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ScienceDirect
Biochemical and Biophysical Research Communications 358 (2007) 130–135
BBRC
www.elsevier.com/locate/ybbrc

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,