Sequential induction of heme oxygenase-1 and manganese superoxide dismutase protects cultured astrocytes against nitric oxide

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Abstract

Nitric oxide (NO) is a widely recognized mediator of physiological and pathophysiological signal transmission. In an attempt to better understand the molecular actions of NO in astrocytes, stress protein expression in response to NO donor sodium nitroprusside was investigated. Heme oxygenase-1 (HO-1) has been identified as an inducer of manganese superoxide dismutase (MnSOD), playing a cytoprotective role under the condition of nitrosative stress. We present evidence that the sequential induction of HO-1 and MnSOD protects astrocytes from NO toxicity: (1) both HO-1 and MnSOD expression were induced by NO; (2) NO-mediated increase in MnSOD activity was partly abolished by HO-1 inhibitor Zn(II) protoporphyrin IX (ZnPP); (3) pretreatment of astrocytes with a nontoxic dose of NO protected the cells against the later treatment with a toxic dose of NO; (4) inhibition of HO-1 by ZnPP sensitized astrocytes to the nontoxic dose of NO resulting in a marked cytotoxicity; and (5) adenovirus-mediated overexpression of MnSOD protected astrocytes from the NO toxicity. The molecular action of NO in astrocytes appears to be dose-dependent. While a high dose of NO exerts cytotoxicity leading to the tissue damage in the central nervous system, a low dose of NO may act as an important signaling molecule in astrocytes with concurrent induction of cytoprotective proteins such as HO-1 and MnSOD.
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1. Introduction

Nitric oxide (NO) is an intra- and extracellular-messenger that mediates diverse signaling pathways in target cells and is known to play an important role in many physiological as well as pathological processes [1–3]. NO is produced from L-arginine and molecular oxygen in a reaction catalyzed by one of three NO synthase (NOS) isoenzymes (eNOS, nNOS, and iNOS). Among these isoforms, iNOS is inducible and is capable of production of high amounts of NO that characterize inflammatory condition. NO can prevent or induce cellular apoptosis depending on its concentration and cellular redox state [4]. Long-lasting production of NO under inflammatory condition acts as a proapoptotic modulator by activating caspase family proteases. However, low or physiological concentrations of NO prevent cells from apoptosis. A direct inhibition of the apoptotic caspase family proteases by S-nitrosylation of the cysteine thiol is thought to be one of the antiapoptotic mechanisms of NO. Also, at low concentration, NO acts as a signaling molecule by stimulating guanylate cyclase in blood vessels, brain, kidney, heart, and many other organs [5]. In contrast, at high concentration, NO may cause tissue damage by negatively influencing the activity of essential biomolecules [6].

In the central nervous system (CNS), astrocytes are one of the major cellular sources of NO production [7,8]. Astrocytes, the most abundant cell type within the CNS, provide mechanical and metabolic support for neurons. Astrocytes are also important for neuronal survival. They release a variety of growth factors and cytokines that

stimulate neuronal survival and protect neurons against excitotoxic and oxidative insults by releasing antioxidants [9–11]. There is also growing evidence that NO produced by activated astrocytes may be involved in neuronal destruction [12–14]. Upon inflammatory stimulation, astrocytes proliferate and produce diverse intercellular mediators such as NO and TNFα [7,8,15–17]. These inflammatory mediators are thought to act as a neurotoxin that causes CNS pathologies. Astrocytes are also a target of NO action. NO exerts diverse effects on astrocytes depending on the experimental conditions. Astrocytes undergo apoptosis upon inflammatory activation, and NO produced by activated astrocytes acts on themselves to induce the auto-regulatory apoptosis via caspase-3 [18]. NO mediates a lipopolysaccharide (LPS)-induced depression of cytochrome P450 (CYP1A) activity in astrocytes [19]. NO induces apoptosis-like cell death in astrocytes by p53- and Bax-dependent mechanisms [20]. Cytoprotective effects of NO have been also reported. NO reacts with superoxide to generate peroxynitrite, which specifically induces nerve growth factor expression and secretion in astrocytes [21]. NO protects cultured astrocytes against apoptosis via a cGMP-dependent mechanism [22]. NO acts as a powerful antioxidant for astrocytes preserving mitochondrial and cellular integrity during oxidative stress [23]. In spite of these previous studies, the precise regulatory role of NO in astrocytes and the molecular mechanisms of NO actions are far from clear, because (i) NO is converted into various derivatives including peroxynitrite and nitrite, (ii) NO reacts with a wide range of cell components, and (iii) NO and its various derivatives can be cytotoxic or cytoprotective depending on the conditions.

In order to better understand the molecular actions of NO in the stress response of brain astrocytes, we assessed the effect of NO on the expression of stress proteins. We have found that NO sequentially induces heme oxygenase-1 (HO-1) and manganese superoxide dismutase (MnSOD), and the induction of these proteins is responsible for the cytoprotective effects of NO at low concentration in mouse astrocytes.

2. Materials and methods

2.1. Reagents and cells

Sodium nitroprusside (SNP) and Zn(II) protoporphyrin IX (ZnPP) were obtained from Sigma (St. Louis, MO). Astrocyte cultures were prepared from the brains of 0–4-day-old ICR mice (Samtako Co.; Osan, Korea) by the method of McCarthy and de Vellis [24]. Briefly, whole brains were dissociated in Dulbecco's modified Eagle's medium (DMEM) (GibcoBRL, Carlsbad, CA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GibcoBRL), 100 U/ml penicillin and 100 μg/ml streptomycin (GibcoBRL). Cells were seeded at 75 cm^2

tissue culture flasks coated with poly D-lysine (Falcon, Becton Dickinson and Company, Franklin Lakes, NJ). Cells were grown at 37 °C in a 5% CO$_2$ humidified atmosphere. The culture medium was changed after 5 days in vitro and then every 3 days. Secondary pure cultures of astrocytes were obtained by shaking mixed glial cultures at 250 rpm overnight and then culture medium was discarded. Astrocytes were dissociated using trypsin-EDTA (GibcoBRL) and then collected by centrifugation at 1000 rpm for 10 min. The cells were resuspended in DMEM with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, seeded at 1×10^5 cells/ml onto six-well plates coated with poly-D-lysine and cultured for 4 days. The purity of astrocyte cultures was greater than 95% as determined by glial fibrillary acidic protein (GFAP) immunocytochemical staining (data not shown). Animals used in the current research have been acquired and cared for in accordance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Two-dimensional gel electrophoresis (2DE) and protein identification

Isoelectric focusing (IEF) was conducted using the IPG-phor/IsoDalt system (Amersham Biosciences, Piscataway, NJ) at 20 °C as described [25] with minor modifications. IPG gel strips (Amersham Biosciences) were rehydrated in a swelling solution (7 M urea, 2 M thiourea, 2% CHAPS, 100 mM DTT, 0.5% IPG buffer, and bromophenol blue) containing 100–500 μg proteins for 12 h at 20 °C. IEF was performed for 1 h at 200 V, 1 h at 500 V, 1 h at 1000 V, 1 h at 1000 V, 30 min at 8000 V, and 45000 V/h. Thereafter, the IPG strips were equilibrated for 15 min in 50 mM Tris–HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 10 mg/ml DTT, and bromophenol blue, and then for 15 min in 50 mM Tris–HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 2% iodoacetamide, and bromophenol blue. For the second dimension, vertical slab gels (12% SDS PAGE) of 1.5 mm were prepared, and then an equilibrated IPG gel strip was laid on top of the gel filled with 0.5% agarose solution. Gel electrophoresis was carried out at 16 °C at 5 mA/cm for 1 h and at 10 mA/cm until the bromophenol blue reached the bottom of the gel. The gel was fixed in 50% methanol and 12% acetic acid for 1.5 h. Proteins were detected by silver-staining, as previously described [26]. The scanned gel image was analyzed using a standard protocol for PDQuest software (Bio-Rad, Hercules, CA). Protein identification was done by Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry using Voyager-DE^{TM} STR Biospectrometry Workstation (Applied Biosystems, Foster City, CA).

2.3. Western blot analysis

The cells were lysed in a single-detergent lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.02% sodium

azide, 1% Nonidet P-40, 1 μg/ml aprotinin, 1 mM phenyl-methylsulfonyl fluoride, 1 mM sodium metavanadate, 5 mM sodium fluoride). The protein concentration in cell lysates was determined using a BCA protein assay reagent kit (Pierce, Rockford, IL). An equal amount of protein for each sample was separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to PROTRAN nitrocellulose transfer membranes (Schleicher and Schuell, Germany). The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween-20 (TBS-T; 20 mM Tris–HCl, pH 7.6, 137 mM NaCl, 0.05% Tween-20), and incubated sequentially with primary antibodies and with HRP-conjugated secondary antibodies (anti-rabbit IgG and anti-mouse IgG; Pierce), followed by ECL detection (Amersham Biosciences). The primary antibodies used were rabbit polyclonal anti-heme-oxygenase-1 antibody (Stressgen, Victoria, BC, Canada), rabbit polyclonal anti-Mn-super-oxide dismutase antibody (Stressgen), and monoclonal anti-α-tubulin clone B-5-1-2 mouse ascites fluid (Sigma).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Cells were plated at a density of 1×10^6 cells/well in 6-well plates and then cells were treated with stimuli. Total RNA was extracted using TRIZOL (Invitrogen, Carlsbad, CA). Reverse transcription was carried out using 1 μg of total RNA, 0.75 pmol of oligo(dT) primer, 1 unit of RNase inhibitor (Promega, Madison, WI), 0.5 mM dNTPs and 20 units of M-MLV reverse transcriptase (Promega) in reaction buffer containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT at 37 °C for 1 h. After heat inactivation, cDNA was stored at −20 °C until PCR reaction. PCR amplification was carried out in 50 μl PCR reaction mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 20 pmol of primer sets, 2 units of Taq DNA polymerase (Roche, Indianapolis, IN), 0.2 mM dNTPs, and 2 μl of the first strand cDNA. Nucleotide sequences of the primers were based on published cDNA sequences: HO-1 forward, 5′-AACAAGCAGAACC-CAGTCTA-3′; HO-1 reverse, 5′-CCTTCTGTGCAATCT-TCTTC-3′; β-actin forward, 5′-ATCCTGAAAGACCTC-TATGC-3′; β-actin reverse, 5′-AACGCAGCTCAGTAA-CAGTC-3′. The PCR reaction was performed for 30 cycles with denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min using a DNA Engine Tetrad Peltier Thermal Cycler (MJ Research, Waltham, MA). For the analysis of PCR products, 10 μl of each PCR reaction was electrophoresed on 1% agarose gel and visualized by ethidium bromide staining. The β-actin was used as an internal control.

2.5. Nitrite quantification

After cells were treated with SNP in 96-well plates, NO_2^- in culture supernatants was measured to assess nitric oxide (NO) production in primary astrocytes. Fifty micro-liters of sample aliquots were mixed with 50 μl of Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2% phosphoric acid) and incubated at 25 °C for 10 min. The absorbance at 540 nm was measured on a microplate reader (Anthos Labtec Instruments GmbH, Salzburg, Austria). $NaNO_2$ was used as the standard to calculate NO_2^- concentrations.

2.6. Assessment of cytotoxicity by MTT assay or trypan blue exclusion assay

After various treatments of the cells in culture, the medium was removed and 0.5 mg/ml 3-[4,5-dimethylthia-zol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added, followed by incubation at 37 °C for 2 h in CO_2 incubator. After a brief centrifugation, supernatants were carefully removed and DMSO was added to the cells. After insoluble crystals were completely dissolved, absorbance at 570 nm was measured using a microplate reader. For trypan blue exclusion assay, cell suspension was mixed with the same volume of 0.4% trypan blue solution (Sigma). Afterwards, the number of stained cells and the total number of cells were counted using a hemocyt-ometer (Marienfeld, Lauda-Koenigshofen, Germany).

2.7. Adenoviral expression of MnSOD and its activity assay

Adenovirus expressing MnSOD (Ad5CMVSOD2) was obtained from Gene Transfer Vector Core, University of Iowa (Iowa City, IA), and was propagated in HEK293 cells. Astrocytes were infected with the adenovirus at a MOI of 50 for the forced expression of MnSOD. MnSOD activity was determined by Superoxide Dismutase Assay Kit (Cayman Chemical, Ann Arbor, MI). The assay kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine [27].

2.8. Statistical analysis

Statistical comparison between different treatments was done by Student's t-test. The $p < 0.05$ was considered statistically significant.

3. Results

3.1. Induction of heme oxygenase (HO)-1 and manganese superoxide dismutase (MnSOD) expression by NO in cultured astrocytes

In order to investigate the stress responses of astrocytes under nitrosative stress condition, we have selected HO-1 and MnSOD as potential targets of NO action on the

following grounds: (1) the expression of HO-1 protein in mouse astrocyte cultures was detected by two-dimensional gel electrophoresis followed by MALDI-TOF mass spectrometry (Fig. 1); (2) both HO-1 and MnSOD are involved in the redox regulation of cells with HO-1 catalyzing degradation of heme into biliverdin, carbon monoxide (CO), and free iron [28], while MnSOD acting as an antioxidant by scavenging superoxide anion [29,30]; (3) it has been previously shown in other cell types that NO induces HO-1 expression [31,32]; (4) NO is known to induce MnSOD protein expression in glomerular mesangial cells [33]; and (5) HO-1 induces MnSOD expression in response to oxidative stress, and inhibition of HO-1 decreases the MnSOD expression [34]. Based on these previous findings and the result of proteomic analysis of astrocytes (Fig. 1), HO-1 protein expression was first assessed after treatment of mouse primary astrocyte cultures with NO donor sodium nitroprusside (SNP). Treatment of astrocyte cultures with 0.2 mM SNP for 24 h, but not 0.1 mM, induced a significant cytotoxicity (Fig. 2A). Production of nitrite was dependent on the concentration and duration of SNP treatment (Fig. 2B). Thus, we employed the 0.1 mM SNP treatment for 24 h as an optimal condition to assess the modulation of protein expression without the apparent cytotoxicity. When the protein level of HO-1 was evaluated by Western blot analysis, a marked induction of HO-1 protein expression was observed following SNP treatment (0.1 mM) with a peak induction at 24 h (Fig. 3A). SNP of 0.05 mM, however, did not induce the HO-1 expression. At mRNA level, a peak induction of the HO-1 expression occurred at 6 h as demonstrated by RT-PCR analysis (Fig. 3B). When
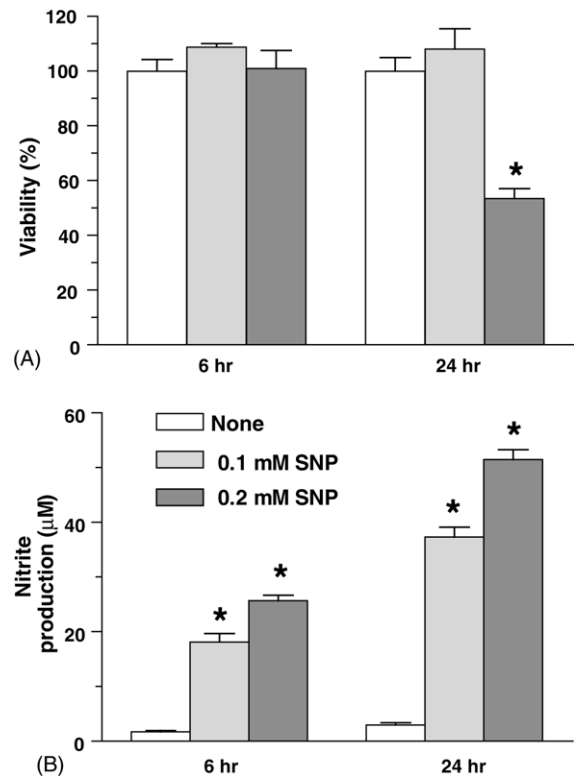

Fig. 2. Effects of SNP treatment on the viability (A) and NO production (B) in astrocytes. After treatment of primary astrocyte cultures with SNP (0.1 or 0.2 mM) for 6 or 24 h, cell viability (A) or nitrite production (B) was measured by MTT assay or Griess reaction, respectively. SNP treatment led to the nitrite production in a dose- and time-dependent manner. The results in this and all similar experiments were repeated several times and one representative done in triplicates is shown. Values represent mean ± S.D. Asterisks indicate statistically significant differences from untreated control ($p < 0.05$).
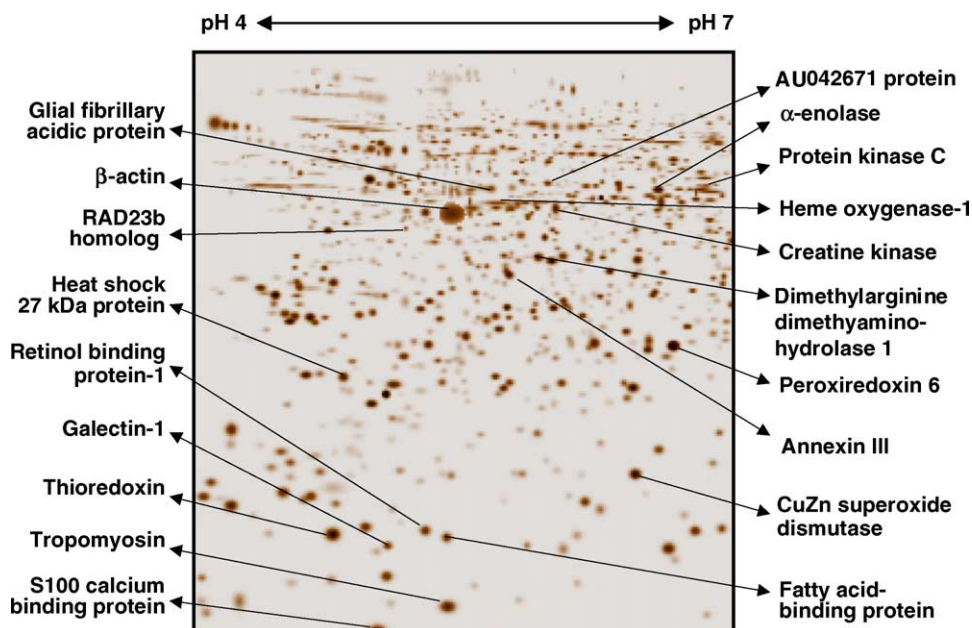

Fig. 1. A representative two-dimensional gel image of astrocytes. Whole cell extracts of mouse astrocyte cultures were separated by two-dimensional gel electrophoresis and visualized by silver staining. Some of the protein spots were identified by MALDI-TOF mass spectrometry and the database searching. The result is a representative of several experiments.
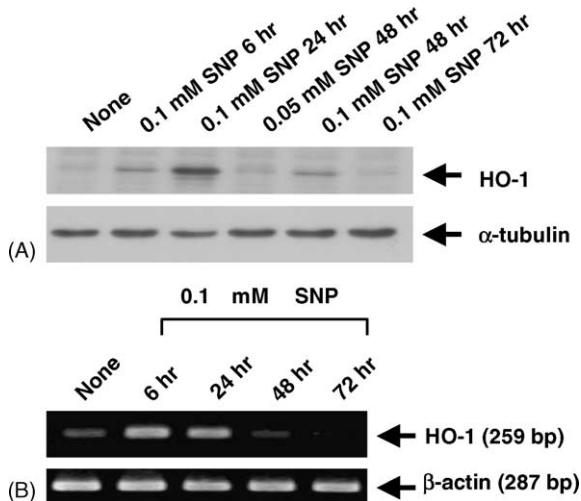
Fig. 3. Evaluation of NO effects on HO-1 expression in astrocyte cultures by Western blot analysis (A) or RT-PCR (B). Astrocytes were treated with the indicated concentrations of SNP for 6–72 h, and then the levels of HO-1 protein or mRNA were assessed by Western blot analysis or RT-PCR, respectively. Detection of α-tubulin or β-actin was done to confirm the equal loading or integrity of the samples. The results are representative of three independent experiments.

MnSOD protein expression was next analyzed by Western blot, a significant induction by SNP occurred as late as 48 h after the treatment (Fig. 4A). A peak induction of MnSOD
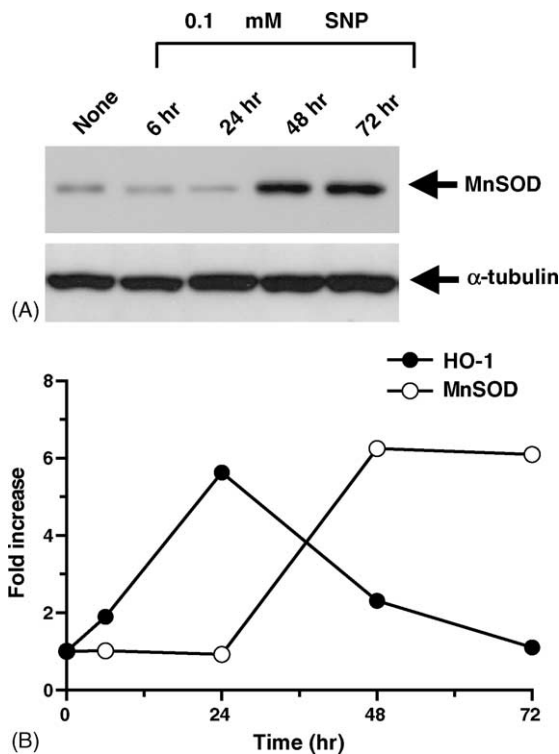

Fig. 4. Induction of MnSOD protein expression by SNP treatment. After treatment of astrocyte cultures with 0.1 mM SNP for the indicated time periods, MnSOD protein levels were assessed by Western blot analysis (A). The fold increase in HO-1 or MnSOD protein levels was plotted against time after SNP treatment (B). A relative increase in the protein levels is based on the Western blot results in Figs. 3A and 4A.
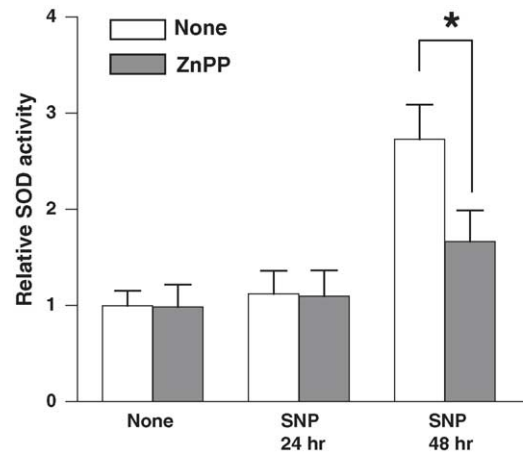

Fig. 5. HO-1-dependent increase in MnSOD activity following SNP treatment. After treatment of primary astrocyte cultures with SNP (0.1 mM) for 24 h or 48 h in the presence or absence of HO-1 inhibitor ZnPP (2.5 μM), MnSOD activity was measured using the SOD assay kit. SNP increased MnSOD activity, which was partially abolished by ZnPP cotreatment. Values are mean ± S.D. The asterisk indicates statistically significant differences between the two treatments ($p < 0.05$).

was preceded by the strong HO-1 induction, suggesting a sequential induction of HO-1 and MnSOD following the SNP treatment (Fig. 4B). The NO-mediated induction of MnSOD protein was further investigated by MnSOD activity assay (Fig. 5). SNP strongly induced MnSOD activity in astrocyte cultures after 48 h. When the astrocyte cultures were treated with SNP in the presence of a specific competitive inhibitor of HO-1, zinc protoporphyrin IX (ZnPP) [35], the SNP-mediated increase in MnSOD activity was abolished, supporting the HO-1-dependent induction of MnSOD.

3.2. Cytoprotective role of HO-1 and MnSOD against NO toxicity

It has been previously shown that HO-1 and MnSOD exert cytoprotective effects under stress conditions, and NO exerts dose-dependent effects being cytoprotective at low concentration and being cytotoxic at high concentration [28,36]. Thus, we hypothesized that the sequential induction of HO-1 and MnSOD by a low-dose NO in astrocytes may protect the cells against toxic effects of the high-dose NO. To test this hypothesis, we extended the SNP dose response study. When the concentration of SNP was gradually increased from 0.1 to 0.4 mM, a pronounced cytotoxicity was observed at 0.2 mM SNP (Fig. 6A). Similar results were obtained by trypan blue assay (data not shown). Potential cytoprotective effects of low-dose NO were tested by a pretreatment experiment, where astrocytes were pretreated with a nontoxic dose of SNP (0.1 mM) for 24 or 48 h prior to the toxic 0.2 mM SNP treatment for 24 h. Compared to astrocytes without the pretreatment, pretreated cells showed a much higher viability (Fig. 6B). To evaluate the role of HO-1 and MnSOD in the cytoprotection, the HO-1 inhibitor ZnPP and the
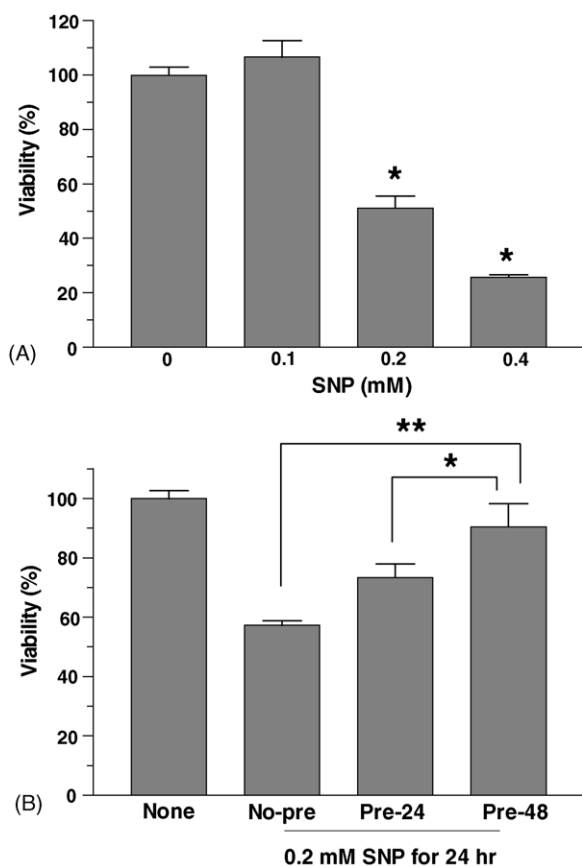
Fig. 6. Cytoprotective effects of pretreatment with a nontoxic dose of NO against NO toxicity. Astrocyte cultures were treated with the increasing concentrations of SNP for 24 h, and then the cell viability was measured by MTT assay (A). Alternatively, astrocyte cultures were pretreated with SNP of 0.1 mM for 24 h (Pre-24) or 48 h (Pre-48), and then treated with SNP of 0.2 mM for 24 h prior to MTT assay (B). The viability of cells without the pretreatment (No-pre) is also shown. Values are mean ± S.D. Asterisks indicate statistically significant differences from untreated control (A) or significant difference between the two treatments (B) ($^*p < 0.05$, $^{**}p < 0.01$).

adenoviral vector expressing MnSOD were employed (Fig. 7). When astrocytes were treated with a nontoxic dose of SNP in the presence of ZnPP, a significant cytotoxicity was observed (Fig. 7A). Adenovirus-mediated overexpression of MnSOD protected astrocytes against the SNP cytotoxicity (Fig. 7B and C). In addition, cotreatment with ZnPP abolished the adenovirus-mediated cytoprotective effects (Fig. 7C). Combined with the results in Fig. 5 (the reduction of MnSOD activity upon the inhibition of HO-1), these results supported our hypothesis that the sequential induction of HO-1 and MnSOD confers a cytoprotection against NO toxicity.

4. Discussion

NO exerts various physiological as well as pathological effects [1–3]. Although the downstream signaling events of NO actions are partly understood with respect to its physiological functions, one of the most puzzling questions

regarding NO actions that has yet to be answered is how NO exerts opposing effects on the cell viability depending on its concentration. At high concentration, NO is known to mediate cytotoxic effects in a variety of cell types. Under many other conditions, however, NO exerts anti-apoptotic effects at low concentration. Our results reported here may provide an answer to this question. To better understand molecular actions of NO in the astrocyte stress responses, we focused on HO-1 and MnSOD in the current work, with a hypothesis that these proteins may be induced by a mild nitrosative condition and may play a cytoprotective role under the severe nitrosative condition in astrocytes. Our hypothesis was supported by the following observations: (1) both HO-1 and MnSOD expression were induced by NO; (2) pretreatment of astrocytes with nontoxic dose of NO protected the cells against the later treatment with toxic dose of NO; (3) HO-1 and MnSOD were required for the cytoprotective activity. These results indicate that the molecular action of NO in astrocytes appears to depend strictly on the concentration of the molecule. When astrocytes are exposed to low dose of NO, they induce cytoprotective proteins such as HO-1 and MnSOD, and then cells survive to use NO as a signaling molecule. In contrast, overwhelming dose of NO seems to rapidly induce cell death without giving the cells a chance to produce cytoprotective proteins, which may lead to the tissue damage in the CNS.

Heme oxygenase is an enzyme that catalyzes the breakdown of heme to biliverdin, with the release of iron ions and CO [28]. Two isoforms of heme oxygenase have been characterized, a constitutive isoform (HO-2) predominantly under normal physiological conditions, and a stress-induced isoform (HO-1), which is identical to the heat shock protein HSP32. The overall effect of heme oxygenase is to remove a pro-oxidant (heme), and to generate a putative antioxidant (bilirubin) and another pro-oxidant (iron). HO-1 protein has been shown to be associated with the protection against oxygen toxicity in cultured fibroblasts [37]. It has been also reported that mice lacking HO-1 are vulnerable to mortality and hepatic necrosis when challenged with endotoxin [38], suggesting that HO-1 is an important enzyme in the protection of cells from oxidative stress both in vitro and in vivo. MnSOD is another antioxidant enzyme [29]. Induction of the enzyme under various stress conditions has been reported, and it has been shown to exert cytoprotective effects against oxidative stress, amyloid-beta peptide, and NO [30]. Although the induction of HO-1 and MnSOD and their cytoprotective role under the stress condition are well documented in multiple cell types, a functional role of these two enzymes and their interrelationship in astrocytes under the nitrosative stress have not been addressed. The sequential induction of HO-1 and MnSOD under the nitrosative condition, as demonstrated in the current study, suggests an important functional link between the antioxidant enzymes and the existence of a time-dependent role assignment among the antioxidant enzymes to cope with the stress condition.
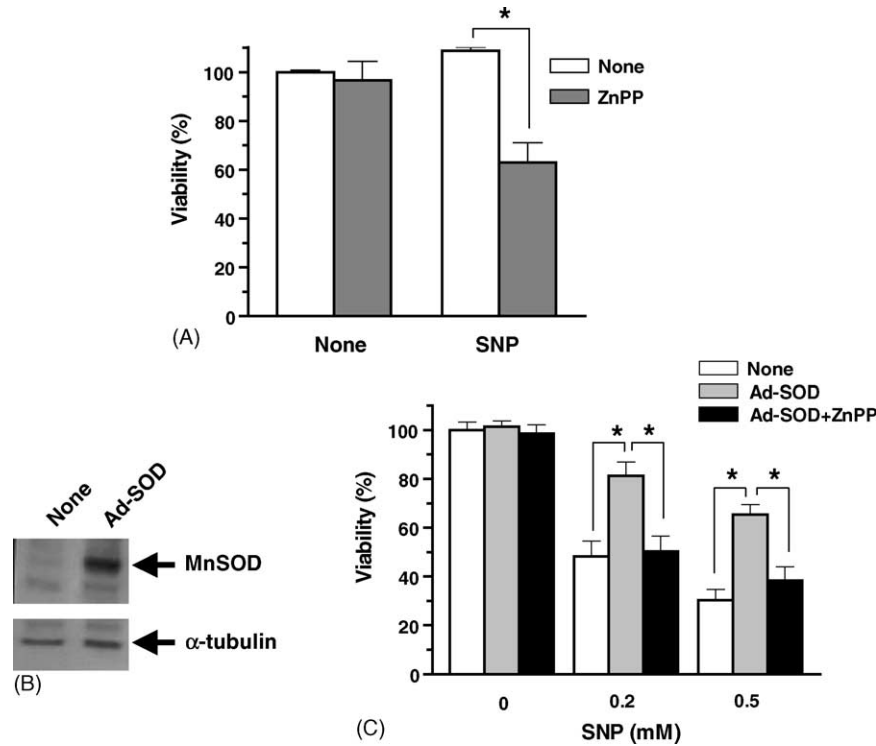
Fig. 7. Cytoprotective effects of the sequential induction of HO-1 and MnSOD against the NO toxicity. Astrocyte cultures were treated with SNP of 0.1 mM in the absence or presence of ZnPP (2.5 μM) for 24 h prior to MTT assay (A). Inhibition of HO-1 by ZnPP sensitized the astrocytes to the nontoxic dose of SNP. For the evaluation of cytoprotective effects of MnSOD, astrocyte cultures were infected with the adenovirus expressing MnSOD (Ad-SOD), and then the expression of MnSOD was evaluated by Western blot analysis at 24 h after the infection (B), or SNP toxicity was measured by MTT assay after 24 h treatment in the absence or presence of ZnPP (2.5 μM) (C). Adenoviral expression of MnSOD attenuated the SNP toxicity, which was reversed by HO-1-inhibiting ZnPP. Values represent mean ± S.D. Asterisks indicate statistically significant differences between the two treatments ($p < 0.05$).

Adenovirus-mediated overexpression of MnSOD protected astrocytes against the SNP cytotoxicity, but cotreatment with ZnPP abolished the cytoprotective effects observed in cells overexpressing MnSOD, suggesting that HO-1 regulates the activity of MnSOD (as shown in Fig. 5) independently of its level of expression (Fig. 7C). Thus, HO-1 appears to influence both the expression and the activity of MnSOD. What is then the mechanism by which HO-1 influences the expression and activity of MnSOD? After all, HO-1 is an enzyme that mediates the conversion of heme into biliverdin, iron, and CO. Therefore, the effect of HO-1 is possibly mediated by these products of the enzymatic reaction. The release of free iron is capable of generating the vicious hydroxyl radical through Fenton reaction. The resulting prooxidant state may induce MnSOD expression, because the induction of the gene expression has been frequently observed following the oxidative stress in many experimental systems. CO exerts its effect by avidly binding to hemoglobin or guanylyl cyclase. CO has been shown to induce general hypoxia, to regulate vasomotor tone as well as neurotransmission, and to exert anti-inflammatory effects [28]. CO may modulate the activity or the function of the proteins via direct interactions, and MnSOD may be one of its targets. The cytoprotective effect of HO-1 and MnSOD does not seem to be restricted to the nitrosative stress condition. Exposure of astrocyte cultures to the endotoxin and cytokine mix-

tures (LPS/IFNγ/TNFα) has been shown to induce a marked cytotoxicity [18]. Pretreatment of astrocyte cultures with 0.1 mM of SNP conferred a cytoprotection against the subsequent treatment with the LPS/IFNγ/TNFα (Suk K, et al., unpublished results).

An increased production of free radical species is related to the development of neurodegenerative diseases, vascular dysfunction, carcinogenesis, and aging [39]. Irrespective of the source and mechanisms that lead to the oxidative or nitrosative challenges, mammalian cells have developed highly refined inducible systems against a variety of stressful stimuli. Each one of these systems, when appropriately activated, has the possibility to restore cellular homeostasis and to rebalance redox equilibrium [40]. Nitrosative stress, defined as excessive or deregulated NO formation [41], leads to an increase of reactive nitrogen species (RNS) production and has been associated with a number of disorders. Although, in the CNS, astrocytes are suspected of being involved in a wide range of pathologies including trauma, ischemia, and neurodegeneration, there are many unanswered questions about the role of nitrosative stress in astrocytes as well as in these CNS disorders. Now, the identification of MnSOD as a downstream effector of HO-1 in astrocytes under the nitrosative condition provides new possibilities for improved therapeutic approaches against the CNS diseases associated with nitrosative stress, in which astrocytes may play a pathogenic role. Moreover, further

characterization of the downstream mediators of NO actions will provide a better understanding of astrocyte responses to the nitrosative stress as well as the markers for astrocyte responses in the pathogenesis of various CNS disorders.

Acknowledgment

References

[1] Prast H, Philippu A. Nitric oxide as modulator of neuronal function. Prog Neurobiol 2001;64:51–68.

[2] Beck KF, Eberhardt W, Frank S, Huwiler A, Messmer UK, Muhl H, et al. Inducible NO synthase: role in cellular signalling. J Exp Biol 1999;202:645–53.

[3] Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991;43:109–42.

[4] Chung HT, Pae HO, Choi BM, Billiar TR, Kim YM. Nitric oxide as a bioregulator of apoptosis. Biochem Biophys Res Commun 2001;282:1075–9.

[5] Schmidt HH, Lohmann SM, Walter U. The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. Biochim Biophys Acta 1993;1178:153–75.

[6] Brune B, von Knethen A, Sandau KB. Nitric oxide and its role in apoptosis. Eur J Pharmacol 1998;351:261–72.

[7] Murphy S. Production of nitric oxide by glial cells: regulation and potential roles in the CNS. Glia 2000;29:1–13.

[8] Dong Y, Benveniste EN. Immune function of astrocytes. Glia 2001;36:180–90.

[9] Barres BA, Barde Y. Neuronal and glial cell biology. Curr Opin Neurobiol 2000;10:642–8.

[10] Mattson MP, Cheng B, Baldwin SA, Smith-Swintosky VL, Keller J, Geddes JW, et al. Brain injury and tumor necrosis factors induce calbindin D-28k in astrocytes: evidence for a cytoprotective response. J Neurosci Res 1995;42:357–70.

[11] Tsacopoulos M, Magistretti PJ. Metabolic coupling between glia and neurons. J Neurosci 1996;16:877–85.

[12] Brown GC, Bal-Price A. Inflammatory neurodegeneration mediated by nitric oxide, glutamate, and mitochondria. Mol Neurobiol 2003;27:325–55.

[13] Becher B, Prat A, Antel JP. Brain-immune connection: immunoregulatory properties of CNS-resident cells. Glia 2000;29:293–304.

[14] Aschner M. Astrocytes as mediators of immune and inflammatory responses in the CNS. Neurotoxicology 1998;19:269–81.

[15] Galea E, Feinstein DL, Reis DJ. Induction of calcium-independent nitric oxide synthase activity in primary rat glial cultures. Proc Natl Acad Sci USA 1992;89:10945–9.

[16] Sawada M, Kondo N, Suzumura A, Marunouchi T. Production of tumor necrosis factor-alpha by microglia and astrocytes in culture. Brain Res 1989;491:394–7.

[17] Simmons ML, Murphy S. Induction of nitric oxide synthase in glial cells. J Neurochem 1992;59:897–905.

[18] Suk K, Kim SY, Kim H. Essential role of caspase-11 in activation-induced cell death of rat astrocytes. J Neurochem 2002;80:230–8.

[19] Nicholson TE, Dibb S, Renton KW. Nitric oxide mediates an LPS-induced depression of cytochrome P450 (CYP1A) activity in astrocytes. Brain Res 2004;1029:148–54.

[20] Yung HW, Bal-Price AK, Brown GC, Tolkovsky AM. Nitric oxide-induced cell death of cerebrocortical murine astrocytes is mediated through p53- and Bax-dependent pathways. J Neurochem 2004;89:812–21.

[21] Vargas MR, Pehar M, Cassina P, Estevez AG, Beckman JS, Barbeito L. Stimulation of nerve growth factor expression in astrocytes by peroxynitrite. In Vivo 2004;18:269–74.

[22] Takuma K, Phuagphong P, Lee E, Enomoto R, Mori K, Baba A, et al. The nitric oxide donor NOC12 protects cultured astrocytes against apoptosis via a cGMP-dependent mechanism. Jpn J Pharmacol 2002;89:64–71.

[23] Robb SJ, Connor JR. Nitric oxide protects astrocytes from oxidative stress. Ann N Y Acad Sci 2002;962:93–102.

[24] McCarthy KD, de Vellis J. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J Cell Biol 1980;85:890–902.

[25] West KA, Yan L, Miyagi M, Crabb JS, Marmorstein AD, Marmorstein L, et al. Proteome survey of proliferating and differentiating rat RPE-J cells. Exp Eye Res 2001;73:479–91.

[26] Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 1996;68:850–8.

[27] Spitz DR, Oberley LW. An assay for superoxide dismutase activity in mammalian tissue homogenates. Anal Biochem 1989;179:8–18.

[28] Otterbein LE, Choi AM. Heme oxygenase: colors of defense against cellular stress. Am J Physiol Lung Cell Mol Physiol 2000;279:1029–37.

[29] Macmillan-Crow LA, Cruthirds DL. Manganese superoxide dismutase in disease. Free Radic Res 2001;34:325–36.

[30] Keller JN, Kindy MS, Holtsberg FW, St Clair DK, Yen HC, Germeyer A, et al. Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: suppression of peroxynitrite production, lipid peroxidation, and mitochondrial dysfunction. J Neurosci 1998;18:687–97.

[31] Foresti R, Sarathchandra P, Clark JE, Green CJ, Motterlini R. Peroxynitrite induces haem oxygenase-1 in vascular endothelial cells: a link to apoptosis. Biochem J 1999;339:729–36.

[32] Pae HO, Choi BM, Oh GS, Lee MS, Ryu DG, Rhew HY, et al. Roles of heme oxygenase-1 in the antiproliferative and antiapoptotic effects of nitric oxide on Jurkat T cells. Mol Pharmacol 2004;66:122–8.

[33] Keller T, Pleskova M, McDonald MC, Thiemermann C, Pfeilschifter J, Beck KF. Identification of manganese superoxide dismutase as a NO-regulated gene in rat glomerular mesangial cells by 2D gel electrophoresis. Nitric Oxide 2003;9:183–93.

[34] Park SY, Chang I, Kim JY, Kang SW, Park SH, Singh K, et al. Resistance of mitochondrial DNA-depleted cells against cell death: role of mitochondrial superoxide dismutase. J Biol Chem 2004;279:7512–20.

[35] Lee TS, Chau LY. Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. Nat Med 2002;8:240–6.

[36] Pfeilschifter J, Eberhardt W, Beck KF. Regulation of gene expression by nitric oxide. Pflugers Arch 2001;442:479–86.

[37] Dennery PA, Sridhar KJ, Lee CS, Wong HE, Shokoohi V, Rodgers PA, et al. Heme oxygenase-mediated resistance to oxygen toxicity in hamster fibroblasts. J Biol Chem 1997;272:14937–42.

[38] Poss KD, Tonegawa S. Reduced stress defense in heme oxygenase 1-deficient cells. Proc Natl Acad Sci USA 1997;94:10925–30.

[39] Halliwell B. Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. Drugs Aging 2001;18:685–716.

[40] Calabrese V, Bates TE, Stella AM. NO synthase and NO-dependent signal pathways in brain aging and neurodegenerative disorders: the role of oxidant/antioxidant balance. Neurochem Res 2000;25:1315–41.

[41] Hausladen A, Stamler JS. Nitrosative stress. Meth Enzymol 1999;300:389–95.