as 100% (black column). Data are mean ± SEM from five different cell batches. $^{***}p < 0.001$, represents means significantly different from control cells; $^{**}p < 0.01$ represents means significantly different from Rot/oligo and Rot/oligo + GUO 1 mM; $^{\#\#}p < 0.01$ represents means significantly different from all other groups.

3.2. Guanosine protects against Rot/oligo-induced cell death via BK channels and adenosine receptors modulation

We previously showed that neuroprotection afforded by guanosine against ischemic damage depends on BK channels activation (Dal-Cim et al., 2011). In order to evaluate if this channel is also involved in the protective effect of guanosine against mitochondrial damage, SH-SY5Y cells were incubated with charybdotxin (100 nM) 30 min prior to the addition of guanosine. Charybdotoxin prevented the protective effect of guanosine against cell damage induced by Rot/oligo (Fig. 2A).

Since it has been suggested that guanosine may also interact with adenosine A_1 receptors in some situations and activation of such receptor subtype is involved in neuroprotective effects (Ciccarelli et al., 2000; Cunha, 2005) , we verified the role of adenosine A_1 receptor in the protective effects triggered by guanosine in SH-SY5Y neuroblastoma cells. The presence of A_1 receptor antagonist (DPCPX, 100 nM) abolished neuroprotection induced by guanosine. Interestingly, the A_{2A} receptor antagonist (ZM241385, 50 nM) also blocked guanosine-induced protective effect (Fig. 2A).

3.3. Guanosine protects against Rot/oligo-induced cell death by activation of the PI3K/Akt cell survival pathway and inactivation of GSK-3β

In order to analyze the signaling pathways participating in the neuroprotective mechanism of guanosine against Rot/oligo-induced cell damage in SH-SY5Y cells, we performed experiments with LY294002, an inhibitor of the PI3K; PD98059, an inhibitor of MEK; cheleritrine, an inhibitor of PKC and SB203580, an inhibitor of $p38^{MAPK}$. As shown in Fig. 2B, only the inhibitor of PI3K (LY294002, 10 μM) partially blocked the neuroprotective effect of guanosine. Therefore these results indicate that PI3K/Akt, but not ERK1/2, PKC or $p38^{MAPK}$ are participating in guanosine-induced neuroprotection.

To further corroborate the participation of Akt, we incubated SH-SY5Y cells during different time periods with guanosine (1 mM) and thereafter protein extracts were obtained to measure p-Akt by Western blot. The results showed guanosine significantly increased by 2.5-fold p-Akt levels after 30 min incubation; this induction was maintained at 60 min, but it decreased after 24 h incubation (Fig. 3A).

GSK-3β is tightly regulated by the survival pathway represented by PI3K and its downstream effector, Ser/Thr protein kinase Akt. Akt targets GSK-3β, also a Ser/Thr protein kinase that phosphorylated at

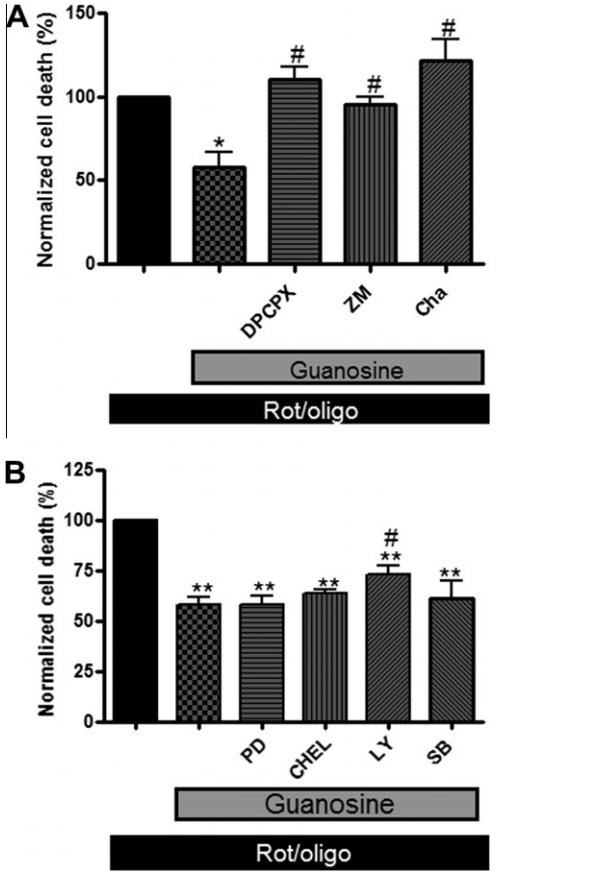

Fig. 2. Evaluation of extracellular interaction sites and signaling pathways involved in guanosine-induced neuroprotection against cell death induced by Rot/oligo in SH-SY5Y neuroblastoma cells. (A) SH-SY5Y neuroblastoma cells were incubated for 24 h with 30 μM rotenone plus 10 μM oligomycin A (Rot/oligo) with or without 1 mM guanosine (GUO) or pre-incubated for 30 min with 100 nM DPCPX (A_1 receptor antagonist), ZM241385 50 nM (A_{2A} receptor antagonist) or Charybdotoxin 100 nM (BK channel blocker) and subsequently co-incubated 24 h with 1 mM guanosine and Rot/oligo. Cell death was normalized in each individual experiment as percentage of the maximum cell death (Rot/oligo) which was considered as 100% (black column). (B) SH-SY5Y neuroblastoma cells were incubated for 24 h with 30 μM rotenone plus 10 μM oligomycin A (Rot/oligo) with or without 1 mM guanosine (GUO) or pre-incubated for 30 min with 10 μM PD98059 (MEK inhibitor), 0.1 μM cheleritrine (chel) (PKC inhibitor), 10 μM LY294002 (PI3K inhibitor) or 10 μM SB SB203580 ($p38^{MAPK}$ inhibitor) and subsequently co-incubated 24 h with 1 mM guanosine and Rot/oligo. Cell death was normalized in each individual experiment as percentage of the maximum cell death (Rot/oligo) which was considered as 100% (black column). Data are mean ± SEM from five different cell batches. $^{*}p < 0.05$ represents means significantly different from Rot/oligo; $^{**}p < 0.01$ represents means significantly different from Rot/oligo; $^{\#}p < 0.05$ represents means significantly different from Rot/oligo + GUO.

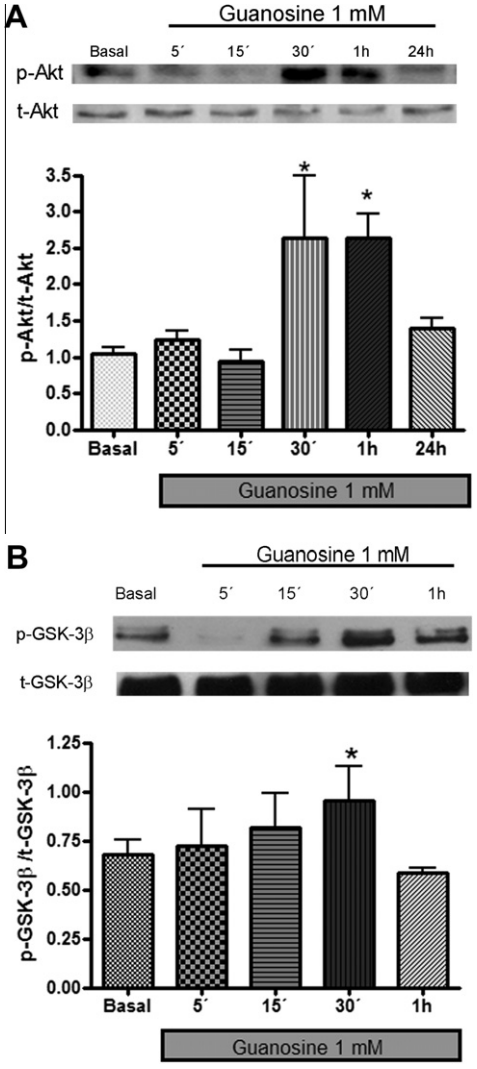

Fig. 3. Guanosine induces Akt phosphorylation and inactivation of GSK-3β by phosphorylation of its Ser-9. (A) Representative immunoblot of Akt phosphorylation. SH-SY5Y neuroblastoma cells were incubated for 5, 15, 30 min, 1 and 24 h with guanosine 1 mM. The histogram presents the densitometric quantification of p-Akt/total-Akt. (B) SH-SY5Y neuroblastoma cells were incubated for 5, 15, 30 min and 1 h with guanosine 1 mM. The histogram presents the densitometric quantification anti-p-Ser9-GSK-3β and anti-total-GSK-3β. Data are mean ± SEM from four different cell batches. $^{*}p < 0.05$ indicates significantly different from basal group.

its Ser-9, is inactivated (Rylatt et al., 1980). GSK-3β phosphorylates and thereby regulates many important metabolic and signaling proteins, structural proteins and transcription factors (Frame and Cohen, 2001). Incubation of SH-SY5Y cells at different time intervals with guanosine (1 mM) gradually increased p-Ser9-GSK-3β, maximum phosphorylation was also achieved after 30 min (Fig. 3B).

3.4. Participation of HO-1 in the protective mechanism of guanosine

GSK-3β has been identified as a key mediator regulating the cross-talk between the survival signal elicited by PI3K/Akt and the antioxidant phase II cell response (Salazar et al., 2006). In this context, HO-1 is found among the phase II detoxification genes. Since induction of a moderate intracellular heme catabolism through HO-1 represents an adaptive and protective response to oxidative injury (Doré et al., 1999; Baranano et al., 2002), we investigated whether guanosine could be regulating this enzyme expression. For these experiments, cells were incubated for 24 h with basal medium alone, guanosine (1 mM), or guanosine in the presence of the PI3K inhibitor LY294002. Then, cell lysates were resolved in SDS–PAGE and analyzed by immunoblotting with anti-HO-1. Twenty-four hours incubation with guanosine increased to almost 3-fold the expression of HO-1. The PI3K inhibitor significantly reduced HO-1 levels induced by guanosine (Fig. 4A). Therefore, guanosine was capable of inducing the antioxidant enzyme HO-1 and PI3K/Akt participated in this induction.

To further analyze the involvement of HO-1 in the protective effect of guanosine, we used a HO-1 inhibitor, Sn(IV) protoporphyrin IX (SnPP) (Marinissen et al., 2006). Indeed, SnPP was able to prevent the protective effect afforded by guanosine (Fig. 4B). Moreover, bilirubin, a HO-1 product, was used to mimic HO-1 activity induction in the neuroprotective effect of guanosine against Rot/oligo. Bilirubin is an end product of heme catabolism and probably the best-known endogenous antioxidant in mammals. Bilirubin (50 nM) also counteracted citotoxicity in SH-SY5Y exposed to Rot/oligo (Fig. 4C), confirming guanosine induced endogenous antioxidant system of HO-1 which contribute to protective effect triggered by this nucleoside.

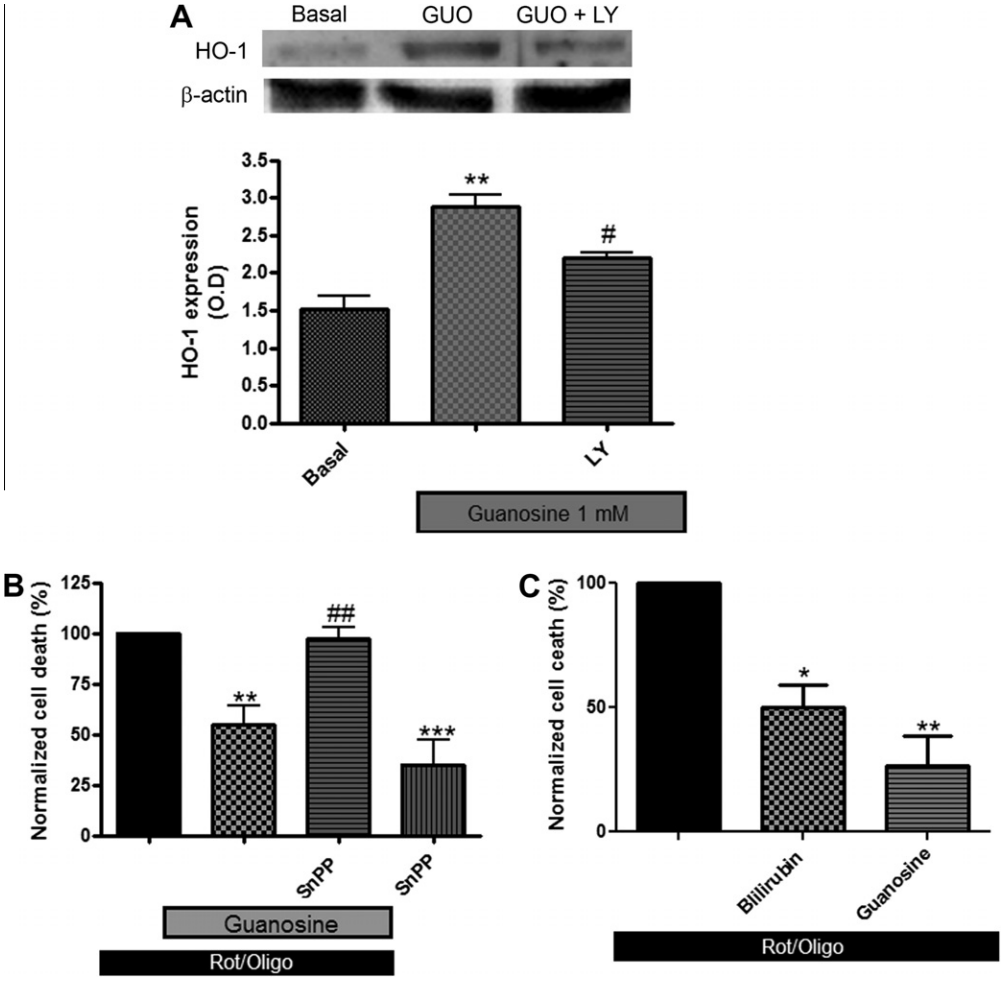

Fig. 4. Guanosine protects SH-SY5Y neuroblastoma cells by inducing HO-1 expression via PI3K/Akt. (A) Representative immunoblot showing HO-1 induction in SH-SY5Y neuroblastoma cells incubated or 24 h with guanosine (GUO) in the presence or in the absence of 10 μM LY294002. The histogram represents the densitometric quantification of HO-1 protein levels. Data are mean ± SEM from four different cell batches. $^{**}p < 0.01$ represents means significantly different from basal groups. $^{\#}p < 0.05$ indicates significantly different from guanosine group. (B) SH-SY5Y neuroblastoma cells were incubated for 24 h with 30 μM rotenone plus 10 μM oligomycin A (Rot/oligo) with or without 1 mM GUO or pre-incubated for 30 min with 30 μM SnPP (HO-1 inhibitor) and subsequently co-incubated 24 h with 1 mM GUO and Rot/oligo. Cell death was normalized in each individual experiment, as percentage of the maximum cell death (Rot/oligo) which was considered as 100% (black column). Data are mean ± SEM from five different cell batches. $^{**}p < 0.01$, $^{***}p < 0.001$, indicates significantly different from Rot/oligo group; $^{\#\#}p < 0.01$ indicates significantly different from Rot/oligo + GUO group. (C) SH-SY5Y neuroblastoma cells were incubated for 24 h with 30 μM rotenone plus 10 μM oligomycin A (Rot/oligo) with 1 mM GUO or with biluribin 50 nM. Cell death was normalized in each individual experiment, as percentage of the maximum cell death (Rot/oligo) which was considered as 100% (black column). Data are mean ± SEM from four different cell batches.$^{*}p < 0.05$, $^{**}p < 0.01$, indicates significantly different from Rot/oligo group.

3.5. Guanosine prevents Rot/oligo-induced ROS production

Since Rot/oligo are potent inhibitors of the complex I and V of the respiratory chain, respectively, and cause oxidative stress (Egea et al., 2007), we measured ROS generation with the fluorescent probe H_2DCFDA. At the end of the 24 h period with Rot/oligo, cells were loaded with 10 μM H_2DCFDA for 20 min. Cells exposed to Rot/oligo increased ROS production to 293%. When cells were co-incubated with guanosine and Rot/oligo for 24 h, the amount of ROS produced by cells was reduced to basal levels. Interestingly, LY294002 abolished the reduction of ROS elicited by guanosine (Fig. 5). Taken together, these results indicate that guanosine protects SH-SY5Y cells from mitochondrial oxidative stress by a mechanism that implicates PI3K/Akt pathway.

4. Discussion

Central to this study is guanosine protects cells from mitochondrial oxidative stress by inducing the antioxidant enzyme HO-1 via PI3K/Akt/GSk-3β pathway Fig. 6.

Mitochondrial dysfunction and bioenergetic deficiency is closely linked to pathogenesis of many neurodegenerative disorders. Mitochondria play a variety of roles by integrating extracellular signals and executing important intracellular events in neuronal survival and death. In this context, the regulation of mitochondrial function via therapeutic approaches may exert some salutary and neuroprotective mechanisms. In this study we have caused interruption of the respiratory chain at complexes I (rotenone) and V (oligomycin-A) that causes mitochondrial depolarization, increases ROS production, and a vicious circle leading to cell death (Egea et al., 2007). The oxidative damage induced by Rot/oligo profoundly affected cellular viability of SH-SY5Y neuroblastoma cells, as observed by a significant impairment in MTT reduction, as well as by morphological alterations consistent with cell death. MTT reduction assay can also be considered an approach to evaluate cell proliferation. However, in a previous study, incubation of SH-SY5Y cells with the same concentration of Rot/oligo and time incubation used in our study, can also induce LDH leakage (Cañas et al., 2007), indicating Rot/oligo induces cell death and does not seem to interfere with cell proliferation. Under these experimental conditions, guanosine was able to reduce cell death in a concentration-dependent manner. Studies evaluating a protective role of guanosine against cell death induced by apoptosis in SH-SY5Y cell culture have shown that this effect is observable at concentrations between 100 μm and 300 μM (Di Iorio et al., 2004; Pettifer et al., 2004, 2007). Here we observed that 1 mM guanosine was the most effective concentration of guanosine against cell death induced by Rot/oligo. This concentration can be justified by the high amount of necrotic cell death induced by Rot/oligo after 24 h of treatment (Egea et al., 2007; Parada et al., 2010) and thus a greater concentration of guanosine was necessary to induced protection.

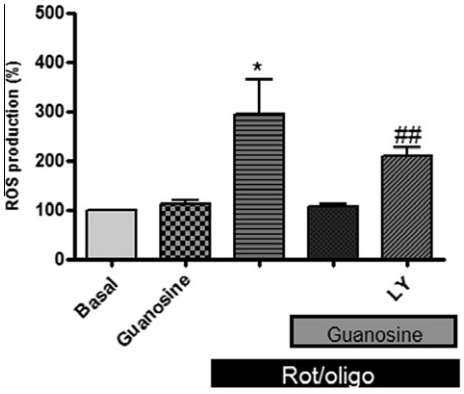

Fig. 5. Guanosine inhibits mitochondrial ROS production elicited by Rot/oligo in SH-SY5Y neuroblastoma cells. SH-SY5Y neuroblastoma cells were incubated for 24 h with 30 μM rotenone plus 10 μM oligomycin A (Rot/oligo) with or without 1 mM guanosine (GUO). Cells were pre-incubated for 30 min with 10 μM LY294002 (PI3K inhibitor) and subsequently co-incubated 24 h with 1 mM guanosine and Rot/oligo. Data are means ± SEM from four different cell batches. $^{*}p < 0.05$ represents means significantly different from all other groups. $^{\#\#}p < 0.01$ represents means significantly different from Rot/oligo group.

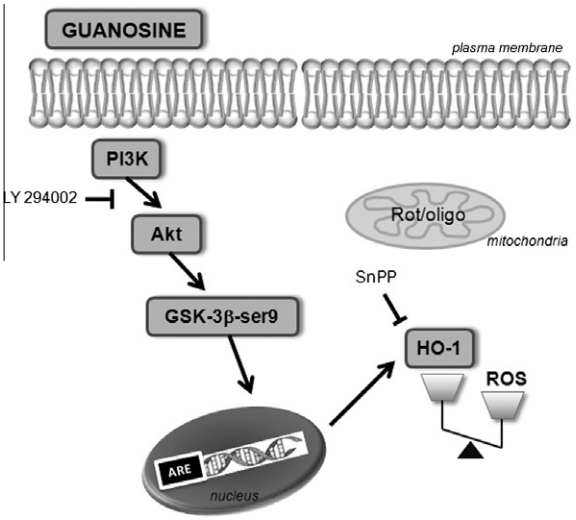

Fig. 6. Schematical representation of putative protective mechanism of guanosine. Guanosine-induced neuroprotection seems to involve modulation of adenosine A_1 and A_{2A} receptors and BK channels activation. Guanosine activates PI3K/Akt/GSK-3β pathway leading to HO-1 induction which protect against ROS production caused by blockade of mitochondrial complexes I and V with rotenone and oligomycin A, respectively. The PI3K inhibitor (LY294002) and HO-1 inhibitor (SnPP) blocked the protective effect induced by guanosine.

Identification of molecular targets for guanosine is of fundamental relevance, since guanosine receptors are poorly characterized (Tasca et al., 1999; Traversa et al., 2002; Volpini et al., 2011). Astrocytic cells subjected to hypoxia/hypoglicemia release guanosine (Ciccarelli et al., 1999), whose levels remain high for several days afterwards (Uemura et al., 1991). Therefore, in a pathological scenario, guanosine could be released locally from astrocytes to provide protection under circumstances involving neuronal injury. Previous evidence showed guanosine protective effects were mediated by potassium channels (Oleskovicz et al., 2008), which were identified as BK channels in guanosine-induced neuroprotection in hippocampal slices subject to oxygen/glucose deprivation (Dal-Cim et al., 2011). The evaluation of a putative interaction site of guanosine in SH-SY5Y cells, in order to trigger this protective effect, also demonstrated dependence on BK channels activity. Since BK channels promote a negative feedback regulation of Ca^{2+} influx via voltage-gated-Ca^{2+} channels, regulation of cellular excitability and neurotransmitter release (Hu et al., 2001; Ghatta et al., 2006), it is feasible guanosine effect on BK channels may contribute to ionic homeostasis and reduction of ROS production.

However, some studies have claimed a putative interaction of guanosine on A_1 adenosine receptors (Thauerer et al., 2012). Adenosine A_1 receptors regulate the activity of cell membrane Ca^{2+} channels, K^+ channels, adenylate cyclase and phospholipase C (Palmer and Stiles, 1995), and it is associated to control of neurodegenerative process (Cunha, 2005). Thus, the possible interaction of guanosine with A_1 receptors observed here could result in an intracellular signaling leading to cellular protection. Surprisingly, it was observed the A_{2A} receptor antagonist, ZM241385, was also able to

prevent guanosine effect. Despite A_1 and A_{2A} receptors trigger opposing signal transduction and these receptors can interact forming A_1-A_{2A} receptor heterodimers with antagonic actions (Ciruela et al., 2006), we have previously observed guanosine-induced trophic effects on cerebellar neurons depend on both A_1 and A_{2A} adenosine receptors activation (Tasca et al., 2010). Therefore, the exact mechanism of interaction or modulation of BK and adenosine receptors through guanosine-induced neuroprotection remains to be unraveled.

In this study, we focused in understanding signaling pathways related to the protective mechanism of action of guanosine. The putative participation of PI3K, MAPKs and PKC cascades was evaluated because they have been described as key elements of signal transduction involved in cell proliferation, differentiation and stress response (Cantley, 2002; Cañas et al., 2007). Guanosine has proven to induce trophic effects by a mechanism that implicates activation of MAPK and PKC (Decker et al., 2007) and promote neuroprotection via PI3K/Akt (Di Iorio et al., 2004; Pettifer et al., 2004; Oleskovicz et al., 2008; Dal-Cim et al., 2011). Based on these evidences, we looked for the involvement of PI3K, MEK, PKC and $p38^{MAPK}$ in the protective effect of guanosine against Rot/oligo-induced cell death. Neither MEK, PKC or $p38^{MAPK}$ inhibition altered the protective effect of guanosine. However, inhibition of PI3K/Akt pathway by LY294002, partially abolished the protective effect of guanosine. Moreover, guanosine induced an increased Akt phosphorylation and LY294002 also prevented reduction of ROS production promoted by guanosine in Rot/oligo treated cells. Taken together, these data reinforce the idea PI3K/Akt pathway is implicated in the protective effect of guanosine.

Akt phosphorylates its substrate GSK-3β at the position Ser-9 and this phosphorylation inactivates GSK-3β (Stambolic and Woodgett, 1994). GSK-3β inhibition has been shown to be protective against a plethora of neurological insults (Manji et al., 1999; Endo et al., 2007), suggesting its inhibition can improve brain cell survival. Guanosine induces increased levels of p-Ser9-GSK-3β, showing inhibition of its activity. These results agree with previous data from our group, which demonstrate that guanosine protects against glutamate-induced cell death in rat hippocampal slices by activation of PI3K/Akt and subsequent inactivation of GSK-3β (Molz et al., 2011).

Inhibition of GSK-3β increases Nrf2 transcriptional activity (Rojo et al., 2008) which, in turn, regulates HO-1 transcription (Alam and Cook, 2003). HO-1 is a stress defense enzyme with anti-inflammatory and antioxidant properties (Lee and Chau, 2002; Salinas et al., 2003). Guanosine increased HO-1 expression, an effect related to guanosine-induced protection, since HO-1 inhibition with SnPP prevented guanosine effect on cell viability. A previous study has also shown that guanosine can promote HO-1 expression, although this effect was related to neurite outgrowth induction in PC12 cells (Bau et al., 2005), but not cellular protection as showed.

Interesting, the upregulation of HO-1 induced by guanosine, as well as its effect on ROS production was fully prevented by a PI3K/Akt inhibitor, indicating this pathway directly participates in induction of this antioxidant enzyme. The participation of PI3K/Akt in regulating HO-1 induction has been previously described (Cañas et al., 2007; Hwang and Jeong, 2010; Parada et al., 2010).

Modest induction of HO-1 activity results in a modest increase in biliverdin/bilirubin levels. Data in the literature have demonstrated a protective role for very low nanomolar concentrations of bilirubin in cultured rat primary hippocampal neurons exposed to H_2O_2 and to ethanol-induced neurotoxicity (Doré et al., 1999; Ku et al., 2006). In this study, bilirubin protected against Rot/oligo-induced cell death, as observed with guanosine. Bilirubin is an important endogenous antioxidant and may be particularly important as a cytoprotectant for tissues with relatively weak endogenous antioxidant defenses such as the nervous system (Ewing et al., 1992). These findings reinforce the idea that guanosine-induced protection against excessive ROS production elicited by Rot/oligo could be related to its ability to induced HO-1 antioxidant activity and, could thus restore balance between ROS production and scavenging.

In conclusion, guanosine can reduce oxidative stress induced by mitochondrial disruption due to a mechanism involving PI3K/Akt/GSK-3β pathway and induction of the antioxidant enzyme HO-1. Thus, endogenous compounds that protect against oxidative stress can be considered as new strategies to protect neurons in CNS pathologies.

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