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Heme oxygenase-1 induction prevents neuronal damage triggered during mitochondrial inhibition: Role of CO and bilirubin

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ABSTRACT

Heme oxygenase (HO) catalyzes the breakdown of heme to iron, carbon monoxide (CO), and biliverdin, the latter being further reduced to bilirubin (BR). A protective role of the inducible isoform, HO-1, has been described in pathological conditions associated with reactive oxygen species (ROS) and oxidative damage. The aim of this study was to investigate the role of HO-1 in the neurotoxicity induced by the mitochondrial toxin 3-nitropropionic acid (3-NP) in primary cultures of cerebellar granule neurons (CGNs). Toxicity of 3-NP is associated with ROS production, and this metabolic toxin has been used to mimic pathological conditions such as Huntington's disease. We found that cell death caused by 3-NP exposure was exacerbated by inhibition of HO with tin mesoporphyrin (SnMP). In addition, HO-1 up-regulation induced by the exposure to cobalt protoporphyrin (CoPP) before the incubation with 3-NP, prevented the cell death and the increase in ROS induced by 3-NP. Interestingly, addition of SnMP to CoPP-pretreated CGNs exposed to 3-NP, abolished the protective effect of CoPP suggesting that HO activity was responsible for this protective effect. This was additionally supported by the fact that CORM-2, a CO-releasing molecule, and BR, were able to protect against cell death and the increase in ROS induced by 3-NP. Our data clearly show that HO-1 elicits in CGNs a neuroprotective action against the neurotoxicity of 3-NP and that CO and BR may be involved, at least in part, in this protective effect. The present results increase our knowledge about the role of HO-1 in neuropathological conditions.

1. Introduction

Reactive oxygen species (ROS) are generated primarily in the mitochondria as by-products of cellular metabolism. When ROS production overwhelms the intrinsic antioxidant capacity, damage to cellular macromolecules such as DNA, proteins, and lipids ensues. This state is known as "oxidative stress" and it is thought to contribute to the pathogenesis of a number of neurodegenerative diseases (Browne et al., 1999; Schipper et al., 1998; Smith et al., 1994). Inhibition of mitochondrial metabolism by the toxin 3-nitropropionic acid (3-NP), is known to induce the generation of ROS, and chronic treatment with this toxin reproduces some of the histopathological, neurochemical and motor alterations associated with Huntington's disease in monkeys and rodents (Beal et al., 1993; Brouillet et al., 1995), implicating mitochondrial dysfunction and oxidative stress in neurodegenerative disorders. The generation of ROS such as superoxide anion ($O_2^{\bullet -}$) and hydroxyl radicals ($OH^{\bullet}$), as well as cellular oxidative damage, have been reported in the rat striatum after 3-NP treatment (Kim and Chan, 2002; La Fontaine et al., 2000; Santamaría et al., 2001). Moreover, both oxidative damage and the size of the striatal lesion induced by 3-NP are reduced by antioxidant administration (Kim et al., 2000; Rodríguez-Martínez et al., 2004). Increased release of hydrogen peroxide (H_2O_2) has also been described in PC12 cells after 3-NP exposure (Mandavilli et al., 2005), and mitochondrial $O_2^{\bullet -}$ generation has been demonstrated in the presence of 3-NP in alveolar bronchial epithelial cells (Bacsi et al., 2006). As a whole, these studies associate ROS production and oxidative damage with 3-NP toxicity, both in neurons and other cell types.

Aerobic cells, including neurons, have a set of antioxidant enzymes that keep balance between ROS production and oxidative damage. Among these enzymes, emerging evidence supports a role for heme oxygenase (HO) enzymes as important components of the cellular antioxidant and cytoprotective defense (Cuadrado and Rojo, 2008); however its role in neuropathology remains controversial. HO catalyzes the conversion of heme into biliverdin, carbon monoxide (CO), and ferrous iron. Biliverdin is subsequently reduced to bilirubin (BR) via biliverdin reductase. Two isoforms of HO have been identified in mammals: the inducible HO-1 and the constitutive HO-2 (Maines, 1988). HO-1 is induced by several

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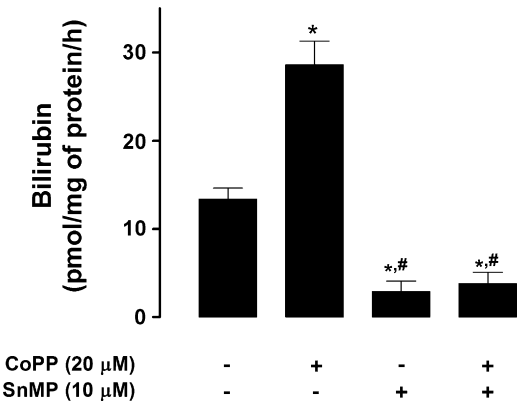

Fig. 1. HO activity in untreated CGNs and after the incubation with CoPP, SnMP or both. CGNs were incubated with CoPP for 3 h in culture medium, CoPP was then removed and cultures were incubated overnight (14 h) before the assay. CGNs were incubated with SnMP 15 min before the assay, $n = 3–5$, $^{*}p < 0.01$ vs. untreated CGNs, $^{\#}p < 0.001$ vs. CoPP.

stimuli such as heme, cytokines, heavy metals and ROS, among others (Sikorski et al., 2004). A protective role of HO-1 has been suggested from knockout mice (Poss and Tonegawa, 1997) and a patient with HO-1 deficiency (Yachie et al., 1999), showing increased susceptibility to inflammatory and oxidative injury. In agreement, HO-1 has recently been described as a critical factor involved in antioxidant, anti-inflammatory and anti-apoptotic defense against several disease states (Abraham and Kappas, 2008; Cuadrado and Rojo, 2008; Kirkby and Adin, 2006; Kruger et al., 2006; Wu et al., 2006; Parfenova et al., 2006). In addition, in vitro assays have shown that HO catalytic-derived products possess antioxidant activity (Matsumoto et al., 2006; Srisook et al., 2006).

Moreover, increased expression of HO-1 protein has been observed in postmortem brain tissue from Alzheimer, Parkinson and Huntington's disease patients (Smith et al., 1994; Schipper et al., 1998; Browne et al., 1999), but the function of HO-1 in the above-mentioned pathological conditions remains unknown. However, there are evidences supporting that HO-1 may either exacerbate (Vaya et al., 2007; Song et al., 2006; Koeppen et al., 2004) or ameliorate (Chen et al., 2000; Chen-Roetling et al., 2005; Panahian et al., 1999; Ahmad et al., 2006; Ku et al., 2006) neuronal damage. The above information suggests that additional experiments are necessary to clarify the role of HO-1 in pathological conditions of the nervous tissue.

Based on this evidence, we decided to investigate the effect of HO-1 induction in the neurotoxicity induced by 3-NP in primary cultures of cerebellar granule neurons (CGNs). Primary cultures of CGNs have been proposed as a suitable in vitro model for studying the mechanisms of neuroprotection, and previous studies show that this neuronal type is vulnerable to 3-NP toxicity (Verdaguer et al., 2002; Fink et al., 2000). Results suggest that the transient overexpression of HO-1 is able to ameliorate 3-NP-induced neurotoxicity, which is associated with the decrease of ROS production. In addition, this protective effect is reproduced by CORM-2, a CO-releasing molecule, and BR.

2. Materials and methods

2.1. Materials

Trypsin, deoxyribonuclease 1 (DNAse I), basal Eagle's medium, L-glutamine, gentamicin, poly-L-lysine, cytosine arabinose, glucose, glucose-6-phospate dehydrogenase, glucose 6 phosphate, hemin, nicotinamide adenine nucleotide phosphate (NADPH), 3-NP, anti-α-tubulin antibodies, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), $[Ru(CO)_3Cl_2]_2$ or CO-releasing molecule (CORM-2) and reagents for polyacrylamide gel electrophoresis were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Soybean trypsin inhibitor, trypan blue stain, fetal bovine serum and penicillin/streptomycin were purchased from Gibco (Gaithersburg, MD, USA). KCl was from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). Tin mesoporphyrin (SnMP), cobalt protoporphyrin (CoPP) and BR were from Frontier Scientific Inc. (Logan, UT, USA). Anti-HO-1 polyclonal antibodies (catalogue number SPA-895) and recombinant rat HO-1 protein (used as controls in a western blot analysis) were acquired from Assay Design (Ann Arbor, MI, USA). Dihydroethidium (DHE), propidium iodide (PI) and 5-(and 6-) carboxy-2,7-dichlorodihydrofluorescein diacetate (carboxy-DHFDA) were purchased from Molecular Probes (Eugene, OR, USA). All other reagents were of analytical grade and commercially available.

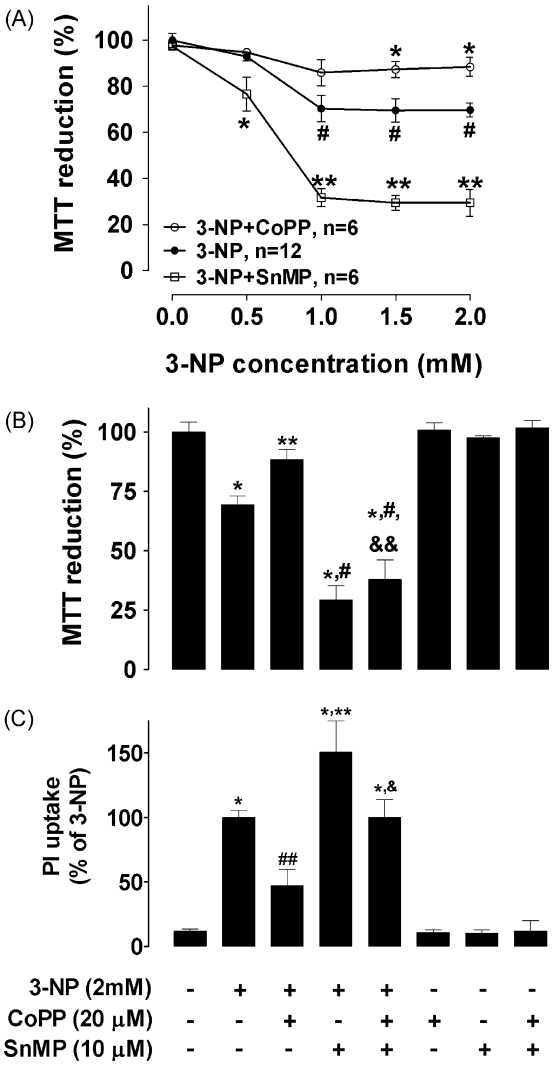

Fig. 2. Effect of CoPP or SnMP on neurotoxicity of 3-NP. (A) CGNs treated with different concentrations of 3-NP (0.5, 1, 1.5 and 2 mM) were pretreated with CoPP or SnMP according to the protocols described in Section 2. $^{*}p < 0.05$ vs. 3-NP, $^{**}p < 0.001$ vs. 3-NP: two-way ANOVA followed by Bonferroni. $^{\#}p < 0.001$ vs. 0 mM: one-way ANOVA followed by Dunnett. (B) MTT reduction and (C) quantitative data of the fluorescent PI assay were assessed 24 h after the onset of 3-NP exposure. CGNs pretreated with CoPP were also treated with SnMP and exposed to 2-mM 3-NP for 2 h. $^{*}p < 0.001$ vs. control, $^{**}p < 0.05$ vs. 3-NP, $^{\#}p < 0.001$ and $^{\#\#}p < 0.01$ vs. 3-NP, $\&p < 0.01$ and $\&\&p < 0.001$ vs. 3-NP + CoPP: one-way ANOVA followed by Bonferroni, $n = 5–6$.