Glutathionylation of Adenine Nucleotide Translocase Induced by Carbon Monoxide Prevents Mitochondrial Membrane Permeabilization and Apoptosis

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The present work demonstrates the ability of CO to prevent apoptosis in a primary culture of astrocytes. For the first time, the anti-apoptotic behavior can be clearly attributed to the inhibition of mitochondrial membrane permeabilization (MMP), a key event in the intrinsic apoptotic pathway. In isolated non-synaptic mitochondria, CO partially inhibits (i) loss of potential, (ii) the opening of a nonspecific pore through the inner membrane, (iii) swelling, and (iv) cytochrome c release, which are induced by calcium, diamide, or atractyloside (a ligand of ANT). CO directly modulates ANT function by enhancing ADP/ATP exchange and prevents its pore-forming activity. Additionally, CO directly modulates ANT function by enhancing ADP/ATP induced by calcium, diamide, or atractyloside (a ligand of ANT). CO directly modulates ANT function by enhancing ADP/ATP exchange and prevents its pore-forming activity. Additionally, CO induces reactive oxygen species (ROS) generation, and its prevention by β-carotene decreases CO cytoprotection in intact cells as well as in isolated mitochondria, revealing the key role of ROS. On the other hand, CO induces a slight increase in mitochondrial oxidized glutathione, which is essential for apoptosis modulation by (i) delaying astrocytic apoptosis, (ii) decreasing MMP, and (iii) enhancing ADP/ATP translocation activity of ANT. Moreover, CO and GSSG trigger ANT glutathionylation, a post-translational process regulating protein function in response to redox cellular changes. In conclusion, CO protects astrocytes from apoptosis by preventing MMP, acting on ANT (glutathionylation and inhibition of its pore activity) via a preconditioning-like process mediated by ROS and GSSG.

Preconditioning (PC) 2 is induced by stimulation below the threshold of injury, resulting in subsequent tissue protection, or tolerance, which is defined as a condition of transiently increased resistance to injury. PC was first found to be triggered by short episodes of ischemia, called ischemic preconditioning; however, the stimuli can also be pharmacological or chemical (1). Understanding the preconditioning phenomenon can be a tool to elucidate the cellular endogenous protective mechanisms. Many factors are involved in the signaling, transducing or executing the PC response. Reactive oxygen species (ROS) are crucial signaling molecules during PC development and are mostly generated in the mitochondria (2). The four protein complexes associated with the respiratory chain are the primary source of ROS by handling the bulk of oxygen metabolism (3). Furthermore, respiratory chain inhibition induces preconditioning and cytoprotection against focal cerebral ischemia via ROS generation (4).

Apoptosis occurs via two distinct pathways: an extrinsic pathway (relying on cell surface membrane receptors) and an intrinsic pathway, which is triggered by several conditions of intracellular stress, leading to mitochondrial membrane permeabilization (MMP). In many models, MMP induces (i) mitochondrial transmembrane potential dissipation, (ii) respiratory chain uncoupling, (iii) ROS overproduction, (iv) ATP synthesis arrest, and (v) the release of several death-regulating molecules (activating proteases and nuclease), making the cell death process irreversible (5, 6). Depending on the cell type and apoptosis stimuli, MMP can occur only in the outer membrane or in both mitochondrial membranes (inner and outer membranes) via the mitochondrial permeability transition pore (PTP). Permeability transition consists of a sudden increase in the inner membrane permeabilization to solutes up to 1500 Da; PTP can include the dynamic interaction between voltage-dependent anion channel, cyclophilin D, and ANT (7, 8). Apoptotic or necrotic cell death due to hypoxia-ischemia and reperfusion injury clearly involves the process of mitochondrial permeability transition (5).

ANT is the most abundant inner membrane protein responsible for the vital function of stoichiometric ADP/ATP exchange on the inner membrane. However, ANT can switch to a lethal function, corresponding to its pore-forming activity. ANT can interact with different proteins, depending on the cell type or in response to apoptotic stimuli. Anti- or proapoptotic members of the Bcl-2 family, such as Bcl-2 or Bax, can physically interact with ANT, facilitating its antiporter or pore-form-
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ing activity, respectively (9, 10). On the other side, glutathione S-transferase (GST) interacts with ANT in normal tissue, in colon carcinoma cells and in vitro. This interaction is lost during apoptosis induction, suggesting that GST behaves as an endogenous repressor of PTP and of ANT pore activity (11). The ANT pore forming property is also modulated by oxidation of critical thiol groups in cysteine residues (cysteine 56, 159, and 256) facing the matrix side (12, 13). Several thiol-cross-linking agents (such as diamide, dithiodyipyridine, or phenylarsine oxide) enforce PTP opening and pore-forming activity of ANT, which is not prevented by Bcl-2 (13, 14).

Carbon monoxide (CO) is an endogenous product of heme degradation by heme oxygenase, which also generates free iron and biliverdin (15). Heme oxygenase plays an important role in cell redox state, acting as an antioxidant enzyme, which can be especially important for tissues with weak endogenous antioxidant defenses, such as the myocardium and the nervous system (16). Heme oxygenase activity has been suggested to modulate and prevent cerebral cell death in several models; this neuroprotective property has been mainly attributed to bilirubin antioxidant activity (17, 18); however, little data are available for CO in the central nervous system.

Low concentrations of CO confer an increased resistance to apoptosis triggered by several stimuli in different models, including endothelial cells, vascular smooth muscle cells, liver, or lung. CO also mediates other biological functions, such as anti-inflammatory, arrest of proliferation, or vasodilatation (15), and this molecule presents a strong potential in therapeutic applications (19). More recently, it has been shown that ROS, generated at mitochondria (20, 21), are imperative signaling molecules for CO biological functions, such as anti-inflammatory, cardioprotection, anti-proliferation, or anti-apoptosis in several systems (22). Carbon monoxide is described as binding to cytochrome c oxidase (complex IV), which slows down the rate of electron transport enabling electron to accumulate, including at complex III. Thus, the lifetime of the ubisemiquinone state of coenzyme Q is prolonged, increasing the propensity to reduce O2 into superoxide (O2−), which is enzymatically converted to other ROS (22–24). In the literature, CO biological properties are prevented by the addition of antioxidants, inhibition of complex III, or the use of respiration-deficient ρ-zero cells (22–25). In neuronal primary cultures, CO exposure provides cytoprotective PC with an increased resistance against apoptosis, and ROS are crucial signaling molecules (25).

On one hand, CO presents antiapoptotic properties in several models. On the other hand, mitochondria are central executors of the programmed cell death process, via the MMP. In the literature, the most described role of CO in mitochondria is the generation of ROS, which are signaling factors. Because no data concerning the direct action of CO on MMP are available, the present work has explored the direct effect of CO on non-synaptic mitochondria (MMP modulation) and, consequently, its ability to prevent apoptosis in astrocytes. The involvement of ROS as signaling molecules for CO-PC triggering was investigated as well as the biochemical mechanisms involved in the MMP control by CO.

EXPERIMENTAL PROCEDURES

Materials—All of the chemicals were of analytical grade and were obtained from Sigma unless stated otherwise. Plastic tissue culture dishes were from Nunc; fetal bovine serum, glutamine, penicillin/streptomycin solution, and Dulbecco’s minimum essential medium were obtained from Invitrogen; and Wistar rats were purchased from Instituto de Higiene e Medicina Tropical (Lisboa, Portugal).

Cell Culture—Primary cultures of astrocytes were prepared from 2-day-old rat cortex, as described (26). Briefly, cerebral hemispheres were carefully freed of the meninges, washed in ice-cold phosphate-buffered saline (PBS), and mechanically disrupted. Single-cell suspensions were plated in T-flasks (three hemispheres/175 cm2) in Dulbecco’s minimum essential medium supplemented with 10% (v/v) fetal bovine serum (heat-inactivated), 100 units/ml penicillin/streptomycin solution, and glucose (to obtain a final concentration of 10 mM). Cells were maintained in a humidified atmosphere of 7% CO2 at 37 °C. After 8 days, the phase dark cells growing on the astrocytic cell layer were separated by vigorous shaking and removed. The remaining astrocytes were detached by mild trypsinization using trypsin/EDTA (0.25%, w/v) and subcultured in T-flasks for another 2 weeks. Growth medium was changed twice a week.

Isolation of Non-synaptic Mitochondria from Cortex—Mitochondria were isolated from 300–350-g male Wistar adult rats according to Ref. 27. Briefly, the cortex was removed and washed in a ice-cold isolation buffer containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, and 5 mM HEPES, pH 7.4. The tissue was minced with scissors and manually homogenized with Potter-Elvehjem in isolation buffer. The homogenate was centrifuged at 1300 × g for 3 min, and the resuspended pellet was recentrifuged a 1300 × g for 3 min. Both supernatants were pooled together and centrifuged at 21,200 × g for 10 min. The remaining pellet was resuspended in 3.5 ml of 15% Percoll solution and layered into centrifuge tubes containing a preformed two-step discontinuous density gradient consisting of 3.7 ml of 24% Percoll on top of 1.7 ml of 40% Percoll. The gradient was centrifuged at 31,700 × g for 9 min. The mitochondrial fraction, located between the layers of 24 and 40% Percoll, was removed, diluted 1:8 in isolation buffer and centrifuged at 16,700 × g for 10 min. The pellet was resuspended in 10 ml of isolation buffer containing 5 mg/ml bovine serum albumin (to remove lipids) and centrifuged at 6800 × g for 10 min. The mitochondrial pellet was resuspended in 100 μl of isolation buffer, and the total amount of protein was quantified using a BCA assay (Pierce). All of the steps were carried out at 4 °C.

All isolated mitochondria analyses were performed on modified brain buffer (27) containing 125 mM KCl, 2 mM K2HPO4, 1 mM MgCl2, 15 μM EGTA, 20 mM Tris, 5 mM glutamate, and 5 mM malate, pH 7.3, unless stated otherwise. All CO treatment in isolated mitochondria was performed with a final concentration of 10 μM for 15 min at room temperature, unless stated otherwise.

Preparation of CO Solutions— Fresh stock solutions of CO gas were prepared each day and carefully sealed. PBS was saturated by bubbling 100% of CO gas for 30 min to produce 10−3 M...
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stock solution. The concentration of CO in solution was determined spectrophotometrically by measuring the conversion of deoxymyoglobin to carbon monoxymyoglobin, as described previously (28). 100% CO was purchased as compressed gas (Linde).

Apoptosis Induction/Prevention—Astrocytes were treated with CO (50 μM) for 3 h or with ethacrynic acid (EA; 50 μM) or carmustine (BCNU; 100 μM) for 1 h, followed by medium exchange. Then apoptosis was induced with diamide at concentrations ranging from 50 to 250 μM or with tert-butylhydroperoxide (t-BHP) at concentrations from 80 to 280 μM for 18 h. In some cases, β-carotene (1 μM) was used to modulate CO effect by applying this antioxidant to cells 1 h prior to CO treatment.

Assessment of Apoptosis-associated Parameters—To detect apoptosis induced by diamide or t-BHP, cell samples were collected by trypsinization, and cells were gated by the forward and side scatter. Two dyes were used: 3,3′-dihexyloxacarbocyanine iodide (DiOC₆(3); 20 nm) (Invitrogen) to quantify the mitochondrial transmembrane potential (ΔΨm) and propidium iodide (PI; 1 μg/ml) (Invitrogen) to determine cell viability, based on plasma membrane integrity. A flow cytometer (Partec, Germany) was used to analyze apoptosis-associated parameters. This cytometer contains a blue solid state laser (488 nm) with CO exchange. Then apoptosis was induced with diamide at concentrations 5 or 15 μM for 30 min at 37 °C and are expressed as a percentage relative to the positive control, 5 μM Ca²⁺ (100%), at the indicated time point.

Inner Membrane Permeabilization Assay—A citrate synthase activity assay is used to assess the inner membrane permeability, as described in Ref. 29. Upon inner mitochondrial membrane permeabilization, acetyl-CoA is able to enter into the mitochondrial matrix, reacting with citrate synthase. A 5,5′-dithiobis-2-nitrobenzoic acid and deacetylated acetyl-CoA reaction gives 5-thio-2-nitrobenzoate, which can be followed by absorbance at 412 nm.

Briefly, 25 μg of protein from isolated mitochondria was incubated with CO (10 μM), GSSG (1 μM), or EA (25 μM), in modified brain buffer containing 100 μM 5,5′-dithiobis-2-nitrobenzoic acid, 300 μM acetyl-CoA, and 1 mM oxaloacetate.

Mitochondrial Depolarization Detection—For depolarization measurements, isolated mitochondria (25 μg) containing 1 μM rhodamine 123 in modified brain buffer were pretreated with 10 μM CO, 5 μM cyclosporine, 1 μM β-carotene, or 10 μM EA. To depolarization assessment by rhodamine 123 dequenching several MMP inducers were added: 5 or 7.5 μM Ca²⁺, 300 μM atractyloside, or 250 μM diamide. The fluorescent measurements (λ ex, 485 nm; λ em, 535 nm) (Biotek Synergy 2 spectrofluorimeter) were followed for 30 min at 37 °C and are expressed as a percentage relative to the positive control, 5 μM Ca²⁺ (100%), at the indicated time point.

Quantification of Mitochondrial Swelling—25 μg of mitochondrial protein was diluted in modified brain buffer containing or not containing 10 μM CO. After 15 min of incubation at room temperature, 5 or 15 μM Ca²⁺ was added, and the decrease in optical density at 540 nm was immediately measured for 30 min at 37 °C, using a Biotek Synergy 2 spectrofluorimeter. 100% of swelling is calculated based on the optical density decrease after 30 min between non-treated and 15 μM Ca²⁺-treated mitochondria.

Cytochrome c Release Detection—25 μg of protein from isolated mitochondria was diluted in modified brain buffer containing or not containing 10 μM CO. After 15 min of incubation at room temperature, 5 or 15 μM Ca²⁺ was added, and mitochondria were incubated for 30 min at 37 °C. Samples were centrifuged for 10 min at 10,000 × g, and the mitochondrial pellet was analyzed by immunoblotting with α-cytochrome c.

Measurement of ROS Generation—ROS generation was followed by the conversion of 2′,7′-dichlorofluorescein diacetate (H₂DCFDA) (Invitrogen) to fluorescent 2′,7′-dichlorofluorescein (DCF). Astrocytes were treated for 3 h with CO, supernatant was removed, and cells were incubated for 20 min with 10 μM H₂DCFDA prepared in PBS. Cells were washed twice, and fluorescence was measured (λ ex, 485 nm; λ em, 530 nm) using a FL500 96-well spectrofluorimeter. β-Carotene (1 μM) was added 1 h prior to CO treatment. In the case of isolated mitochondria, 25 μg of mitochondrial protein was incubated with 5 μM H₂DCFDA and 10, 50, or 250 μM CO in modified brain buffer. Fluorescence (λ ex, 485 nm; λ em, 530 nm) was measured using a Biotek Synergy 2 spectrofluorimeter for 30 min at 37 °C.

ROS generation was calculated as an increase over base-line levels, determined for untreated cells (100%). In some cases, β-carotene (1 μM) was added to isolated mitochondria 10 min prior to CO treatment.

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tion of GSH with 2-vinylpyridine (31). The values are expressed as the GSSG/GSH ratio.

Mitochondrion Isolation from Primary Culture of Astrocytes—Primary cultures of astrocytes were washed with ice-cold PBS collected by trypsinization. The samples were centrifuged at 200 × g for 10 min, and cells were washed in PBS by centrifugation at 200 × g for 10 min. The supernatant was discarded, and the pellet (cells) was incubated in 3.5 ml of hypotonic buffer (0.15 mM MgCl₂, 10 mM KCl, 10 mM Tris-HCl, pH 7.6) at 4 °C for 5 min. After the addition of an equal volume of homogenization buffer (0.15 mM MgCl₂, 10 mM KCl, 10 mM Tris-HCl, 0.4 mM phenylmethylsulfonyl fluoride, 250 mM saccharose, pH 7.6) twice concentrated, samples were manually homogenized with a Dounce Potter homogenizer. Cell extracts were centrifuged at 100,000 g for 5 min. After the addition of an equal volume of homogenization buffer containing 0.5% of Triton X-100 in the presence of 20 μl of α-GSH (1 mg/ml; ViroGen) or 20 μl of α-ANT monoclonal antibody (Mitosciences) for 90 min at 37 °C, followed by immunoprecipitation with 15 μl of protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) for 30 min at 37 °C. After 10 min of 10,000 × g centrifugation, supernatant was discarded, and pellet was washed four times with PBS. Proteins attached to the beads were solubilized by Laemmli buffer for further Western blot analysis.

Immunoprecipitation—100 μg of mitochondrial protein (isolated from astrocytes or from rat cortex) was incubated in 100 μl of homogenization buffer containing 0.5% of Triton X-100 in the presence of 20 μl of α-GSH (1 mg/ml; ViroGen) or 20 μl of α-ANT monoclonal antibody (Mitosciences) for 90 min at 37 °C, followed by immunoprecipitation with 15 μl of protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) for 30 min at 37 °C. After 10 min of 10,000 × g centrifugation, supernatant was discarded, and pellet was washed four times with PBS. Proteins attached to the beads were solubilized by Laemmli buffer for further Western blot analysis.

Immunoblotting—Several samples (from cell extracts, mitochondria, or immunoprecipitated protein) were separated under reducing electrophoresis on a 1-mm NuPAGE® Novex BisTris gel (Invitrogen) and electrically transferred to a nitrocellulose membrane (Hybond™-C extra, Amersham Biosciences). ANT, caspase-3, GST, or cytochrome c protein was stained with α-ANT monoclonal antibody (Mitosciences), α-caspase-3 (Sigma), α-GST (GE Healthcare), or α-cytochrome c (Abcam), all of them at a 1:1000 dilution and 1 h of room temperature incubation. Blots were developed using the ECL (enhanced chemiluminescence) detection system after incubation with horseradish peroxidase-labeled anti-mouse IgG antibody (Amersham Biosciences) at 1:5000 dilution and 1 h of room temperature incubation. The area and intensity of bands were quantified by densitometry analysis (GraphPad Prism 4) and are presented as a percentage relative to the positive control (100%). These experiments have been repeated three times with similar results.

Statistical Analyses—The data concerning intact cells were from experiments carried out in at least three independent preparations (cell isolation). All data related to isolated mitochondria were derived in triplicate from at least three independent animals. Mitochondrial data are presented as a representative result of three independent assays. For the Western blot technique, a representative image of three independent assays is shown. All values are mean ± S.D., n ≥ 3. Error bars, corresponding to S.D., are represented in the figures. Statistical comparisons were performed using analysis of variance, single factor with replication, with p < 0.05, n ≥ 3. p < 0.05 means that samples are significantly different at a confidence level of 95%.

RESULTS

Carbon Monoxide Prevents Apoptosis in Astrocytes—Astrocytic apoptosis was induced by oxidative stress with thiol cross-linker diamide (Fig. 1, A and B) and pro-oxidant tert-butylnitroperoxide (Fig. 1, C and D) treatment during 18 h. Both reagents have been shown to induce cell death by acting at the mitochondrial level (14). Primary cultures of cortical astrocytes were treated with CO-saturated PBS with a final concentration of 50 μM 3 h prior to apoptosis induction. CO partially prevents the dissipation of ΔΨm (quantified by DiOC₆(3); Fig. 1, A and C), followed by plasma membrane permeabilization (detected by propidium iodide fluorescence, Fig. 1, B and D), a marker for loss of viability. These features were assessed by flow cytometry. CO is not toxic up to 100 μM; however, at this concentration, its cytoprotection decreases (supplemental Fig. 1). In addition, carbon monoxide prevents astrocytic cell death up to 48 h after induction (supplemental Fig. 2). Thus, the role of CO is not limited to delaying apoptosis in a short time window.

In addition, carbon monoxide prevents caspase-3 activation induced by diamide (Fig. 1E). In conclusion, CO does protect astrocytes from cell death induced by oxidative stress; furthermore, CO presents an extended time window of action.

ROS Generation Is Crucial for CO-induced Cytoprotection—Astrocytes were treated with 50 μM carbon monoxide, and after 3 h, intracellular ROS generation (specifically H₂O₂) was measured by the conversion of H₂DCFDA to fluorescent DCF. CO induces an increase in intracellular ROS levels of about 20%, which is prevented by 1 h of pretreatment with the antioxidant β-carotene at 1 μM (Fig. 2A). In order to verify the role of ROS and, thus, the preconditioning mode of action of CO, 1 μM β-carotene was added to primary culture of astrocytes previous to CO treatment. Indeed, inhibition of ROS generation decreases the antiapoptotic effect of CO for diamide (Fig. 2B) and for t-BHP (Fig. 2C) inductions. Thus, in intact cells, ROS are imperative signaling molecules for prevention of apoptosis by CO, indicating that a preconditioning-like mechanism is involved.

CO Inhibits MMP in Isolated Non-synaptic Mitochondria—Several different approaches were used to assess direct or indirectly mitochondrial membrane permeabilization: mitochondrial depolarization, inner membrane permeabilization, cytochrome c release, and mitochondrial swelling. Non-synaptic mitochondria isolated from brain cortex (27) were treated with 10 μM CO during 15 min prior to the addition of diamide, atracyloside (a ligand of ANT that prevents ADP/ATP translocation and induces its pore forming function), or calcium to induce MMP. Loss of ΔΨm, or mitochondrial depolarization, was measured using the methodology based on dequenching of the fluorescent probe rhodamine 123 (29). Loss of ΔΨm induced by atracyloside was prevented by the prior addition of CO (Fig. 3A). CO also inhibits mitochondrial depolarization induced by diamide and Ca²⁺ (Fig. 3B). To quantify this effect, Ca²⁺ at 5 μM was normalized to 100% of depolarization (Fig.
Moreover, cyclosporine A also prevents ΔΨm loss, indicating the involvement of the permeability transition pore (Fig. 3B) (32). Changes in the inner membrane permeability (the opening of a large channel for molecules up to ~800 Da) were assessed via an enzymatic assay based on the accessibility of citrate synthase, which is a soluble matrix enzyme (29). The atracyloside induction of inner membrane permeabilization is partially prevented by CO (Fig. 3C). Finally, CO also inhibits mitochondrial swelling triggered by 5 or 15 μM Ca²⁺ (Fig. 3D), and there is a partial prevention of cytochrome c release from mitochondria pretreated with CO when challenged with Ca²⁺ at 15 μM (Fig. 3E). CO treatment at 10 μM (alone) in isolated mitochondria had no effect on swelling, mitochondrial depolarization, or pore formation through the inner membrane (Fig. 3). In conclusion, CO inhibits mitochondrial swelling, cytochrome c release, loss of ΔΨm, and inner membrane permeabilization. Thus, CO prevents apoptosis via a direct effect on mitochondria (i.e. by reducing MMP).

ROS Are Important Molecules for CO Prevention of MMP—In isolated non-synaptic mitochondria, treatment with CO at 10, 50, or 250 μM induced ROS generation in a dose-response manner, and pretreatment with β-carotene (1 μM) prevented ROS formation by CO (Fig. 4A). Inhibition of mitochondrial depolarization by CO was lost when mitochondria were treated with the antioxidant β-carotene prior to CO exposure (Fig. 4B). Still, CO became unable to prevent the atracyloside-induced opening of a large channel in the inner membrane when β-carotene was added to mitochondria before CO treatment (Fig. 4C). These findings support the hypothesis that ROS generation by CO is necessary for its MMP prevention in isolated mitochon-
Therefore, ROS are also imperative CO-signaling molecules at the mitochondrial level.

**CO Facilitates ADP/ATP Translocation Function of ANT**—In order to clarify the mechanisms involved in CO inhibition of MMP, the influence of this gas on ANT activity was measured using an enzyme-based assay (29). ANT is a double function protein located in the mitochondrial inner membrane. The physiological role of ANT consists in exchanging ADP against ATP in a stoichiometric manner; in contrast, in response to diverse stimuli, its activity can switch to that of a pore-forming protein, modulating MMP (9). Mitochondria treated with CO present an increased ANT translocase activity (Fig. 5), providing evidence that CO prevents the opening of a nonspecific pore through the inner membrane by directly acting on ANT and enforcing its physiological activity.

**CO Augments GSSG/GSH Ratio in Isolated Mitochondria**—Based on the fact that (i) CO generates ROS in isolated mitochondria and (ii) glutathione is one of the most efficient antioxidant systems in the cell, as well as at the mitochondrial level (33), GSH and GSSG were measured after CO treatment in isolated mitochondria. The total amount of mitochondrial GSH is not altered in the presence of carbon monoxide; this result correlates with the fact that glutathione synthesis occurs in the cytosol, whereas at the mitochondrial level, only GSH recycling takes place. In contrast, mitochondrial levels of GSSG increase after CO exposure in a dose-response manner, which enhances the GSSG/GSH ratio (Fig. 6). In addition, mitochondrial pretreatment with \( \beta \)-carotene prevents GSSG/GSH ratio augmentation due to the presence of CO (Fig. 6). These data suggest that GSSG is also a candidate factor for signaling preconditioning and apoptosis prevention triggered by CO.

**GSSG Signaling and Protein Glutathionylation Are Involved in the Modulation of ANT Activity and MMP by CO**—In order to challenge the hypothesis of GSSG as a signaling molecule in the CO cytoprotective pathway, three distinct approaches were studied: (i) the effect of GSSG on ADP/ATP translocation by ANT, (ii) modulation of inner membrane permeability by small amounts of GSSG, and (iii) the covalent modification of ANT by glutathionylation of thiol groups. Small amounts of oxidized glutathione (1 \( \mu \)M) increase the translocase activity of ANT (Fig. 7A), which is similar to the CO
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Effect upon this enzyme. Furthermore, mitochondria treated with EA, which prevents glutathione recycling (34) and increases GSSG levels, also facilitates ADP/ATP translocation by ANT (Fig. 7A). Thus, there might be a direct effect of GSSG on ANT. However, higher concentrations of GSSG (100 μM) prevent ADT/ATP exchange activity (Fig. 7A). Still, oxidized glutathione (1 μM) and EA partially prevent inner membrane permeabilization challenged by atractyloside and Ca2+ (Fig. 7A, B, C). Thus, small amounts of GSSG seem to modulate ANT activity and prevent MMP.

The GSSG/GSH ratio was calculated for different conditions in order to correlate the effect of CO on glutathione levels and the addition of 1 μM GSSG. GSSG/GSH ratio in non-treated mitochondria is 0.080, and in CO (10 μM)-treated mitochondria, it increases to 0.135, whereas the addition of 1 μM GSSG in a mitochondrial preparation corresponds to a GSSG/GSH ratio of 0.462, which is 3 times higher than the ratio induced by 10 μM CO. However, oxidized glutathione is added into the media, and it does not correspond to glutathione concentration in the mitochondrial matrix. Because of this, and based on these similar values (the same order of magnitude), one can consider that the addition of GSSG at 1 μM might mimic the augmentation of the GSSG/GSH ratio due to CO induction. These data are an additional support for the hypothesis that GSSGs are signaling molecules in the CO-induced prevention of MMP.

In addition, an increase of GSSG levels allows the formation of protein mixed disulfides, or protein glutathionylation. Reversible protein glutathionylation is a post-translational process involved in the cellular response to redox changes, which can protect cysteine residues against irreversible damage due to oxidative stress (35). In addition, ANT presents critical thiol residues (14) that are important for the control of protein function and are candidates to be glutathionylated. Purified mitochondria from control astrocytes or from CO-treated astrocytes were incubated with α-GSH to immunoprecipitate glutathionylated proteins, followed by immunodetection of ANT in a Western blot assay (Fig. 7D). Moreover, purified mitochondria from non-treated astrocytes were incubated with small amounts of GSSG (1 μM), followed by immunoprecipita-
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In order to verify if diamide-targeted thiol residues of ANT are protected by glutathionylation, a competition assay was performed. Isolated mitochondria were treated with diamide and followed by CO exposure. In fact, diamide pretreatment decreased the ANT glutathionylation levels induced by CO (Fig. 7E), suggesting that diamide acts on the same cysteine residue(s) that is glutathionylated by CO. Taken all together, these data provide evidence that CO increases GSSG levels, which covalently modifies ANT by glutathionylation, facilitating ADP/ATP translocation and preventing inner membrane permeabilization.

In contrast, and to further confirm the functional role of ANT glutathionylation by CO, the same experimental conditions used to test CO modulation of MMP (Fig. 3) were used to detect ANT glutathionylation. Isolated non-synaptic mitochondria were first pretreated with CO (10 or 50 \( \mu M \)) or GSSG (1 \( \mu M \)) at room temperature for 15 min, followed by diamide addition (100 \( \mu M \)) at 37 °C for 30 min. Immunoprecipitation was performed, showing that ANT glutathionylation still occurs after diamide treatment (supplemental Fig. 3).

Role of GST—In the literature, GST is described to physically interact with ANT and to be an endogenous repressor of apoptosis (11). ANT has been co-purified with GST from rat brain and co-immunoprecipitated from a colon carcinoma cell line, and the functional cooperation between both proteins has been

FIGURE 4. Influence of ROS on CO effect at mitochondrial level. In A, mitochondria were treated with 10, 50, or 250 \( \mu M \) CO in the presence or absence of 1 \( \mu M \) \( \beta \)-carotene, followed by ROS quantification using H$_2$DCFDA (\( \lambda_{ex} 485 \text{ nm}; \lambda_{em} 530 \text{ nm} \)). The values are expressed in percentage relative to control (100%). All values are mean ± S.D., \( n = 4 \). *, \( p < 0.05 \) compared with control; **, \( p < 0.05 \) compared with control; #, \( p < 0.05 \) compared with control; ##, \( p < 0.05 \) compared with 10 \( \mu M \) CO; ###, \( p < 0.05 \) compared with 50 \( \mu M \) CO. B, mitochondria were pretreated with 1 \( \mu M \) \( \beta \)-carotene and 10 \( \mu M \) CO, and then atractyloside at 300 \( \mu M \) or diamide at 250 \( \mu M \) was added. The fluorescent measurements (\( \lambda_{ex} 485 \text{ nm}; \lambda_{em} 535 \text{ nm} \)) are expressed in relative percentage to 5 \( \mu M \) Ca$^{2+}$ (100%) at 15 min of incubation. All values are mean ± S.D., \( n = 3 \). *, \( p < 0.05 \) compared with control and with \( \beta \)-carotene and CO-treated mitochondria. C, inner membrane permeabilization was assessed according to Ref. 29. Measurements were performed at 412 nm in the absence or presence of 10 \( \mu M \) CO and 300 \( \mu M \) atractyloside for 20 min at 37 °C. All values are mean ± S.D. (error bars), \( n = 3 \).

FIGURE 5. Carbon monoxide effect on ADP/ATP translocase activity of ANT. The results were obtained using isolated non-synaptic mitochondria treated with 10 \( \mu M \) CO, and ADP/ATP translocation was assessed according to Ref. 29. ADP was added to mitochondria and diffused into the intermembrane space through the voltage-dependent anion channel. Once into the intermembrane space, ADP can be transformed by adenylate kinase (AK) in AMP and ATP or exchanged against ATP by adenine nucleotide translocator (ANT). The values are expressed in relative percentage to control (100%) at 15 min of incubation and are mean ± S.D. (error bars), \( n = 3 \). *, \( p < 0.05 \) compared with control mitochondria.
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**DISCUSSION**

The present study has demonstrated that CO confers protection against oxidative stress-induced apoptosis in primary culture of astrocytes (Fig. 1). For the first time, it has been shown that there is a direct antiapoptotic effect of CO upon mitochondria, by preventing MMP, which is a key event in the intrinsic apoptotic pathway. The data reported here revealed that CO inhibits mitochondrial swelling, cytochrome c release, dissipation of $\Delta \Psi m$, and the opening of a nonspecific pore through the inner membrane in isolated non-synaptic mitochondria (Fig. 3).

Previously, we have shown that CO prevented neuronal apoptosis by inducing a preconditioning-like effect, a model in which reactive oxygen species appeared to be signaling molecules (25). Herein, it has been demonstrated that (i) CO induces ROS production in astrocytes and (ii) inhibition of ROS generation by an antioxidant addition ($\beta$-carotene) reverses the antiapoptotic effect of CO. In intact cells, ROS are necessary for CO to delay apoptosis (Fig. 2). Also, at the subcellular level (isolated mitochondria), ROS appear to be critical for CO to reduce MMP (Fig. 4). Thus, ROS generation is crucial for CO signaling.

ANT is a key protein involved in the control of the permeability transition pore, leading to the release of proapoptotic factors into the cytosol (36). ANT interacts with either the proapoptotic protein Bax or the antiapoptotic protein Bcl-2, which both influence ANT in opposite ways. Bax facilitates the opening of a pore through the inner membrane, whereas Bcl-2 increases translocation activity of ANT (9, 10, 37). CO increases the ADP/ATP translocation by ANT (Fig. 5), which is comparable with the Bcl-2–ANT model of MMP regulation. CO appears to change ANT conformation, which stimulates translocation activity and inhibits channel function. This conformation might be the c-conformation first described by Vignais and co-workers (38). This is in agreement with the literature because it has been demonstrated that heme oxygenase-1 expression increases the activity of ADP/ATP transporter in renal mitochondria in experimental diabetes (39). Therefore, CO modulates MMP by acting on ANT.

CO augments the mitochondrial GSSG/GSH ratio in a dose-response manner (Fig. 6). This GSSG/GSH ratio increase is prevented by $\beta$-carotene addition. Additionally, glutathione redox changes can lead to the regulated formation of mixed disulfides between protein thiol and glutathione disulfide (protein glutathionylation). The progressive glutathionylation of key proteins is proposed as a molecular switch by which cells respond in an immediate and reversible fashion to oxidative stress (35). Protein glutathionylation can be considered as a physiological sig-

![FIGURE 6. Carbon monoxide effect on mitochondrial GSSG/GSH ratio. After CO treatment (10 or 50 $\mu$M) in the presence or absence of $\beta$-carotene (1 $\mu$M), oxidized and reduced glutathione quantification was performed using a microtiter plate assay, as described under “Experimental Procedures.” The values are mean ± S.D. (error bars), n = 3. * p < 0.05 compared with control; ** p < 0.05 compared with mitochondria treated with 10 $\mu$M CO; #, p < 0.05 compared with mitochondria without $\beta$-carotene treatment and 10 $\mu$M CO treatment; ##, p < 0.05 compared with mitochondria without $\beta$-carotene treatment and 50 $\mu$M CO treatment.](image-url)
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A

B

C

D

E

FIGURE 7. Role of ANT glutathionylation in MMP modulation. A, ADP/ATP translocation was followed in isolated mitochondria in the presence of 1 or 10 μM GSSG or 10 μM EA. The values are expressed in relative percentage to control (100%) at 15 min of incubation at 37 °C and are mean ± S.D. (error bars), n = 3. *, p < 0.05 compared with control. B and C, isolated non-synaptic mitochondria were treated with GSSG at 1 μM (B) or with EA at 25 μM (C) for 10 min, followed by atracyloside (Atra; 300 μM) or Ca²⁺ (5 μM) addition in order to induce inner membrane permeabilization, which was assessed according to Ref. 29. Measurements were performed at 412 nm for 20 min at 37 °C. All values are mean ± S.D., n = 3. D, primary cultures of astrocytes were treated with CO following mitochondria isolation; additionally, 1 μM GSSG was added to mitochondria isolated from control astrocytes. Glutathionylated proteins (α-GSH) were immunoprecipitated in mitochondria isolated from astrocytes, and ANT was immunodetected by Western blot from the immunoprecipitated proteins. The area and intensity of bands were quantified by densitometry analysis (GraphPad Prism 4) and are presented as relative percentage to the positive control (100%). This experiment was repeated three times with similar results. 

E, isolated non-synaptic mitochondria were treated in the presence or absence of diamide at 100 μM for 15 min, followed by CO (10 μM) incubation for 15 min, and then glutathionylated proteins were immunoprecipitated, and ANT was immunodetected by Western blot from the immunoprecipitated proteins. This experiment was repeated three times with similar results.
order of magnitude. Moreover, the addition of GSSG is done into the medium, and the GSSG/GSH ratio is calculated in the mitochondrial matrix. In conclusion, the addition of a small amount of GSSG mimics endogenous GSSG generation triggered by CO, acting as signaling factors.

A slight increase in mitochondrial GSSG levels is also demonstrated to be important for cell signaling in functional approaches. Partial inhibition of GSH recycling at the mitochondrial level, by EA addition, protects astrocytes from cell death induced by oxidative stress (Fig. 9A) and protects mitochondria from MMP induced by atractyloside (Fig. 9B). However, when a general inhibitor of GSH recycling (BCNU) is used, no protection is found (Fig. 9B). Furthermore, EA increases ANT glutathionylation levels (Fig. 9C). Taken together, these data suggest that little amounts of mitochondrial GSSG can act as signaling molecules, in much the same way as ROS do.

Several examples of protein glutathionylation or deglutathionylation involved in apoptosis control can be found in the literature: (i) glutathionylation prevents caspase-3 activation (41); (ii) glutathionylation of complex II decreases after myocardial ischemia and reperfusion, limiting the electron transfer activity of this complex (42); or (iii) reversible glutathionylation of complex I increases mitochondrial superoxide formation (43). In accordance with our data, Piantadosi et al. (44) have found that rats exposed to small amounts of CO presented higher levels of protein mixed disulfides in liver mitochondria. Further data are necessary to clarify the existence of other target proteins to be glutathionylated in the CO modulation of apoptosis. Some possible targets are Bcl-2 family proteins or the different isoforms of ANT because ANT1 and ANT3 have been identified as pro-apoptotic isoforms, whereas ANT2 is antiapoptotic (45).

GST is considered as an endogenous repressor of apoptosis because its interaction with ANT is lost during the apoptotic process and, on the other hand, cancer cell lines present higher levels of GST interacting with ANT (11). Ablation of GST expression increases apoptosis induced by oxidative stress with 4-hydroxynonenal (46). Moreover, GST is also a promising candidate enzyme to be involved in the ANT glutathionylation process. By immunoprecipitation assays, it was confirmed that ANT physically interacts with GST; however, CO does not alter this interaction (Fig. 8). Whether CO induces an augmentation of GST activity remains to be determined.

In this work, CO protects astrocytes from cell death by inhibiting MMP, and the process can be divided into at least three main steps: (i) ROS as signaling molecules, (ii) mitochondrial GSSG as transducing factors, and (iii) ANT as effector protein. CO appears to trigger a preconditioning-like event, by activating the cellular endogenous protective mechanisms. It is important to highlight that the mechanisms described in the present study are related to early preconditioning response because the
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time frame window analyzed is short. ROS generation, mitochondrial GSSG increase, and ANT glutathionylation were assessed between 5 minutes and 3 h after CO treatment. However, late preconditioning is not excluded when apoptosis is induced 24 h after CO treatment, this molecule is still able to confer cell protection.\(^3\) In addition, it is expected that late preconditioning involves the expression of cellular antioxidant enzymes. Indeed, hypoxia-induced PC increases expression of superoxide dismutase and/or glutathione peroxidase (47, 48), and continuous CO exposure in rats increases SOD2 expression (44). Thus, in future work, the cellular antioxidant machinery behavior in response to CO will be explored. Still, it can be speculated that oxidized glutathione increases locally in mitochondria in a first time window of cellular response to CO, and in a second step (late preconditioning), cellular reduced glutathione levels might increase to prevent oxidative injury.

In summary, CO presents antiapoptotic properties in astrocytes by directly preventing mitochondrial membrane permeabilization, with GSSG as transducing factor, and acting on ANT function via critical thiol residue glutathionylation. CO is a potential antiapoptotic factor against cerebral hypoxia-ischemia and reperfusion; furthermore, it can be used as a tool to disclose the cellular pathways involved in the preconditioning phenomenon able to confer cytoprotection.

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\(^3\) C. S. F. Queiroga, A. S. Almeida, C. Martel, C. Brenner, P. M. Alves, and H. L. A. Vieira, unpublished data.