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Neuroprotective effect of sulforaphane against methylglyoxal cytotoxicity

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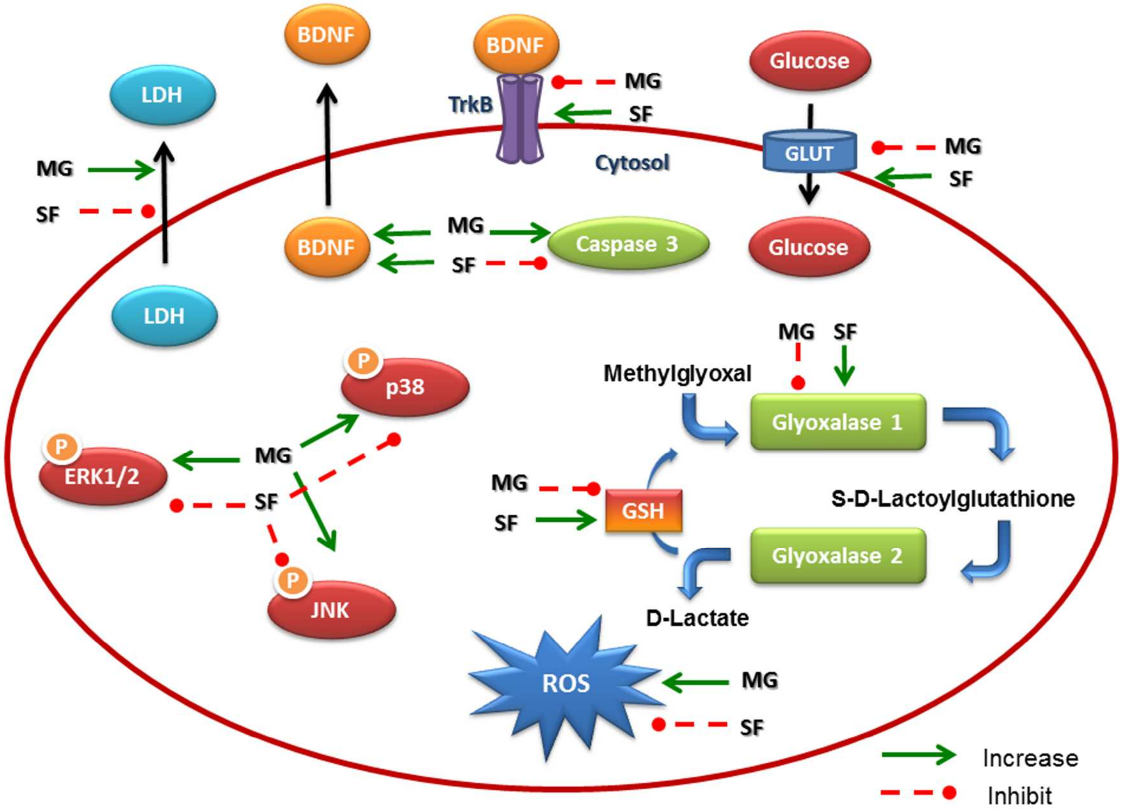
KEYWORDS: methylglyoxal; glyoxalase; oxidative stress; sulforaphane; brain derived neurotrophic factor; Alzheimer’s disease.

TABLE OF CONTENT GRAPHIC



ABSTRACT

Glycation, an endogenous process that leads to the production of advanced glycation end products (AGEs), plays a role in the etiopathogenesis of different neurodegenerative diseases such as Alzheimer's disease (AD). Methylglyoxal is the most potent precursor of AGEs and high levels of methylglyoxal have been found in the cerebrospinal fluid of AD patients. Methylglyoxal may contribute to AD both inducing extensive protein cross-linking and as mediator of oxidative stress. Aim of this study was to investigate the role of sulforaphane, an isothiocyanate found in Cruciferous vegetables, in counteracting methylglyoxal induced damage in SH-SY5Y neuroblastoma cells. Data demonstrated that sulforaphane protected cells against glycative damage by inhibiting the activation of caspase-3 enzyme, reducing the phosphorylation of MAPK signaling pathways (ERK1/2, JNK, and p38), reducing oxidative stress and increasing intracellular GSH levels. For the first time we demonstrated that sulforaphane enhanced methylglyoxal detoxifying system increasing the expression and activity of glyoxalase I. Sulforaphane modulated brain derived neurotrophic factor and its pathway, whose dysregulation is related to AD development. Moreover, sulforaphane was able to revert the reduction of glucose uptake caused by methylglyoxal. In conclusion, sulforaphane demonstrated a pleiotropic behavior thanks to its ability to act on different cellular targets, suggesting its potential role in preventing/counteracting multifactorial neurodegenerative diseases such as AD.

INTRODUCTION

Neurodegenerative diseases are multifactorial pathologies with a dramatically increasing incidence rate worldwide [1, 2]. The mechanisms triggering neurodegeneration are complex and may involve various characteristics such as mitochondrial dysfunction, excitotoxicity, abnormal protein aggregation, and inflammation [3-7]. In particular AD is characterized by an accumulation of misfolded proteins especially in the cerebral cortex and hippocampus [8, 9]. Accumulation of the β-amyloid (Aβ) peptide and neurofibrillary tangles in the brain are pathological hallmarks of AD [2, 10-12]. Besides protein aggregation, oxidative damage to lipids and proteins is closely linked to cognitive impairment and cellular dysfunction displayed in AD patients [13, 14] and correlates with the severity of the disease [15]. In addition, it has been suggested that glycation may have a key role in both extensive protein cross-linking and oxidative stress in AD [16, 17]. Glycation is a process of post-translational modification of proteins, in which free reducing sugars and toxic aldehydes such as methylglyoxal (MG) and glyoxal react non-enzymatically with amino groups, leading to the formation of advanced glycation end products (AGEs) [18]. AGEs are known to accumulate in the ageing brain [19] and in the brains of AD individuals, suggesting their pathological role in this context of neurodegeneration [20, 21]. High levels of AGE immunoreactivity are present in AD plaques and neurofibrillary tangles [22]. Elevated levels of AGEs represent a common pathological marker of both type 2 diabetes and AD and diabetic patients could have an increased risk of AD via AGE production [22]. Moreover impaired glucose consumption and energy metabolism have been observed in AD patients [23] and the decrease in glucose metabolism is an important hallmark of the clinical severity of the disease [24]. This scenario characterizes AD as “brain-type diabete”[25].

MG, formed endogenously as a by-product of the glycolytic pathway or nonenzymatically by sugar fragmentation reactions, is the most potent precursor of AGE formation [26], and high levels of MG have been found in the cerebrospinal fluid of AD patients and in plasma of diabetic individuals [27, 28]. It has been also suggested that MG may contribute to AD as mediator of oxidative stress [29-31].

MG is mainly detoxified by the glyoxalase system, which consist of the glutathione, Zn2+-dependent glyoxalase (GLO) 1 and glyoxalase 2 [32]. In healthy human brains, glyoxalase I decreases in old age [33] which could be one reason for cell damage and AGE accumulation during aging. Moreover, MG reduces glucose uptake in Hep G2 hepatoma cells [34].

In patients with severe memory dysfunction such as AD, levels of brain-derived neurotrophic factor (BDNF), a member of the mammalian neurotrophin family, are markedly depressed [35, 36]. Interestingly, Wang et al. observed a down-regulation of BDNF protein expression by AGEs in neural stem cells [37].

Nowadays no drugs are available to stop the progression of neurodegenerative disorders such as AD and only agents that could alleviate or delay these disorders have been developed. Phytochemicals are a promising source of potentially beneficial agents and have been proposed as an alternative form of treatment for neurodegenerative diseases [38-40].

Sulforaphane (isothiocyanato-4-(methylsulfinyl)-butane) (SF), abundantly found in broccoli, has been demonstrated to have neuroprotective effects in different model of AD [41-43]. We previously demonstrated SF ability to prevent cell death induced by oxidative stress in neuroblastoma cell line [44] and to protect primary cortical neurons against 5-S-cysteinyl-dopamine induced neuronal injury [45]. Moreover we have recently demonstrated that SF elicits a multi-target behavior against MG-induced damage in primary culture of neonatal rat cardiomyocytes [46].

In this study we investigated the protective effects of SF against glycative stress in neuroblastoma SH-SY5Y cells focusing on MG induced apoptosis and oxidative stress, the modulation of MG- detoxifying system and BDNF expression. Moreover due to the importance of glucose metabolism in neurodegeneration, the effect of SF on glucose uptake has been evaluated.

MATERIALS AND METHODS

MATERIALS

Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), penicillin/streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), 2-deoxy-glucose (DOG), acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC), monochlorobimane (MCB), phloretin, CelLytic M, mammalian protease inhibitor mixture, anti-β-actin, methylglyoxal (MG), bovine serum albumin (BSA), and all other chemicals of the highest analytical grade were purchased from Sigma-Aldrich. PhosSTOP was purchased from Roche Diagnostics (Mannheim, Germany). D,L-Sulforaphane (SF) was obtained from LKT Laboratories (Minneapolis, MN, USA). All other reagents were of the highest grade of purity commercially available.

CELL CULTURES

The SH-SY5Y human neuroblastoma cell line was obtained from Sigma-Aldrich (Milan, Italy). Cells were grown in DMEM supplemented with 10% (v/v) FBS, 2 mM glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin and maintained at 37 °C in a humidified incubator with 5% CO_2, as reported in [47]. Cells were used for experiments after reaching 80–90% confluence.

MTT AND LACTATE DEHYDROGENASE ACTIVITY ASSAYS

Cells were treated with different concentrations of MG (0.1-5 mM) for 24 h and/or pre-treated with 2.5 μM SF before MG exposure. Cell viability was evaluated by measuring MTT reduction as previously reported [48]. Briefly, at the end of each experiment, 0.5 mg/ml MTT was added and incubated for 1 h at 37°C. After incubation, MTT solution was removed, 200 µl DMSO was added, and the absorbance was measured at λ=595 nm using a microplate spectrophotometer (VICTOR3 V Multilabel Counter; Perkin-Elmer, Wellesley, MA, USA). Lactate dehydrogenase (LDH) activity was evaluated in the culture medium and the test was performed by using the Lactate Dehydrogenase Activity Assay Kit (SIGMA) according to the manufacturer's instructions.

DETERMINATION OF GSH LEVELS

Reduced GSH levels were determined by the MCB fluorometric assay as previously reported [49].

After treatments, culture medium was removed, and cells were washed twice with cold PBS and incubated for 30 min at 37°C in 0.1 ml fresh PBS containing 50 µM MCB. After incubation, fluorescence was measured at 355 nm (excitation) and 460 nm (emission) with a microplate spectrofluorometer (VICTOR3 V Multilabel Counter; Perkin-Elmer).

CASPASE-3 ACTIVITY ASSAY

Caspase-3 activity was measured by using Ac-DEVD-AMC peptide as a substrate as previously reported [46]. Briefly, cells were pre-treated with 2.5 µM SF for 24 h subsequently exposed to 0.5 mM MG for further 24 h. At the end of the treatment cells were scraped off and incubated on ice in lysis buffer (50 mM HEPES pH 7.4, 5 mM CHAPS and 5 mM DTT) for 20 min. Samples were centrifuged at 15 000 g for 15 min at 4°C and protein concentration in supernatants was determined according to the Bradford method[50]. Reaction mix (20 mM HEPES pH 7.4, 0.1% CHAPS, 5 mM DTT and 2 mM EDTA) containing Ac-DEVD-AMC was added to each sample and fluorescence intensity was recorded by a microplate spectrofluorometer (λex/em= 360/460 nm) (VICTOR3 V Multilabel Counter; Perkin-Elmer). Caspase activity was calculated using a AMC standard curve and results were expressed as nmol AMC/min/mg protein.

INTRACELLULAR ROS PRODUCTION ASSAY

The formation of intracellular ROS was evaluated using the fluorescent probe DCFH-DA, as previously reported [49]. Briefly, SH-SY5Y cells were treated with 5 μM SF and after 24 h were incubated with 5 μM DCFH-DA in PBS for 30 min. After removal of DCFH-DA, cells were incubated with 0.5 mM MG for 6 h. Cell fluorescence was measured using 485 nm excitation and 535 nm emission with a microplate spectrofluorometer (VICTOR3 V Multilabel Counter).

IMMUNOFLUORESCENCE STAINING

Cells were seeded on glass cover-slips and treated with 2.5 μM SF before the addition of 0.5 mM MG, then washed twice with PBS, fixed with 3% paraformaldehyde, washed with 0.1 M glycine in PBS, and permeabilized in 70% ice cold ethanol.

After fixing, the cells were incubated with anti-8-hydroxy-deoxyguanosine (8-OHdG) (StressMarq Biosciences, Victoria, CA) overnight at 4 °C. Subsequently the samples were washed with 1% BSA in PBS and incubated with FITC conjugated secondary antibody for 1 h at room temperature. DAPI was used for labeling nuclei. Preparations were embedded in Mowiol and images were acquired using an IX50-Olympus inverted microscopy (Olympus, Tokyo, Japan).

WESTERN IMMUNOBLOTTING

After treatments, SH-SY5Y cells were collected and homogenized in CelLytic M cell lysis reagent, with mammalian protease inhibitor mixture and PhosSTOP. Samples were boiled for 5 min before separation on 10% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (Hybond-C; GE Healthcare, Buckinghamshire, UK) in tris-glycine buffer at 110 V for 90 min. Membranes were then incubated in a blocking buffer containing 5% (w/v) skimmed milk and incubated with anti-phospho-p38, anti-p38, anti-phsopho-ERK1/2, anti-ERK1/2, anti-phosho-JNK, anti-JNK, anti- TrkB (Cell Signaling Technology, Beverly, MA), anti-BDNF (Sigma), anti-GLO1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). and anti-βactin (Sigma) as internal normalizer, overnight at 4°C on a three-dimensional rocking table. The results were visualized by chemiluminescence using ECL advance reagent according to the manufacturer's protocol (GE Healthcare, Buckinghamshire, UK). Semiquantitative analysis of specific immunolabeled bands was performed using a Fluor S image analyzer (Bio-Rad, Hercules, CA).

GLO1 ACTIVITY ASSAY

GLO1 activity was assessed by a spectrophotometric method according to [34] with minor modifications. Briefly, SH-SY5Y cells were scraped off in 10 mM PBS, lysed by freezing and thawing repeatedly, and protein concentrations were estimated using the Bradford's method [50], GLO1 activity

assay was performed by addition of 20 µl of cell lysate to a reaction mixture containing 2 mM MG and 2 mM GSH, which had been equilibrated for 10 min before sample addition. The reaction was monitored spectrophotometrically by following the formation of S-D-lactoylglutathione at 240 nm.

GLUCOSE TRANSPORT ASSAYS

SH-SY5Y cells were pre-treated with SF 2.5 μM for 24 h before MG 0.5 mM addition and glucose uptake was evaluated with two different methods. The first method was performed as previously reported [51]. Briefly, cells were washed twice in PBS and treated for 10 min with a mixture of DOG (0.8 μCi/assay) and 1.0 mM unlabeled glucose analogue, under conditions where the uptake was linear at least for 20 min. The transport was stopped by adding phloretin (final concentration 0.3 mM), a potent inhibitor of glucose transport activity. Radioactivity was measured by liquid scintillation counting (Tri-Carb liquid scintillation analyser, PerkinElmer).

Glucose uptake was also measured using a fluorescent D-glucose analogue 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG). At the end of each experiments, cells were washed twice and incubated with 100 µM 2-NBDG in glucose-free culture medium for 1 h. Cells cultured in medium lacking 2-NBDG were used as a negative control. The cells were washed twice prior to fluorescence detection using a microplate reader (VICTOR3 V Multilabel Counter) at a fluorescence excitation of 488 nm and emission of 520 nm.

STATISTICAL ANALYSIS

Each experiment was performed at least three times, and all values are represented as means ± SD. One-way ANOVA was used to compare differences among groups followed by Dunnett’s or Bonferroni’s test (Prism 5; GraphPad Software, San Diego, CA). Values of $P < 0.05$ were considered as statistically significant.

RESULTS

EFFECTS OF SF AGAINST MG-INDUCED DAMAGE IN SH-SY5Y CELLS

To determine the potential cytotoxic effect of MG, SH-SY5Y cells were treated with increasing concentrations (0.1-5 mM) of MG for 24 h after which cell viability was assessed by MTT assay (Fig. 1 A). MG caused a marked dose-dependent increase in neuronal mortality, identifying 0.5 mM (corresponding to ~50% of cell viability) as the best dose to proceed with the following experiments.

To identify the possible protective effect of SF against MG induced glycative stress, cells were pre-treated with 2.5 μM SF for 24 h and then exposed to 0.5 mM MG for further 24 h (Fig. 1 B). SF pre-treatment significantly improved cell viability compared to MG treated cells by ~30%, evidencing a potential protective role of SF in counteracting MG-induced damage in SH-SY5Y cells. For additional assessment of cell viability, LDH release in the culture medium was quantified in the same experimental conditions (Fig. 1 C). As expected, MG treatment induced a significant and strong increase of LDH release, while SF pre-treatment was able to maintain LDH release at level comparable to control cells. Moreover, to elucidate whether the neuroprotection conferred by SF involves protection from apoptosis, we evaluated the activity of the pro-apoptotic enzyme caspase-3 (Fig. 1 D). MG significantly increased caspase-3 activity in SH-SY5Y cells indicating that at least part of the cells undergo apoptotic cell death, while SF pre-treatment significantly reduced caspase-3 activity compared to MG. Interestingly, in MG treated cells SF was able to maintain caspase-3 activity to value comparable to control cells.

EFFECT OF SF AGAINST MG-INDUCED OXIDATIVE STRESS IN SH-SY5Y

We investigated SF ability to counteract MG-induced intracellular ROS production by the DCFH-DA assay. As illustrated in Fig. 2, incubation of SH-SY5Y cells with 0.5 mM MG for 6 h resulted in a significant increase in intracellular ROS production. SF treatment, in contrast, significantly counteracted the intracellular ROS production compared to MG. There was no change in ROS production after treatment with SF alone.

To confirm the oxidative stress status in MG-treated SH-SY5Y cells, we assessed the formation of 8-OHdG, a marker of oxidative damage to DNA In control and SF treated cells, positive staining for 8-OHdG was hardly detectable in the cytoplasm or nucleus. As expected, there was a strong positive staining for 8-OHdG in the cytoplasm and nucleus of MG treated cells, meanwhile staining of 8-OHdG was strongly attenuated by SF pre-treatment as shown in Fig. 3.

MODULATION OF THE GLYOXALASE SYSTEM BY MG AND SF

As the glyoxalase system is the most important pathway for the detoxification of MG, we investigated the effect of 2.5 μM SF for different time points (1, 6, 24, 48 h) on GLO1 protein level and activity in SH-SY5Y cells (Fig. 4 A, B). SF treatment caused a significant increase in GLO1 protein level after 24 and 48 h. Accordingly, SF treatment triggered a significant increase in GLO1 activity at the same time points. In Figures 4 C and 4 D, the effect of SF pre-treatment on GLO1 protein levels and activity in SH-SY5Y cells exposed to MG is reported. Interestingly, MG induced a significant reduction of both GLO1 protein levels and activity, meanwhile SF was able to maintain GLO1 levels to values comparable to control cells.

As a fundamental player in the glyoxalase system is GSH we evaluated the effect of SF pre-treatment on intracellular GSH levels by the MCB assay. Cells were pre-treated with 2.5 μM SF for 24 h and then exposed to 0.5 mM MG (Fig. 5). SF alone was able to increase GSH levels compared to control cells. Exposure to MG caused a significant decrease in GSH levels, meanwhile pre-treatment with SF significantly increased the amount of GSH to levels comparable to control cells.

MAPK MODULATION BY MG AND SF IN SH-SY5Y CELLS

Since we observed that MG triggers apoptotic death in SH-SY5Y cells, we conducted immunoblotting analysis to investigate the effects of MG on the main signal transduction pathways involved in the induction of apoptosis in neuronal cells. Neuroblastoma cells were treated with 0.5 mM MG and after different time points the phosphorylation of ERK1/2, p38 MAPK, and JNK was evaluated with specific antibodies (Fig. 6 A). Interestingly, after only 30 min a strong phosphorylation of ERK1/2, p38 MAPK, and JNK was observed. To verify the role of SF pre-treatment in the modulation of MAPK phosphorylation induced by MG, SH-SY5Y cells were exposed to SF for 24 h before the addition of MG for 30 min (Fig. 6 B). SF exposure did not influenced MAPK activation, meanwhile was able to partially reduce MG-induced phosphorylation of all the MAPKs evaluated, confirming the role of SF in counteracting apoptotic cell death induced by MG.

To better characterized the role of each MAPK in MG-induced damage, we investigated the effect of specific inhibitors of p38 (SB203489), JNK (SP600125) and ERK1/2 (PD98059) on cell viability and LDH release in SH-SY5Y cells exposed to 0.5 mM MG for 24 h (Fig. 7). As already shown, MG induced a significant reduction of cell viability and an increase of LDH release in respect to control cells. MAPK inhibitor treatment before MG exposure caused a significant increase of cell viability and a decrease of LDH release in comparison to MG, suggesting that p38, JNK and ERK1/2 are involved in MG induced cell death.

EFFECT OF MG AND SF ON BDNF EXPRESSION AND ITS TRANSDUCTIONAL PATHWAY

To examine whether SF regulates BDNF expression in MG-treated cells, immunoblot analyses were performed (Fig. 8). BDNF was significantly increased by SF and surprisingly by MG alone, and the highest BDNF levels were observed in cells pre-treated with SF before MG exposure. In order to better understand the functional role of the up-regualation of this neurotrophin, the BDNF-triggered signal transduction pathway was investigated. MG significantly reduced TrkB protein level in respect to control cells, whereas SF pretreatment was able to maintain TrkB protein levels to values comparable to control cells. SF alone did not influenced TrkB.

EFFECT OF MG AND SF ON GLUCOSE UPTAKE

We investigated the effect of SF treatment on glucose uptake in SH-SY5Y cells exposed to MG (Fig. 9). MG significantly decreased glucose uptake in neuroblastoma cell line as measured by both a radioisotopic method (Fig. 9 A) and an ELISA assay (Fig. 9 B). SF treatment significantly increased glucose uptake compared to control cell, while SF pre-treatment before MG exposure maintained glucose uptake to level comparable to control cells.

DISCUSSION

Accumulating evidence suggests that AGEs formation is an important pathway leading to neuronal impairment in many degenerative disease such as AD [52-55]. The reactive carbonyl compound MG is the main contributor to AGEs formation [56] and vitro studies demonstrated that MG triggers cellular injury and toxicity to neuronal cells [57-61]. Our data confirmed MG deleterious effect in SH-SY5Y cells and demonstrated that SF protects cells from necrotic and apoptotic cell death modulating in a pro-survival direction ERK1/2, p38 MAPK and JNK, enhancing the expression of the MG detoxifying system, up-regulating BDNF and its receptor, and increasing glucose uptake.

There are convincing evidence that free radicals may be involved in the etiopathogenesis of AD [62]. MG metabolism leads to the production of ROS [46, 63-66] and, in particular, it has been shown that MG accumulation induces deleterious effects on hippocampal neuron viability mediated by ROS production [67]. As we previously demonstrated that SF prevents the increase of oxidative stress induced by 6-hydroxydopamine challenge in neuronal cells [44], we investigated the antioxidant effects of SF pre-treatment in SH-SY5Y cells exposed to MG. Our results confirmed the increased oxidative stress triggered by MG in neuronal cells and demonstrated that SF significantly counteracts MG induced ROS production as measured by DCHF-DA and by immunostaining of 8-OHdG. To have a deeper knowledge of the mechanisms by which SF counteracts oxidative stress, we measured the level of the antioxidant molecule GSH. MG markedly reduced GSH intracellular level, in agreement with the data obtained in different cell lines [58, 68, 69], while SF pre-treatment significantly counteracted MG induced GSH depletion. Interestingly, GSH is also substrate of GLO1 that converts MG into the corresponding α-hydroxy acids and thus reducing MG neurotoxicity and apoptosis [30]. It has been shown that GLO1 increases in the early stages of AD, meanwhile decreases with the progression of the disease [70]. Thus enhancing glyoxalase expression in the late phase of AD could be an important therapeutic target. Our data demonstrated, for the first time, that SF treatment increases GLO1 protein expression and activity in SH-SY5Y cells, extending our previous results obtained with cardiomyocytes [46]. On the other hand, MG down-regulates GLO1 protein expression and activity, in agreement with the results of Di Loreto et al. [58]. SF pre-treatment abolishes GLO1 down-regulation induced by MG.

MAPK signaling pathways play pivotal roles in cell survival, differentiation, development and growth [71, 72]. Three main subfamilies have been identified: ERK1 ⁄ 2, JNK and p38 kinases. It has been widely demonstrated the involvement of JNK and p38 MAPK in cell death, whereas ERK1⁄2 opposes cell death [72]. However, in the last years, different studies have suggested a death-promoting role also for ERK1⁄2 in both in vitro and in vivo models of neuronal death [73]. Since MAPKs play a critical role in cellular processes that are affected in AD, the importance of MAPKs as pathological modulators has been widely recognized. In particular, it has been observed that all MAPK pathways are activated in AD [74-76]. Thus, we investigated the activation state of ERK1/2, JNK and p38 MAPK signaling pathways in MG-induced damage in SH-SY5Y cells. As expected, all the MAPKs were phosphorylated already after 30 min MG exposure, confirming the role of this di- carbonyl compound in triggering apoptotic cell death, as demonstrated by other in vitro studies in neuronal cells [57, 58, 60]. Our data are partially in agreement with the results of Huang et al. [57] that observed the activation of JNK and p38, but not of ERK1/2 in Neuro-2A cells exposed to MG. This discrepancy could be due to the different MG exposure time as they studied the activation state of the MAPK pathways after 24 h MG incubation. Interestingly, immunoblot analysis revealed that SF-pre-treatment significantly inhibited MG induced phosphorylation of ERK1/2, JNK and p38, highlighting the ability of SF to directly modulate the MAPK signaling pathways. The involvement of ERK1/2, JNK and p38 in MG induced apoptosis was further confirmed by the use of specific inhibitors of the MAPK pathways. These results show that the inhibition of ERK1/2, JNK and p38 phosphorylation significantly counteracted MG induced cell death suggesting that SF protective effect could be partially mediated by the inhibition of the activation of these pro-apoptotic signaling pathways.

BDNF is a member of the neurotrophin family of growth factors and can promote neuronal survival, neurite outgrowth and synaptic plasticity interacting with the tropomyosin-related kinase B (TrkB) and p75 cellular receptors [77, 78]. Growing evidence suggests that BDNF levels play an important role in AD pathophysiology [79]. BDNF expression levels are reduced in the brains of AD or aged patients compared with healthy subjects [80, 81]. In neuronal cultures, it has been shown that BDNF counteracts neuronal toxicity induced by $A\beta_{1-42}$ and $A\beta_{25-35}$ [82] and triggers de-phosphorylation of tau protein [83]. For these reasons, we investigated the effect of SF and MG on BDNF. Our data demonstrated that both SF and MG up-regulate BDNF protein levels. This is an unexpected result, as BDNF triggers pro-survival messages whereas MG challenge induces cell death. Interestingly, other Authors showed that MG increases BDNF level [58, 65] without inducing hippocampal neuroprotection, strengthening our results. We also observed that SF pre-treatment before MG exposure had the highest effect on BDNF up-regulation, showing an additive effect of SF and MG. As Di Loreto et al. demonstrated that MG induces BDNF but, at the same time, reduces TrkB expression explaining the lack of neuroprotection, we focused also on TrkB. Our results confirmed the reduction of TrkB protein expression by MG exposure, and demonstrated that SF pre-treatment significantly up-regulates this receptor, evidencing another mechanisms by which SF counteracts MG induced damage.

Brain energy metabolism is mainly dependent upon glucose consumption, so a decline in glucose metabolism and a shift to alternative substrates can result in neurotoxic by-products [84]. There are convincing clinical and experimental evidence which demonstrate that brain glucose hypometabolism and reduced glucose transport are a metabolic phenotype characteristic of the Alzheimer's brain [85]. Chronic MG administration to Sprague-Dawley rats for 28 days induced glucose intolerance, reduced GLUT4 and reduced GLUT2 expression [86]. In addition, we previously demonstrate that MG reduces glucose uptake in SH-SY5Y cells [51]. To verify the potential role of SF in counteracting glucose hypometabolism induced by MG we measured glucose uptake by a radioisotopic and an ELISA method. Interestingly, SF alone was able to increase glucose uptake in respect to control cells and SF-pretreatment totally counteracted MG induced reduction of glucose uptake, suggesting a potential role of SF in preserving glucose availability in the brain of AD patients.

In conclusion, our results reveal a novel role of SF as anti-glycative agent in the cellular response to MG challenge, and provide new insight for understanding the detailed mechanisms of MG induced-cell death. SF acts on different cellular targets reverting the deleterious effects induced by MG (Fig. 10). Taking into account the emerging role of glycation in neurodegenerative diseases and in particular its role in protein cross-linking and oxidative stress in AD, SF may be considered a promising nutraceutical compound in preventing/counteracting a multifactorial disease like AD.

FIGURES

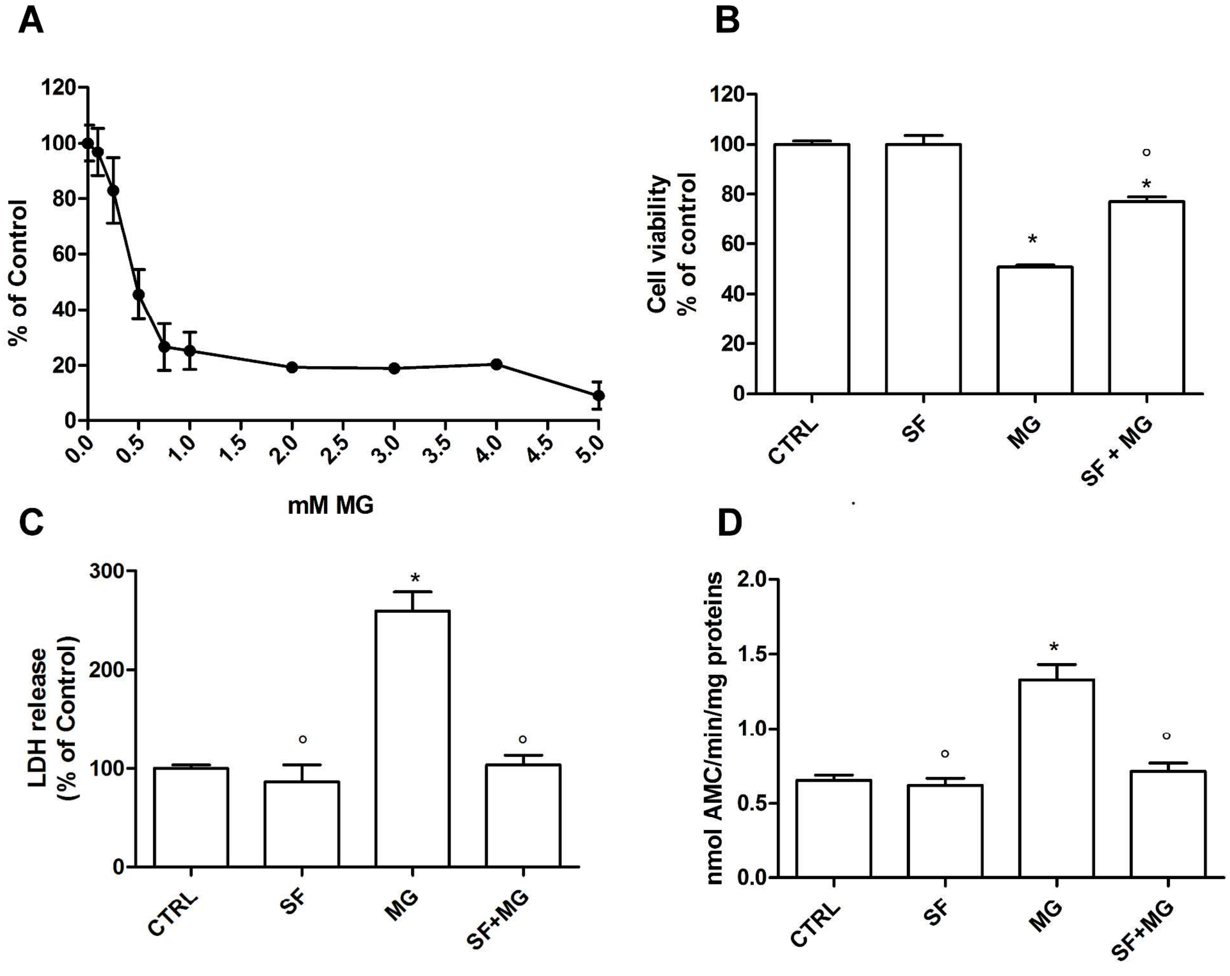


Figure 1. SF protection against MG-induced damage. SH-SY5Y cells were treated with 0.1-5 mM MG for 24 and cell viability was assessed by MTT assay (A). SH-SY5Y cells were pre-treated with 5 µM SF for 24 h and then exposed to 0.5 mM MG and cell viability was assessed by MTT assay (B) and LDH release (C); apoptotic cell death was evaluated measuring caspase-3 activity (D) as reported in Material and Methods. Each bar represents means ± SD of 4 independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. *p <0.05 with respect to control; °p< 0.05 with respect to MG.

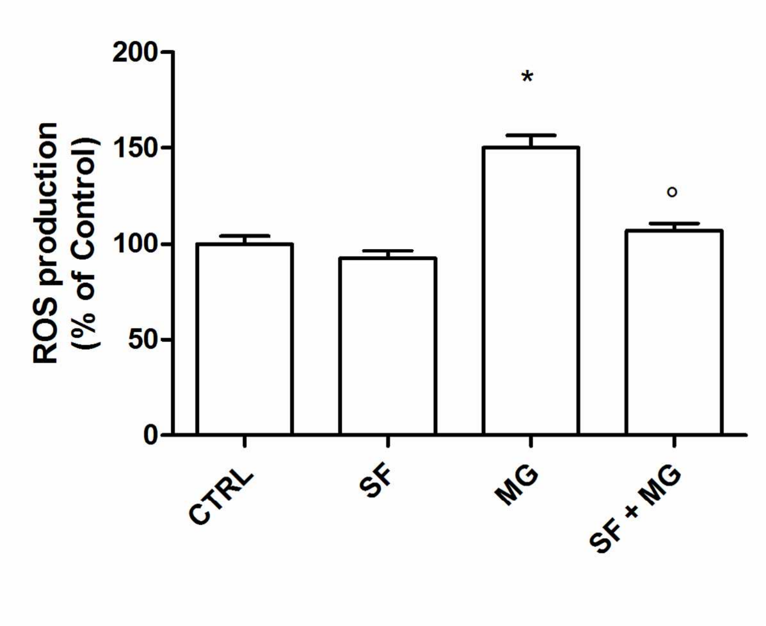


Figure 2. Fluorimetric assays of SF effect on MG-induced ROS generation. SH-SY5Y cells were pre-treated with 2.5 µM SF for 24 h before addition of 0.5 mM MG. Intracellular ROS levels were determined with the peroxide-sensitive probe DCFH-DA as reported in Material and Methods. Data are expressed as a percentage compared to control and are presented as mean ± SD, n = 4 in each group,* $p<0.05$ vs Control; ° $p<0.05$ vs MG.

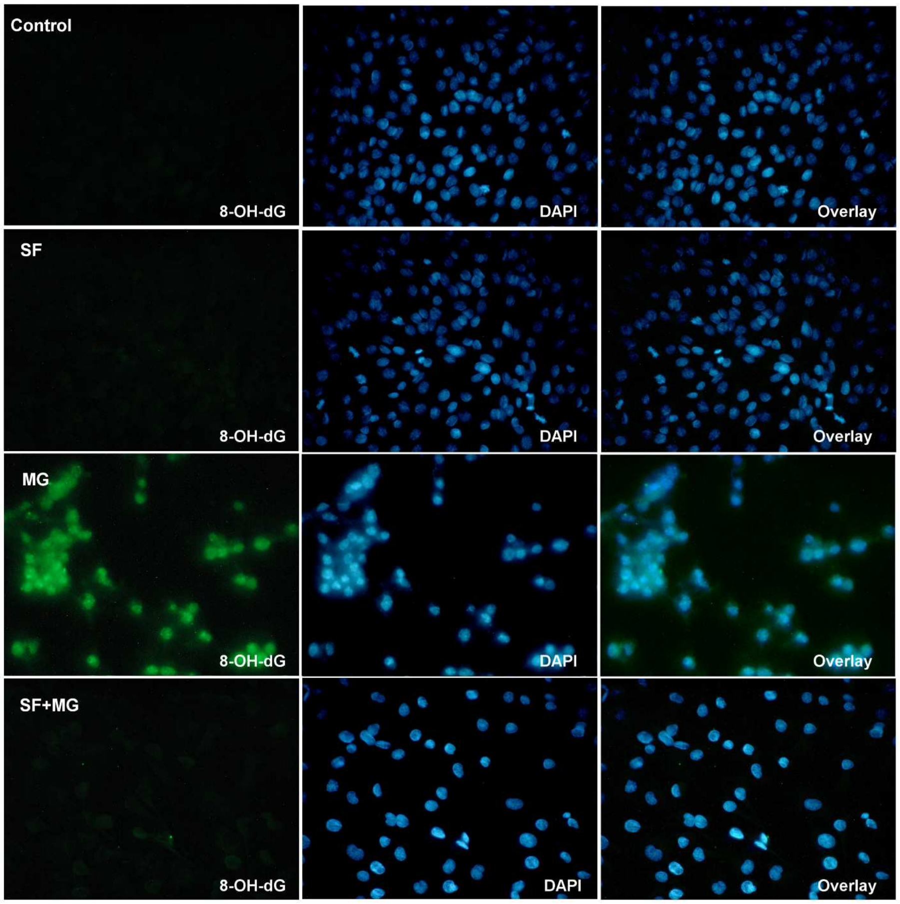


Figure 3. Effect of SF on oxidative DNA damage in MG-treated SH-SY5Y cells. SH-SY5Y cells were incubated with 0.5 mM MG in the presence or absence of SF. Intracellular oxidative DNA damage was detected using an immunofluorescence staining with anti-8-OHdG antibody as reported in Material and Methods. Middle panels indicate DAPI nuclear staining as a counterstain.

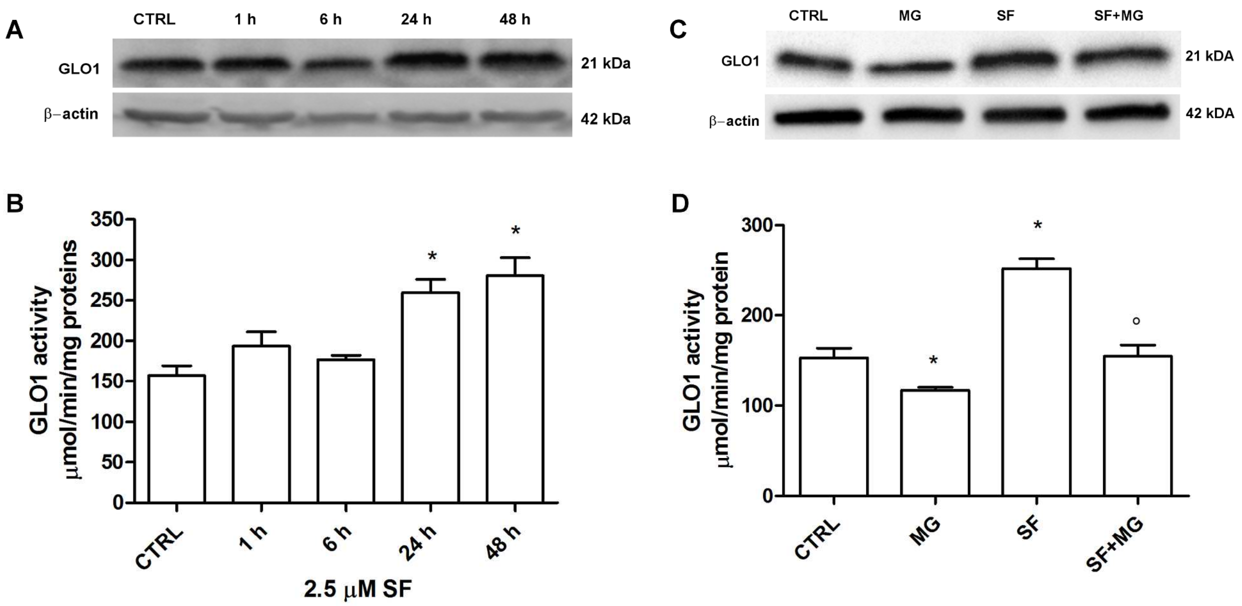


Figure 4. Effect of MG and SF on GLO1 protein level and activity. SH-SY5Y cells were treated with 2.5 μM SF for 1-48 h followed by western blot analysis for GLO1 (A) or evaluation of GLO1 enzymatic activity (B) as reported in Material and Methods. SH-SY5Y cells were pre-treated with 2.5 μM SF for 24 h before exposure to 0.5 mM MG followed by western blot analysis for GLO1 (C) or evaluation of GLO1 enzymatic activity (D). Each bar represents means ± SD of 4 independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. *p <0.05 with respect to control; °p< 0.05 with respect to MG

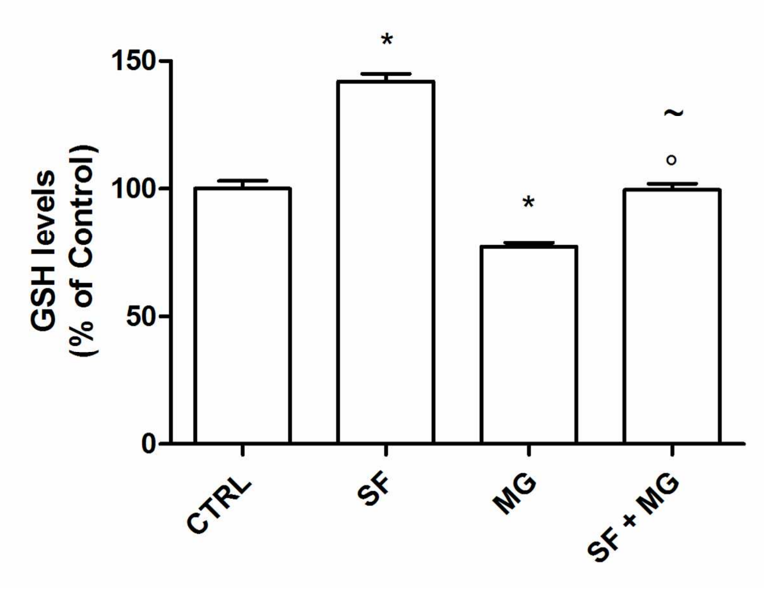


Figure 5. Effect of SF treatment on GSH levels in SH-SY5Y cells exposed to MG. Cells were pre-treated with 2.5 μM SF for 24 h and then treated with 0.5 mM MG for further 24 h. GSH levels were measured using the fluorescence probe MCB as reported in Material and Methods. Each bar represents the mean ± SD of 4 independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's test. * $p < 0.05$ with respect to control; ° $p < 0.05$ with respect to MG; ~ $p < 0.05$ with respect to SF.

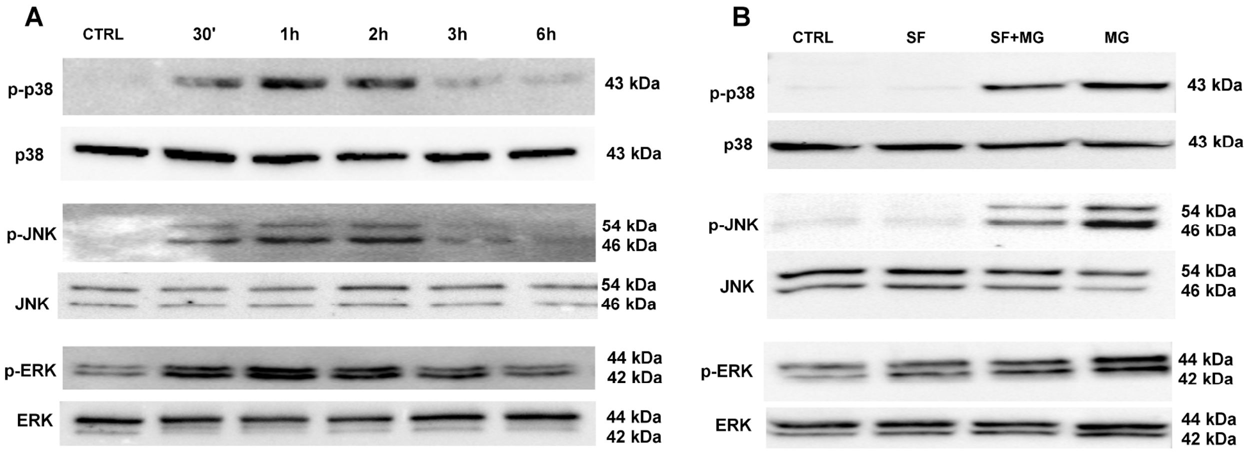


Figure 6. Effect of MG and SF on protein expression of total and phosphorylated MAPKs. Proteins were separated by SDS–PAGE electrophoresis and immunoblotted and probed for total and phosphorylated forms of p38, JNK and ERK1/2 as reported in Materials and Methods. SH-SY5Y cells were treated with MG 0.5 mM for different time periods (30 min- 6 h) (A). SH-SY5Y cells were pre-treated with 2.5 μM SF for 24 h and then exposed to 0.5 mM MG for 30 min (B).

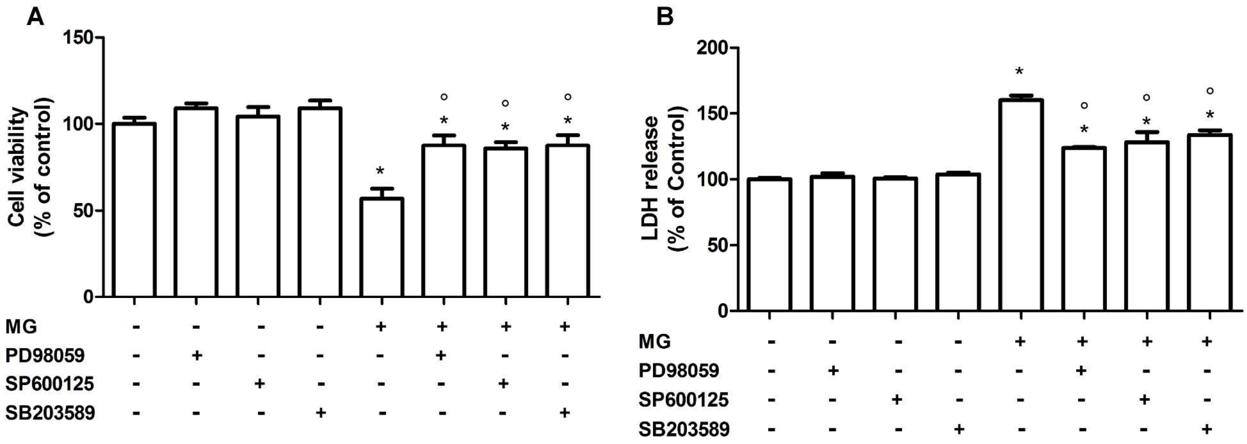


Figure 7. Effect p38, ERK and JNK inhibitors on MG-induced damage. SH-SY5Y cells were treated with 10 μM PD98059, 10 μM SP600125, or 10 μM SB203489 1 h before exposure to 0.5 mM MG for 24 and cell viability was assessed by MTT assay (A) and LDH release (B) as reported in Materials and Methods. Each bar represents means ± SD of 3 independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. *$p < 0.05$ with respect to control; °$p < 0.05$ with respect to MG.

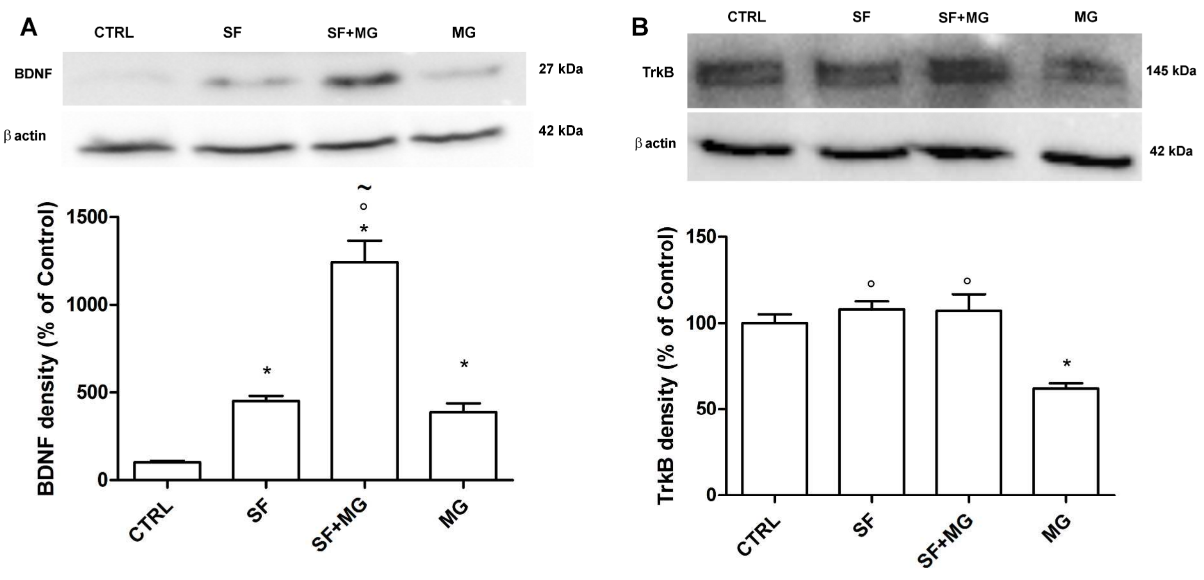


Figure 8. Effect of MG and SF on BDNF and TrkB expression. SH-SY5Y cells were pre-treated with SF for 24 h and then exposed to 0.5 mM MG for 24 h. Proteins prepared by SDS–PAGE electrophoresis were immunoblotted and probed for BDNF (A) and TrkB (B). Representative bands and densitometric values analysis are shown. Relative amounts were normalized to the intensity of the corresponding β-actin band and represented as % of Control. Data were analyzed by one-way ANOVA followed by Bonferroni's test. *$p < 0.05$ with respect to control cells; °$p < 0.05$ with respect to MG; ~ $p<0.05$ with respect to SF.

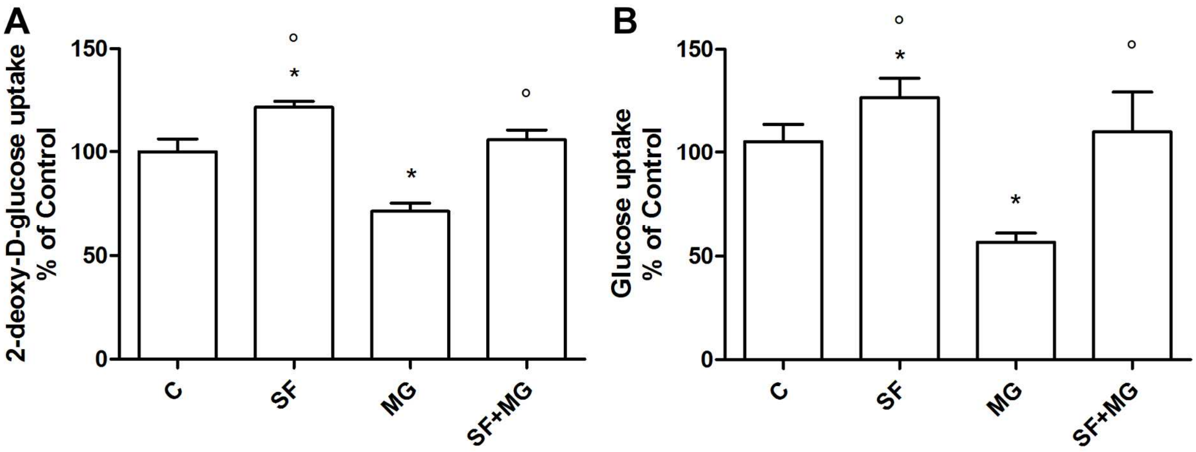


Figure 9. Effect MG and SF on glucose uptake. SH-SY5Y cells were pre-treated with 2.5 µM SF before the addition of 0.5 mM MG. A) Glucose uptake was assayed by a radioisotope method using 2-deoxy-D-[2, 3] glucose (A) and using the fluorescent probe 2-NBDG (B) as reported in Materials and Methods. Each bar represents means ± SD of 3 independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. *p <0.05 with respect to control; °p< 0.05 with respect to MG.

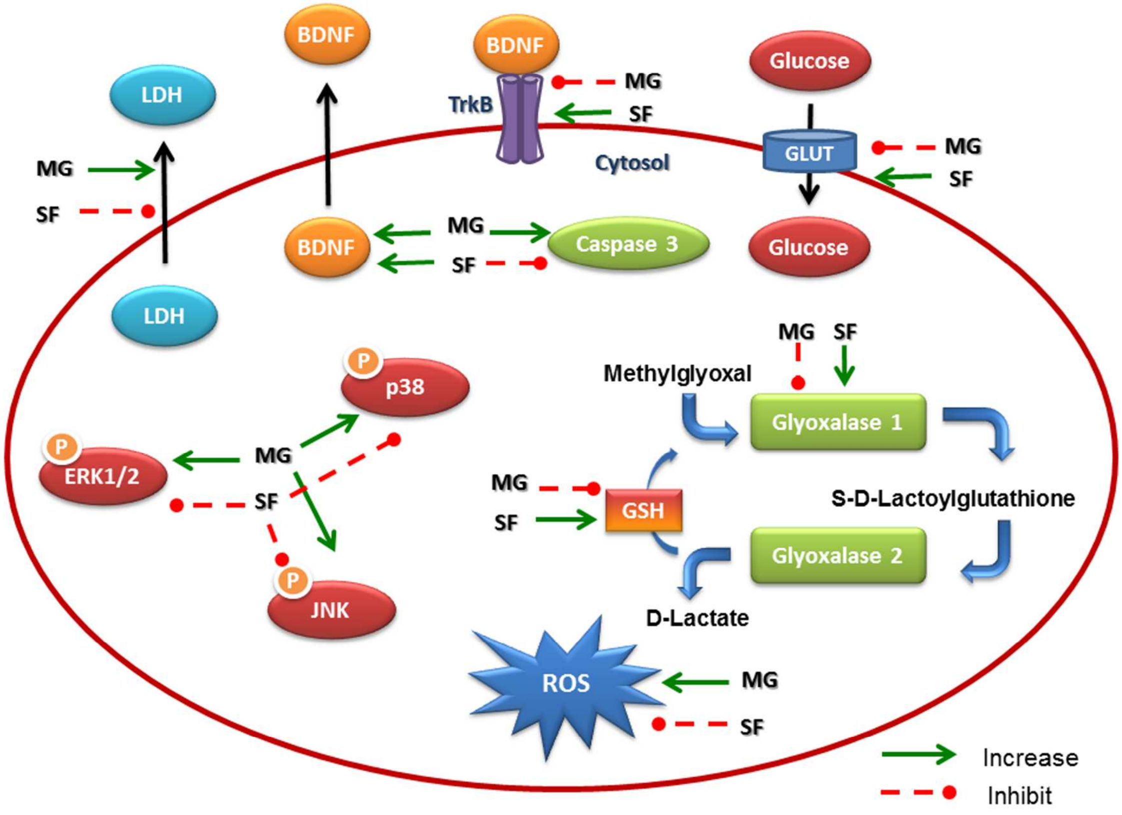


Figure 10. Diagrammatic representation of the potential pathways underlying the pleiotropic neuroprotective effects of SF against MG-induced injury.

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ABBREVIATIONS

2-NBDG, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose; 8-OHdG, 8-hydroxy-deoxyguanosine; Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin; AD, Alzheimer's disease; AGEs, advanced glycation end products; Aβ, Amyloid β; BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; DOG, 2-deoxy-glucose; ERK, extracellular-signal-regulated kinase; FBS, foetal bovine serum; GLO, glyoxalase; GLUT, glucose transporter; GSH, glutathione; JNK, c-Jun N-terminal kinase; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MCB, monochlorobimane; MG, methylglyoxal; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; SF, sulforaphane; TrkB, tropomyosin receptor kinase B;.

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