Neurotoxicity Research (2019) 36:491–502
https://doi.org/10.1007/s12640-019-00091-1

ORIGINAL ARTICLE

Tanshinone I Induces Mitochondrial Protection by a Mechanism Involving the Nrf2/GSH Axis in the Human Neuroblastoma SH-SY5Y Cells Exposed to Methylglyoxal

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Received: 9 May 2019 / Revised: 2 July 2019 / Accepted: 18 July 2019 / Published online: 29 July 2019

Abstract

Methylglyoxal (MG) is a dicarbonyl molecule exhibiting high reactivity and is a major responsible for glycation in human cells. Accumulation of MG is seen in certain diseases, including metabolic disturbances and neurodegeneration. Among other effects, MG promotes mitochondrial dysfunction, leading to bioenergetic decline and redox impairment in virtually any nucleated human cells. The detoxification of MG is dependent on the availability of reduced glutathione (GSH), a major antioxidant that is also utilized in phase II detoxification reactions. The synthesis of GSH is mainly controlled by the transcription factor nuclear factor (erythroid-derived 2)–like 2 (Nrf2). The activation of Nrf2 is stimulated by several reactive compounds, including natural molecules produced by plants. Tanshinone I (T-I) is obtained from *Salvia miltiorrhiza* Bunge and exerts potent cytoprotective actions in different cell types. Thus, we have investigated here whether and how T-I would be able to protect mitochondria of the human neuroblastoma SH-SY5Y cell line exposed to MG. The cells were pretreated with T-I at 2.5 μM for 2 h before the challenge with MG at 500 μM. T-I significantly attenuated the MG-induced loss of cell viability, bioenergetic decline, and redox impairment in SH-SY5Y cells. The inhibition of the GSH synthesis by buthionine sulfoximine (BSO) at 100 μM suppressed the mitochondrial protection promoted by T-I. The silencing of Nrf2 by small interfering RNA (siRNA) abrogated the synthesis of GSH and the mitochondrial protection stimulated by T-I in SH-SY5Y cells. Therefore, T-I induced mitochondrial protection by a mechanism involving the Nrf2/GSH axis in MG-challenged SH-SY5Y cells.

Keywords Tanshinone I · Methylglyoxal · Mitochondria · SH-SY5Y cells · Glutathione · Nrf2

Introduction

Accumulation of reactive agents, such as reactive metabolites and reactive oxygen and nitrogen species (ROS and RNS, respectively), has been seen as an important part of the mechanism associated with aging and several human diseases, such as neurodegeneration, cardiovascular diseases, and metabolic disturbances (Brownlee 2001; Sies et al. 2017). In that context, the reactive dicarbonyl molecule methylglyoxal (MG; also known as pyruvaldehyde) is originated from the glycolysis as a by-product (Allaman et al. 2015). It was estimated that 0.1–0.4% of the glycolytic flux would lead to the production of MG (Kalapos 2008). Nonetheless, MG binding to macromolecules occurs at a very high rate, and studies have indicated that MG may reach total concentrations as high as 310 μM (Allaman et al. 2015; Chaplen et al. 1998). MG exhibits potent ability to glycate nucleic acid, proteins, and lipids, leading to the formation of the advanced glycation end products (AGE), which are central players in diabetes

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12640-019-00091-1) contains supplementary material, which is available to authorized users.

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mellitus and neurodegenerative disorders, such as Alzheimer's disease (AD) (Angeloni et al. 2014; Lo et al. 1994; Robertson 2017; Westwood et al. 1997). The production of MG is enhanced in conditions of hyperglycemia and in the case of triosephosphate isomerase enzyme deficiency, among others (Ahmed et al., 2003; Allaman et al. 2015). MG causes disruption of bioenergetics systems and redox impairment, at least in part, by promoting mitochondrial dysfunction in virtually any nucleated human cell (Neviere et al. 2016; Pun and Murphy, 2012; Roy et al. 2003; Seo et al. 2014).

MG detoxification is mediated by the glyoxalase system and is highly dependent on glutathione (GSH), the major non-enzymatic antioxidant agent in human cells (Allaman et al. 2015). Moreover, it was described that the glyoxalase I enzyme may be inhibited by nitric oxide ($NO^{\bullet}$, a free radical) and *S*-nitrosoglutathione, which is an adduct formed by GSH and $NO^{\bullet}$ (Mitsumoto et al. 2000). Therefore, GSH depletion and/or increased free radical production may reduce the effectiveness of the glyoxalase system in MG detoxification. The synthesis of GSH is dependent on the rate-limiting enzyme γ-glutamate-cysteine ligase (γ-GCL), which contains the catalytic (GCLC) and regulatory/modifier (GCLM) subunits (Lu 2013). The expression of γ-GCLC and GCLM is controlled mainly by the transcription factor nuclear factor (erythroid-derived 2)–like 2 (Nrf2), among others (Lu 2013). Actually, Nrf2 is a major regulator of the expression of antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), peroxiredoxins (PRX), and heme oxygenase-1 (HO-1) (Tonelli et al. 2018). The activation of Nrf2 is promoted by reactive species and xenobiotics that cause the dissociation of Nrf2 from Kelch-like ECH–associated protein 1 (Keap1) in the cytosol and posterior translocation of the transcription factor to the cell nucleus, causing the upregulation in the expression of specific genes (Itoh et al. 1999; Tonelli et al. 2018). There are several natural compounds exhibiting the ability to upregulate Nrf2 and causing cytoprotection, as demonstrated in in vitro and in vivo experimental models (Chen et al. 2012; de Oliveira et al. 2016; de Oliveira et al. 2018a,b). In this regard, tanshinone I (T-I; $C_{18}H_{12}O_3$) has been isolated from *Salvia miltiorrhiza* Bunge (Danshen) and presents antioxidant, anti-inflammatory, and antitumor activities (Tian and Wu, 2013). The antioxidant effects caused by T-I seem to be associated with its ability in attenuating mitochondrial impairment in chemically stressed cells, as previously demonstrated by us (de Oliveira et al. 2017a,b). Moreover, T-I is a potent inducer of Nrf2 and of the GCLC and GCLM subunits of γ-GCL, as reported by Jing et al. (2016). In this context, T-I have upregulated GSH levels in both cytoplasm and mitochondria of SH-SY5Y cells (De Oliveira et al., 2017a).

However, it remains to be examined whether T-I would be able to promote mitochondrial protection in cells exposed to MG. Therefore, we have investigated here whether and how T-I would be able to promote mitochondrial protection in the human neuroblastoma SH-SY5Y cell line exposed to MG. We have analyzed whether GSH, which is an important substrate in the reactions of MG detoxification, would also play a role in the mitochondrial protection caused by T-I. Furthermore, the involvement of the transcription factor Nrf2 in the modulation of GSH synthesis was analyzed in the SH-SY5Y cells.

Material and Methods

Materials

We have purchased the plastic material used for cell culture from Corning, Inc. (NY, USA) and Becton Dickson (NJ, USA). The reagents utilized in the maintenance of cell culture were obtained from Sigma-Aldrich (MO, USA). Other chemicals and assay kits utilized here were acquired as mentioned below.

Cell Culture and Treatments

The human dopaminergic neuroblastoma SH-SY5Y cells, which were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA), were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 HAM nutrient medium (1:1 mixture; presenting 10% fetal bovine serum, 2 mM L-glutamine, 1000 units/mL penicillin, 1000 μg/mL streptomycin, and 2.5 μg/mL amphotericin B) in a 5% CO_2-humidified incubator at 37 °C.

Aiming to induce cellular impairment and mitochondrial dysfunction, the cells were challenged with MG at 500 μM for 24 h, as described before (Angeloni et al. 2015; de Oliveira et al. 2015; Nishimoto et al. 2017). A treatment with T-I at 2.5 μM or vehicle (DMSO) for 2 h was performed before the exposure of the cells to MT at 500 μM for an additional 24 h. In order to inhibit the synthesis of GSH, the cells were treated with buthionine sulfoximine (BSO) at 100 μM for 24 h prior to the administration of T-I at 2.5 μM for further 24 h.

Evaluation of Cell Viability and Cytotoxicity

Cell viability was analyzed by performing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (REF). The lactate dehydrogenase (LDH) leakage assay was performed to evaluate cytotoxicity, following the protocol of the manufacturer (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega) (de Oliveira et al. 2017c).

Quantification of Intracellular ROS Production

We performed the quantification of the intracellular production of ROS by using the nonpolar compound 2′-7′-

dichlorodihydrofluorescein diacetate (DCFH-DA) assay, as previously described (LeBel et al. 1992; REF de Oliveira et al. 2016).

Measurement of Hydrogen Peroxide Production

The generation of hydrogen peroxide (H_2O_2) by the SH-SY5Y cells was assessed by using the OxiRed probe after deproteinization of the samples, as indicated by the manufacturer of a commercial assay kit (Abcam, MA, USA) (de Oliveira et al. 2016).

Analysis of Nitric Oxide Production

We have measured the nitric oxide (NO•) production in the SH-SY5Y cells by using a commercial assay kit, as indicated by the manufacturer (Abcam, MA, USA). This assay is dependent on the conversion of nitrate into nitrite by the nitrate reductase enzyme. Then, the nitrite generated in that reaction reacts with the fluorescence probe DAN (2,3-diaminonaphthalene) (de Oliveira et al. 2016).

Isolation of Mitochondria

Briefly, we have performed mitochondrial isolation after washing the cells and re-suspending it in a buffer presenting 250 mM sucrose, 10 mM KCl, 1 mM EGTA, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM pepstatin A, 10 mg/mL leupeptin, 2 mg/mL aprotinin, and 20 mM HEPES (pH 7.4), as previously described (de Oliveira et al. 2017c; Wang et al., 2014).

Extraction of Submitochondrial Particles and Quantification of Radical Anion Superoxide

We have isolated submitochondrial particles (SMP) from SH-SY5Y cells by utilizing a buffer presenting 230 mM mannitol, 70 mM sucrose, 10 mM Tris-HCl, and 1 mM EDTA (pH 7.4) and by performing differential centrifugations, as previously reported (de Oliveira et al. 2017c; Poderoso et al. 1996). The SMP obtained by performing this protocol was utilized to assay the production of anion superoxide ($O_2^{-\bullet}$) and also to quantify the levels of oxidative and nitrosative stress in mitochondrial membranes (Poderoso et al. 1996).

Evaluation of MDA, Protein Carbonyl, Protein Thiol Groups, and Protein Nitration

We have quantified the levels of MDA, protein carbonyl, and protein thiol groups by utilizing commercial assay kits, as recommended by the manufacturer (Abcam, MA, USA). The levels of 3-nitrotyrosine in mitochondrial membranes were analyzed by performing an indirect ELISA assay utilizing a polyclonal antibody to 3-nitrotyrosine (Calbiochem, Germany) diluted (1:2000) in phosphate-buffered saline (PBS, pH 7.4) containing 5% albumin (de Oliveira et al. 2017c).

Quantification of Enzyme Activities

We have analyzed the activities of complexes I and V by using commercial assay kits, as indicated by the manufacturer (Abcam, MA, USA) (de Oliveira et al. 2017c).

Measurement of ATP Levels

The levels of ATP were quantified by performing a method based on the instructions of the manufacturer of a commercial assay kit (Abcam, MA, USA) (de Oliveira et al. 2017c).

Determination of MMP

We have quantified matrix metalloproteinase (MMP) by using the lipophilic cationic dye tetraethylbenzimidazolylcarbocyanide iodine (JC-1), as described elsewhere (de Oliveira et al. 2017c).

Evaluation of Cell Death-Associated Parameters

The quantification of the amounts of Bcl-2, Bax, cytochrome c (mitochondrial and cytosolic), and cleaved PARP was performed through ELISA assay kits, as indicated by the manufacturer (Abcam, MA, USA). The activities of the enzymes caspase-9 and caspase-3 were assayed by using fluorimetric assay kits, following the instructions of the manufacturer (Abcam, MA, USA). The levels of DNA fragmentation in the cell lysates were evaluated by using an ELISA assay kit based on the manufacturer's instructions (Roche, Germany).

Analyses of Inflammation-Related Parameters

We have performed the quantification of the levels of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) by using ELISA assay kits, as indicated by the manufacturer (Abcam, MA, USA). The immunocontents of cyclooxygenase-2 (COX-2) were determined by performing an ELISA assay kit, as indicated by the manufacturer (Sigma-Aldrich, MO, USA).

Quantification of GSH

The levels of GSH were measured by utilizing a Thiol Green Indicator present in a commercial assay kit based on the instructions of the manufacturer (Abcam, MA, USA) (de Oliveira et al. 2017c).

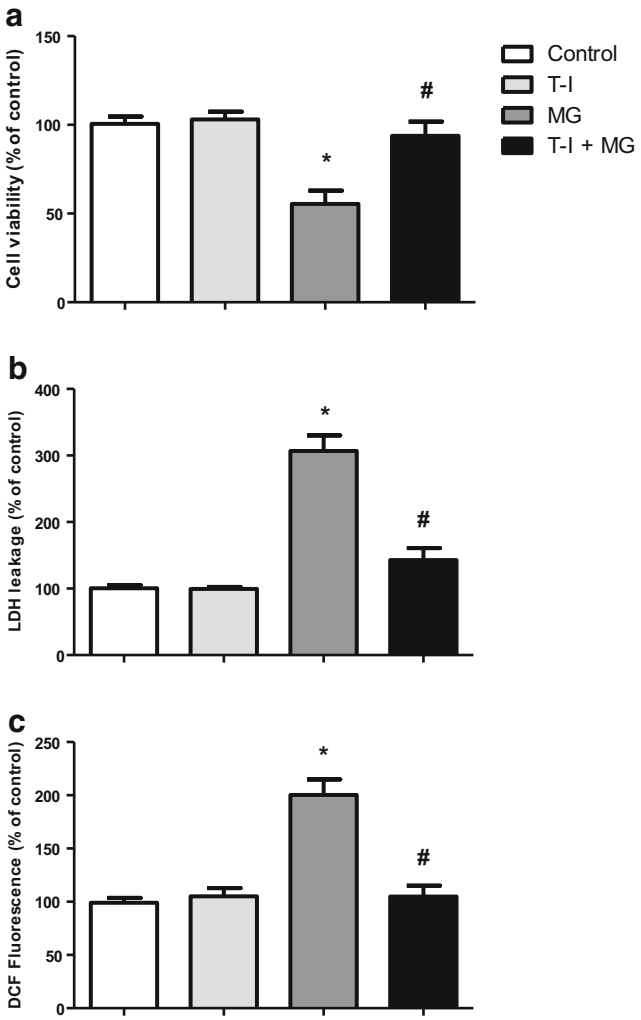

Fig. 1 T-I alleviates the MG-induced loss of viability (**a**), cytotoxicity (as assessed through the quantification of the LDH leakage from the cells) (**b**), and ROS production (**c**) in SH-SY5Y cells. The human neuroblastoma SH-SY5Y cells were administrated with T-I at 2.5 μM for 2 h and then challenged with MG at 500 μM for a further 24 h. Data are demonstrated as the mean ± SEM of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test, *$p < 0.05$ vs the control group, #$p < 0.05$ vs MG-treated cells

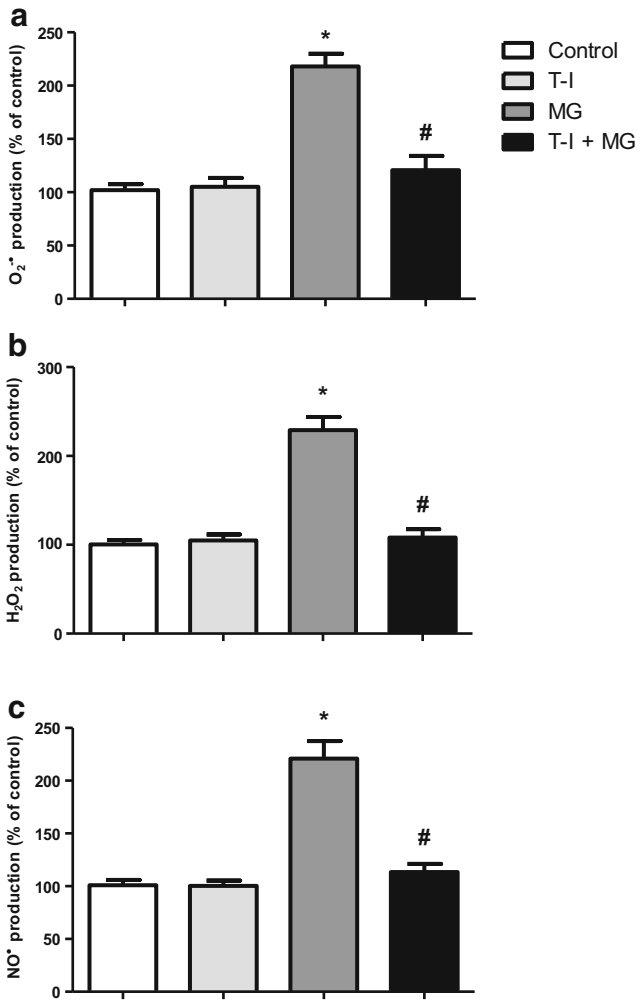

Fig. 2 T-I alleviates the effects of MG on the mitochondria-related cell death–associated parameters caspase-9 activity (**a**), caspase-3 activity (**b**), cleaved PARP levels (**c**), and DNA fragmentation (**d**) in SH-SY5Y cells. The human neuroblastoma SH-SY5Y cells were administrated with T-I at 2.5 μM for 2 h and then challenged with MG at 500 μM for a further 24 h. Data are demonstrated as the mean ± SEM of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test, *$p < 0.05$ vs the control group, #$p < 0.05$ vs MG-treated cells

Isolation of the Cell Nucleus

We performed the isolation of cell nucleus by using the Nuclear Extraction Kit, as described by the manufacturer (Cayman Chemical, MI, USA). The cells (1×10^7; 80–90% confluence) were collected in phosphate-buffered saline (PBS, ice-cold, pH 7.4). A centrifugation was carried out at 300×*g* for 5 min at 4 °C, and the cells were pelleted and resuspended in ice-cold hypotonic buffer, leading to cellular swelling. We used the Nonidet P-40 at 10% reagent to dissolve the membranes of the cells in order to access the cytoplasmic fraction without promoting nuclear membrane damage. Another centrifugation (13,000×*g* for 30 s at 4 °C) was performed, rendering purified nuclei, which were lysed after the exposure to an ice-cold extraction buffer. Then, we performed a centrifugation (14,000×*g* for 10 min at 4 °C) that resulted in purified nuclear extracts, which were used in the Nrf2 activity assay (de Oliveira et al. 2017c).

Silencing of Nrf2

Nrf2 knockdown was performed by using siRNA against the Nrf2 sequence based on the instructions of the manufacturer (Santa Cruz, CA, USA) (de Oliveira et al. 2017c).

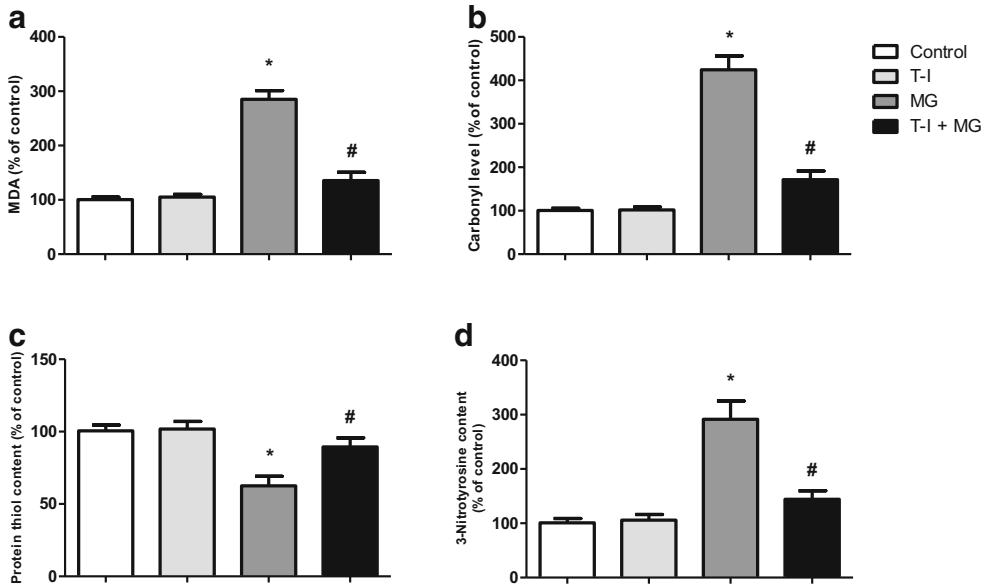

Fig. 3 T-I alleviates the MG-induced increase in the mitochondrial production of O_2^{-}• (**a**), and the cellular generation of H_2O_2 (**b**) and NO• (**c**). The human neuroblastoma SH-SY5Y cells were administrated with T-I at 2.5 μM for 2 h and then challenged with MG at 500 μM for a further 24 h. Data are demonstrated as the mean ± SEM of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test, *$p < 0.05$ vs the control group, #$p < 0.05$ vs MG-treated cells

Evaluation of the Activity of Nrf2

We performed the analysis of the activity of Nrf2 by utilizing a commercial assay kit after the isolation of cell nucleus, following the protocol of the manufacturer (Active Motif, CA, USA).

Measurement of the Nuclear Levels of Nrf2

Aiming to quantify the nuclear levels of Nrf2, we have utilized a commercial assay kit following the instructions of the manufacturer (Active Motif, CA, USA) and as described by us (de Oliveira et al. 2017a) and by others

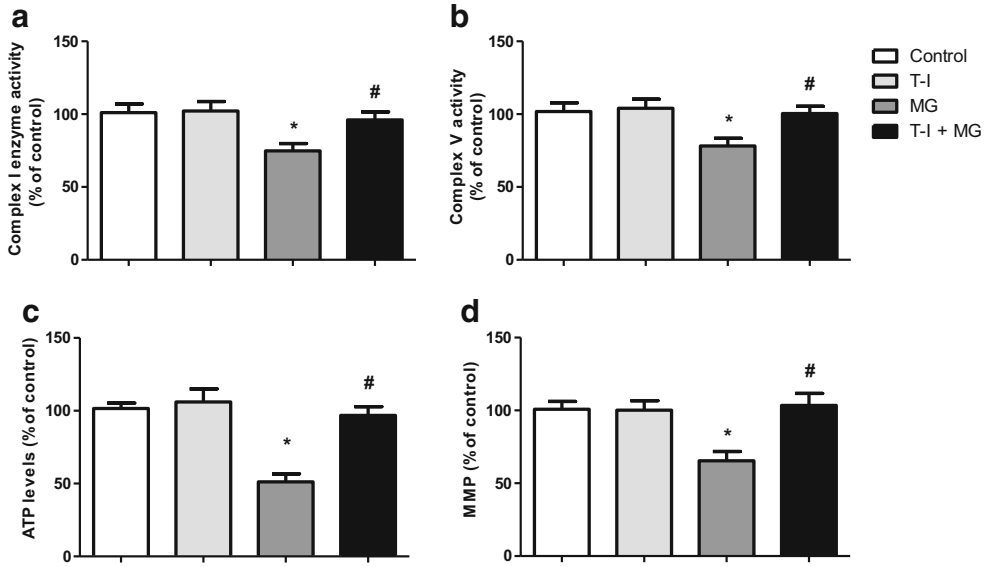

Fig. 4 T-I alleviates the MG-induced lipid peroxidation (**a**), protein carbonylation (**b**), oxidation of protein thiol groups (**c**), and protein nitration (**d**) in the mitochondrial membranes in the SH-SY5Y cells. The human neuroblastoma SH-SY5Y cells were administrated with T-I at 2.5 μM for 2 h and then challenged with MG at 500 μM for further 24 h. Data are demonstrated as the mean ± SEM of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test, *$p < 0.05$ vs the control group, #$p < 0.05$ vs MG-treated cells

(Afonyushkin et al. 2011; Kang et al. 2010). This assay was also used to verify whether siRNA targeting Nrf2 would be effective in the present work.

Statistical Analyses

We have performed statistical analyses by utilizing the GraphPad 5.0 software. Data are exhibited as the mean ± standard error of the mean (S.E.M.) of three or five independent experiments each done in triplicate; p values were considered significant when $p < 0.05$. Differences between the experimental groups were determined by one-way ANOVA followed by the post hoc Tukey's test.

Results

T-I Pretreatment Protected SH-SY5Y Cells Against the Cytotoxic Effects Induced by MG

According to Fig. S1, MG at 500 μM for 24 h caused a 50% decline in the viability of the SH-SY5Y cells, as previously reported by us (de Oliveira et al. 2015,2017d) and by others (Angeloni et al. 2015; Nishimoto et al. 2017). Also in Fig. S1, it may be observed that T-I pretreatment (for 2 h) at 2.5 μM caused potent cytoprotection in SH-SY5Y cells exposed to MG at 500 μM ($p < 0.05$). Therefore, we have utilized this concentration of T-I in the other assays. Accordingly, T-I prevented loss of cell viability ($p < 0.05$; Fig. 1a), cytotoxicity ($p < 0.05$; Fig. 1b), and general production of ROS ($p < 0.05$; Fig. 1c) in MG-challenged SH-SY5Y cells.

We also evaluated whether T-I pretreatment would modulate the MG-induced mitochondria-related apoptotic signals in the SH-SY5Y cells. As demonstrated in Fig. S2A, T-I prevented the MG-induced decline in the levels of the antiapoptotic protein Bcl-2 in SH-SY5Y cells ($p < 0.05$). T-I also significantly attenuated the MG-elicited upregulation in the levels of the pro-apoptotic protein Bax ($p < 0.05$; Fig. S2B). Accordingly, T-I alleviated the release of cytochrome c from the mitochondria to the cytosol ($p < 0.05$; Fig. S2C), preserving the levels of this protein in the organelles ($p < 0.05$; Fig. S2D). In this context, the pretreatment with T-I reduced the activity of the pro-apoptotic caspase-9 ($p < 0.05$; Fig. 2a) and caspase-3 ($p < 0.05$; Fig. 2b) in MG-challenged SH-SY5Y cells. Consequently, T-I pretreatment attenuated the levels of cleaved PARP ($p < 0.05$; Fig. 2c) and DNA fragmentation ($p < 0.05$; Fig. 2d), which are hallmarks of apoptosis, in MG-treated cells.

We next examined the effect of T-I on the production of specific reactive species in MG-treated cells. As depicted in Fig. 3a, T-I prevented the MG-induced increase in the generation of $O_2^{-\bullet}$ by SMP obtained from SH-SY5Y cells ($p < 0.05$). In the cellular context, T-I pretreatment was efficient in reducing the production of H_2O_2 ($p < 0.05$; Fig. 3b) and NO• ($p < 0.05$; Fig. 3c) in MG-challenged SH-SY5Y cells.

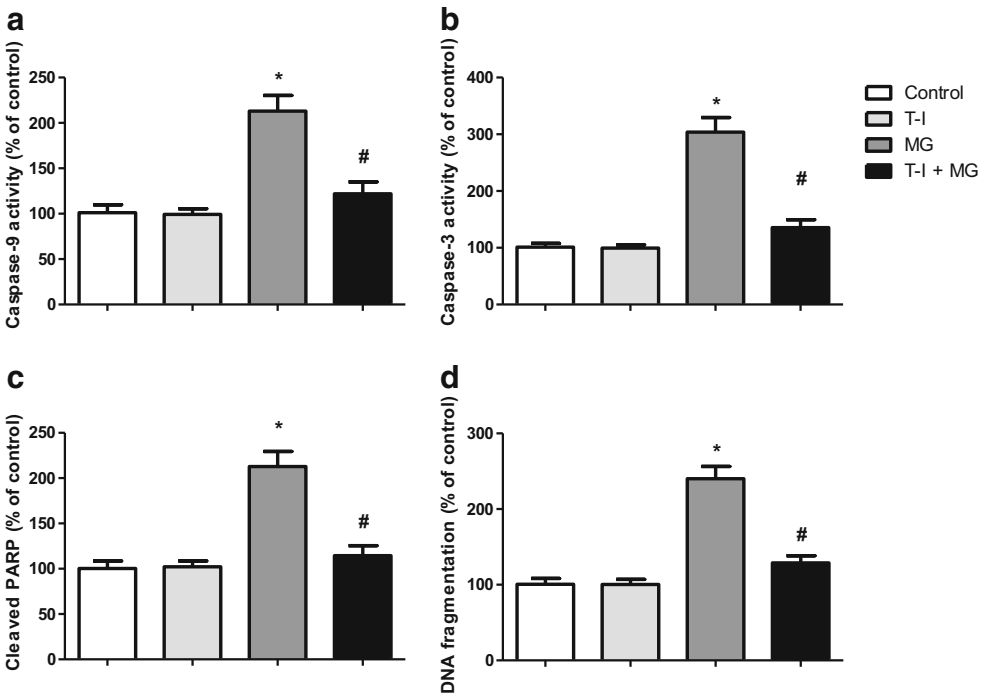

Fig. 5 T-I alleviates the effects of MG on complex I activity (**a**), complex V activity (**b**), ATP levels (**c**), and MMP (**d**) in SH-SY5Y cells. The human neuroblastoma SH-SY5Y cells were administrated with T-I at 2.5 μM for 2 h and then challenged with MG at 500 μM for further 24 h. Data are demonstrated as the mean ± SEM of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test, *$p < 0.05$ vs the control group, #$p < 0.05$ vs MG-treated cells

T-I Prevented the Redox Impairment and Bioenergetics Disruption Caused by MG in the Mitochondria of SH-SY5Y Cells

We next examined whether T-I would be able to promote mitochondrial protection in MG-treated SH-SY5Y cells. According to Fig. 3, T-I pretreatment abrogated the lipid peroxidation ($p < 0.05$; Fig. 4a), protein carbonylation ($p < 0.05$; Fig. 4b), oxidation of protein thiol groups ($p < 0.05$; Fig. 4c), and protein nitration ($p < 0.05$; Fig. 4d) in the membranes of mitochondria obtained from the SH-SY5Y cells exposed to MG. T-I also attenuated the effects of MG on the activity of the mitochondrial complexes I ($p < 0.05$; Fig. 5a) and V ($p < 0.05$; Fig. 5b), consequently preserving the levels of ATP in this experimental model ($p < 0.05$; Fig. 5c). Moreover, T-I pretreatment alleviated the loss of MMP in the SH-SY5Y cells administrated with MG ($p < 0.05$; Fig. 5d).

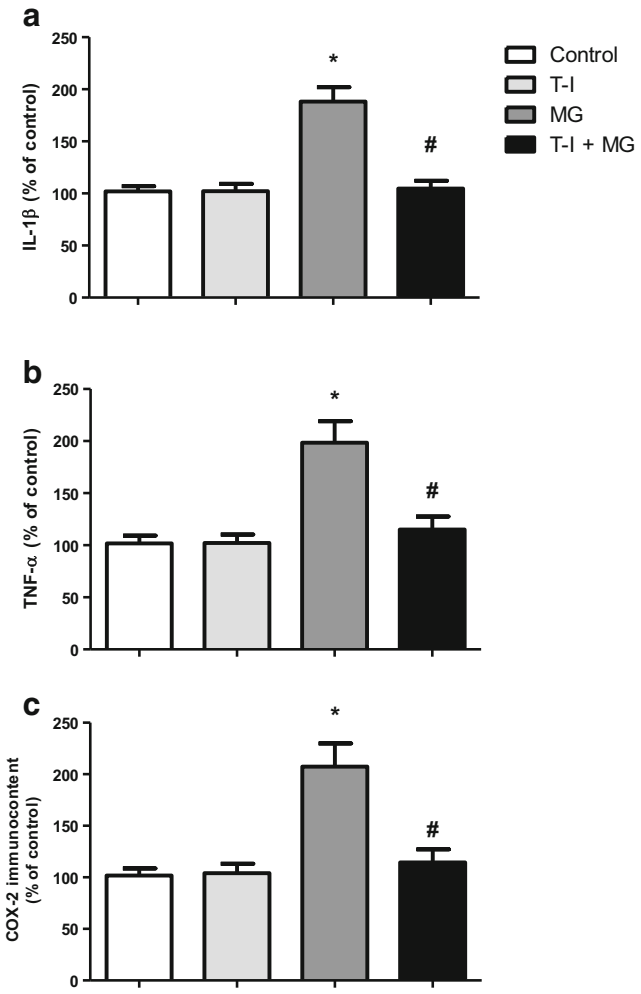

Fig. 6 T-I alleviates the effects of MG on the levels of IL-1β (**a**), TNF-α (**b**), and COX-2 (**c**) in MG-treated SH-SY5Y cells. The human neuroblastoma SH-SY5Y cells were administrated with T-I at 2.5 μM for 2 h and then challenged with MG at 500 μM for further 24 h. Data are demonstrated as the mean ± SEM of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test, *$p < 0.05$ vs the control group, #$p < 0.05$ vs MG-treated cells

T-I Pretreatment Induced Anti-Inflammatory Effects in the MG-Treated SH-SY5Y Cells

T-I prevented the MG-induced inflammation in SH-SY5Y cells, as may be observed in Fig. 7. T-I pretreatment efficiently reduced the levels of IL-1β ($p < 0.05$; Fig. 6a), TNF-α ($p < 0.05$; Fig. 6b), and COX-2 ($p < 0.05$; Fig. 6c), demonstrating a potent anti-inflammatory activity in this experimental model.

T-I Promoted Mitochondrial Protection and Cytoprotection by a Mechanism Associated with the Nonenzymatic Antioxidant GSH

We next examined the possible mechanism of action by which T-I have prevented the mitochondrial impairment

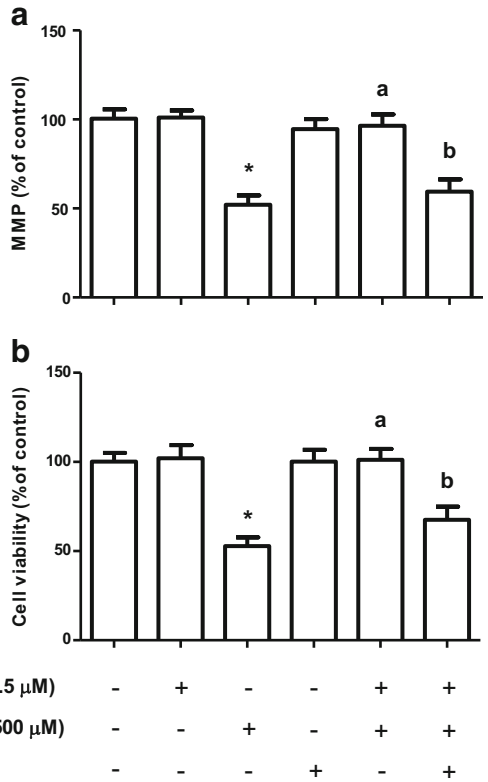

Fig. 7 The GSH synthesis inhibitor BSO suppresses the effects of a pretreatment with T-I on cell viability (**a**) and MMP (**b**) in SH-SY5Y cells challenged with MG. The cells were treated with T-I at 2.5 μM for 2 h and then challenged with MG at 500 μM for additional 24 h. BSO at 100 μM was administrated to the cells for 24 h before the treatment with T-I. Data are demonstrated as the mean ± SEM of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test, *$p < 0.05$ different from the control group; a different from MG-treated group; **b** $p < 0.05$ different from T-I + MG-treated SH-SY5Y cells

and toxic effects of MG in SH-SY5Y cells. We observed that the incubation of the SH-SY5Y cells with BSO (a specific inhibitor of the synthesis of GSH) abrogated the T-I-induced protection regarding mitochondrial function ($p < 0.05$; Fig. 7a) and cell survival ($p < 0.05$; Fig. 7b), suggesting a role for GSH in the modulation of such parameters in T-I-pretreated cells exposed to MG. We also evaluated whether T-I would affect the synthesis of GSH in SH-SY5Y cells (Fig. 8). We have found that T-I at 1–2.5 μM for 24 h upregulated the levels of GSH in SH-SY5Y cells ($p < 0.05$; Fig. 8a). Also, we observed that BSO efficiently blocked the T-I-stimulated GSH production in this experimental model ($p < 0.05$; Fig. 8b). Moreover, the utilization of siRNA targeting Nrf2 revealed that the T-I-induced synthesis of GSH is associated with this transcription factor ($p < 0.05$; Fig. 9). According to Fig. 10a, the utilization of siRNA targeting Nrf2 efficiently suppressed the activity of this transcription factor in the present experimental design ($p < 0.05$). Furthermore, the T-I-induced upregulation in the nuclear levels of Nrf2 was abrogated by Nrf2 silencing ($p < 0.05$; Fig. 10b), reinforcing that the knockdown of this transcription factor by using siRNA was efficient in the present work.

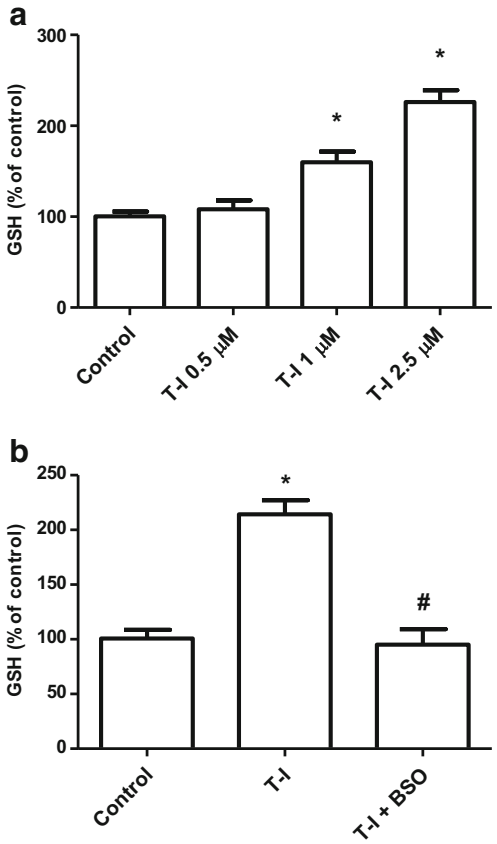

Fig. 8 The effects of a treatment with T-I (**a**) and T-I and/or BSO (**b**) on the levels of GSH in the SH-SY5Y cells (**a**). The levels of GSH were quantified in the cells after a treatment with T-I at 0.5–2.5 μM for 24 h. The GSH synthesis inhibitor BSO was administrated at 100 μM for 24 h prior to the addition of T-I at 2.5 μM to the cells. Data are demonstrated as the mean ± SEM of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test; *$p < 0.05$ vs the control group, #$p < 0.05$ vs T-I-treated cells

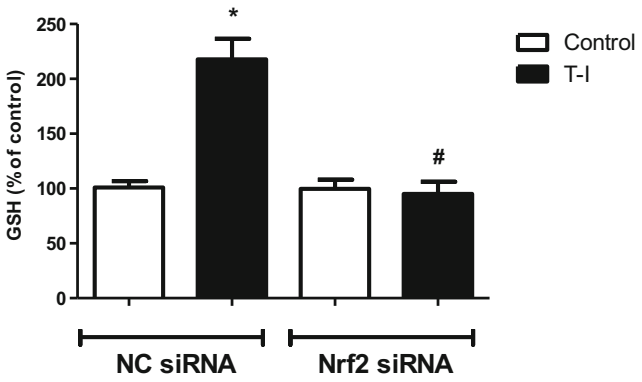

Fig. 9 The effects of Nrf2 silencing with siRNA (for 48 h) on the levels of GSH in T-I-treated SH-SY5Y cells. Data are demonstrated as the mean ± SEM of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test, *$p < 0.05$ vs the control group; #$p < 0.05$ vs T-I-treated cells transfected with negative control (NC) siRNA

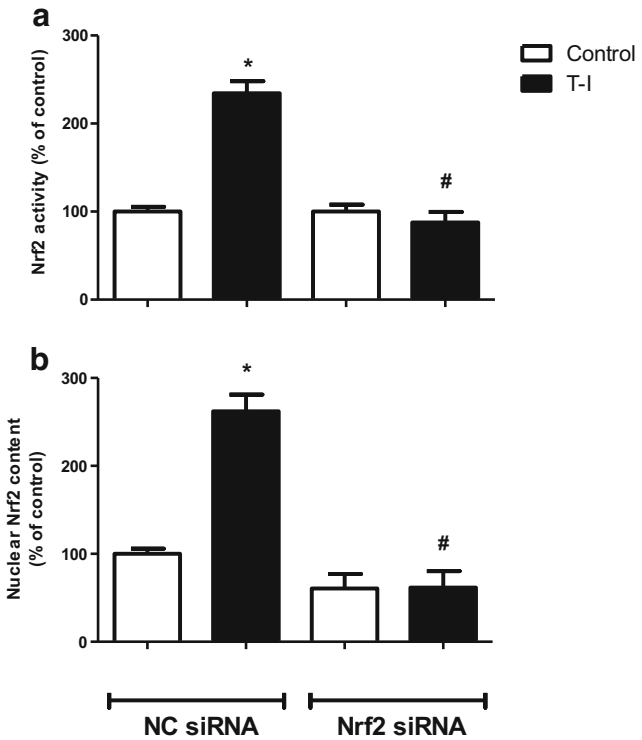

Fig. 10 The effects of Nrf2 silencing with siRNA (for 48 h) on the activity (**a**) and on the nuclear levels (**b**) of Nrf2 in T-I-treated SH-SY5Y cells. Data are demonstrated as the mean ± SEM of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test, *$p < 0.05$ vs the control group; #$p < 0.05$ vs T-I-treated cells transfected with negative control (NC) siRNA

T-I Attenuated the MG-Induced Effects on Mitochondrial Function and Cell Viability by a Mechanism Involving Nrf2

As demonstrated in Fig. 11a, silencing of Nrf2 suppressed the effects of T-I on the levels of ATP in MG-treated cells ($p < 0.05$). In a similar way, Nrf2 knockdown significantly attenuated the effect of a pretreatment with T-I on the MMP levels in the cells that were exposed to MG ($p < 0.05$; Fig. 11b). In agreement with such findings, Nrf2 silencing abrogated the cytoprotection caused by T-I in SH-SY5Y cells challenged with MG ($p < 0.05$; Fig. 12).

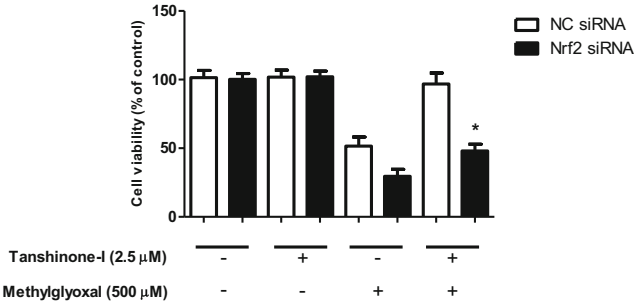

Fig. 12 The effects of Nrf2 silencing with siRNA (for 48 h) on the viability of SH-SY5Y cells treated with MG and/or T-I. Data are demonstrated as the mean ± SEM of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test, *$p < 0.05$ vs the T-I + MG-treated cells transfected with negative control (NC) siRNA

Discussion

In the present work, we have demonstrated that T-I prevented mitochondrial dysfunction in SH-SY5Y cells exposed to MG by a mechanism involving Nrf2 activation and enhanced GSH synthesis. T-I pretreatment decreased the production of free radicals by the mitochondria (as is the case of O_2^-•) and by the cells as a whole (total production of H_2O_2 and NO•), attenuating the formation of markers of redox impairment in the mitochondria of the SH-SY5Y cells exposed to MG. Besides, T-I protected mitochondria against the bioenergetic decline induced by MG. It is very likely that the protection caused by T-I regarding the activity of complexes I and V takes a role in the decreased formation of reactive species by the organelles, since it has been reported that complex I dysfunction enhances the production of O_2^-• by the mitochondria (Grivennikova and Vinogradov, 2006; Hernansanz-Agustín et al. 2017). Moreover, failure to produce ATP results in increased amounts of adenosine diphosphate (ADP) and/or adenosine monophosphate (AMP), which may directly or indirectly increase the flux of electrons in the respiratory chain, causing an increase in the generation of O_2^-• (Murphy 2009; Turrens 2003). Also, the free radical NO• may cause glyoxalase I inhibition, favoring accumulation of MG (Mitsumoto et al. 2000). Enhanced production of NO• in microenvironments of the cells (as well as in parts of some organelles, such as the mitochondria) can contribute to the amplification of reactive species generation by inhibiting electron transfer in the respiratory chain, therefore causing mitochondrial dysfunction (Poderoso et al. 1996; Riobó et al. 2001; Zhang et al. 1990). BSO, a specific inhibitor of the synthesis of GSH, suppressed the mitochondria-related protection caused by T-I. Besides, silencing of Nrf2 abrogated the T-I-induced GSH synthesis and mitochondrial protection. In fact, the Nrf2/GSH axis is crucial in the glyoxalase system during MG detoxification (Allaman et al. 2015; Thornalley 1993).

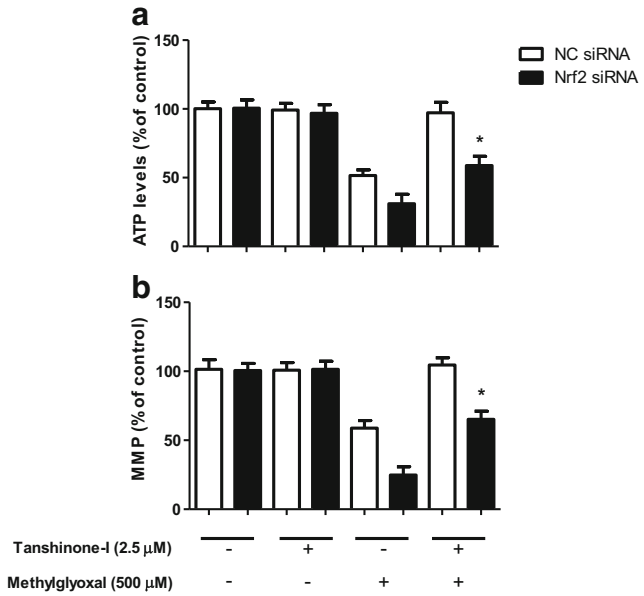

Fig. 11 The effects of Nrf2 silencing with siRNA (for 48 h) on the levels of ATP (**a**) and MMP (**b**) in T-I-treated SH-SY5Y cells. Data are demonstrated as the mean ± SEM of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test, *$p < 0.05$ vs the T-I + MG-treated cells transfected with negative control (NC) siRNA

Impaired electron flux in the respiratory chain may be a cause for the loss of MMP (de Arriba et al., 2006,2007). Additionally, damaged mitochondrial membranes would not be a barrier against loss of protons, which are the major component of the electrochemical gradient across the inner mitochondrial membrane (Satoh et al., 1997). The consequences of the decline in MMP include increased reactive species production and release of cytochrome c to the cytosol, triggering apoptosis (Green et al., 2014). In this regard, the T-I-induced activation of the Nrf2/GSH axis abrogated loss of MMP and the consequences it would be able to cause in the mitochondria of MG-treated SH-SY5Y cells.

On the other hand, T-I attenuated the levels of the pro-inflammatory cytokines IL-1β and TNF-α, as well as of the COX-2 enzyme, which also mediates inflammation in

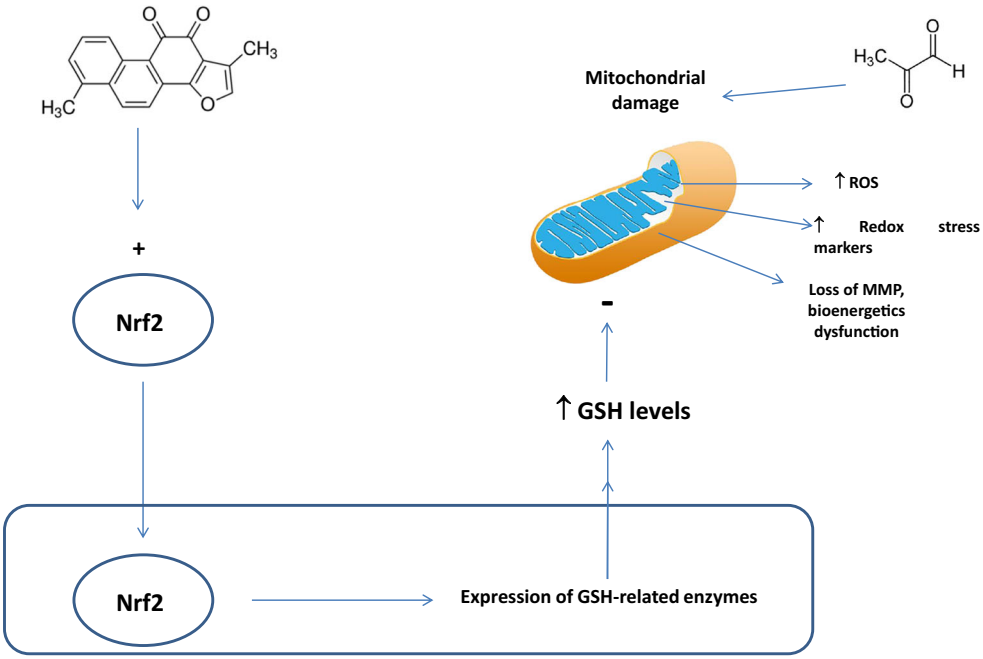

Fig. 13 A summary of the possible mechanism involved in the mitochondrial protection mediated by tanshinone I. The activation of the transcription factor Nrf2 caused an increase in the synthesis of reduced glutathione (GSH), which may be involved in the protection regarding both redox and bioenergetics mitochondrial parameters

mammalian tissues (Ricciotti and FitzGerald 2011). It has been demonstrated that mitochondrial dysfunction may be associated with inflammation by promoting the synthesis of pro-inflammatory cytokines (Di Filippo et al. 2010; Naik and Dixit 2011; Witte et al. 2010). Vaamonde-García et al. (2012) have shown that the inhibition of the mitochondrial complexes III and V enhanced the production of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in normal human chondrocytes. Thus, it is very likely that the mitochondrial protection promoted by T-I would lead to decreased levels of pro-inflammatory cytokines. However, it remains to be better understood.

It was previously reported that T-I stimulates the expression of the components of the γ-GCL enzyme, leading to increased GSH production in SH-SY5Y cells (de Oliveira et al. 2017a; Jing et al. 2016). Nonetheless, it was not previously examined whether this GSH would take a role during the mitochondrial protection induced by T-I. The link between GSH and the maintenance of mitochondrial function is focus of research by certain research groups. Fernández-Checa et al. (1997) have published that the transport of GSH into mitochondria would be a crucial step in the cell resistance against the redox impairment induced by TNF-α or alcohol. On the other hand, Circu et al. (2008) have described that the blockade of the GSH uptake by mitochondria resulted in increased susceptibility of colonic epithelial NCM460 cell line to menadione. Besides, Wilkins et al. (2013) have reported that a reduction in the mitochondrial levels of GSH would facilitate death of primary cultures of rat cerebellar granule neurons and cerebellar astrocytes exposed to conditions of oxidative and nitrosative stress. Accordingly, T-I failed to suppress the MG-induced bioenergetic decline and mitochondrial dysfunction in SH-SY5Y treated with BSO, as demonstrated here. In fact, the mitochondrial levels of GSH are increased in T-I-treated SH-SY5Y cells, as previously reported by us (de Oliveira et al. 2017a). Nonetheless, the exact mechanism involved in the increased mitochondrial susceptibility of BSO-treated SH-SY5Y cells to MG remains to be better studied. It may not be discarding the direct involvement of GSH in the detoxification reactions of MG. Also, GSH plays a central role in the conversion of H_2O_2, whose production is stimulated by MG, into water by the GPx enzyme (Giorgio et al. 2007; Sies et al. 2017). Therefore, inhibition of GSH would affect, at least in part, two important routes associated with the cell response against the intoxication with MG.

Overall, the herein presented data indicates a role for the Nrf2/GSH axis in the mitochondrial protection promoted by T-I in MG-challenged SH-SY5Y cells (Fig. 13). Since the T-I bioavailability is limited in mammals (Xing et al. 2017), it is recommended checking whether T-I would be able to cause mitochondria-related neuroprotection in animal models associated with impaired metabolism of glucose or exposed directly to MG.

Funding Information MRO receives a "Bolsa de Produtividade em Pesquisa 2 - PQ2" fellow from the Conselho Nacional de Pesquisa e Desenvolvimento Tecnológico (CNPq) (protocol number 301273/2018-9). This research was supported by CNPq (protocol numbers 400216/2016-7 and 460903/2014-4).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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