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Inhibition of the Nrf2/HO-1 Axis Suppresses the Mitochondria-Related Protection Promoted by Gastrodin in Human Neuroblastoma Cells Exposed to Paraquat

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Abstract

Mitochondria are double-membrane organelles involved in the transduction of energy from different metabolic substrates into adenosine triphosphate (ATP) in mammalian cells. The oxidative phosphorylation system is comprised by the activity of the respiratory chain and the complex V (ATP synthase/ATPase). This system is dependent on oxygen gas (O_2) in order to maintain a flux of electrons in the respiratory chain, since O_2 is the final acceptor of these electrons. Electron leakage from this complex system leads to the continuous generation of reactive species in the cells. The mammalian cells exhibit certain mechanisms to attenuate the consequences originated from the constant exposure to these reactive species. In this context, the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) and one of the enzymes whose expression is modulated by Nrf2, heme oxygenase-1 (HO-1), take a central role in inducing cytoprotection in humans. Mitochondrial abnormalities are observed during intoxication and in certain diseases, including neurodegeneration. Mitochondrial protection promoted by natural compounds has attracted the attention of researchers due to the promising effects these agents induce experimentally. In this regard, we examined here whether and how gastrodin (GAS), a phenolic glucoside, would prevent the paraquat (PQ)-induced mitochondrial impairment in the SH-SY5Y cells. The cells were exposed to GAS (25 μM) for 4 h prior to the challenge with PQ at 100 μM for additional 24 h. The silencing of Nrf2 by siRNA or the inhibition of HO-1 by ZnPP IX suppressed the GAS-elicited cytoprotection. Therefore, GAS promoted mitochondrial protection by an Nrf2/HO-1-dependent manner.

Keywords Gastrodin · Paraquat · Mitochondria · Bioenergetics · Nrf2 · HO-1

Introduction

The high demand for adenosine triphosphate (ATP) is met by the aerobic metabolism, which depends on the integrity of the mitochondria in mammals [1, 2]. Mitochondria are double-membrane organelles that consume oxygen gas (O_2) in order to maintain the oxidation and conservation of energy obtained from different nutrients, such as carbohydrates, lipids, amino acids (utilized in the form of α-ketoacids), and ketone bodies [3, 4]. The oxidative phosphorylation system comprises the respiratory chain and the complex V (ATP synthase/ATPase) enzyme) [5]. The complexes I (NADH dehydrogenase), II (succinate dehydrogenase), III (ubiquinol:cytochrome c oxidoreductase), and IV (cytochrome c oxidase) are the components of the respiratory chain [6, 7]. The flux of electrons in the respiratory chain releases energy that is utilized by the complexes (with exception to the complex II) to pump protons from the mitochondrial matrix into the intermembrane space (IMS), which is an area between the inner mitochondrial membrane (IMM) and the outer mitochondrial membrane

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(OMM) [5]. The accumulation of protons in the IMS originates an electrochemical gradient that is widely measured as the mitochondrial membrane potential (MMP) [5, 8]. The return of the protons to the mitochondrial matrix occurs through the complex V enzyme, which utilizes this movement of protons to produce ATP from adenosine diphosphate (ADP) and inorganic phosphate (Pi) [3]. In order to maintain a constant flux of electrons in the respiratory chain, it is necessary to the cells a certain O_2 concentration and mitochondrial integrity [3, 9]. The high consumption of O_2 by mammalian cells leads to the formation of reactive species by the mitochondria, as well as by other redox-dependent systems [10, 11]. Actually, mitochondria are the main source of reactive species in mammalian cells physiologically [12–14]. Furthermore, mitochondrial dysfunction, which may be caused by exposure to toxicants and by genetic and sporadic diseases, causes an exacerbation in the production of such reactive agents [15–23].

The redox environment, including the mitochondria-related redox biology, is constantly regulated by a myriad of signaling pathways [14]. However, there is a master regulator of the redox environment, the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), which is kept in an inactive form in the cytoplasm, forming a complex with the Kelch-like ECH-associated protein 1 (Keap1) [24]. The release of Nrf2 from Keap1 is triggered by electrophiles and other reactive molecules [25]. After being released from the Keap1-containing complex, Nrf2 migrates to the nucleus of the cells and, after binding to specific proteins (such as the small musculoaponeurotic fibrosarcoma—sMaf), activates the antioxidant response element (ARE) or electrophile responsive element (EpRE), which is located in the genes whose products mediate reactions involved in the antioxidant defense and detoxification in virtually any nucleated cell [26–28]. Nrf2 activation causes the upregulation of antioxidant enzymes, such as the manganese-containing superoxide dismutase (Mn-SOD), glutathione peroxidase (GPx), glutathione reductase (GR), γ-glutamate-cysteine ligase (γ-GCL) catalytic and regulatory (GCLC and GCLM, respectively) subunits, heme oxygenase-1 (HO-1), and NAD(P)H:quinone dehydrogenase 1 (NQO-1) [24, 29]. The expression of the thioredoxin-thioredoxin reductase system, which is not involved only in the antioxidant defense in the cells, is also stimulated by Nrf2 [30]. Nrf2 also controls the expression of glutathione-*S*-transferase (GST), responsible for phase II detoxification reactions by mediating the conjugation of reduced glutathione (GSH) with toxicants in order to increase their solubility in water and posterior excretion from the cells [26]. The upregulation of Nrf2 and, consequently, of the expression of the enzymes that are controlled by this transcription factor is an important pharmacological strategy based on the principle of hormesis [31, 32]. In that context, the exposure of the cells to certain mild/moderate stressors leads to the activation of signaling pathways involved in cytoprotection and cell survival [33]. Actually, this is the main mechanism by which natural compounds induce beneficial effects in mammalian cells, as widely reported by several research groups [34–36]. Mitochondrial protection may also be promoted by the hormetic mechanism, and the number of natural compounds exhibiting this ability has increased [37–46].

In this regard, the enzyme HO-1 has attracted the attention of several research groups worldwide [47, 48]. This enzyme mediates the degradation of heme into free iron, carbon monoxide (CO), and biliverdin, which is converted into bilirubin by biliverdin reductase (BVR) [49]. The products originated from the HO-1 and BVR reactions exert a myriad of cytoprotective actions, including antioxidant and anti-inflammatory effects [50]. Bilirubin, for example, is a potent antioxidant, as reported in different cell types [51]. CO exhibits anti-inflammatory effects by downregulating signaling pathways involved in the activation of the nuclear factor-κB (NF-κB), a major regulator of inflammation [52–54]. There is strong evidence pointing to a mitochondria-related effect induced by the activation of the Nrf2/HO-1 axis, as demonstrated by our research group [45, 55].

Gastrodin (GAS; 4-hydroxybenzyl alcohol 4-O-beta-D-glucoside), which is a phenolic glucoside, has been isolated from the roots of the plant *Gastrodia elata* Blume, a herbal Chinese medicine [56]. GAS presents strong antioxidant and anti-inflammatory activities, as assessed in both in vitro and in vivo experimental models [57]. Our research group has recently demonstrated that GAS induced cytoprotection in SH-SY5Y cells exposed to hydrogen peroxide (H_2O_2), a non-radical pro-oxidant agent, by an Nrf2-associated mechanism [58]. However, the antioxidant ability of a natural compound would depend, at least in part, on the chemical nature of the stressor [59]. Taking it into account, we investigated here whether and how GAS would cause cellular and mitochondrial protection in human neuroblastoma SH-SY5Y cells exposed to paraquat (PQ), an agro-chemical presenting pro-oxidant capacity by the generation of radical anion superoxide ($O_2^{-\bullet}$) by redox cycling [60, 61]. PQ is widely utilized in experimental models mimicking neurodegeneration, namely Parkinson's disease (PD), since this toxicant is able to inhibit the mitochondrial complex I, causing both bioenergetics decline and redox abnormalities, among other effects, in mammalian cells [62].

Materials and Methods

Materials

We acquired the plastic materials used in cell culture from Corning, Inc. (NY, USA) and Beckton Dickson (NJ, USA). The culture analytical grade chemicals were obtained from Sigma (MO, USA). Other chemicals, as well as assay kits

we utilized to perform the present work, were acquired as described whenever necessary.

Cell Culture and Chemical Treatment

The SH-SY5Y cells, which are a human neuroblastoma cell line, were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 HAM nutrient medium (1:1 mixture), which was supplemented with 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 (37 °C).

We utilized PQ at 100 μM in order to induce loss of cell viability and mitochondrial dysfunction in SH-SY5Y cells, as previously reported [63, 64]. A pretreatment with GAS at 0.5–25 μM was performed for 4 h before challenging the cells with PQ for further 6 or 24 h, depending on the assay. ZnPP IX (10 μM) was administrated to the cells for 1 h prior to the treatment with GAS.

Cell Viability and Cytotoxicity Assays

The viability of the cells was tested by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [65]. As an index of cytotoxicity, we utilized the assay in which the release of the lactate dehydrogenase (LDH) enzyme is assessed through the quantification of the activity of the enzyme in the medium, as stated in the protocol of the manufacturer of the kit (CytoTox 96-Non-Radioactive Cytotoxicity Assay, Promega).

Malondialdehyde, Protein Carbonyl, Protein Thiol Groups, and 8-Oxo-dG Level Quantification

We evaluated the levels of malondialdehyde (MDA), protein carbonyl, and thiol content, as well as the amounts of nuclear 8-oxo-dG, through the utilization of commercial kits, based on the instructions of the manufacturer (Abcam, MA, USA), as previously reported [66, 67].

3-Nitrotyrosine and HO-1 Levels Measurement

The levels of 3-nitrotyrosine and HO-1 were assayed by using a polyclonal antibody to 3-nitrotyrosine (Calbiochem, Germany) in an indirect ELISA assay, as previously published [68].

Mitochondrial Isolation

We obtained mitochondria from SH-SY5Y cells by utilizing a protocol described by Wang et al. [69]. Firstly, the cells were washed and re-suspended in a buffer (250 mM sucrose, 10 mM KCl, 1 mM EGTA, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride—PMSF, 1 mM benzamidine, 1 mM pepstatin A, 10 mg/mL leupeptin, 2 mg/mL aprotinin, and 20 mM HEPES—pH 7.4). After differential centrifugations, samples with purified mitochondria were collected and utilized in further assays.

Submitochondrial Particle Isolation

After isolating mitochondria by performing the protocol described above, the organelles were frozen and thawed (three times), leading to the disruption of mitochondrial membranes and consequent leakage of mitochondrial matrix-located enzymes, including Mn-SOD. The SMP were washed (twice) with a buffer (140 mM KCl, 20 mM Tris-HCl—pH 7.4), causing the complete release of Mn-SOD from the mitochondria. Thus, we assessed $O_2^{-\bullet}$ production by the mitochondria and redox impairment in mitochondrial membranes after obtaining SMP, as previously reported [70].

Intracellular Reactive Oxygen Species Generation Quantification

We analyzed the generation of intracellular ROS by utilizing the nonpolar compound 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA) experimental assay, which is widely utilized to this end [71].

Production of $O_2^{-\bullet}$ Determination

The $O_2^{-\bullet}$ generation by the mitochondria was studied by quantifying the auto-oxidation of adrenaline to adrenochrome at 480 nm at 32 °C, as reported elsewhere [70].

Nitric Oxide Generation Analysis

We investigated the generation of $NO^{\bullet}$ through the utilization of a commercial assay kit, as described by the manufacturer (Abcam, MA, USA).

Enzyme Activity Quantification

We analyzed the activity of the mitochondria-located enzyme α-ketoglutarate dehydrogenase (α-KGDH), complex I, and complex V by using commercial kits following the instructions of the manufacturer (Abcam, MA, USA).

ATP Level Evaluation

We checked the levels of ATP by using an assay kit, according to the instructions of the manufacturer (Abcam, MA, USA).

MMP Analysis

We evaluated MMP by performing an assay based on the reactivity of tetraethylbenzimidazolylcarbocyanide iodine (JC-1) with mitochondria, according to the protocol described by the manufacturer (Abcam, MA, USA).

Nrf2 Knockdown

The transcription factor Nrf2 was silenced by performing transient transfection of the human neuroblastoma SH-SY5Y cells with siRNA against Nrf2, as stated by the manufacturer (Santa Cruz, CA, USA) and as previously described [72, 73].

Statistical Analyses

The GraphPad 5.0 software was used in order to perform statistical evaluation of the data obtained in the present work. Data are shown 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 $p < 0.05$. The differences between the experimental groups were checked by one-way ANOVA followed by the post hoc Tukey's test.

Results

Gastrodin Attenuated the Effects of PQ on the Viability and Apoptosis of SH-SY5Y Cells by a Mechanism Associated with HO-1

As demonstrated in Fig. 1, gastrodin at 5–25 μM prevented the PQ-induced decline in the viability of SH-SY5Y cells ($p < 0.05$; Fig. 1a). As depicted in Fig. 1b, gastrodin pretreatment at 25 μM attenuated the PQ-mediated cytotoxicity, as assessed through the measurement of LDH leakage from SH-SY5Y cells ($p < 0.05$). According to Fig. 2, the effect of gastrodin on the viability of PQ-treated SH-SY5Y cells was abolished by ZnPP IX, a specific inhibitor of HO-1 enzyme activity ($p < 0.05$).

Based on the data described above, we next examined whether HO-1 would be involved in the anti-apoptotic effects induced by gastrodin. As may be observed in Fig. 3, the inhibition of HO-1 by ZnPP IX suppressed the anti-apoptotic effects triggered by gastrodin. ZnPP IX blocked the effects of gastrodin on the levels of the pro-apoptotic protein Bax ($p < 0.05$; Fig. 3a) and on the levels of the anti-apoptotic protein Bcl-2 ($p < 0.05$; Fig. 3b). ZnPP IX also abrogated the effects of gastrodin pretreatment on the release of cytochrome c to the cytosol ($p < 0.05$; Fig. 3c). Consequently, the mitochondrial amounts of cytochrome c were found reduced in the gastrodin and PQ-treated cells in which the activity of HO-1 was inhibited by ZnPP IX ($p < 0.05$; Fig. 3d). Gastrodin also prevented the PQ-induced upregulation in the activity of the pro-apoptotic enzymes caspase-9 ($p <$

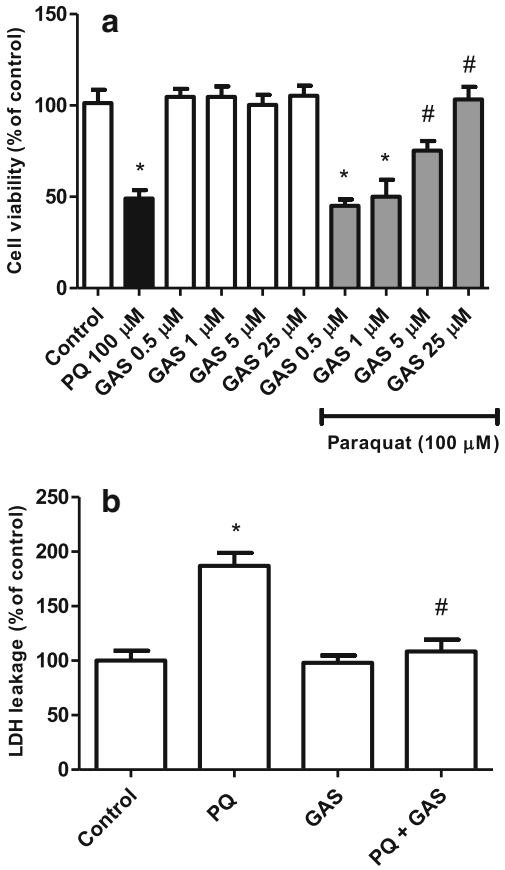

Fig. 1 The effects of paraquat (PQ) and/or gastrodin (GAS) on cell viability (**a**) and cytotoxicity (**b**) in SH-SY5Y cells. The cells were treated with GAS at 0.5–25 μM for 4 h prior to the exposure to PQ at 100 μM for further 24 h. Data are shown as the mean ± SEM of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test, *$p < 0.05$ different from the control group; #$p < 0.05$ different from PQ-treated group

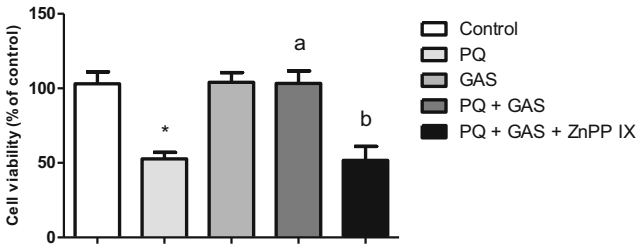

Fig. 2 The effects of the inhibition of HO-1 by ZnPP IX on the viability of gastrodin (GAS)-treated SH-SY5Y cells exposed to paraquat (PQ). Data are shown as the mean ± SEM of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test, *$p < 0.05$ different from the control group; a $p < 0.05$ different from PQ-treated group; b $p < 0.05$ different from PQ + GAS-treated cells

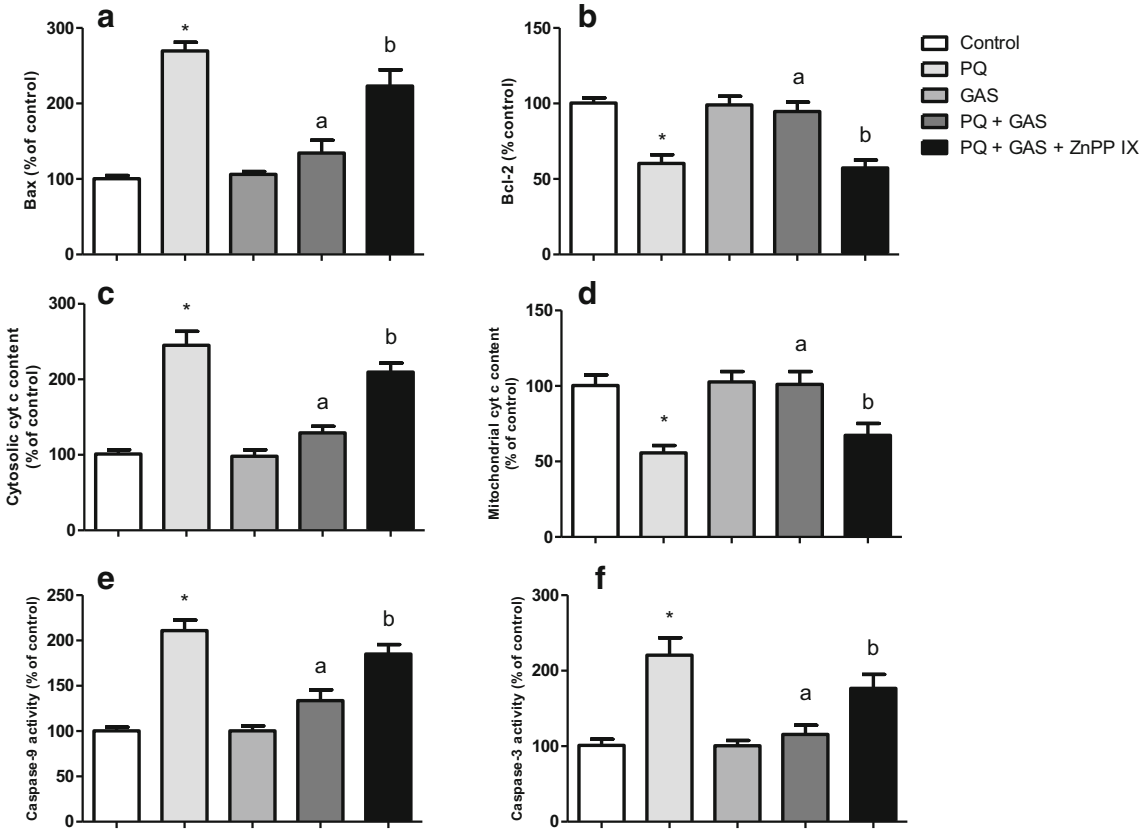

Fig. 3 The effects of the inhibition of HO-1 by ZnPP IX on the levels of Bax (**a**), the levels of Bcl-2 (**b**), the cytosolic cytochrome c content (**c**), the mitochondrial cytochrome c content (**d**), the activities of caspase-9 (**e**), and caspase-3 (**f**) in gastrodin (GAS)-treated SH-SY5Y cells exposed to paraquat (PQ). Data are shown 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 $p < 0.05$ different from PQ-treated group; b $p < 0.05$ different from PQ + GAS-treated cells

0.05; Fig. 3e) and caspase-3 ($p < 0.05$; Fig. 3f) by an HO-1-dependent manner, since the inhibition of this enzyme suppressed the effect induced by gastrodin ($p < 0.05$). We also measured the levels of DNA fragmentation, a hallmark of the apoptotic cell death, in this experimental model. We found that the effect induced by gastrodin on this parameter was abolished by the ZnPP IX-mediated HO-1 inhibition ($p < 0.05$; Fig. 4).

Gastrodin Induced an HO-1-Dependent Antioxidant Effect in SH-SY5Y Cells

As depicted in Fig. 5a, gastrodin decreased the general production of reactive species in the PQ-treated SH-SY5Y cells by a mechanism associated with HO-1 ($p < 0.05$). A similar effect was seen regarding the production of $O_2^{-\bullet}$ by SMP isolated from SH-SY5Y cells ($p < 0.05$; Fig. 5b) and on the generation of $NO^{\bullet}$ ($p < 0.05$; Fig. 5c). We next evaluated whether gastrodin would be able to prevent the redox impairment induced by PQ on mitochondrial membranes. Gastrodin attenuated the effects elicited by PQ on levels of lipid peroxidation ($p < 0.05$; Fig. 6a), oxidation of protein thiol groups ($p < 0.05$; Fig. 6b), protein carbonylation ($p < 0.05$; Fig. 6c), and protein nitration ($p < 0.05$; Fig. 6d) in the membranes of mitochondria obtained from SH-SY5Y cells.

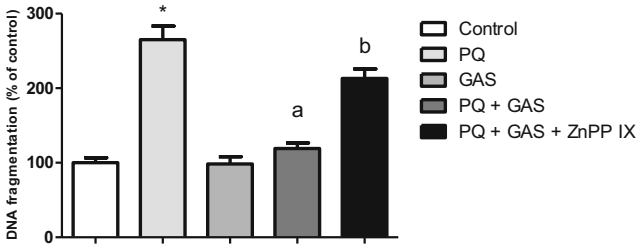

Fig. 4 The effects of the inhibition of HO-1 by ZnPP IX on levels of DNA fragmentation in gastrodin (GAS)-treated SH-SY5Y cells exposed to paraquat (PQ). Data are shown as the mean ± SEM of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test, $*p < 0.05$ different from the control group; a $p < 0.05$ different from PQ-treated group; b $p < 0.05$ different from PQ + GAS-treated cells

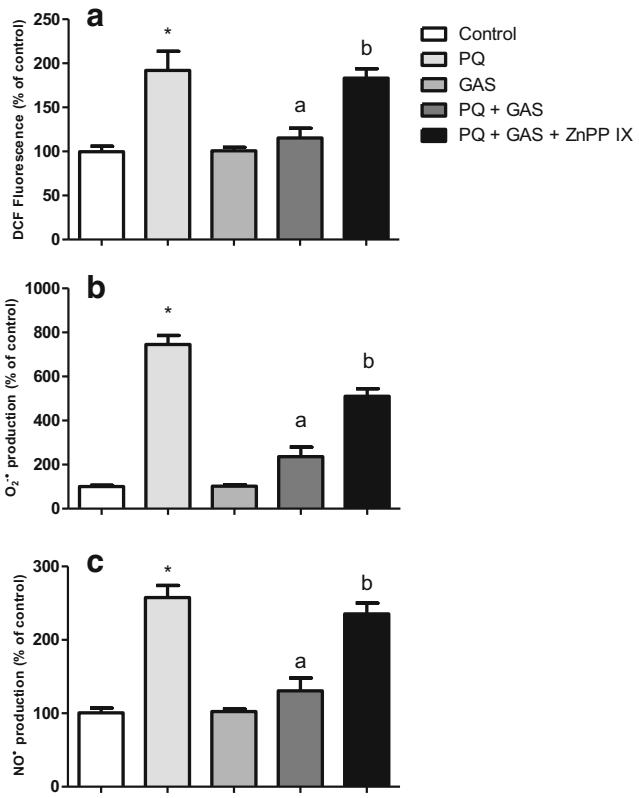

Fig. 5 The effects of the inhibition of HO-1 by ZnPP IX on the production of reactive species (**a**), $O_2^{-\bullet}$ (**b**), and $NO^{\bullet}$ (**c**) in gastrodin (GAS)-treated SH-SY5Y cells exposed to paraquat (PQ). Data are shown as the mean ± SEM of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test, $*p < 0.05$ different from the control group; a $p < 0.05$ different from PQ-treated group; b $p < 0.05$ different from PQ + GAS-treated cells

Gastrodin Alleviated the Effects of PQ on the Mitochondria-Related Bioenergetics Parameters

Gastrodin prevented the inhibition of the complexes I ($p < 0.05$; Fig. 7a) and V ($p < 0.05$; Fig. 7b) in the SH-SY5Y cells exposed to PQ by an HO-1-associated mechanism. Moreover, gastrodin blocked the PQ-induced decline in the levels of ATP by an HO-1-dependent manner ($p < 0.05$; Fig. 7c). Gastrodin also reduced the impact of the exposure of SH-SY5Y cells to PQ on the activity of the TCA enzyme α-KGDH by a similar way ($p < 0.05$; Fig. 8). The PQ-elicited loss of MMP was also attenuated by gastrodin through a mechanism involving HO-1 ($p < 0.05$; Fig. 9).

Gastrodin Promoted Cytoprotection by an Nrf2-Dependent Mechanism in SH-SY5Y Cells Exposed to PQ

Since HO-1 enzyme expression is modulated, at least in part, by the transcription factor Nrf2, we evaluated whether this protein would be involved in the cytoprotection induced by gastrodin. We found that the silencing of Nrf2 suppressed the beneficial effects induced by gastrodin on the MMP ($p < 0.05$; Fig. 10a) and on the viability of PQ-treated SH-SY5Y cells ($p < 0.05$; Fig. 10b). We confirmed that gastrodin is a potent inducer of Nrf2 and HO-1 in SH-SY5Y cells, as demonstrated in Fig. S1A and B, respectively. Additionally,

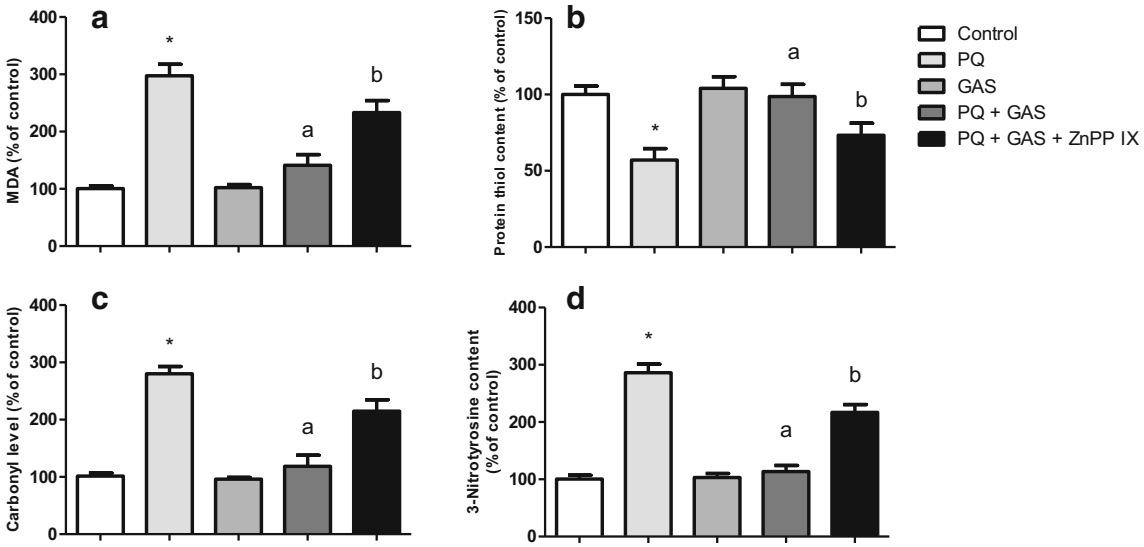

Fig. 6 The effects of the inhibition of HO-1 by ZnPP IX on the levels of lipid peroxidation (**a**), protein thiol groups (**b**), protein carbonylation (**c**), and protein nitration (**d**) in the membranes of mitochondria obtained from gastrodin (GAS)-treated SH-SY5Y cells exposed to paraquat (PQ). Data are shown 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 $p < 0.05$ different from PQ-treated group; b $p < 0.05$ different from PQ + GAS-treated cells

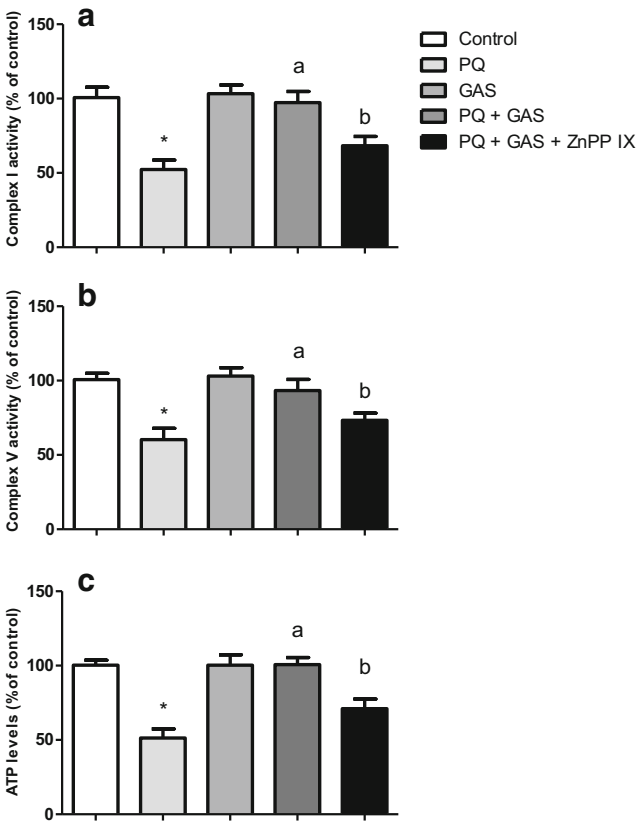

Fig. 7 The effects of the inhibition of HO-1 by ZnPP IX on the activity of the complexes I (**a**) and V (**b**) and on the levels of ATP (**c**) in gastrodin (GAS)-treated SH-SY5Y cells exposed to paraquat (PQ). Data are shown as the mean ± SEM of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test, $*p < 0.05$ different from the control group; a $p < 0.05$ different from PQ-treated group; b $p < 0.05$ different from PQ + GAS-treated cells

the effect of the knockdown of Nrf2 on the levels of HO-1 is shown in Fig. S2, demonstrating that Nrf2 mediated the gastrodin-induced HO-1 upregulation ($p < 0.05$). Altogether, these data indicate a role for Nrf2 in mediating the upregulation of HO-1 in the gastrodin-treated cells.

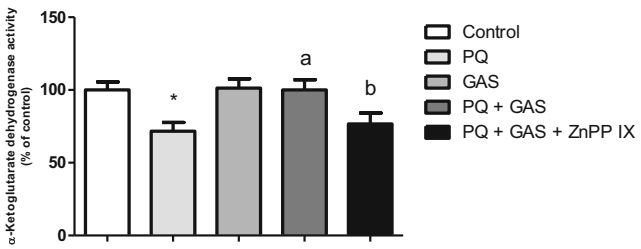

Fig. 8 The effects of the inhibition of HO-1 by ZnPP IX on the activity of α-ketoglutarate dehydrogenase in gastrodin (GAS)-treated SH-SY5Y cells exposed to paraquat (PQ). Data are shown as the mean ± SEM of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test, $*p < 0.05$ different from the control group; a $p < 0.05$ different from PQ-treated group; b $p < 0.05$ different from PQ + GAS-treated cells

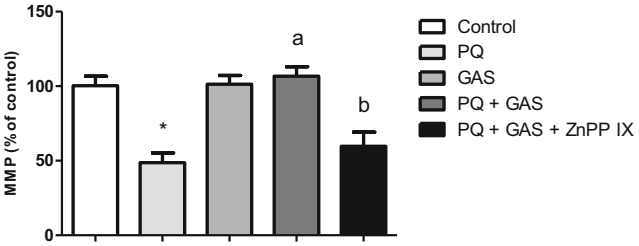

Fig. 9 The effects of the inhibition of HO-1 by ZnPP IX on the mitochondrial membrane potential (MMP) in gastrodin (GAS)-treated SH-SY5Y cells exposed to paraquat (PQ). Data are shown as the mean ± SEM of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test, $*p < 0.05$ different from the control group; a $p < 0.05$ different from PQ-treated group; b $p < 0.05$ different from PQ + GAS-treated cells

Discussion

Even though there is evidence demonstrating that gastrodin is an inducer of HO-1 and Nrf-2-related signaling pathways, it was not investigated yet whether the gastrodin-dependent HO-1 induction would be associated with mitochondrial protection [74–78]. Based on the data exhibited here, gastrodin promoted mitochondrial protection by a mechanism associated with the upregulation of the Nrf2/HO-1 axis, since the silencing of Nrf2 or the inhibition of HO-1 suppressed the beneficial effects seen in the mitochondria of gastrodin-treated cells.

The interest in the investigation of new drugs presenting the ability to induce mitochondrial protection has increased throughout the years due to the role these organelles play in several diseases, including neurodegeneration [79]. There is no cure for neurodegenerative diseases, such as Alzheimer's diseases, Parkinson's disease, and Huntington's disease [80–84]. Importantly, sporadic origin, i.e., the onset of the diseases is not related with genetic abnormalities, is a major cause for triggering the neurodegenerative events that will lead to neuronal loss and motor and cognitive decline, among other symptoms, depending on the disease [81]. Thus, drugs able to promote mitochondrial protection would attenuate the consequences of mitochondrial impairment on both bioenergetics and redox environment homeostasis, as well as on the triggering of the intrinsic apoptotic pathway, which is dependent on the release of cytochrome c by the mitochondria. In this context, gastrodin significantly alleviated the bioenergetics and redox impairments elicited by PQ on the mitochondria of SH-SY5Y cells. Moreover, gastrodin blocked the PQ-induced triggering of apoptosis in this experimental model. The effects were mediated by HO-1, which degrades heme groups generating biliverdin, CO, and free iron [47]. Biliverdin is converted by BVR into bilirubin, a potent antioxidant [49]. CO has been viewed as an anti-inflammatory agent due to

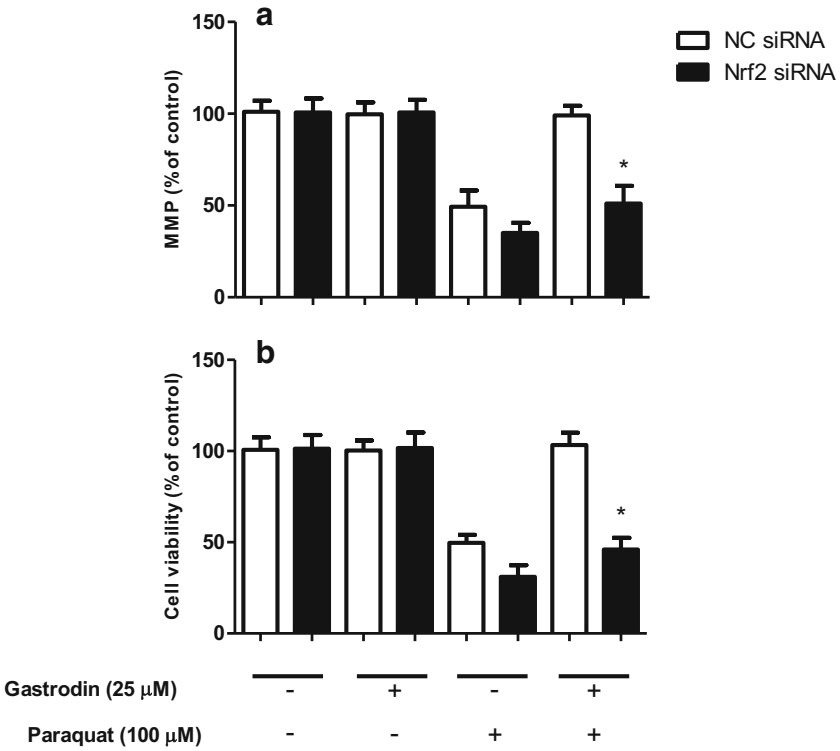

Fig. 10 The effects of the silencing of Nrf2 by siRNA (48 h) on the mitochondrial membrane potential (MMP) (**a**) and viability (**b**) of SH-SY5Y cells treated or not with gastrodin and/or paraquat. Data are shown as the mean ± S.E.M. of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test, $*p < 0.05$ vs the gastrodin and paraquat-treated cells transfected with negative control (NC) siRNA

its ability in inhibiting the transcription factor NF-κB [85, 86]. The link between the gastrodin-induced antioxidant and anti-inflammatory actions remains to be elucidated, but it is possible to be mediated by HO-1, as previously demonstrated in previously reported works [74, 76]. In other works, it was reported that the upregulation of Nrf2 by natural compounds caused an increase in the expression of other cytoprotective enzymes, such as Mn-SOD and GPx, which mediate antioxidant defense in the mitochondria [44, 87, 88]. Therefore, it may not be discarded when interpreting the protective effect elicited by gastrodin in SH-SY5Y cells. However, additional studies would be necessary in order to confirm the involvement of other antioxidant enzymes in the gastrodin-mediated mitochondrial protection seen here.

Overall, gastrodin-induced mitochondrial protection in PQ-treated SH-SY5Y cells by a mechanism associated with the Nrf2/HO-1 signaling pathway. Further research is necessary to investigate whether gastrodin would trigger beneficial effects in the mammalian brain mitochondria in in vivo experimental models. It is necessary also due to the limited bioavailability the natural compounds present in the mammalian organism [89–91]. Moreover, there is evidence that gastrodin upregulates other pro-survival signaling pathways in other cell types [57]. Importantly, the exact association between Nrf2 activation and mitochondria-related bioenergetics maintenance during stressful conditions needs to be completely elucidated, since there are data indicating a role for Nrf2 in the modulation of mitochondria-associated metabolic pathways in mammalian cells [92–95]. Thus, it may be taken into account when investigating the mitochondria-related effects elicited by this glucoside in the future.

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Compliance with Ethical Standards

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

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