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Biochemical and Biophysical Research Communications 358 (2007) 130–135
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Relationship between reactive oxygen species and heme metabolism during the differentiation of Neuro2a cells

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Received 31 March 2007
Available online 19 April 2007

Abstract

Although neuronal cells are highly vulnerable to oxidative stress, recent studies suggest that production of reactive oxygen species (ROS) increases during and is essential for neuronal differentiation. In addition, we have previously found that heme biosynthesis is up-regulated during retinoic acid-induced differentiation of Neuro2a cells. In the current study, we showed that this up-regulation of heme biosynthesis during differentiation is ROS-dependent. Furthermore, we found that ROS-dependent induction of heme oxygenase, which degrades heme and acts as an anti-oxidant, and catalase, another anti-oxidant enzyme that contains heme as a prosthetic group, occurs during differentiation. These results suggest that heme biosynthesis following the degradation of heme protects Neuro2a cells from oxidative stress caused by ROS during differentiation.

Keywords: Neuron; Differentiation; ROS; Heme; Heme oxygenase; Catalase

Heme is an essential prosthetic group in many proteins and plays a regulatory role in cells [1]. Several groups have reported that heme is important in the nervous system. For example, a deficiency in heme causes neuronal cell death and the suppression of key neuronal genes [2,3]. Also, altered heme metabolism may be related to aging and Alzheimer's disease [4].

In addition, heme is a substrate of heme oxygenase (HO), which degrades heme to biliverdin, CO, and Fe^{2+}. Bilirubin, metabolite of biliverdin, is a potent radical scavenger and protects neuronal cells from oxidative stress [5]. In a previous study, we found that heme biosynthesis is up-regulated during retinoic acid (RA)-induced differentiation of Neuro2a cells [6], but we did not determine the significance or identify the regulatory mechanism of this up-regulation.

Recent reports show that neuronal cells produce high levels of ROS [7,8]. ROS, which includes free radicals and peroxides, are generally highly reactive molecules and could cause significant damage to the neuronal cells. It is therefore expected that anti-oxidant systems are indispensable for neuronal survival. In general, cells possess several strategies to avoid damage by ROS, including ROS-degrading enzymes and low-molecular weight anti-oxidants. Two of the enzymatic systems, HO and catalase, require heme for activity.

In this study, we examined the relationship between the up-regulation of heme biosynthesis and ROS production during the differentiation of Neuro2a cells [6]. We specifically focused on the role of HO and catalase in the relationship between heme metabolism and ROS.

Materials and methods

Cell culture. Neuro2a cells were cultured as described previously [6] in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, St. Louis,

Abbreviations: ALAS-1, 5-aminolevulinic acid synthase-1; 3-AT, 3-aminotriazole; CPG, coproporphyrinogen; DSP, downstream primer; HO, heme oxygenase; NAC, *N*-acetyl cysteine; PMP70, peroxisomal membrane protein 70; RA, retinoic acid; ROS, reactive oxygen species; SA, succinyl-acetone; USP, upstream primer; ZnPP IX, zinc protoporphyrin IX.

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doi:10.1016/j.bbrc.2007.04.071

MO, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL Life Technologies, Paisley, Scotland), 100 U/ml penicillin, 100 μg/ml streptomycin, and 292 μg/ml glutamine at 37 °C in a humidified 5% (v/v) CO_2 incubator. Differentiation was induced by treating the cells with 20 μM RA in DMEM containing 2% (v/v) FBS.

Detection of intracellular H_2O_2. Cells were seeded on coverslips (Grace Bio-Lab., Bend, OR) and incubated as described above (see Cell culture). H_2O_2 was detected using the fluorescent probe BES–H_2O_2 (Commercial name) (Wako, Nagoya Japan), which is converted to a fluorescent product by reaction with H_2O_2. The medium was replaced with fresh medium containing 10 μM BES-H_2O_2 with or without 10 mM *N*-acetyl cysteine (NAC) and incubated for 15 min at 37 °C in a humidified 5% (v/v) CO_2 incubator. Prior to observation, the BES-H_2O_2-containing medium was removed, and the cells on the coverslips were washed with phosphate-buffered saline (pH 7.4). Next pre-warmed medium (2% FBS/DMEM) without BES-H_2O_2 was added, and the fluorescence was detected using a NIKON ECLIPSE E600 fluorescence microscope with a 465- to 495-nm excitation filter, a 505-nm dichroic mirror, and a 515- to 555-nm emission filter.

Detection of peroxisomes by immunofluorescence microscopy. Peroxisomes were detected using a Select Alexa Fluor 488 Peroxisome Labeling kit (Molecular probes), which detects peroxisomal membrane protein 70 (PMP70). Cells were grown on coverslips, and peroxisome staining was performed according to the manufacturer's instructions. Fluorescence from Alexa Fluor 488 was detected using a NIKON ECLIPSE E600 fluorescence microscope as described above (see Detection of intracellular H_2O_2).

Quantitative real-time PCR. Total RNA was isolated from cells using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA was reverse-transcribed, and complementary DNAs were synthesized using an oligo (dT) primer. Real-time PCR was performed using a LC real-time PCR apparatus (Roche Diagnostics, Mannheim, Germany) and a Quantitect SYBR-Green RT-PCR Kit (Qiagen, Hilden, Germany) in a 20-μl volume containing 0.5 μM of each upstream primer (USP) and downstream primer (DSP) according to the manufacturer's instructions. The primers used were as follows: for β-actin, β-actin-USP (5′-tggaatcctgtggcatccatgaaac-3′), and β-actin-DSP (5′-taaaacgcagctcagtaacagtccg-3′); for 5-aminolevulinic acid synthase-1 (ALAS-1), ALAS-1-USP (5′-gtcaagcttctgaggc-3′), and ALAS-1-DSP (5′-cctggtcatcaactc-3′); for coproporphyrinogen oxidase (CPG oxidase, EC 1.3.3.3), CPG oxidase-USP (5′-ctccaggatccaggatc-3′), and CPG oxidase-DSP (5′-cctttggatggcgcaac-3′); for porphobilinogen deaminase (PBG deaminase, EC 2.5.1.61), PBG deaminase-USP (5′-ccgtagcagtgcatacagtg-3′), and PBG deaminase-USP (5′-ctggatggtggcctgcatag-3′); for catalase, catalase-USP (5′-ccagtgcgctgtagatg-3′) and catalase-DSP (5′-caatgttctcacacaggc-3′); for HO, HO-1-USP (5′-gacacctgaggtcaagc-3′) and HO-1-DSP (5′-ctctgacgaagtgacg-3′). The PCR was carried out as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation for 10 s at 95 °C, elongation for 20 s (60 °C for β-actin and PBG deaminase; 52 °C for ALAS-1, and 58 °C for CPG oxidase, catalase, and HO-1), and annealing for 10 s at 72 °C. The mRNA levels were normalized according to the level of β-actin mRNA.

Measurement of catalase activity. Collected cells were suspended in potassium phosphate (50 mM) buffer (pH 7.0) containing 1 mM EDTA, homogenized, and centrifuged at 10,000*g* for 15 min at 4 °C. Catalase activities in the supernatants were measured using an Amplex Red reagent-based H_2O_2 detection system (Amplex Red Catalase Assay Kit, Molecular Probes) according to the manufacturer's instructions.

Measurement of HO activity. HO activity was measured by the bilirubin generation method [9,10]. In brief, cells were collected by centrifugation (1000*g* for 10 min at 4 °C), and the cell pellet was suspended in a buffer of 2 mM $MgCl_2$ in 100 mM potassium phosphate (pH 7.4), sonicated on ice, and centrifuged at 18,800*g* for 10 min at 4 °C. The supernatant was added to the reaction mixture (100 μl), which contained rat liver cytosol (0.5 mg/ml), hemin (20 μM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 U), and NADPH (0.8 mM), and incubated for 1 h at 37 °C in the dark. The formed bilirubin was extracted with 300 μl of chloroform, and the change in optical density between 464 and 530 nm was measured (extinction coefficient = 40 mM^{-1} cm^{-1} for bilirubin).

Statistical analysis. Each experiment was performed three times. The data were plotted as the means ± SD. Student's *t*-test was used for comparisons. Differences were considered significant at $P < 0.01$ or 0.05 as indicated in the figure legends.

Results

ROS production during differentiation of Neuro2a cells'

Because neuronal cells may produce high levels of ROS [7,8], we examined whether Neuro2a cells produce ROS during RA-induced differentiation. In these experiments, we used the probe BES-H_2O_2 to measured the production of H_2O_2, which is a relatively stable molecule and thought to be a major form ROS [8].

We found that the fluorescence intensity produced by BES-H_2O_2 was significantly higher in RA-treated differentiating cells than in untreated control cells (Supplement 1a). The fluorescence was almost completely abolished by inclusion of the radical scavenger NAC (Supplement 1b), confirming that the observed fluorescence was ROS-dependent. The higher fluorescence levels were observed from around 6 h after the treatment with RA and continued thereafter (data not shown), indicating that ROS is produced in differentiating Neuro2a cells.

Effect of the radical scavenger NAC on the expression of heme biosynthetic enzymes in Neuro2a cells during RA-induced differentiation

Because we previously observed that heme biosynthesis is up-regulated during differentiation of Neuro2a cells [6] and because heme is essential for the activity of anti-oxidative enzymes, we postulated that the up-regulation of heme biosynthesis is related to the increase in ROS levels during differentiation. Therefore, we examined the effect of the radical scavenger NAC on heme biosynthesis.

We first measured the effect of NAC on the mRNA levels for rate-limiting enzymes, namely, ALAS-1 and CPG oxidase, which we previously found to be up-regulated in Neuro2a cells during RA-induced differentiation, and on the level of PBG deaminase, which did not change significantly during differentiation [6]. As shown in Fig. 1, the up-regulation of mRNA levels for ALAS-1 and CPG oxidase by RA was inhibited by NAC, whereas the mRNA level for PBG deaminase was not affected by RA or NAC. This result suggests that the up-regulation of heme biosynthesis during Neuro2a differentiation is ROS-dependent.

Alteration in the level of HO-1 mRNA and HO enzymatic activity during RA-induced differentiation in Neuro2a cells

HO plays an anti-oxidative role by converting heme into the anti-oxidant bilirubin. HO-1, the inducible form of HO,

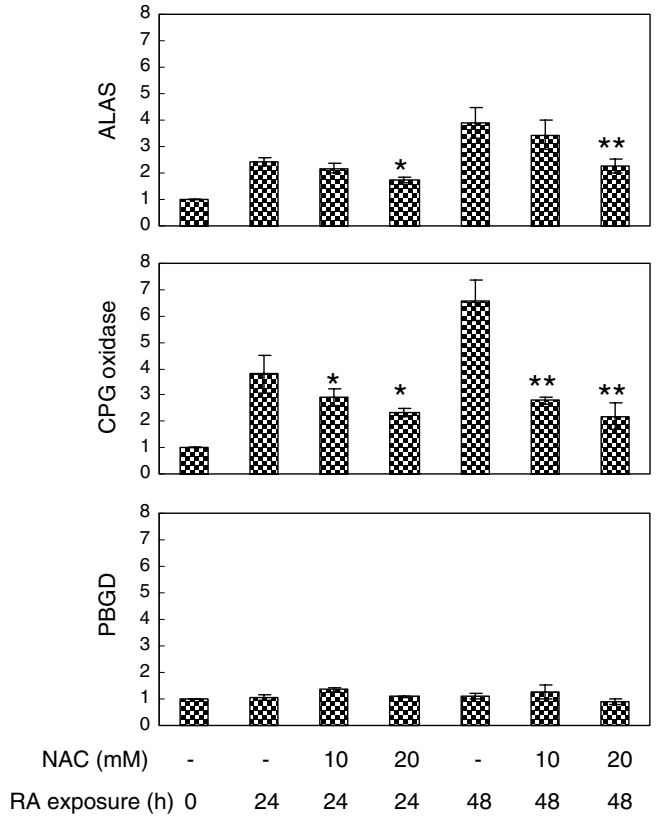

Fig. 1. Effect of NAC on the mRNA levels for heme biosynthetic enzymes. Cells were exposed to RA in the presence or absence of NAC (10 and 20 mM). The mRNA levels for heme biosynthetic enzymes ALAS-1, CPG oxidase, and PBG deaminase were measured using quantitative RT-PCR. Results represent the means ± SD ($n = 3$). $^{*}P < 0.05$ vs. 24 h, $^{**}P < 0.05$ vs. 48 h without NAC.

responds to various stimuli including ROS [11,12]. Because our results suggested that ROS up-regulates heme biosynthetic enzymes, we suspected that ROS increases the demand for bilirubin and therefore heme. For this reason, we examined the effect of RA on the level of HO-1 mRNA and HO enzymatic activity in Neuro2a cells.

We treated the Neuro2a cells for up to 48 h with RA, isolated total RNA, and examined the level of HO-1 mRNA by RT-PCR. As shown in Fig. 2A, the level of HO-1 mRNA increased during differentiation and was approximately 10-fold higher than in untreated control cells 12 h after the induction of differentiation with RA. The level of HO-1 mRNA did not increase thereafter. We also examined the effect of the radical scavenger NAC (10 mM) on the changes in HO-1 mRNA levels. NAC greatly suppressed the increase in HO-1 mRNA levels during differentiation (Fig. 2A). We further measured the HO enzymatic activity in Neuro2a cells exposed to RA for 12 h in the presence or absence of 10 mM NAC (Fig. 2B). HO activity increased after the induction of differentiation, and the activation of HO was greatly reduced by NAC, similar to the changes in the HO-1 mRNA level (Fig. 2A). These results show that HO is activated through the ROS-dependent induction of HO-1 during RA-induced differentiation of Neuro2a cells.

Alteration in the level of catalase in Neuro2a cells during RA-induced differentiation

Catalase is a heme-containing enzyme that catalyzes the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen. Because catalase is an important anti-oxidative enzyme, we examined whether it is also induced during differentiation in Neuro2a cells.

Differentiation was induced in the presence or absence of NAC, and catalase mRNA levels were examined by RT-PCR. As shown in Fig. 2C, the expression of catalase was increased during differentiation, and this induction was suppressed by NAC. We also examined the changes in the enzymatic activity of catalase (Fig. 2D). Catalase was activated approximately 2.5-fold after 48 h of RA-induced differentiation. The activation of catalase was suppressed by NAC. These results suggested that during differentiation, like HO, catalase is activated by ROS. Compared with its effects on HO induction, NAC had a moderate effect on catalase induction. Thus, 10 mM NAC was apparently not sufficient to obtain strong inhibition, although the inhibition was more obvious at 20 mM NAC (Fig. 2D).

Effect of the heme biosynthesis inhibitor succinyl-acetone (SA) on catalase activity and peroxisomal distribution in Neuro2a cells

Because catalase contains heme as a prosthetic group, heme biosynthesis may be essential for the activation of catalase. Thus, we examined whether the inhibition of heme biosynthesis affects the activation of catalase during RA-induced differentiation. Differentiation was induced in the presence or absence of 1 mM SA, an inhibitor of heme biosynthesis, and the catalase activity was measured in cell lysates. We found that SA prevented the increase in catalase activity (Fig. 3).

Catalase is a peroxisomal protein in animal cells, and its importance in the detoxification of ROS has been described previously [13]. In addition, peroxisomes are known to be critical for the function of neurons and the nervous system [14]. Therefore, we examined whether the administration of SA affects the distribution of peroxisomes. Differentiation was induced in the presence or absence of 1 mM SA, and the peroxisomes were stained using an antibody against the peroxisomal protein PMP70. Peroxisomes were abnormally distributed in SA-treated cells. Specifically, we observed many aggregated or dot-like signals in the neurites of SA-treated cells (Supplement 2). Similar changes were also observed in cells treated with the catalase inhibitor 3-aminotriazole (3-AT) (Supplement 3).

Effect of the HO inhibitor zinc protoporphyrin IX (ZnPP IX) on the level of ALAS-1 mRNA in Neuro2a cells

As shown in Fig. 2A, the induction of HO-1 mRNA occurred in the early stage of differentiation (6–12 h) before

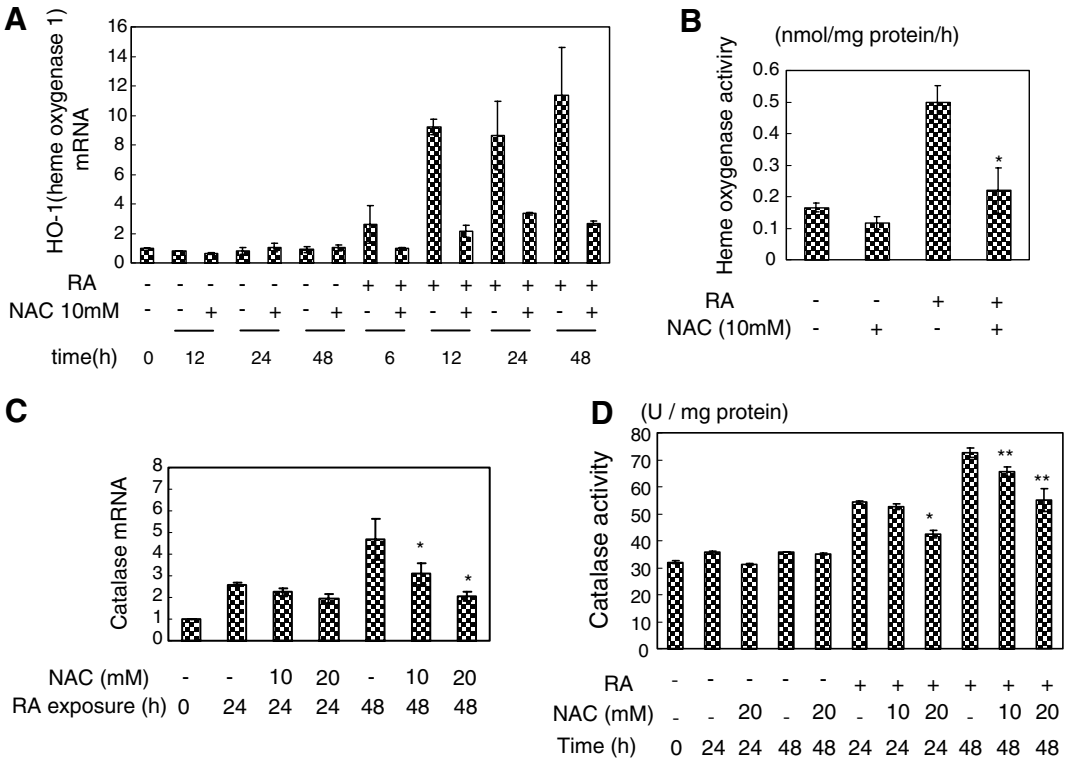

Fig. 2. (A) Changes in the level of HO-1 mRNA during RA-induced differentiation in the presence or absence of NAC. Cells were exposed to RA with or without 10 mM NAC, and HO-1 mRNA levels were measured by quantitative RT-PCR. Signals were normalized by the signal for β-actin mRNA, and the values relative to those at 0 h are presented. (B) HO activity in the presence or absence of NAC. Following a 12 h exposure to RA in the presence or absence of 10 mM NAC, cells were lysed, and HO activity was measured in the cell lysates. Results represent the means ± SD ($n = 3$). The statistical significance was evaluated using Student's t-test ($^{*}P < 0.01$ vs. 12 h with RA). (C) Changes in the level of catalase mRNA during differentiation in the presence or absence of NAC. Differentiation of Neuro2a cells was induced with RA in the presence or absence of NAC (10 and 20 mM). The level of mRNA for catalase was measured by quantitative RT-PCR. Results represent the means ± SD ($n = 3$). $^{*}P < 0.05$ vs. 24 h, $^{**}P < 0.05$ vs. 48 h without NAC. (D) Changes in catalase activity during differentiation in the presence or absence of NAC. Cell lysates were prepared, and catalase activity was measured using Amplex Red. Results represent the means ± SD ($n = 3$). $^{*}P < 0.01$ vs. 24 h, $^{**}P < 0.01$ vs. 48 h without NAC.

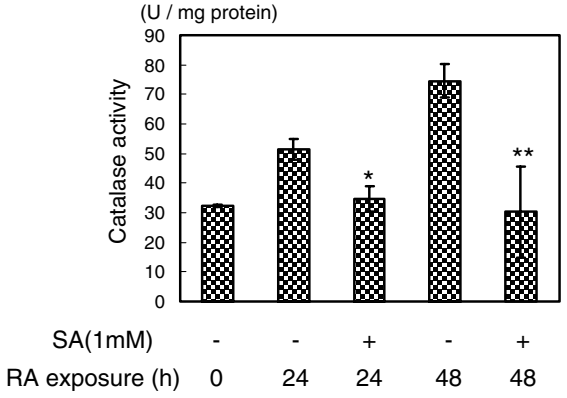

Fig. 3. Effect of SA on the changes in catalase activity. Cells were treated with RA in the presence or absence of 1 mM SA, and catalase activity was measured in cell lysates. Results represent the means ± SD ($n = 3$). $^{*}P < 0.01$ vs. 24 h, $^{**}P < 0.01$ vs. 48 h without SA.

the up-regulation of heme biosynthesis (~48 h) [6], indicating that the HO-1 is a primary target of ROS. To clarify the relationship between the up-regulation of heme biosynthesis and the induction of HO activity, we examined the effect of ZnPP IX, a competitive inhibitor of HO, on the induction of ALAS-1. We found that the increase in ALAS-1 mRNA was significantly reduced by ZnPP IX (Fig. 4A), although the mRNA encoding another heme biosynthetic enzyme, CPG oxidase, was not affected by ZnPP IX (data not shown). ZnPP IX caused a significant reduction in the level of ALAS-1 mRNA at concentrations above 1 μM, which corresponded with the dose-dependence of the effects of ZnPP IX on HO activity (Fig. 4B).

Discussion

In this study, we examined the relationship between ROS and heme metabolism during the differentiation of Neuro2a cells. It has been suggested that heme plays an essential role in the differentiation of neuronal cells [2–4], but the functions of heme are diverse [15] and their significance in neuronal cells has not been fully elucidated. Based on our previous observation that heme biosynthesis is up-regulated during the differentiation of Neuro2a cells [6], our current results suggest that this is related to the activation of anti-oxidative enzymes HO and catalase.

Catalase is one of the major proteins in peroxisomes [16]. Because catalase contains heme as an essential pros-

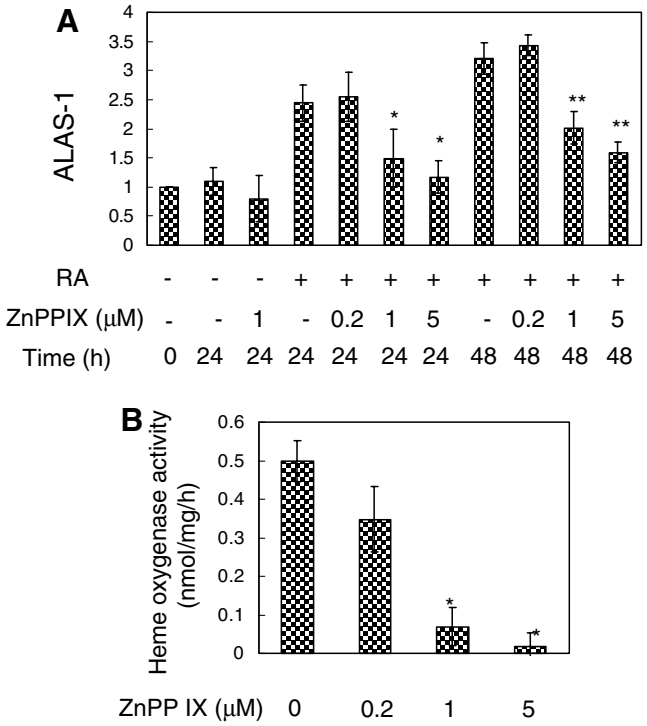

Fig. 4. (A) Effect of ZnPP IX on the level of mRNA for the heme biosynthetic enzyme ALAS-1. Cells were treated with ZnPP IX (0.2, 1, and 5 mM) and the mRNA level for ALAS-1 was measured by quantitative RT-PCR. Results represent the means ± SD ($n = 3$). $^{*}P < 0.01$ vs. 24 h, $^{**}P < 0.01$ vs. 48 h without ZnPP IX. (B) Effect of ZnPP IX on HO activity. Cells were exposed to RA for 12 h in the presence or absence of ZnPP IX, and HO activity was measured in cell lysates. The activity was presented as nmol bilirubin/mg protein/h. Results represent the means ± SD ($n = 3$). $^{*}P < 0.01$ vs. without ZnPP IX.

thetic group, our finding of simultaneous ROS-dependent up-regulation of heme biosynthesis and catalase activity suggests that heme biosynthesis plays an indispensable role in catalase activation. Thus, oxidative damage due to a deficiency in catalase activity could explain the abnormal distribution of peroxisomes observed in SA-treated cells (Fig. 3B). In fact, in the nematode *Caenorhabditis elegans* [13,17], a lack of peroxisomal catalase causes a progeric phenotype and the appearance of aggregated peroxisomes with altered morphologies [13].

Another important finding in this study was that HO-1 is induced by a ROS-dependent mechanism during differentiation. Analysis of the changes in the level of HO-1 mRNA indicated that this enzyme is induced during the very early stage of differentiation and before the induction of catalase and heme biosynthetic enzymes. HO is an anti-oxidative enzyme, and its inducible form, HO-1, is an integral part of the antioxidant system in cells [18] and is up-regulated by a variety of factors, including ROS [11,12]. It is likely that HO-1 induction is the primary target of ROS and that it contributes to the up-regulation of heme biosynthesis, possibly through the consumption of heme [19]. This may help meet demand for heme needed for catalase activation. In fact, the up-regulation of ALAS-1 expression was suppressed by the HO inhibitor ZnPP IX but unaffected by the catalase inhibitor 3-AT (data not shown).

In this report, we investigated the relationship between ROS and heme metabolism during RA-induced differentiation of Neuro2a cells. Previous reports have demonstrated that ROS participate in the differentiation of nonneuronal cells and that NADPH oxidase helps produce ROS [20,21]. Elevation of the level of ROS also seems to be a common phenomenon in neuronal differentiation [7,8], although the source of ROS and the signaling mechanism leading to neuronal differentiation remains to be clarified. RA is essential not only for neuronal differentiation but also for neuronal regeneration [22,23]. Therefore, the findings in this study might also be relevant to neuronal regeneration.

Acknowledgments

This study was supported by a grant-in-aid for scientific research on priority areas, 21st Century COE program and for Creative Scientific Research from the Japanese Ministry of Education, Science, Culture, and Sports (13226015, 13854011, 17209013, 17590368, 18073004, and 18GS0314).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.04.071.

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