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ORIGINAL PAPER

Carvacrol Depends on Heme Oxygenase-1 (HO-1) to Exert Antioxidant, Anti-inflammatory, and Mitochondria-Related Protection in the Human Neuroblastoma SH-SY5Y Cells Line Exposed to Hydrogen Peroxide

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Abstract

The link between mitochondrial dysfunction, redox impairment, and inflammation leads to increased rates of brain cells loss in neurodegenerative diseases and in affective disorders. Carvacrol (CAR) is a component of essential oils found in Labiatae. CAR exerts antioxidant and anti-inflammatory effects in different cell types, as assessed in both in vitro and in vivo experimental designs. Nonetheless, it was not previously investigated whether and how CAR would prevent mitochondrial impairment in human cells exposed to a pro-oxidant challenge. Therefore, we analyzed here whether a pretreatment (for 4 h) with CAR (10–1000 μM) would promote mitochondrial protection in the human neuroblastoma cells SH-SY5Y exposed to hydrogen peroxide (H_2O_2). We found that CAR at 100 μM prevented the H_2O_2-induced decline in the activity of the complexes I and V, as well as on the levels of adenosine triphosphate (ATP). CAR also prevented the H_2O_2-elicited decrease in the activity of the mitochondrial enzymes aconitase, α-ketoglutarate dehydrogenase, and succinate dehydrogenase. Moreover, CAR induced an antioxidant action by decreasing the levels of lipid peroxidation, protein carbonylation, and protein nitration in the mitochondrial membranes. Interestingly, CAR prevented the pro-inflammatory action of H_2O_2 by downregulating the transcription factor nuclear factor-κB (NF-κB). The inhibition of the heme oxygenase-1 (HO-1) enzyme by zinc protoporphyrin IX (ZnPP IX, 10 μM) suppressed the preventive effects caused by CAR regarding mitochondrial function and inflammation. Thus, it is suggested that CAR caused cytoprotective effects by an HO-1-dependent manner in SH-SY5Y cells exposed to H_2O_2.

Keywords Carvacrol · Mitochondria · Bioenergetics · Antioxidant · Anti-inflammatory · HO-1

Introduction

Neurodegenerative diseases present several common aspects, such as impairment in the redox environment, neuroinflammation, and disruption in the bioenergetics state, mainly affecting mitochondrial function and dynamics [1–3]. Moreover, alterations in signaling pathways mediating cell survival lead to increase death rates of brain cells (both neuron and glia) [4]. Similar routes leading to neuronal loss are also observed in the brain of patients suffering from major depression, bipolar disorder, and schizophrenia, which are affective disorders [5–7]. Since there is not a cure for those brain diseases, prevention of neuronal and glial dysfunction in the sporadic cases is an interesting strategy to decrease the number of individuals affected by such maladies [8]. In

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that context, mitochondria are an interesting pharmacological target when aiming to ameliorate brain cells function in the case of neurodegeneration [9].

The mitochondria present the molecular apparatus necessary to produce more than 90% of the adenosine triphosphate (ATP) in the nucleated human cells [10]. The electron transfer chain (ETC) contains the complexes I (NADH dehydrogenase), II (succinate dehydrogenase, SDH), III (coenzyme Q:cytochrome c—oxidoreductase), and IV (cytochrome c oxidase), as well as the electron transfer components ubiquinone (the so-called coenzyme Q_{10}) and cytochrome c (a heme protein) [11, 12]. The flux of electrons in the ETC is utilized by the complexes to generate a proton (H^+) gradient across the inner mitochondrial membrane, which is measured as the mitochondrial membrane potential (MMP) experimentally [13]. The H^+ gradient is utilized by the complex V (ATP synthase/ATPase) protein to produce ATP [11]. Mitochondrial damage leads to decreased ability to sustain the production of ATP in the organelles, as well as enhances the production of reactive oxygen and nitrogen species (ROS and RNS, respectively) by the mitochondria [14]. Actually, the mitochondria are the main site of ROS production in the mammalian cells [14]. The ETC generates radical anion superoxide ($O_2^{-\cdot}$) due to electron leakage in the complexes I–III and IV [14]. Then, $O_2^{-\cdot}$ is converted into hydrogen peroxide (H_2O_2) by the manganese-dependent superoxide dismutase (Mn-SOD), the mitochondrial form of SOD [15]. Catalase (CAT) or glutathione peroxidase (GPx) generates water by consuming H_2O_2 in the mitochondria or in the cytosol [15]. H_2O_2 is not a free radical, but it can react with iron or cupper ions leading to the formation of the hydroxyl radical ($^{\cdot}OH$), the most powerful free radical generated in human cells [15]. It would particularly important in the mitochondria because these organelles contain high concentrations of iron and cupper due to the work of ETC [15]. Therefore, mitochondria are susceptible to a pro-oxidant impairment (i.e., lipid peroxidation and the consequences of this deleterious action) generated by H_2O_2.

In this regard, several natural compounds have been described as potential neuroprotective agents, as evaluated in both in vitro and in vivo experimental models, by promoting mitochondrial protection. We have previously demonstrated that carnosic acid, pinocembrin, naringenin, and tanshinone I, for example, attenuated the effects of different chemical stressors on both redox and functional parameters related to mitochondria in the dopaminergic cell line SH-SY5Y [16–21]. At least in part, the benefits resulting from the pretreatment of SH-SY5Y cells with such bioactive molecules are dependent on the cytoprotective enzyme heme oxygenase-1 (HO-1) [21–23]. HO-1 generates free iron ions, carbon monoxide (CO), and biliverdin during the degradation of heme [24]. The enzyme biliverdin reductase (BVR) consumes biliverdin producing bilirubin, a potent antioxidant [25]. On the other hand, CO has been associated with an anti-inflammatory effect in animal cells due to the ability in inhibiting the transcription factor nuclear factor-κB (NF-κB), the master regulator in the immune response [26, 27]. Nonetheless, some studies have indicated a pro-oxidant and pro-apoptotic role for HO-1 in some cell types [28, 29]. The mechanisms underlying the pro-survival and cytotoxic actions of HO-1 are focus of intense research [29].

In this context, carvacrol (CAR; 5-isopropyl-2-methylphenol; $C_{10}H_{14}O$) has been demonstrated to be an antioxidant, anti-inflammatory, and anti-apoptotic agent experimentally [30]. CAR is found in the essential oil of some plants such as *Origanum vulgare* L. and *Rosmarinus officinalis* L. [31]. It was previously shown that CAR protected SH-SY5Y cells exposed to iron ions by a mechanism involving the inhibition of NF-κB [32]. Moreover, CAR alleviated the effects of cisplatin in the mice kidney by a mechanism involving HO-1 modulation [33]. It was shown that CAR caused neuroprotection in mice subjected to focal cerebral ischemia and reperfusion, indicating that CAR presents the ability to cross the blood–brain barrier (BBB) [34]. Nonetheless, it was not previously reported whether CAR would be able to promote mitochondrial protection in experimental models of neuronal dysfunction. Thus, we investigated here whether would exert mitochondrial protection in SH-SY5Y cells exposed to H_2O_2, a pro-oxidant stressor that is produced by brain cells at high rates and that induces mitochondrial dysfunction by several ways [35].

Materials and Methods

Materials

The plastic materials utilized in the maintenance of cell culture were acquired from Corning, Inc. (NY, USA) and Beckton Dickson (NJ, USA). The chemicals and other materials necessary to cell culture have been obtained from Sigma (MO, USA). Other reagents and assay kits utilized in this work were purchased from different manufacturers, as indicated whenever necessary.

Chemical Assays

Epinephrine Autoxidation

The autoxidation of epinephrine was assayed in alkaline pH (7.4) according to a protocol previously published [36]. The autoxidation of epinephrine was detected at 480 nm in a spectrophotometer.

DPPH Assay

The assay using the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free-radical has been performed based on the protocol published by others [37]. CAR at different concentrations was incubated with DPPH and the absorbance was read at 518 nm in a spectrophotometer.

Biological Assays

Cell Culture and Treatments

We utilized the human neuroblastoma SH-SY5Y cell line as an in vitro experimental model of dopaminergic cells. The SH-SY5Y cell line was acquired from the American Type Culture Collection (Manassas, VA, USA) and was cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 HAM nutrient medium (1:1 mixture) containing fetal bovine serum (FBS, 10%), L-glutamine (2 mM), penicillin (1000 units/mL), streptomycin (1000 µg/mL), and amphotericin B (2.5 µg/mL) in a 5% CO_2 humidified incubator (37 °C).

H_2O_2 at 300 µM was used as a pro-oxidant stressor for 3 h or 24 h according to each specific assay. CAR (dissolved in dimethyl sulfoxide, DMSO) at 10–1000 µM was administrated for 4 h prior exposing the cells to H_2O_2 (pretreatment experimental model). The specific inhibitor of HO-1, zinc protoporphyrin IX (ZnPP IX, 10 µM), was administrated to the cells for 1 h before the treatment with CAR. Additional information is described in the figure legends. The data are shown as the mean ± S.E.M. of three or five independent experiments each done in triplicate.

Cell Viability and Cytotoxicity Assays

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to analyze the effect of specific treatments on the viability of the SH-SY5Y cells [38]. At the end of each experiment, the cells were exposed to MTT for 1 h at 37 °C. After this period, the culture medium was removed and the wells were washed twice with PBS (pH 7.4). The insoluble formazan formed intracellularly was dissolved by the administration of 100 µL DMSO to each well and incubated for 30 min. The absorbance was read at 570 nm in a spectrophotometer.

The leakage of the cytoplasmic lactate dehydrogenase (LDH) enzyme was quantified in the culture medium as an index of membrane integrity based on the protocol indicated by the manufacturer (CytoTox 96-NonRadioactive Cytotoxicity Assay, Promega).

Isolation of Mitochondria

Mitochondria were isolated from the SH-SY5Y cells by washing the and re-suspending in a buffer with 250 mM sucrose, 10 mM KCl, 1 mM EGTA, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl floride (PMSF), 1 mM benzamidine, 1 mM pepstatin A, 10 mg/mL leupeptin, 2 mg/mL aprotonin, and 20 mM HEPES (pH 7.4). The samples were centrifuged at 1000×*g* for 10 min at 4 °C in order to remove cell debris and nuclei, as well as unbroken cells. The mitochondrial fraction was obtained after centrifuging the resulting supernatant at 11,000×*g* for 20 min at 4 °C [39].

Isolation of Submitochondrial Particles

We obtained submitochondrial particles (SMP) after isolating mitochondria from the SH-SY5Y cells. The solution presenting mitochondria was frozen and thawed (three times), leading to the rupture of mitochondrial membranes and to the release of the components of the mitochondrial matrix. This solution was washed (twice) with a buffer containing 140 mM KCl, 20 mM Tris–HCl (pH 7.4) in order to generate SMP without the enzyme Mn-SOD (which would interfere in the quantification of reactive species by the organelles, as described below). We have also utilized this protocol to determine the production of $O_2^{-\cdot}$ and to evaluate the effects of chemical stressors (H_2O_2, in this work) and/or CAR on the levels of markers of redox impairment in the membranes of mitochondria, since SMP are mitochondrial membranes without any component of the matrix of these organelles [36].

Quantification of the Production of $O_2^{-\cdot}$ and $NO^{\cdot}$

We quantified the generation of $O_2^{-\cdot}$ by using the SMP obtained from the SH-SY5Y cells in a reaction medium containing 230 mM mannitol, 70 mM sucrose, 10 mM HEPES-KOH (pH 7.4), 4.2 mM succinate, 0.5 mM KH_2PO_4, 0.1 µM catalase, and 1 mM epinephrine, as previously published [36]. The levels of $NO^{\cdot}$ were quantified in the cellular level following the protocol of the manufacturer of a commercial kit (Abcam, MA, USA).

Examination of the Mitochondria-Related Apoptotic Factors and Cell Death-Associated Parameters

The immunocontents of cytochrome c (mitochondrial and cytosolic) and of the cleaved form of PARP were examined through the utilization of ELISA assay kits, based on the instructions of the manufacturer (Abcam, MA, USA). Caspase-9 and caspase-3 enzyme activities were measured by

using fluorimetric assay kits following the instructions of the manufacturer (Abcam, MA, USA) [16].

Quantification of Enzyme Activities

The enzymatic activity of aconitase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, complex I, and complex V were quantified by using commercial kits according to the instructions of the manufacturer (Abcam, MA, USA).

Evaluation of the Levels of ATP

The levels of ATP were evaluated by following the protocol of a commercial kit (Abcam, MA, USA). After the deproteinization of the samples, it was centrifuged and the levels of ATP were determined in the supernatants [20].

Measurement of the Mitochondrial Membrane Potential (MMP)

The MMP was measured by the use of a commercial kit utilizing tetraethylbenzimidazolylcarbocyanide iodine (JC-1) as a lipophilic cationic dye that can cross mitochondrial membranes, accumulating in the organelles according to the changes in the membrane potential (Abcam, MA, USA).

Examination of the Levels of Malondialdehyde (MDA), Protein Carbonyl, and 8-Oxo-2′-Deoxyguanosine (8-Oxo-dG)

We quantified the levels of MDA, protein carbonyl, and 8-oxo-dG by following the instructions of the manufacturer of commercial kits (Abcam, MA, USA), as previously described [20]. MDA and protein carbonyl levels were quantified in both mitochondrial and total samples.

Determination of the Levels of 3-Nitrotyrosine

We quantified the levels of 3-nitrotyrosine in the membranes of the mitochondria through the utilization of a polyclonal antibody (Calbiochem, Germany), which was diluted 1:2000 in phosphate-buffered saline (PBS containing albumin at 5%, pH 7.4), as previously published [40].

Isolation of the Cell Nucleus

The cell nucleus was isolated by using the Nuclear Extraction Kit, following the instructions of the manufacturer of a commercial assay kit (Cayman Chemical, MI, USA) and as previously published by us [16]. The protein determination was performed through the Bradford method.

Measurement of the Levels of Interleukin-1β (IL-1β) and Tumor Necrosis Factor-α (TNF-α)

The levels of the pro-inflammatory cytokines IL-1β and TNF-α were measured based on the instructions of the manufacturer of a commercial ELISA assay kit (Abcam, MA, USA).

Quantification of the Activity of the Nuclear Factor-κB (NF-κB)

We quantified the activity of the p65 subunit of NF-κB according to the protocol of the manufacturer of a commercial assay kit (Abcam, MA, USA) [21].

Statistical Analyses

We performed the statistical analyses by using the GraphPad 5.0 software. 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 examined by one-way ANOVA, followed by the post hoc Tukey's test.

Results

CAR Attenuated the Effects of H_2O_2 on the Viability of SH-SY5Y Cells by an HO-1-Dependent Fashion

According to Fig. 1, CAR at 10 and 100 μM did not affect the viability of SH-SY5Y cells. However, CAR at 500 and 1000 μM induced a significant decrease in the cell viability in this experimental model ($p < 0.05$). In this regard, only

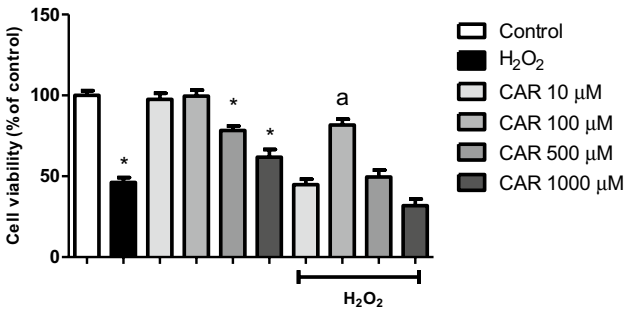

Fig. 1 The effects of carvacrol (CAR) on the viability of SH-SY5Y cells exposed to H_2O_2. The cells were treated with CAR at 10–1000 μM during 4 h prior to the challenge with H_2O_2 at 300 μM for further 24 h. Data are shown as the mean ± SEM of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test, *$p < 0.05$ different from the control group; [a]$p < 0.05$ different from H_2O_2-treated group

CAR at 100 µM attenuated the H_2O_2-induced decline in the viability of SH-SY5Y cells ($p < 0.05$). Therefore, this CAR concentration was utilized in the other experiments that were performed in this work. In order to evaluate whether the HO-1 enzyme would be involved in the cytoprotection caused by CAR in H_2O_2-treated SH-SY5Y cells, ZnPP IX was administrated to the cells before the treatment with CAR. Thus, as depicted in Fig. 2A, the inhibition of HO-1 by ZnPP IX abolished the protection mediated by CAR in H_2O_2-treated cells ($p < 0.05$). Moreover, CAR attenuated the H_2O_2-induced cytotoxicity (as assessed through the measurement of LDH leakage from the cells) by an HO-1-associated manner (Fig. 2B, $p < 0.05$).

CAR also alleviated the pro-apoptotic effects induced by H_2O_2 in SH-SY5Y cells by a mechanism dependent on HO-1. CAR significantly blocked the release of cytochrome c to the cytosol (Fig. 3A, $p < 0.05$). Consequently, CAR prevented the loss of cytochrome c in the mitochondria

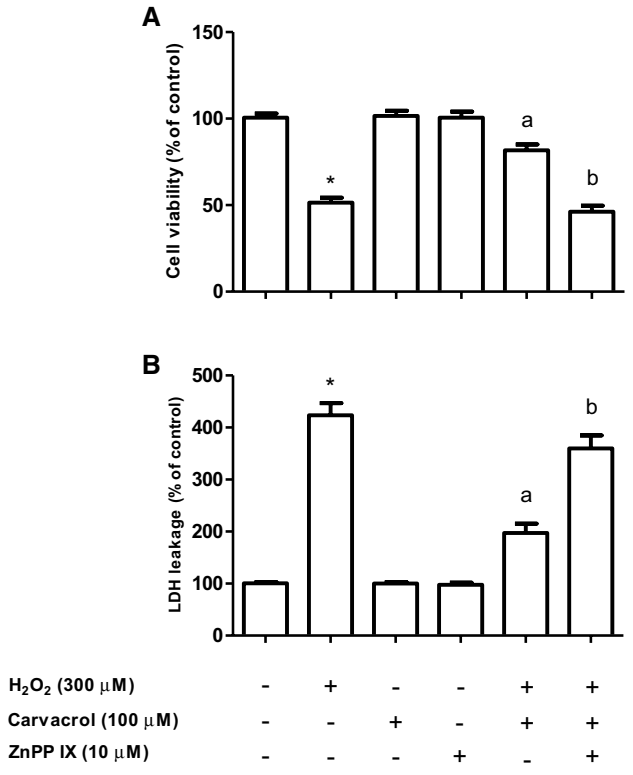

Fig. 2 The effects of heme oxygenase-1 (HO-1) inhibition by zinc protoporphyrin IX (ZnPP IX) on the viability (**A**) and leakage of lactate dehydrogenase (LDH) (**B**) in SH-SY5Y cells treated with carvacrol (CAR) and/or H_2O_2. ZnPP IX at 10 µM was administrated to the cells for 1 h before the exposure to CAR. The cells were treated with CAR at 100 µM during 4 h prior to the challenge with H_2O_2 at 300 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; [a]$p < 0.05$ different from H_2O_2-treated group; [b]$p < 0.05$ different from the CAR + H_2O_2-treated group

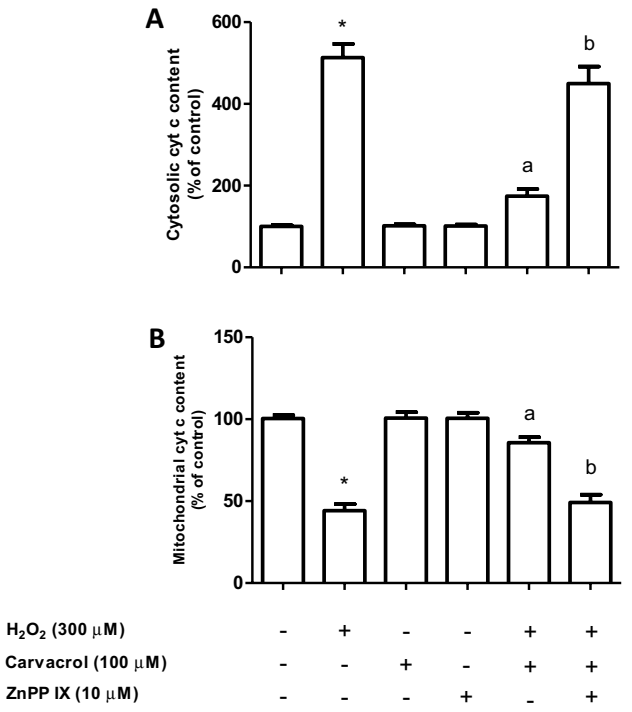

Fig. 3 The effects of heme oxygenase-1 (HO-1) inhibition by zinc protoporphyrin IX (ZnPP IX) on the cytosolic (**A**) and mitochondrial (**B**) contents of cytochrome c (cyt c) in SH-SY5Y cells treated with carvacrol (CAR) and/or H_2O_2. ZnPP IX at 10 µM was administrated to the cells for 1 h before the exposure to CAR. The cells were treated with CAR at 100 µM during 4 h prior to the challenge with H_2O_2 at 300 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; [a]$p < 0.05$ different from H_2O_2-treated group; [b]$p < 0.05$ different from the CAR + H_2O_2-treated group

of H_2O_2-treated cells (Fig. 3B, $p < 0.05$). The activation of the pro-apoptotic caspases-9 and -3 was decreased by CAR in the cells exposed to H_2O_2 (Fig. 4A, B, respectively; $p < 0.05$). Also, the cleavage of PARP, a target of caspase-3 during apoptotic cell death, was attenuated by CAR in H_2O_2-treated cells (Fig. 4C, $p < 0.05$). The anti-apoptotic effects induced by CAR were suppressed by the inhibition of HO-1, showing a role for this enzyme in the CAR-induced mitochondria-related blockade of cell death during the exposure to H_2O_2.

CAR Promoted Mitochondrial Protection by a HO-1-Dependent Mechanism in SH-SY5Y Cells Exposed to the Pro-oxidant Agent H_2O_2

CAR significantly attenuated the H_2O_2-induced impairment in the function of the complex I by a mechanism dependent on HO-1, since the inhibition of this enzyme by ZnPP IX blocked this effect in SH-SY5Y cells (Fig. 5A, $p < 0.05$). Similarly, ZnPP IX abrogated the mitochondrial

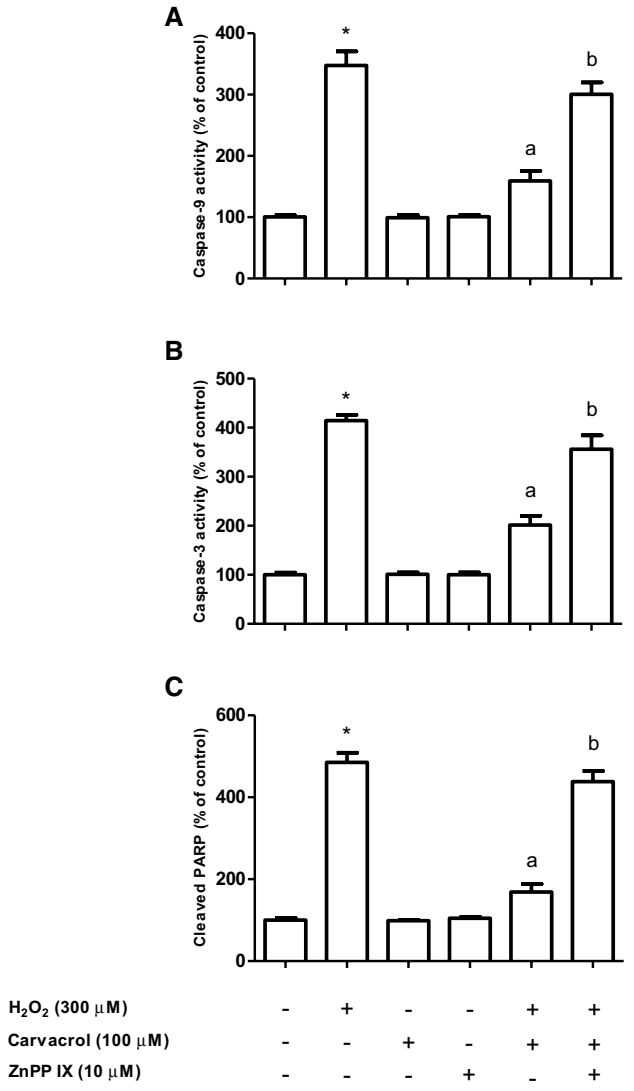

Fig. 4 The effects of heme oxygenase-1 (HO-1) inhibition by zinc protoporphyrin IX (ZnPP IX) on the activity of the pro-apoptotic enzymes caspase-3 (**A**) and caspase-9 (**B**), and on the cleavage of poly [ADP-ribose] polymerase (PARP) (**C**) in SH-SY5Y cells treated with carvacrol (CAR) and/or H_2O_2. ZnPP IX at 10 μM was administrated to the cells for 1 h before the exposure to CAR. The cells were treated with CAR at 100 μM during 4 h prior to the challenge with H_2O_2 at 300 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; [a]$p < 0.05$ different from H_2O_2-treated group; [b]$p < 0.05$ different from the CAR + H_2O_2-treated group

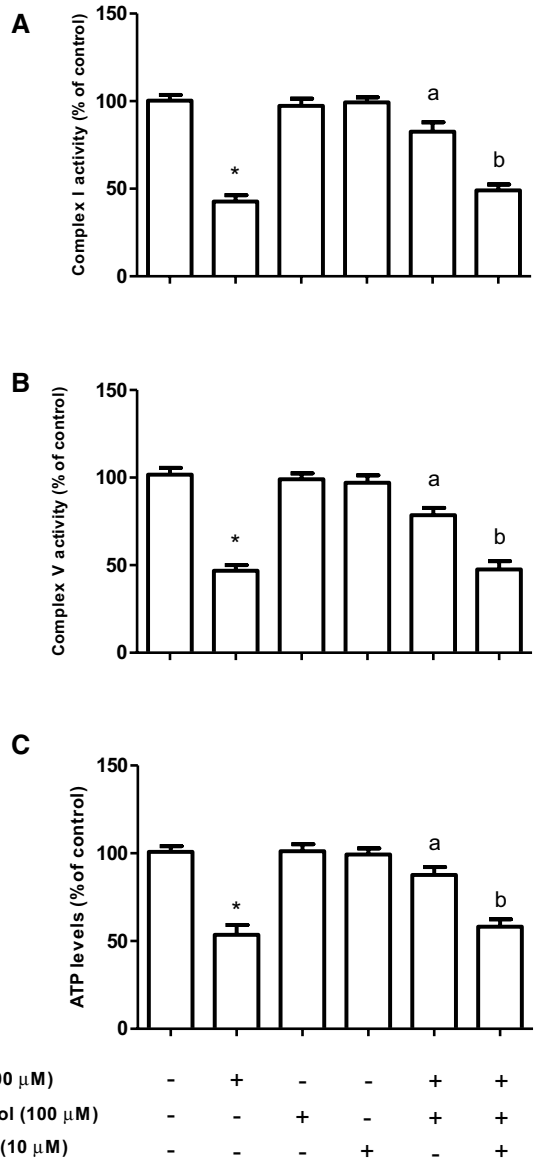

Fig. 5 The effects of heme oxygenase-1 (HO-1) inhibition by zinc protoporphyrin IX (ZnPP IX) on the activity of the complexes I (**A**) and V (**B**) and on the levels of ATP (C) in SH-SY5Y cells treated with carvacrol (CAR) and/or H_2O_2. ZnPP IX at 10 μM was administrated to the cells for 1 h before the exposure to CAR. The cells were treated with CAR at 100 μM during 4 h prior to the challenge with H_2O_2 at 300 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; [a]$p < 0.05$ different from H_2O_2-treated group; [b]$p < 0.05$ different from the CAR + H_2O_2-treated group

protection mediated by CAR regarding the function of complex V in the mitochondria of the cells exposed to H_2O_2 (Fig. 5B, $p < 0.05$). As expected, CAR prevented the decline in the ATP content in the mitochondria of H_2O_2-treated cells by an HO-1-dependent manner (Fig. 5C, $p < 0.05$). Moreover, CAR efficiently prevented the H_2O_2-induced loss of MMP in SH-SY5Y cells by a mechanism involving the HO-1 enzyme (Fig. 6; $p < 0.05$). The mitochondria-related protection caused by CAR was also observed regarding the function of the TCA cycle in SH-SY5Y cells exposed to H_2O_2. As depicted in Fig. 7A, CAR significantly attenuated the H_2O_2-induced impairment in the activity of aconitase ($p < 0.05$). Similar effects

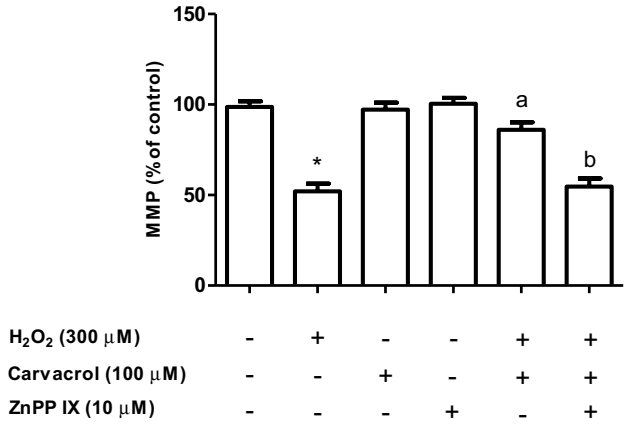

Fig. 6 The effects of heme oxygenase-1 (HO-1) inhibition by zinc protoporphyrin IX (ZnPP IX) on the mitochondrial membrane potential (MMP) in SH-SY5Y cells treated with carvacrol (CAR) and/or H_2O_2. ZnPP IX at 10 μM was administrated to the cells for 1 h before the exposure to CAR. The cells were treated with CAR at 100 μM during 4 h prior to the challenge with H_2O_2 at 300 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; [a]$p < 0.05$ different from H_2O_2-treated group; [b]$p < 0.05$ different from the CAR + H_2O_2-treated group

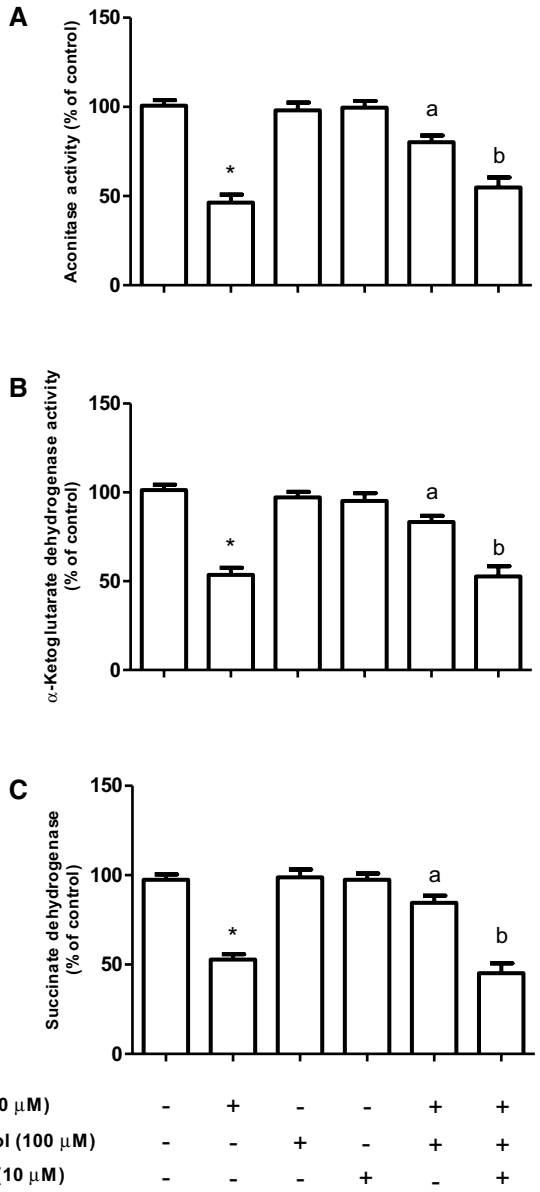

Fig. 7 The effects of heme oxygenase-1 (HO-1) inhibition by zinc protoporphyrin IX (ZnPP IX) on the activity of the tricarboxylic acid cycle (TCA) enzymes aconitase (**A**), α-ketoglutarate dehydrogenase (**B**), and succinate dehydrogenase (**C**) in SH-SY5Y cells treated with carvacrol (CAR) and/or H_2O_2. ZnPP IX at 10 μM was administrated to the cells for 1 h before the exposure to CAR. The cells were treated with CAR at 100 μM during 4 h prior to the challenge with H_2O_2 at 300 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; [a]$p < 0.05$ different from H_2O_2-treated group; [b]$p < 0.05$ different from the CAR + H_2O_2-treated group

were seen regarding the function of α-KGDH and SDH (Fig. 7B, C, respectively; $p < 0.05$). Nonetheless, the inhibition of HO-1 by ZnPP IX suppressed the TCA cycle-related protection caused by CAR in cells challenged by H_2O_2 ($p < 0.05$).

CAR also prevented both cellular and mitochondrial redox impairment elicited by H_2O_2 in SH-SY5Y cells. CAR pretreatment reduced the levels of lipid peroxidation and protein carbonylation in the SH-SY5Y cells (Fig. 8A, B, respectively; $p < 0.05$). Moreover, CAR efficiently attenuated the levels of oxidative stress in the DNA of SH-SY5Y cells, as assessed through the formation of 8-oxo-dG (Fig. 8C, $p < 0.05$). In spite of this, the inhibition of HO-1 blocked the antioxidant effects caused by CAR in the cells exposed to H_2O_2 ($p < 0.05$). Similar antioxidant effects were seen in the mitochondria obtained from the SH-SY5Y cells. CAR decreased the lipid peroxidation levels in the membranes of mitochondria isolated from the H_2O_2-treated cells (Fig. 9A, $p < 0.05$). Also, protein carbonylation in mitochondrial membranes was reduced by CAR in the H_2O_2-treated cells (Fig. 9B, $p < 0.05$). CAR was also effective in decreasing the levels of protein nitration in the membranes of mitochondria obtained from SH-SY5Y cells facing H_2O_2 (Fig. 9C, $p < 0.05$). The antioxidant effects caused by CAR in the mitochondrial membranes obtained from the H_2O_2-challenged cells were suppressed by ZnPP IX, indicating a role for HO-1 in mediating this protective effects elicited by CAR ($p < 0.05$).

The production of free radicals was also assessed in this work in SH-SY5Y cells treated with CAR and/or H_2O_2. As demonstrated in Fig. 10A, CAR reduced the production of $O_2^{-\cdot}$ in the SMP obtained from the H_2O_2-treated cells

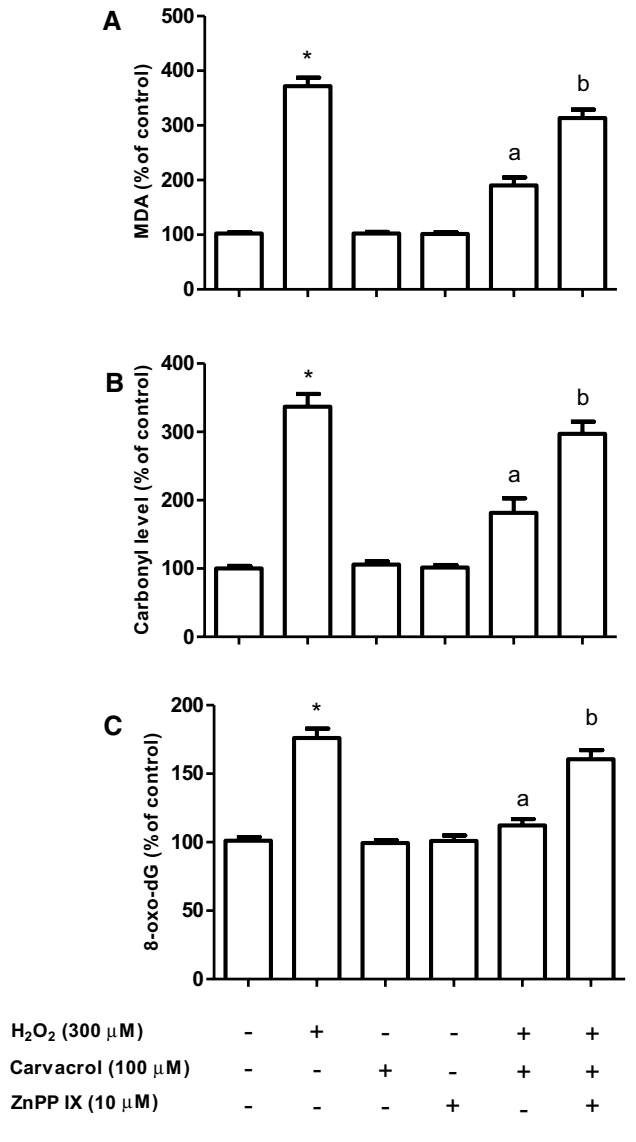

Fig. 8 The effects of heme oxygenase-1 (HO-1) inhibition by zinc protoporphyrin IX (ZnPP IX) on the total levels of malondialdehyde (MDA) (**A**), protein carbonyl (**B**), and 8-oxo-2′-deoxyguanosine (8-oxo-dG) (**C**) in SH-SY5Y cells treated with carvacrol (CAR) and/or H_2O_2. ZnPP IX at 10 μM was administrated to the cells for 1 h before the exposure to CAR. The cells were treated with CAR at 100 μM during 4 h prior to the challenge with H_2O_2 at 300 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; $^{a}p < 0.05$ different from H_2O_2-treated group; $^{b}p < 0.05$ different from the CAR + H_2O_2-treated group

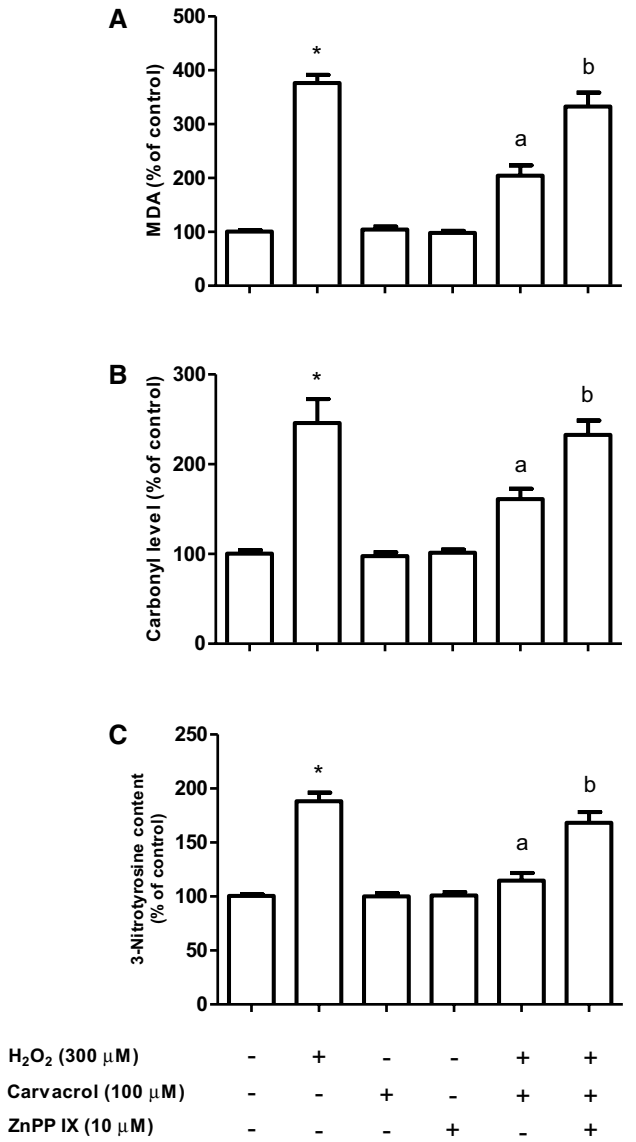

Fig. 9 The effects of heme oxygenase-1 (HO-1) inhibition by zinc protoporphyrin IX (ZnPP IX) on the levels of malondialdehyde (MDA) (**A**), protein carbonyl (**B**), and 3-nitrotyrosine (**C**) in the membranes of mitochondria obtained from SH-SY5Y cells treated with carvacrol (CAR) and/or H_2O_2. ZnPP IX at 10 μM was administrated to the cells for 1 h before the exposure to CAR. The cells were treated with CAR at 100 μM during 4 h prior to the challenge with H_2O_2 at 300 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; $^{a}p < 0.05$ different from H_2O_2-treated group; $^{b}p < 0.05$ different from the CAR + H_2O_2-treated group

by a mechanism involving the enzyme HO-1 ($p < 0.05$). Similarly, HO-1 played a role in mediating the CAR-induced decrease in the cellular production of $NO^{\cdot}$ in the H_2O_2-treated SH-SY5Y cells (Fig. 10B, $p < 0.05$). Interestingly, CAR was not effective in inhibiting the autoxidation of epinephrine, as may be observed in Fig. 11. On the other hand, CAR presented a concentration-dependent antioxidant effect by inhibiting the oxidation of DPPH, as depicted in Fig. 12 ($p < 0.05$). These data indicate that CAR may fail as a direct antioxidant depending on the type of redox stressor present in a particular environment.

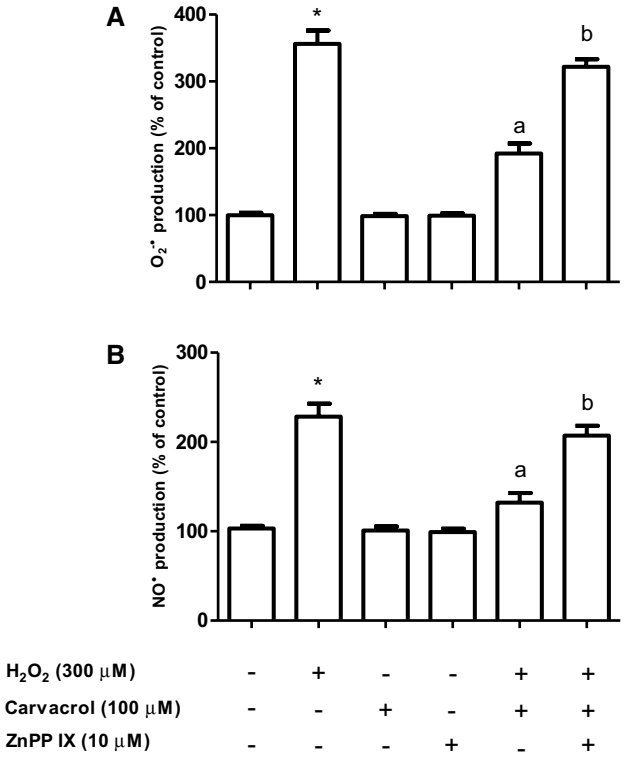

Fig. 10 The effects of heme oxygenase-1 (HO-1) inhibition by zinc protoporphyrin IX (ZnPP IX) on the production of radical anion superoxide ($O_2^{\bullet-}$) (**A**) and nitric oxide ($NO^{\bullet}$) (**B**) by SH-SY5Y cells treated with carvacrol (CAR) and/or H_2O_2. ZnPP IX at 10 μM was administrated to the cells for 1 h before the exposure to CAR. The cells were treated with CAR at 100 μM during 4 h prior to the challenge with H_2O_2 at 300 μM for further 24 h. $O_2^{\bullet-}$ production was determined by using submitochondrial particles (SMP) obtained from SH-SY5Y cells, as described in the "Materials and Methods". 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 H_2O_2-treated group; [b]$p < 0.05$ different from the CAR + H_2O_2-treated group

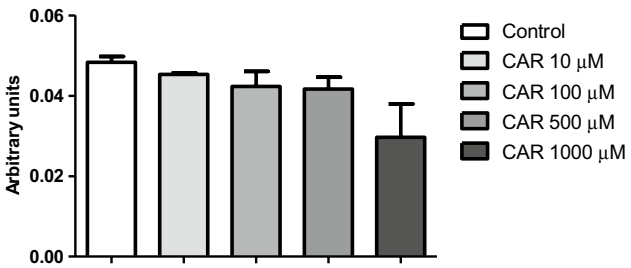

Fig. 11 The effects of carvacrol (CAR) at different concentrations on the autoxidation of adrenaline. Adrenaline was incubated with CAR at 10–1000 μM and the autoxidation of the catecholamine was assessed as described in "Materials and Methods". Data are shown as the mean ± SEM of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test

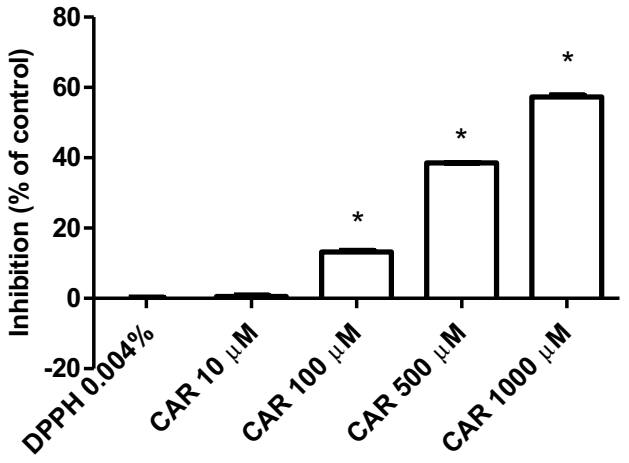

Fig. 12 The effects of carvacrol (CAR) at different concentrations on the oxidation of 2,2-diphenyl-1-picrylhydrazyl (DPPH). DPPH was incubated with CAR at 10–1000 μM and the oxidation of this free radical was assessed as described in "Materials and Methods". Data are shown as the mean ± SEM of three or five independent experiments each done in triplicate. One-way ANOVA followed by the post hoc Tukey's test

CAR Induced Anti-inflammatory Effects in H_2O_2-treated SH-SY5Y Cells by a Mechanism Associated with HO-1

Since pro-oxidant agents are able to cause redox impairment, mitochondrial dysfunction, and inflammation, we tested here whether CAR would be able to modulate the pro-inflammatory state elicited by H_2O_2 in SH-SY5Y cells. As depicted in Fig. 13A, CAR prevented the increase in the levels of IL-1β in the SH-SY5Y cells exposed to H_2O_2 ($p < 0.05$). Similarly, CAR attenuated the H_2O_2-induced increase in the levels of TNF-α in SH-SY5Y (Fig. 13B, $p < 0.05$). CAR pretreatment was also effective in inhibiting the activation of the transcription factor NF-κB in the cells treated with H_2O_2 (Fig. 13C, $p < 0.05$). The anti-inflammatory effects elicited by CAR were blocked by ZnPP IX ($p < 0.05$).

Discussion

In the present work, CAR pretreatment attenuated the deleterious effects induced by the pro-oxidant agent H_2O_2 by a mechanism involving the enzyme HO-1. The cytoprotective actions caused by HO-1 have been reported by several research groups, showing that this enzyme mediates antioxidant, anti-apoptotic, and anti-inflammatory effects in mammalian cells [41–44]. Nonetheless, it was not previously demonstrated whether HO-1 would mediate the beneficial effects induced by CAR experimentally. The role of HO-1 in promoting mitochondrial protection is still on debate,

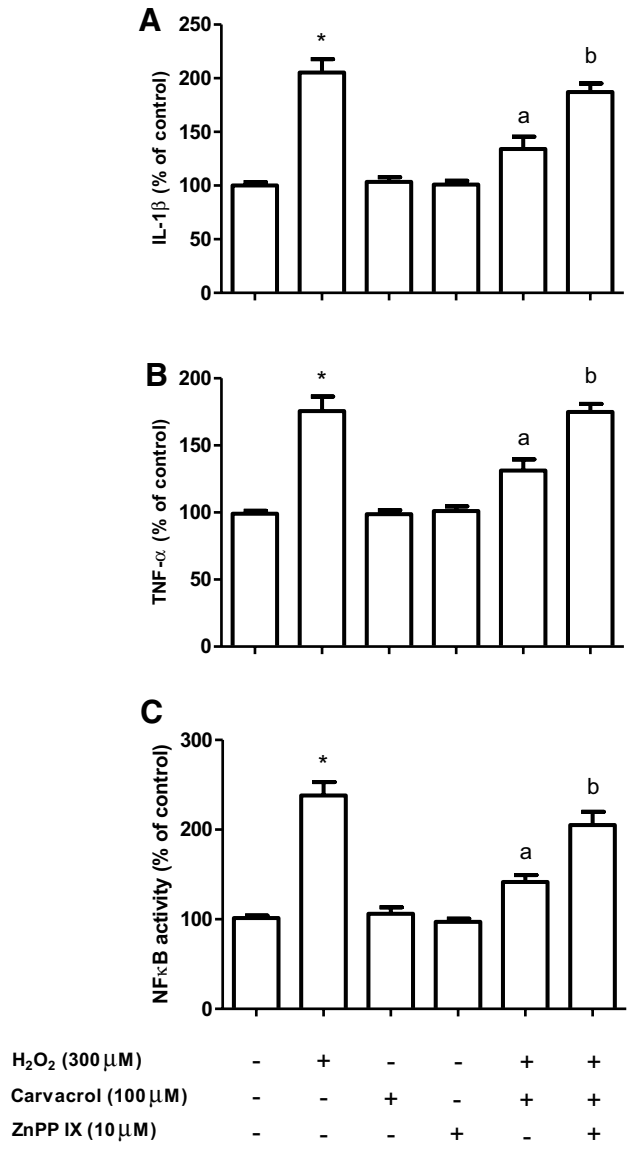

Fig. 13 The effects of heme oxygenase-1 (HO-1) inhibition by zinc protoporphyrin IX (ZnPP IX) on the production of the pro-inflammatory cytokines interleukin-1β (IL-1β) (**A**) and tumor necrosis factor-α (TNF-α) (**B**), and on the activity of the transcription factor nuclear factor-κB (NF-κB) (**C**) in SH-SY5Y cells treated with carvacrol (CAR) and/or H_2O_2. ZnPP IX at 10 µM was administrated to the cells for 1 h before the exposure to CAR. The cells were treated with CAR at 100 µM during 4 h prior to the challenge with H_2O_2 at 300 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; $^{a}p < 0.05$ different from H_2O_2-treated group; $^{b}p < 0.05$ different from the CAR + H_2O_2-treated group

since it was found that HO-1 also works as a pro-oxidant in some cases, causing cellular injury and altering physiological parameters [28]. In this regard, the modulation of HO-1 may serve as a therapeutic target in the case of neurodegenerative diseases, as recently reviewed [29]. We have demonstrated that several natural compounds can modulate HO-1 through the activation of signal pathways related to cell survival, leading to the mitochondrial protection, which is crucial to suppress the pro-apoptotic actions of different chemical stressors [18, 20, 45].

HO-1 generates CO, biliverdin, and free iron as products of the heme degradation [46]. Biliverdin is converted into bilirubin, a potent antioxidant, by BVR [47]. CO, on the other hand, has been viewed as an inhibitor of the master regulator of inflammation, the transcription factor NF-κB [26, 48]. Therefore, it is expected that the combination of direct antioxidant actions exerted by bilirubin and the attenuation of inflammation by CO would cause mitochondrial protection by decreasing the pro-oxidant signal of pro-inflammatory cytokines on the mitochondria. During inflammation, the production of ROS by the mitochondria is enhanced [49]. Also, depending on the duration of the pro-inflammatory stimulus, the intrinsic apoptotic pathway, which presents the mitochondria as central players, is activated during inflammation, causing increased rates of cell death [50]. This is particularly important in brain cells, which are very sensitive to redox impairment and mitochondrial dysfunction [51]. Thus, the modulation of the HO-1/CO/NF-κB signaling pathway by CAR is expected to act both directly and indirectly on the maintenance of mitochondrial function and dynamics. HO-1 is an inducible enzyme whose expression is controlled by the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), among others [52]. Nrf2 is upregulated by certain natural compounds in mammalian cells, and the activation of this transcription factor leads to the expression of several cytoprotective proteins [53]. These effects have been associated with prevention of cellular dysfunction in different experimental models using chemical stressors [22, 23, 42]. Even though we have not analyzed whether the link between HO-1 and Nrf2 in the present work, it may be suggested that such regulator would be associated with the cytoprotection seen here. Further research would be useful in investigating the role of Nrf2 in the HO-1-mediated preventive effects induced by CAR in mammalian cells.

Prevention of mitochondrial dysfunction is central when considering neurodegenerative diseases, since alterations in the activity of these organelles appear in Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD), as well as in affective disorders [54, 55]. Indeed, the use of anti-depressants by patients suffering from major depression or bipolar disorder may not be sufficient to downregulate the pro-inflammatory status observed in different brain areas [56–59]. Sustained pro-inflammatory signal leads to neuronal loss, an event that would amplify the impact of major depression of the life quality of the patients [60]. On the other hand, some environmental factors may cause mitochondrial dysfunction, as is the case of

agrochemicals that have been associated with the induction of PD, for example [61, 62]. By impairing mitochondrial function, these neurotoxic agents enhance the production of ROS by the organelles, leading to posterior inflammation [63]. A similar mechanism has been seen in the pathophysiology of some diseases in in vivo experimental models [64, 65]. Thus, the mitochondrial impaired function may be a cause or a consequence of inflammation. Therefore, targeting mitochondria during neuroinflammation may be an interesting pharmacological strategy to decrease the loss of brain cells in the case of neurodegeneration and/or affective disorders, among others [1, 66].

Overall, CAR promoted mitochondrial protection by a mechanism related to HO-1 in SH-SY5Y cells exposed to H_2O_2. The antioxidant effects CAR exerted in mitochondrial membranes are crucial to maintain both mitochondrial function (mainly the OXPHOS system) and dynamics (during events such as mitochondrial fusion and fission, as well as mitochondrial biogenesis). In spite of the results seen here, future research is necessary to investigate whether CAR would exert similar effects in in vivo experimental models, since the concentration of this natural compound that induced beneficial effects here is considered high when analyzing its bioavailability [67]. Furthermore, CAR at high concentrations induced toxic effects in the SH-SY5Y cell line, as assessed here through the cell viability assay. Thus, it should be studied in the future to avoid intoxication when using CAR therapeutically.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

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