Free Radical Research, March 2009; 43(3): 214–223

informa
healthcare

Neuroprotective role of heme-oxygenase 1 against iodoacetate-induced toxicity in rat cerebellar granule neurons: Role of bilirubin

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(Received 5 November 2008; revised 20 November 2008)

Abstract

Heme oxygenase (HO) catalyses the breakdown of heme to iron, carbon monoxide and biliverdin, the latter being further reduced to bilirubin. A protective role of the inducible isoform, HO-1, has been described in pathological conditions associated with the production of reactive oxygen species (ROS). The aim of this study was to investigate the role of HO-1 in the neurotoxicity induced by iodoacetate (IAA) in primary cultures of cerebellar granule neurons (CGNs). IAA, an inhibitor of the glycolysis pathway, reduces cell survival, increases ROS production and enhances HO-1 expression in CGNs. Furthermore, the induction of HO-1 expression by cobalt protoporphyrin (CoPP) prevented cell death and ROS production induced by IAA, whereas the inhibition of HO activity with tin mesoporphyrin exacerbated the IAA-induced neurotoxicity. The protective effect elicited by CoPP was reproduced by bilirubin addition, suggesting that this molecule may be involved in the protective effect of HO-1 induction in this experimental model.

Keywords: *Iodoacetate, heme oxygenase-1, oxidative stress, metabolic inhibition*

Introduction

Excessive production of reactive oxygen species (ROS) is associated with cell damage involved in several human pathologies, including neurodegenerative disorders [1–3]. Aerobic cells, including neurons, have several antioxidant enzymes that keep the balance between ROS production and oxidative damage. Among these enzymes, emerging evidence supports a role for heme oxygenase (HO) as important components of the cellular antioxidant and cytoprotective defense [4]. HO cleaves the heme molecule yielding free iron, carbon monoxide (CO) and biliverdin, which is converted enzymatically to bilirubin (BR). Two isoforms of HO have been identified in mammals: the inducible HO-1 and the constitutive HO-2 [5]. The HO-1 isozyme is transcriptionally regulated by a large variety of stimuli including its substrate heme, oxidative stress and phenolic compounds [6]. HO-1 has recently been described as a critical factor involved in anti-oxidant, anti-inflammatory and anti-apoptotic defense against several disease states [4,7–11]. In addition, *in vitro* assays have shown that HO catalytic-derived products possess antioxidant activity [12,13].

On the other hand, iodoacetate (IAA) is an alkylating agent and an irreversible inhibitor of glyceraldehyde-3-phosphate dehydrogenase (GAPD H), a glycolytic enzyme, that produces some of the

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ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760802676670

biochemical and pathologic features associated with hypoglycaemia [14–16]. It has been demonstrated that exposure of cultured neurons to IAA induces a rapid decrease in ATP levels [17–19] and the generation of ROS in cultured hippocampal and cerebellar granule neurons (CGNs) [19,20] as well as in *in vivo* models [21]. Consistently, antioxidant treatment decreases IAA-induced ROS generation in CGNs [20], hippocampal cultured neurons [17,19] and cultured glial cells [22].

Taking into account the above information, we decided to study the role of HO-1 on ROS production and cell death induced by IAA in primary cultures of CGNs. It was found that the activity and the content of HO-1 were increased in CGNs exposed to IAA. In addition, the transient overexpression of HO-1 induced by cobalt protoporphyrin (CoPP), a known inductor of HO-1 expression [11], was able to ameliorate IAA-induced neurotoxicity, an effect associated with decreased ROS production. In contrast, inhibition of HO with tin mesoporphyrin (SnMP) exacerbated IAA-induced cell damage and abolished the protective effect of CoPP. Furthermore, the protective effect of HO-1 induction was reproduced by bilirubin. These data clearly show that induction of HO-1 exerts a neuroprotective effect against IAA-induced neurotoxicity in CGNs by a mechanism probably involving bilirubin.

Materials and methods

Reagents

Trypsin, deoxyribonuclease type I (DNAse I), cytosine arabinoside, glutamine, glucose, gentamicin, Basal Eagle's Medium, IAA, 3-[4,5-dimethylthiazol-2-yl)]-2,5-diphenyl-tetrazolium bromide (MTT), manganese chloride, poly-L-lysine and anti-α-tubulin antibodies were purchased from Sigma Chemical Co. (St Louis, MO). Trypsin inhibitor, penicillin-streptomycin, trypan blue and foetal bovine serum were purchased from Gibco (Gaithersburg, MD). SnMP and CoPP were from Frontier Scientific Inc. (Logan, UT). Anti-HO-1 antibodies and recombinant rat HO-1 protein (used as controls in a western blot analysis) were acquired from Assay Design (Ann Arbor, MI). 5-(and 6-) carboxy-2,7-dichlorodihydrofluorescein diacetate (carboxy-DHFDA) and the Live/death kit were purchased from Molecular Probes (Eugene, OR). All other reagents were of analytical grade and commercially available.

Primary cultures of CGNs

Animals were handled and cared for according to the NIH guide for care and use of laboratory animals. All efforts were made in order to minimize animal suffering. Primary cell cultures greatly enriched ($>90\%$) in CGNs were prepared from 7-day-old Wistar rats as previously described [23]. Briefly, after removing cerebella from the skulls, tissue was freed from meninges and incubated in 0.25% trypsin solution for 10 min at 37°C. Tissue was dissociated by trituration using a solution containing 0.08% DNAse I and 0.52% soybean trypsin inhibitor and by filtration through a sterile polyester mesh. Cells were suspended in basal Eagle's medium supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamicin and 25 mM KCl. The number of living cells was estimated by trypan blue exclusion. Cells were plated at a density of $\sim 300 \times 10^3/\text{cm}^2$ in Costar plates pre-coated with poly-L-lysine (5 μg/ml). Cytosine arabinose (10 μM) was added 24 h after plating and glucose (5 mM) was added to the cultures at 4 days *in vitro* (DIV).

Culture treatments

The studies were performed in CGNs after 9 DIV. Cell cultures were exposed to several concentrations of IAA (0–40 μM) in Krebs Ringer buffer (KRB) for 30 min. No foetal bovine serum was present during the exposure to IAA. After this time, IAA was removed and CGNs were incubated in culture medium for 24 h to determine cell viability or for 8, 16 or 24 h to determine HO-1 expression. In order to study the effect of HO inhibition on IAA induced neurotoxicity, the HO specific inhibitor SnMP (10 μM) [24] was added to the culture medium 15 min before the exposure to IAA. HO inhibition was sustained through the addition of SnMP (10 μM) to the culture medium after IAA treatment. Cell viability was evaluated 24 h after incubation with the toxic agent. In order to evaluate the effect of the transient induction of HO-1 expression on neuronal viability, CGNs were treated with 20 μM CoPP (a well-known inductor of HO-1) for 3 h in culture medium, it was then removed and cultures incubated overnight in culture medium before the exposure to IAA according to Parfenova et al. [11]. Cell viability, HO-1 protein levels and HO-1 activity were assessed 24 h after the onset of IAA treatment. ROS production was evaluated 4 h after IAA exposure.

To evaluate the effect of some products of HO-1 activity on IAA-induced neurotoxicity, we exposed cultures to CO and BR. To evaluate the role of CO, we used the CO-releasing molecule CORM-2 in 20, 30 and 40 μM 30 min before IAA exposure. BR (20–80 nm) was also added 30 min before IAA exposure. These compounds were added to the medium after IAA removal. Cell viability was evaluated 24 h after the addition of the toxic agent.

Determination of cell viability

The number of viable cells (percentage of control) was estimated using the colorimetric MTT assay [25].

MTT is transformed to formazan blue by the activity of mitochondrial dehydrogenases and absorbance is directly proportional to the number of viable cells. MTT (1 mg/ml) was added to the culture medium and cells incubated during 1 h at 37°C. After removal of the medium containing the remaining MTT, the blue formazan product was extracted with 2-propanol and quantified spectroscopically at 570 nm. Data are expressed as a percentage of MTT reduction in control cells. In addition, a live/death assay was performed according to Hernández-Fonseca et al. [19]. This assay is based on the specific stain of dead neurons by ethidium homodimer-1, a red-fluorescent marker which permeates only through damaged membranes; living neurons are marked with calcein-AM which emits green fluorescence after its hydrolysation by the activity of cytoplasmic esterases. Cell cultures were incubated with calcein-AM and ethidium homodimer-1 in KRB for 25 min a 37°C. Red- and green-fluorescent neurons were observed under an epifluorescent microscope (using the fluorescent cubes G-2A—excitation 510–560 nm—and B-2A/C—excitation 450 to 490 nm—from Nikon Co. for ethidium homodimer-1 and calcein AM detection, respectively). Bright field images were obtained by phase contrast microscopy to compare cell morphology in all experimental conditions with the data obtained by the live/death kit.

Western blot

Cells were harvested in 50 mM phosphate buffer (pH 7.4) with 1% triton and the total protein content was determined by the Lowry method. Fifty micrograms protein samples were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham, Piscataway, NJ). Membranes were incubated with 5% milk in phosphate buffered saline (pH 7.4) with 0.05% Tween-20 (PBS-T) during 2 h. After washing with PBS-T, membranes were incubated with 1:2000 dilution of rabbit anti-HO-1 or mouse anti-α-tubulin antibodies at 4°C overnight. Membranes were then washed and probed with horseradish peroxidase-conjugated donkey anti-rabbit or goat anti-mouse IgG (Amersham) at a 1:3000 dilution. Chemiluminescence was detected with the Amersham ECL detection kit according to manufacturer's instructions and densitometric analysis was performed throughout the Sigma ScanPro software [26]. As positive control HO-1 recombinant protein was used.

Determination of HO activity

HO activity was determined as previously described [27]. Briefly, 6×10^6 cells were harvested in 50 mM phosphate buffer (pH 7.4) with 1% triton and total protein content was determined by the Lowry method. About 500 μg of protein were incubated with a reaction mixture consisting of mouse liver cytosol, a source of BR reductase (25 μl), hemin (20 μM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 U/ml) and nicotinamide adenine dinucleotide phosphate (NADPH) (0.8 mM). The reaction was conducted at 37°C in the dark for 1.5 h and terminated by the addition of chloroform (0.5 ml). The extracted BR was calculated by the difference in absorbance between 464 and 530 nm ($\varepsilon = 40\ mM^{-1}\ cm^{-1}$). Enzyme activity was expressed as pmol of BR/mg of protein/h.

Determination of ROS production

The fluorescent marker carboxy-DHFDA was used to assess ROS production, according to Hernández-Fonseca et al. [19]. Carboxy-DHFDA is deacetylated, oxidized by ROS and reactive nitrogen species and converted to the fluorescent compound 5- (and 6-) carboxy-2,7-dichlorofluorescein (carboxy-DCF), staining the cell cytoplasm with bright green fluorescence. After incubation, fluorescent probe was loaded in KRB for 20 min. Cells were examined under an epifluorescence microscope (using the fluorescent cube B-2A/C—excitation 450 to 490 nm—from Nikon Co.). The intensity of carboxy-DCF fluorescence was measured in five different fields per well per condition in three independent experiments using the NIS Elements Imaging software (Nikon Co).

Statistics

Data are expressed as mean ± SEM. They were analysed with the software Prism 5 (GraphPad, San Diego, CA) by two-way analysis of variance (ANOVA) followed by Bonferroni Multiple Comparison test or by one-way ANOVA followed by Bonferroni or Dunnet tests, as appropriate; $p < 0.05$ was considered significant.

Results

Neurotoxic effect of IAA

In order to establish the effect of IAA on cell viability, CGNs were incubated with several concentrations of IAA (0–40 μM). IAA caused a dose-dependent decrease in cell viability in CGNs (50% of neuronal death was reached at 19.3 μM). Cell death was significant at IAA concentrations of 15 μM or higher (Figure 1).

HO-1 expression in IAA-treated CGNs

Next we wanted to investigate whether HO-1 was modulated by IAA. It was found that HO-1 protein levels, measured by western blot, increased in a concentration- and time-dependent manner after IAA exposure (Figure 2). HO-1 content increased

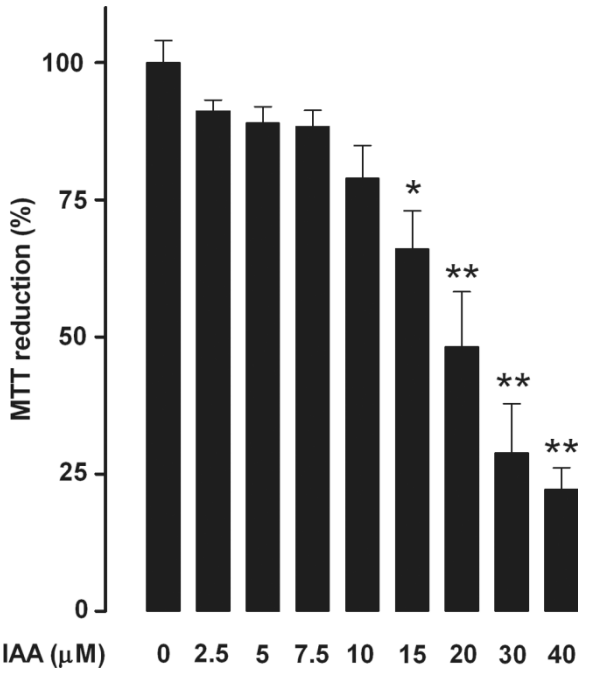

Figure 1. Dose-dependence of IAA-induced neurotoxicity in rat CGNs. Cultures were exposed to IAA (0–40 μM) in KRB for 30 min followed by recovery in growth medium for 24 h. After this time viability was assessed by MTT reduction. Data are mean ± SEM ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.001$ vs 0.

significantly from 15 μM IAA (Figure 2A) and after 16 h IAA exposure (Figure 2B).

Effect of HO inhibition and HO-1 over-expression on CGNs viability

To study the functional role of HO in IAA-induced toxicity, CGNs were incubated in the presence of SnMP, an HO inhibitor, or pre-incubated with CoPP, an HO-1 inductor, and then exposed to increasing concentrations of IAA. Cell viability data are presented in Figure 3A. It is clear that, whereas the incubation with CoPP or SnMP alone had no effect on cell viability, co-incubation with SnMP or CoPP with IAA had a profound influence on CGNs viability. Cell death was clearly exacerbated when cultures were exposed to IAA during HO inhibition and this effect was significant from 10 μM IAA. Neuronal damage was significantly higher in SnMP + IAA treated cells than in CGNs treated with IAA alone. In contrast, pre-incubation with CoPP was able to significantly reduce IAA-induced cell death, which was significantly lower in CoPP + IAA-treated CGNs than in CGNs treated with 20 μM IAA alone. Furthermore, we found that addition of SnMP to CoPP + IAA-treated CGNs abolished the protective effect of CoPP (Figure 3B). MTT data were corroborated using the live/death kit (Figure 4). We detected a high number of dead cells in CGNs treated with IAA, which was reduced by pre-incubation with CoPP. In contrast, CGNs treated with

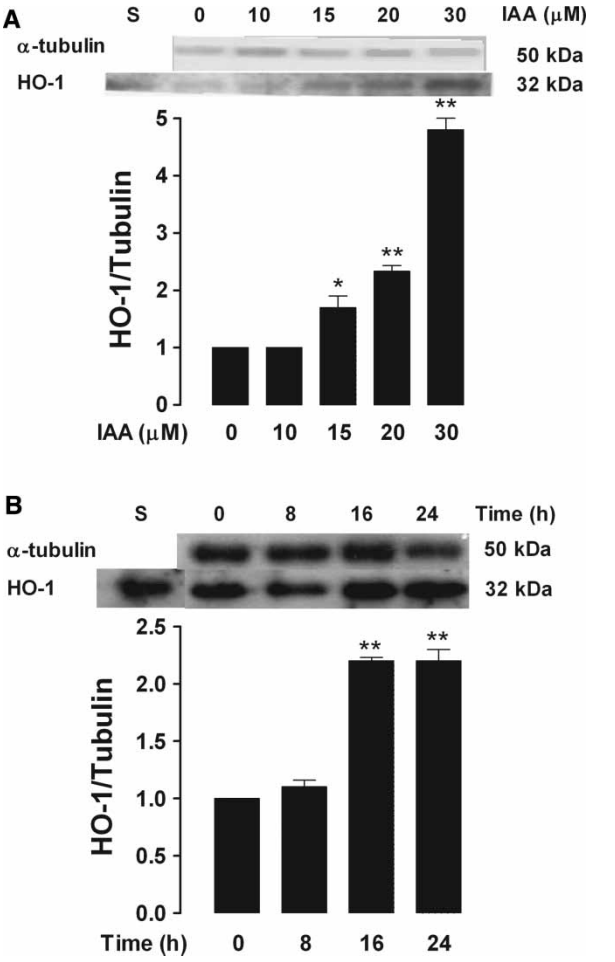

Figure 2. Western blot analysis of HO-1 protein levels in CGNs exposed to IAA (0–30 μM for 30 min) and harvested 24 h after the exposure (A) and CGNs exposed to IAA (20 μM for 30 min) and harvested 8, 16 or 24 h after the exposure (B). HO-1 was identified as a 32 kDa protein. Recombinant rat HO-1 protein (5 ng) was used as positive control, whereas α-tubulin (50 kDa) was used as a loading control. Graphs show the densitometric analysis (HO-1/tubulin) from each band for three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.001$ vs 0.

SnMP + IAA with or without CoPP pre-treatment showed a higher number of damaged neurons. This result supports the exacerbating effect of SnMP on IAA neurotoxicity and the abolishing effect of this inhibitor on the protective action of CoPP.

In addition, bright field micrographs were taken from the same fields as calcein-AM and ethidium homodimer-1 (Figure 4, right panel). Neurons untreated or treated only with SnMP or CoPP look round and dark and a network of processes is visible throughout the field. After IAA incubation, neurons were damaged as evidenced by a decrease in the number of normal dark cell bodies and the presence of thin and fragmented neurites. When CGNs were co-incubated with IAA and SnMP, cell damage was more pronounced. In contrast, when CGNs were exposed to CoPP prior to IAA exposure, cultures were preserved and normal somata and intact processes were visible. CGNs exposed to CoPP and

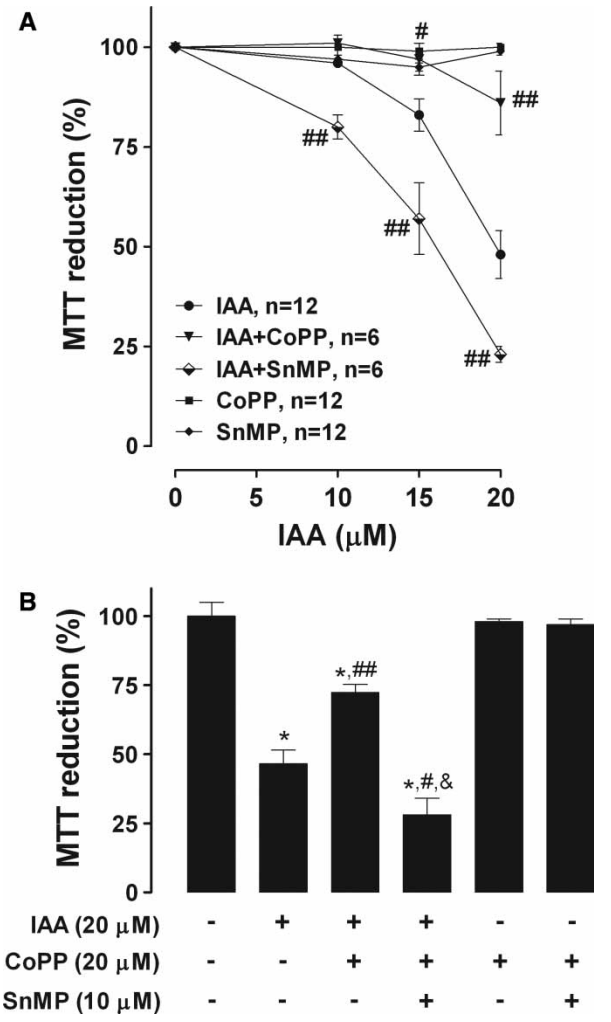

Figure 3. Effect of CoPP or SnMP on the neurotoxicty induced by IAA. (A) CGNs treated with different concentrations of IAA (0–20 μM) were pre-treated with CoPP or SnMP according to the protocols described in the Material and methods section. $^{\#}p<0.05$ vs IAA' $^{\#\#}p<0.001$ vs IAA (two way ANOVA followed by Bonferroni Multiple Comparison Test). (B) CGNs pre-treated with CoPP were also treated with SnMP and exposed to 20 μM IAA for 30 min. $^{\star}p<0.001$ vs Control, $^{\#}p<0.05$ vs IAA, $^{\#\#}p<0.001$ vs IAA, $^{\&}p<0.001$ vs IAA+CoPP (one way ANOVA followed by Bonferroni test). $n=9$. MTT reduction was assessed 24 h after the onset of IAA exposure.

SnMP prior to IAA exposure also show more pronounced cell damage than cultures treated with IAA alone, confirming the abolishing effect of SnMP of CoPP neuroprotection.

Effect of IAA, CoPP and SnMP on HO activity and HO-1 levels

HO-1 protein content and activity were measured 24 h after IAA treatment to investigate whether the potentiating effect of SnMP on IAA neurotoxicity and the protective effect exerted by CoPP are related to changes in HO levels and/or activity.

It was found that both CoPP as well as IAA were able to induce an increase in HO-1 protein content (Figure 5). Interestingly, the HO-1 content secondary to the addition of CoPP + IAA was significantly higher than that observed with the individual treatments, suggesting independent mechanisms of HO-1 induction by IAA and CoPP. This was corroborated by determining HO activity, which was increased in cultures treated with CoPP and IAA, but was significantly higher in CGNs incubated with both compounds (Figure 5B). Additionally, we found that exposure to SnMP was able to decrease basal and IAA-induced HO-1 activity, as well as that stimulated by co-incubation of CoPP + IAA (Figure 5B).

Effect of IAA, CoPP and SnMP on ROS production

In agreement with previous reports [19,20], IAA induced an increase in ROS production, as evidenced by an augmented number of cells positive to carboxy-DCF (Figure 6). ROS production was prevented by CoPP pre-treatment, as evidenced by the absence of carboxy-DCF-positive cells in this condition. This effect was abolished by incubating CGNs in the presence of SnMP (Figures 6 and 7). This data strongly suggest that the protective effect of CoPP is associated with reduced IAA-induced ROS production.

Effect of HO products on IAA-induced cell death and ROS production

In order to investigate if any product of the HO reaction could be responsible of preventing the cell death induced by IAA, CGNs treated with 20 μM IAA were exposed to BR or CORM-2 (a CO releasing molecule). The cell death induced by IAA was not prevented by CORM-2 (data not shown). In contrast, BR was able to prevent in a concentration-dependent way cell death induced by IAA (Figure 8).

Discussion

HO-1 is a catalyst for heme degradation, but it has also been described as a critical factor involved in anti-oxidant, anti-inflammatory and anti-apoptotic defense against several disease states [4,7–10], including some models of neuronal death [11]. Taking into account that high ROS production causes neuronal damage [1–3] and that evidence suggests that protection by HO-1 is associated with its antioxidant properties, it is important to deeply understand the role of this enzyme in the neurotoxicity of ROS-producing agents. One interesting model of neurotoxicity is the metabolic inhibition induced by IAA, which is an alkylating agent and an irreversible inhibitor of GAPDH, an enzyme of the glycolytic pathway. Neurotoxicity of IAA reproduces some of the biochemical and pathologic features associated with ischemia and hypoglycemia [14–16].

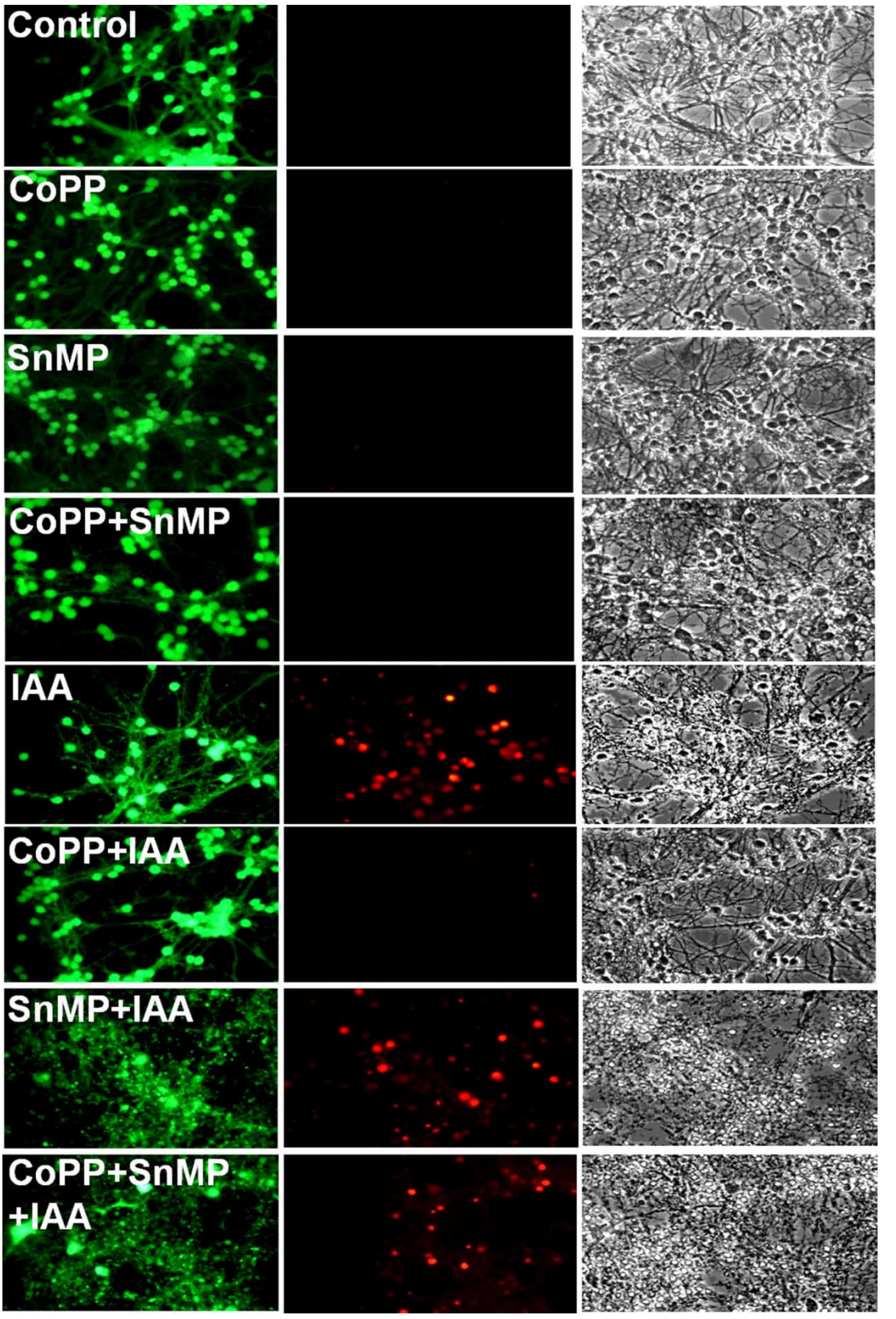

Figure 4. Representative images of the fluorescent Live/death assay. CGNs were treated with IAA (20 μM for 30 min), SnMP (10 μM) and CoPP (20 μM). Before the exposure of CGNs to IAA, cells were treated with CoPP (20 μM) for 3 h and incubated overnight in culture medium. SnMP (10 μM) was added 15 min before the exposure to IAA and added again after IAA removal. Living neurons positive to calcein-AM are shown in the left panel, death neurons positive to ethidium homodimer-1 are in the middle panel and bright field micrographs of the same field are in the right panel.

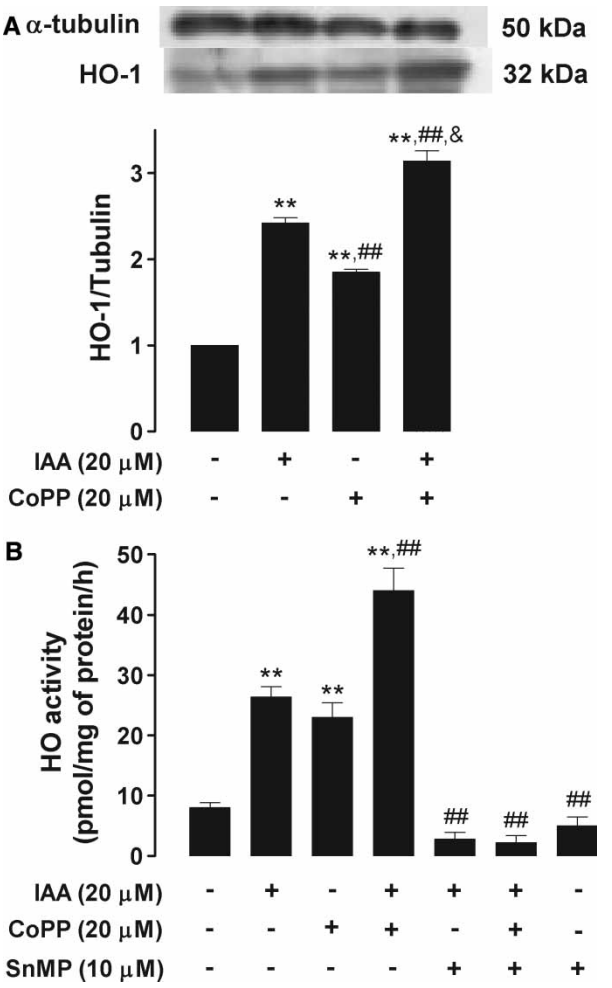

Figure 5. Effect of IAA, CoPP or SnMP on HO-1 levels and HO activity. (A) Western blot analysis of HO-1 protein levels in CGNs exposed to 20 μM IAA with or without a pre-treatment with CoPP according to the protocols described in the Materials and methods section, 24 h after the onset to the IAA exposure. HO-1 was identified as a 32 kDa protein. Recombinant rat HO-1 protein (5 ng) was used as a control. α-tubulin (50 kDa) was used as a loading control. (B) HO activity in untreated CGNs and after the IAA exposure in the presence of with CoPP, SnMP or both according to the Materials and methods section, 24 h after the onset to the IAA exposure. $n=5$. ** $p<0.001$ vs control, $^{\#\#}p<0.001$ vs IAA, $^{\&}p<0.001$ vs CoPP.

It has been demonstrated that IAA induces energy depletion and the production of ROS in neuronal and glial cultures, which is effectively reduced by various antioxidants [17–20,22]. These observations suggest that the generation of ROS is associated with the metabolic inhibition produced by IAA. In addition, IAA is involved in other phenomena associated with energy failure such as activation of the N-methyl-D-aspartate glutamate receptor sub-type and the disruption of the intracellular calcium homeostasis [20,28–30].

Novel findings from this study strongly suggest that HO-1 plays a key role in neuroprotection in CGNs against IAA toxicity. This conclusion is based on the experiments using the HO-1 inductor CoPP and the HO inhibitor SnMP or the combination of both chemicals. We show that both IAA and CoPP effectively increase HO-1 protein levels in CGNs. The incubation with SnMP clearly exacerbates cell death induced by IAA, suggesting a protective role of HO in this model. Conversely, HO-1 transient upregulation induced by CoPP reduces IAA-induced cell death. These data strongly suggest that induction of HO-1 exerts a neuroprotective action in cultured CGNs under metabolic stress. To further confirm the role of HO-1 in the protective effect exerted by CoPP, CGNs pre-treated with CoPP were incubated with both SnMP and IAA. It was found that SnMP effectively abolished CoPP-induced protection, further suggesting that the inducible HO-1 isoform is responsible for protection. These results agree with previous observations showing protection by HO-1 against glutamate-mediated neuronal death [31]. CoPP and SnMP addition were unable to modify by themselves CGNs viability under basal conditions in spite of the changes induced in HO activity (see Figure 5C). In fact, concentrations of both CoPP and SnMP were chosen according to previous experiments to avoid any effect on CGNs viability. These data suggest that the role of HO-1 in this type of neurons is evident only in stressful conditions which agree with previous reported data (reviewed in Cuadrado and Rojo [4]).

The over-expression of HO-1 induced by CoPP elicited a cytoprotective effect on IAA-induced neurotoxicity which was clearly associated with a decrease in ROS production. This supports the hypothesis that the cytoprotective effect of HO-1 is mediated by a decrease in ROS production. This is consistent with previous findings suggesting an association between the protective effect of HO-1 over-expression and the decrease in ROS production and oxidative damage in several experimental models [31–33]. Decreased ROS generation secondary to HO-1 over-expression may be due to the action of the products generated by HO activity, such as biliverdin/BR and CO. Indeed, it has been found that both CO and biliverdin/BR may mediate the cytoprotective effects of HO-1 [12,13,32]. It has been shown that BR is an antioxidant and a scavenger of reactive nitrogen species including peroxynitrite [34,35] and protects from oxidative damage in several models [12,33,36,37]. Interestingly, in spite of the fact that CO has been implied in the protective effect of HO-1 in several models [12,13,32], our data suggest that in our experimental condition CO is not involved in the protective effect of HO-1 on IAA-induced neurotoxicity. In contrast, BR addition was able to ameliorate IAA-induced neurotoxicity, suggesting that the protective effect of CoPP may be mediated, at least in part, by the production of this antioxidant compound.

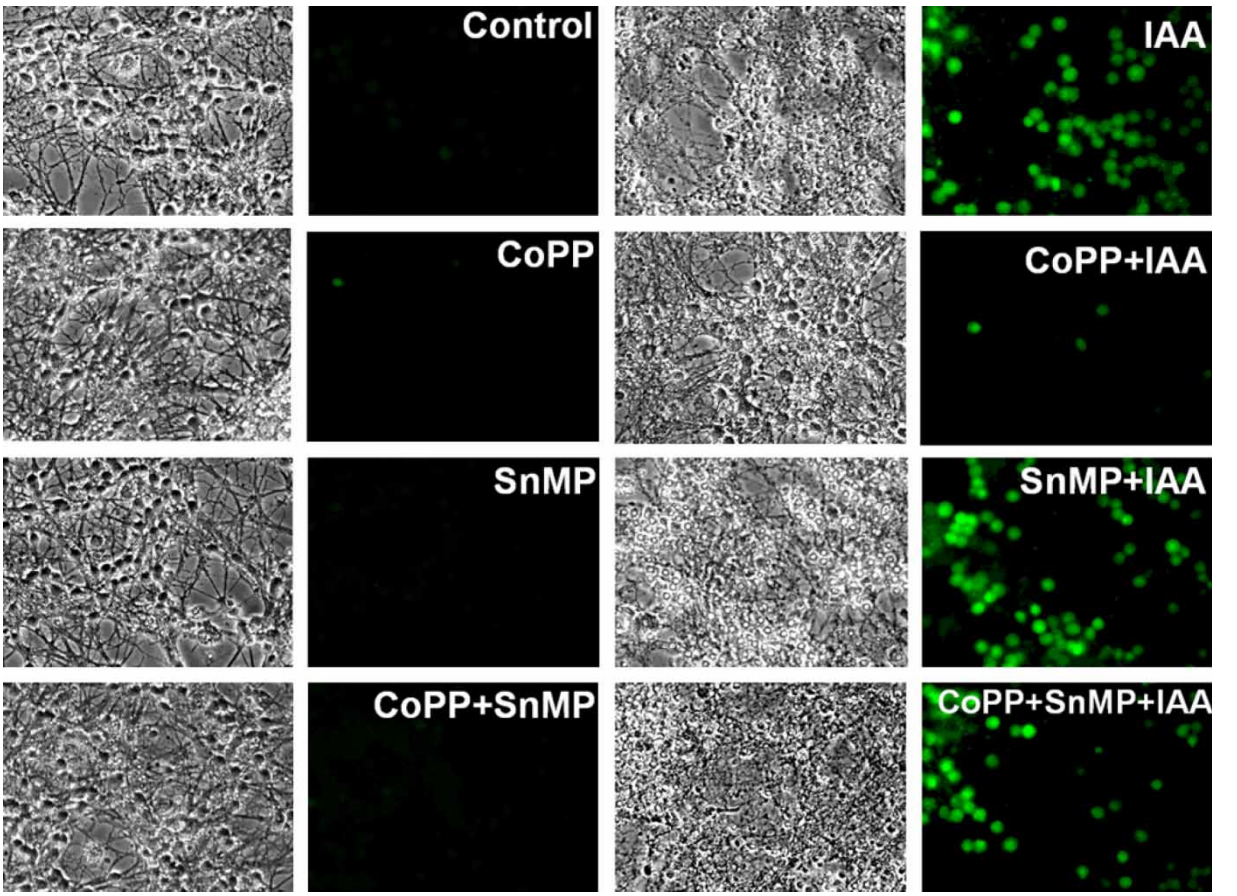

Figure 6. Effect of CoPP and SnMP on 20 μM IAA-induced ROS production in CGNs. Images from one representative experiment showing the effect of CoPP on ROS levels 4 h after IAA exposure. Carboxy-DCF images are shown in the right panel and bright-field images are shown in the left panel. The same field is shown for each condition.

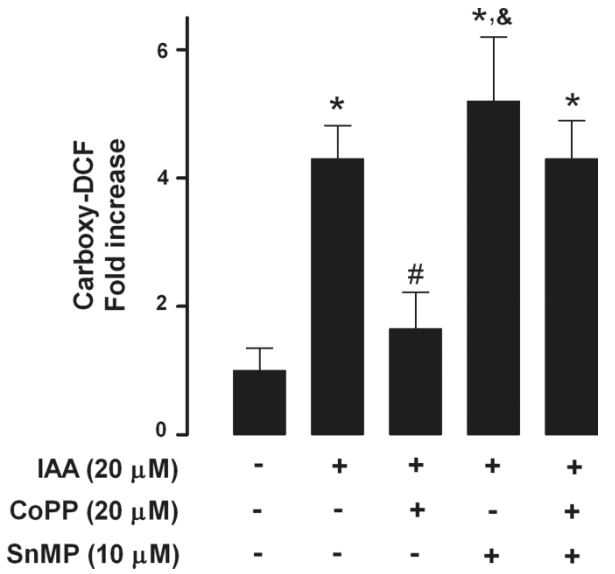

Figure 7. Effect of CoPP and SnMP on 20 μM IAA-induced ROS production in CGNs. ROS levels were estimated 4 h after the onset of IAA exposure. The intensity of fluorescence was measured in five different fields per well per condition in three independent experiments. Fluorescence in the control condition was normalized to 1 unit and the changes in fluorescence were expressed as fold increase relative to control. $^{\star}p<0.05$ vs control, $^{\#}p<0.05$ vs IAA, $^{\&}p<0.05$ vs IAA+CoPP.

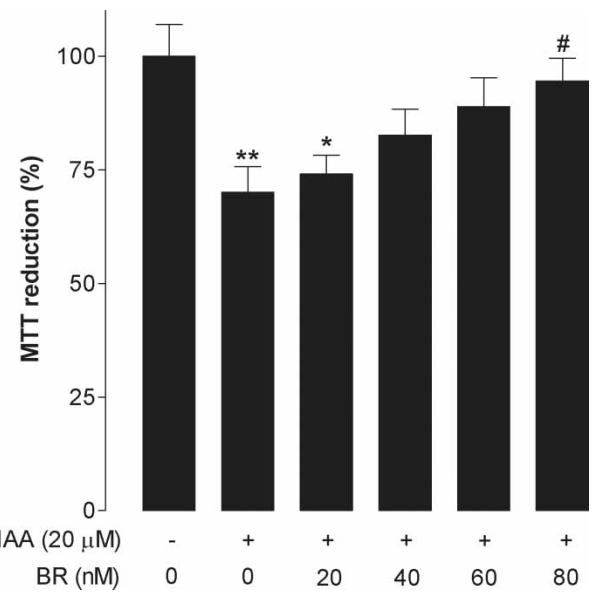

Figure 8. Protective effect of BR against IAA-induced neurotoxicity. BR (20–80 nm) was added 30 min before IAA exposure. After IAA removal, BR was added to the medium. Cell viability was evaluated 24 h after the addition of the toxic agent by the MTT assay. $^{\star}p<0.05$, $^{\star\star}p<0.001$ vs Control and $^{\#}p<0.05$ vs IAA. $n=3$–5.

The main contributions of the present study are that metabolic inhibition is able to increase HO-1 levels in CGNs and that a sustained induction of this enzyme can reduce ROS production and increase cell survival. These observations suggest that HO-1 might have a role as an endogenous defense against oxidative insults in the nervous tissue. This observation is relevant since oxidative stress has been proposed as a mechanism of damage common to diverse neuropathologies.

Acknowledgements

This work was supported by CONACYT 48812M and 48645-Q to JPC and LM. We thank MVZ Atonatiu Gómez for the continuous support with the rats and Dr Ismael Torres Saldaña and Enrique Pinzón Estrada (Facultad de Medicina, UNAM) for their technical assistance with the rats.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on iFirst on 28 January 2009.