Original Article

Involvement of hemoxygenase-1 in di(2-ethylhexyl) phthalate (DEHP)-induced apoptosis of Neuro-2a cells

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ABSTRACT — A widely-used plasticizer di(2-ethylhexyl) phthalate (DEHP) is known to induce apoptosis in neurons, although the mechanisms responsible for DEHP-induced apoptosis is not well explored yet. We recently showed that exposure to DEHP increases the expression of hemoxygenase (HO)-1, an oxidative stress related enzyme, in the mice brain. In this study, we investigated whether HO-1 is involved in DEHP-induced apoptosis using a mouse neuroblastoma cell line Neuro-2a, which forcibly express SCAT3, a fluorescent indicator of caspase-3 activity. The doses of DEHP at 1, 10 or 100 μ M were used in the present study to mimic the level of human exposure to DEHP. Live image analysis of SCAT3-expressing Neuro-2a cells revealed that caspase-3 activity in the cells was significantly increased by DEHP at 100 μ M but not 1 or 10 μ M. We measured HO-1 mRNA level in Neuro-2a cells exposed to DEHP and found significant increase in HO-1 mRNA level by DEHP at 100 μ M but not 1 or 10 μ M. Live image analysis of SCAT3-expressing Neuro-2a cells was further performed to determine the effects of HO-1 siRNA in DEHP-induced apoptosis via caspase-3 activation. We found that knockdown of HO-1 gene nullifies the effects of DEHP to activate caspase-3. These results suggest that HO-1 is involved in DEHP-induced apoptosis. Moreover, this study demonstrates that high-dose DEHP exposure induces caspase-3-dependent apoptosis, which is at least partially mediated by the up-regulation of HO-1 gene, in Neuro-2a cells.

Key words: DEHP, Apoptosis, Hemoxygenase-1, Caspase-3, Neuro-2a, Live imaging

INTRODUCTION

Phthalates are widely used as plasticizers in the production of goods made of polyvinylchloride (PVC) such as food packaging, toys, cosmetics, medical devices, etc. Exposure to phthalates is thought to be increasing as the result of ingestion, inhalation, and dermal contact via plastic-made products. It is therefore raising concerns about the health risks of phthalates. Animal studies have shown that developmental exposure to di(2-ethylhexyl) phthalate (DEHP), a commonly used phthalate plasticizers, causes reproductive tract abnormalities compatible with the disruption of androgen-dependent development and impaired testicular functions in rodents (Klinefelter *et al.*, 2012;

Xi *et al.*, 2011). DEHP is also known to have developmental neurotoxicity, because spontaneous motor activity in young adult rats is increased by neonatal exposure to DEHP (Masuo *et al.*, 2004). Moreover, it was reported that perinatal DEHP exposure impaired motor coordination of adolescent rats (Arcadi *et al.*, 1998) and reduced the number of dopaminergic neurons in the midbrain and brain weight of adolescent mice (Tanida *et al.*, 2009). Epidemiological studies have demonstrated that developmental exposure to phthalate decreases the intelligence of school-age children (Cho *et al.*, 2010) and increases the risk of neuropsychiatric disorders (Testa *et al.*, 2012). Accordingly, it is suspected that phthalate exposure causes detrimental effects on the development of the brain.

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However, little is known about the mechanism underlying DEHP-induced developmental neurotoxicity.

Apoptosis, which can often be seen when cells are exposed to toxic chemicals, is partly considered to be the mechanisms underlying the pathogenesis of diseases including neurodevelopmental and neurodegenerative disorders. It was reported that apoptosis occurs in a mouse neuroblastoma cell line Neuro-2a with exposure to DEHP (Lin *et al.*, 2011) and in neurons differentiated from mouse embryonic stem cells with exposure to mono (2-ethylhexyl) phthalate (MEHP), a metabolite of DEHP (Lim *et al.*, 2009). Moreover these reports showed that DEHP and MEHP stimulate the activity of caspase-3 (Lin *et al.*, 2011; Lim *et al.*, 2009). Caspase-3 is a major executioner protease of apoptotic processes in the central nervous system (Pettmann and Henderson, 1998). An abnormal increase in the number of cells with consequent enlargement of brain, morphogenetic change and premature death occurred in the mice lacking caspase-3 gene (Kuida *et al.*, 1996). These indicate that caspase-3 plays essential roles in brain development and that apoptosis of neurons through activation of caspase-3 normally occurs in developing brain to construct appropriate brain structures with removing unadapted neurons. Taken together, it was suggested that exposure to DEHP and its metabolites during developmental period exert deleterious effects on brain by inducing apoptosis through caspase-3 activation at an abnormal level.

Heme oxygenase 1 (HO-1) is an enzyme that catalyzes the degradation of heme to biliverdin, iron, and carbon monoxide (CO). The expression of HO-1 is induced in response to stress such as oxidative stress, hypoxia, heavy metals, and cytokines (Imuta *et al.*, 2007; Kietzmann *et al.*, 2003; Alam *et al.*, 2000; Elbirt *et al.*, 1998; Hambleton *et al.*, 1996; Mitani *et al.*, 1993). Several lines of evidence have revealed that HO-1 has dual effects under oxidative stress (Suttner and Dennery, 1999; Suttner *et al.*, 1999; da Silva *et al.*, 1996). It was reported that induction of HO-1 protects cells against oxidative injury through antioxidant activities of bilirubin (Ryter *et al.*, 2007; Otterbein *et al.*, 1995). In contrast, HO-1 is also known to mediate adverse effects of oxidative stress, because there are reports showing that overexpression of HO-1 increase the cellular concentration of reactive iron, followed by induction of apoptosis in neurons and fibroblasts (Suttner and Dennery, 1999; Hsieh *et al.*, 2008a). Thus, HO-1 has cytoprotective and anti-inflammatory actions to protect cells against oxidative stress and cytotoxic actions killing cells by apoptosis, although it remains to determine the mechanisms responsible for switching HO-1 actions.

We recently reported that DEHP exposure increases the expression of HO-1 in the brain of mice (Win-Shwe *et al.*, 2013). This suggests that HO-1 is related to DEHP-induced neurotoxicity. However, it is not clear how HO-1 is involved in DEHP-induced neurotoxicity, because HO-1 has not only cytoprotective and anti-inflammatory actions but also cytotoxic actions. In the present study, we aimed to determine the role of HO-1 in DEHP-induced apoptosis using Neuro-2a cells, a mouse neuroblastoma cell line. We previously showed that live imaging of *in vitro* cultured cells is a useful tool for toxic evaluation of environmental chemicals (Aung *et al.*, 2013; Koike-Kuroda *et al.*, 2010). First, we therefore monitored Neuro-2a cells expressing SCAT3, a fluorescent indicator of caspase-3 activity, by live imaging and examined the effects of DEHP in caspase-3-dependent apoptosis. Next we measured the mRNA levels of HO-1 in Neuro-2a cells exposed to DEHP to examine whether HO-1 gene expression in neuronal cells is changed by DEHP exposure. Finally, we performed live imaging analysis of SCAT3-expressing Neuro-2a cells to investigate the effects of knockdown of HO-1 gene by its siRNA in DEHP-induced apoptosis.

MATERIALS AND METHODS

Cell culture and reagent preparation

Neuro-2a cells were cultured in minimum essential medium (MEM; Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 0.1 mM non-essential amino acid (NEAA; Gibco-Invitrogen) and 10% fetal bovine serum (FBS; Gibco-Invitrogen), hereafter referred to as the cell maintenance medium. Cells were passaged every 2-3 days, and a passage number of 7-20 was used for all the experiments.

For each experiment, Neuro-2a cells were cultured in a medium containing MEM (Gibco-Invitrogen) supplemented with 0.1 mM NEAA (Gibco-Invitrogen), 1% FBS (Gibco-Invitrogen), and 0.3 mM N6, 2'-O-dibutyl-adenosine cyclic monophosphate sodium salt (dibutyl-adenosine cyclic AMP; Sigma-Aldrich, St. Louis, MO, USA), hereafter referred to as the experimental medium. Neuro-2a cells cultured with the experimental medium were simultaneously exposed to DEHP at a concentration of 0, 1, 10, or 100 μ M. The doses of DEHP used in this study are relevant to the environmental level of DEHP exposed to human. According to the study of Latini *et al.*, 2003, the concentration of DEHP in the blood of human umbilical cord is approximately 1 μ g/ml that is equivalent to a concentration of 2.5 μ M. Moreover, neonatal exchange transfusion with PVC-made catheters could increase the blood level of DEHP to the range from 13.2 to

84.9 $\mu\text{g/ml}$ (approximately 30 to 200 μM) (Kavlock *et al.*, 2002). We therefore considered the doses of DEHP at 1, 10, and 100 μM used in this study as low-, moderate- and high-dose of DEHP exposure, respectively. Because of the poor solubility of DEHP in water, DEHP was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mM. DEHP dissolved in DMSO was then diluted with the experimental medium to yield the above-mentioned doses. The medium used for the control group only contained DMSO, and the final concentration of DMSO in all experimental groups was set at 0.1%.

Cell viability assay

The effects of DEHP on the viability of Neuro-2a cells were assessed using a colorimetric assay based on the reduction of tetrazolium salt to formazan by mitochondrial dehydrogenase activity. Neuro-2a cells were seeded on 96-well plates (1×10^4 cells/well), which were pre-coated with 0.25% polyethyleneimine (Sigma-Aldrich), and cultured in 100 μl of cell maintenance media. The plate was then kept in an incubator overnight at 37°C and in an atmosphere of 5% CO_2 . On the next day, the cell maintenance medium was changed to fresh experimental medium containing DEHP at a concentration of 0, 1, 10, or 100 μM . Cell viability was assessed at 24 hr after the initiation of DEHP by the addition of 10 μl of tetrazolium reagent WST-1 (Roche Applied Bioscience, Indianapolis, IN, USA) to each well of the culture plate followed by incubation for 3 hr at 37°C and 5% CO_2 . Absorbance at 450 nm was then measured using a reference wavelength of 650 nm. The absorbance at 650 nm was subtracted from the absorbance at 450 nm, and this value was then expressed as a percentage of the value obtained for Neuro-2a cells from the control group, the viability of which was set at 100%. Each experimental group was performed in triplicate for each experiment, and the data was obtained from four independent experiments.

DNA transfection and live imaging analysis

Neuro-2a cells ($2\text{--}4 \times 10^4$ cells/well) were seeded onto an 8-well chambered glass coated with 0.25% polyethyleneimine (Sigma-Aldrich) and cultured with 300 μl of cell maintenance medium. The cells were then transfected with pcDNA SCAT3 vector, provided by Dr. M. Miura (The University of Tokyo, Tokyo, Japan), using FuGENE HD transfection reagents (Promega, Madison, WI, USA). SCAT3 contains a caspase-3 cleavage peptide fused to enhanced cyan fluorescence protein (ECFP) and Venus, an altered green fluorescence protein, and the life and death of cell can be monitored by measuring the fluorescent intensities of ECFP and Venus (Takemoto *et al.*, 2003).

According to the manufacturer's protocol, we transfected Neuro-2a cells with 0.25 μg of pcDNA-SCAT3 in 300 μl of cell maintenance medium in an atmosphere containing 5% CO_2 at 37°C for 8 hr. After transfection, Neuro-2a cells were washed with phosphate-buffered saline to remove the transfection reagent and were kept in 300 μl of cell maintenance medium in an atmosphere containing 5% CO_2 at 37°C until the start of live imaging.

One or two days after transfection, SCAT3-expressing Neuro-2a cells were cultured in the experimental medium with or without DEHP (0, 1, 10, or 100 μM). One hour after the initiation of DEHP exposure, live imaging of SCAT3-expressing Neuro-2a cells was started using a fluorescence time-lapse microscope (BioRevo9000; Keyence Co., Osaka, Japan). SCAT3-expressing Neuro-2a cells were placed in an incubation chamber assembled within the microscope (Microscope Incubation System INU-KI-F1; Tokai Hit, Shizuoka, Japan), in which the temperature and gas concentration were controlled at 37°C and 5% CO_2 , respectively. A 440AF21 excitation filter, a 455DRLP dichroic mirror, and two emission filters (480AF30 for ECFP and 535AF25 for Venus; Opto Science Inc., Tokyo, Japan) were used for fluorescent time-lapse imaging. Images of the SCAT3-expressing Neuro-2a cells were captured using an objective lens (Plan Fluor ELWD DM 20xC, NA 0.45; Nikon, Tokyo, Japan) and a CCD camera attached to the fluorescent microscope every 30 min within a 24-hr period. Four independent live imaging experiments were performed in this study.

To examine the effect of DEHP exposure on apoptosis in SCAT3-expressing Neuro-2a cells, the fluorescence intensity of ECFP and Venus emissions was measured using digital image data, as previously described (Aung *et al.*, 2013; Koike-Kuroda *et al.*, 2010). Briefly, the colors representing ECFP (cyan blue) and Venus (yellow green) in the digital images of the SCAT3-expressing Neuro-2a cells were converted into monochromatic color images of red and green, respectively. These images were merged at each time point to measure the fluorescence intensities of ECFP and Venus simultaneously. After the brightness of the red color for ECFP and the green color for Venus was measured, the ECFP/Venus emission ratio was calculated by dividing the brightness value of the red color by that of the green color. The calculated values for each time point were calibrated with the ratio at the same time in the control group, which was set at a value of 1.

In the experiment for evaluating the effects of HO-1 gene knockdown in DEHP-induced apoptosis, Neuro-2a cells were transfected with the SCAT3 expression vector using the above-mentioned method and were subse-

quently transfected with rodent HO-1 siRNA (5'-GCC GAG AAU GCU GAG UUC A-3') (Hsieh *et al.*, 2008b) or control nucleotide sequences (control siRNA: 5'-UAA GGC UAU GAA GAG AUA C-3') (Yan *et al.*, 2007). siRNA transfection was performed using Lipofectamine RNAiMAX reagent (Gibco-Invitrogen) according to the manufacturer's protocol. Two or three days after siRNA transfection, Neuro-2a cells were exposed to DEHP (0 or 100 μ M), and live imaging analyses were then performed using the above-mentioned procedures.

Quantification of specific-gene expression

The effects of DEHP on the mRNA level of HO-1 and a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured by a real-time PCR analysis. The mRNA level of GAPDH was used to normalize HO-1 gene expression. The specific primer sets for HO-1 (forward primer: 5'-CAGATGGCGTCACTTCGT-3'; reverse primer: 5'-ATTCCCA CTGCC ACTGTT-3') and GAPDH (forward primer: 5'-CACTGCCACCCA-GAAGA-3'; reverse primer: 5'-TCCACGA CGGACA-CATT-3') were designed with LightCycler Probe Design Software 2.0 (Roche Diagnostics, Mannheim, Germany) using the mRNA sequences downloaded from the genetic sequence database at the National Center for Biotechnical Information (NCBI) Genbank to obtain a melting temperature of $\approx 60^{\circ}\text{C}$, a GC content of $\approx 50\%$, and amplicon lengths from 150 to 200 bp.

Neuro-2a cells seeded on 0.25% polyethyleneimine-coated 12-well plates (10^5 cells/well) were grown in the cell maintenance medium in an atmosphere of 5% CO_2 at 37°C overnight. Neuro-2a cells were then exposed to DEHP (0, 1, 10, and 100 μ M) for 24 hr in 1 ml of the experimental medium. Afterwards, the total RNA was extracted and purified using an RNeasy Mini kit (Qiagen, Valencia, CA, USA) and was reverse transcribed into cDNA using the PrimescriptTM RT Reagent kit (TaKaRa Bio, Inc., Otsu, Japan). For each sample, the total RNA (500 ng) was reverse transcribed to first-strand cDNA in a final volume of 20 μ l of PrimeScript buffer containing PrimeScript RT Enzyme Mix I (1 μ l), random hexamers (100 pmol), and oligo dT primer (50 pmol) for 15 min at 37°C . The reaction was terminated by incubation at 85°C for 5 sec. The reaction mixtures containing cDNA were stored at -80°C until the real-time PCR analysis.

To determine the efficiency of HO-1 siRNA for knock-down of HO-1 gene, we measured the mRNA level of HO-1 after Neuro-2a cells were transfected with HO-1 siRNA. Neuro-2a cells seeded on 0.25% polyethyleneimine-coated 12-well plates (10^5 cells/well) were trans-

fected with 10 nM of HO-1 siRNA or control siRNA, and total RNA isolation was performed at 2, 3, 4 days after transfection. Total RNA extraction from the cells and cDNA synthesis were performed using the same procedures described above.

The products of the reverse transcription were used to determine the mRNA levels of HO-1 and GAPDH using Light Cycler 1.5 (Roche Diagnostics). To prepare the standard samples for each gene transcript, an equal amount of each cDNA sample was combined and then serially diluted with EASY Dilution (TaKaRa Bio). The cDNA samples were diluted 10-fold in DNase- and RNase-free water. Two microliters of the standards and diluted unknown samples were amplified in a 20- μ l reaction mixture containing 200 nM of each primer and 10 μ l of $2 \times$ SYBR Premix Ex Taq (TaKaRa Bio). The conditions for the real-time PCR were an initial step to activate Taq polymerase for 10 sec at 95°C , followed by 50 cycles at 95°C for 5 sec for denaturation and 60°C for 20 sec for annealing and extension, with a temperature transition rate of $20^{\circ}\text{C}/\text{sec}$. After real-time PCR, a melting curve analysis was performed to confirm the specificity of the PCR products. The melting curve for the PCR products targeting each gene transcript showed a single peak (data not shown).

The amount of mRNAs for HO-1 gene in each sample was normalized by dividing each value with the amount of GAPDH mRNA in the same sample. The normalized mRNA levels of HO-1 were then expressed relative to that of the control (0 μ M DEHP) or intact group without transfection of siRNA, which were set at 100% for each experiment. The percentage was averaged from six or three independent experiments.

Statistical analyses

A two-way factorial analysis of variance (ANOVA) for repeated measures was used to examine the effects of DEHP exposure on the temporal changes in the ECFP/Venus emission ratio among the groups. A one-way ANOVA was performed to determine the differences in the mean of the ECFP/Venus emission ratio and mRNA expression level of HO-1 among groups. When significant overall effects were detected using a one-way ANOVA, the Fisher protected least significant difference test was performed as a post-hoc analysis. In the post hoc analysis, a probability value of $p < 0.05$ was considered significant.

RESULTS

Effects of DEHP on cell viability of Neuro-2a cells

Neuro-2a cells were exposed to various concentrations of DEHP (0, 1, 10, or 100 μM) for 24 hr and cell viability was assessed using a WST-1 assay (Fig. 1). We found that the viability of Neuro-2a cells was significantly decreased by a high-dose DEHP at 100 μM ($p < 0.01$), compared with that of control group (0 μM). However, the cell viability of DEHP-exposed groups at lower doses (1 and 10 μM) did not significantly differ from that of the control group.

Effects of DEHP in SCAT3-expressing Neuro-2a cells

Live imaging analysis resulted in that the morphology and fluorescence intensities of ECFP and Venus of SCAT3-expressing Neuro-2a cells were altered over time by DEHP exposure at a dose of 100 μM , but not at 0, 1, or 10 μM (Fig. 2; Supplementary material: movies 1-4). In Neuro-2a cells exposed to 100 μM of DEHP, cells exhibiting a shrunken morphology and apoptotic body forma-

tion were observed with time after DEHP exposure was started (Fig. 2D). In addition, the fluorescence intensity of ECFP was increased and that of Venus was decreased with time after DEHP exposure, and the signals of both fluorescence proteins were then extremely decreased until the end of live imaging. The temporal pattern of ECFP/Venus emission ratio, which was useful end point to evaluate the effects of chemicals in apoptosis via caspase-3 activation (Koike-Kuroda *et al.*, 2010), in Neuro-2a cells exposed to DEHP at 100 μM was increased with time compared to Neuro-2a cells of the control group, but not in Neuro-2a cells exposed to lower doses of DEHP (Fig. 3A). A two-way ANOVA for repeated measures indicated that the pattern of the ECFP/Venus emission ratio significantly differed among groups ($F_{3,564} = 94.09$, $p < 0.001$) and with time ($F_{46,564} = 2.56$, $p < 0.001$). The interactive effect of the main factors, groups and time, was also significant ($F_{138,564} = 2.19$, $p < 0.01$).

A one-way ANOVA indicated that there was no significant difference in the mean of ECFP/Venus emission ratio during 1.5-8 hr and 8.5-16 hr after initiation of DEHP exposure (Fig. 3B). However, the mean of the ratio during 16.5-24 hr after DEHP exposure differed significantly among groups ($F_{3,12} = 7.01$, $p < 0.05$). A post-hoc analysis showed that the mean of ECFP/Venus emission ratio during 16.5-24 hr after DEHP exposure was significantly increased by high-dose DEHP at 100 μM , but not by lower doses of DEHP at 1 or 10 μM , compared to the control group (Fig. 3B).

Effects of DEHP in HO-1 gene expression of Neuro-2a cells

Live imaging analysis showed that DEHP increased the ECFP/Venus emission of SCAT3-expressing Neuro-2a cells until 24 hr after DEHP exposure was initiated. We therefore measured the mRNA levels of HO-1 in Neuro-2a cells 24 hr after DEHP exposure (Fig. 4). A one-way ANOVA indicated that there was significant difference in the mRNA level of HO-1 among groups ($F_{3,20} = 11.63$, $p < 0.01$). A post-hoc analysis showed that the mRNA level of HO-1 in Neuro-2a cells exposed to DEHP at 100 μM was significantly higher than that of the control group. However, no significant increase of the HO-1 mRNA levels in Neuro-2a cells exposed to DEHP at 1 or 10 μM was observed compared to that of the control group.

Effect of HO-1 gene knockdown in DEHP-exposed Neuro-2a cells

To confirm the efficiency of HO-1 siRNA for gene knockdown, we measured the mRNA level of HO-1 in Neuro-2a cells at 2 to 4 days after cells were transfected

Fig. 1. Effects of DEHP on the viability of Neuro-2a cells. Neuro-2a cells were exposed to the indicated concentration of DEHP for 24 hr. The cell viability was expressed relative to the viability of the control group (0 μM DEHP), the value of which was set at 100%. Values are the means $\pm$ S.E.M. of four independent experiments. * $p < 0.01$ vs. control group.

Fig. 2. Time-lapse images of SCAT3-expressing Neuro-2a cells, which were captured using transmitted light (TL), emissions of Venus and ECFP, and images of Venus and ECFP were merged after the original fluorescent colors were converted into pseudo colors (Venus: yellow-green to green; ECFP: cyan blue to red). Digital images were obtained from SCAT3-expressing Neuro-2a cells exposed to DEHP at concentrations of 0 μM (A), 1 μM (B), 10 μM (C), or 100 μM (D) from 1 hr to 24 hr after initiation of DEHP exposure. Scale bars indicate 20 μm .

ed with HO-1 siRNA. The mRNA level of HO-1 was significantly reduced when Neuro-2a cells were transfected with HO-1 siRNA and cultured for 2, 3, or 4 days after transfection (Fig. 5). On the other hand, there was no significant effect of control siRNA in the HO-1 mRNA level. The HO-1 mRNA level in Neuro-2 cells transfected with HO-1 siRNA was approximately 40% of Neuro-2a cells without siRNA transfection (intact group), and the reduced level was not changed with days after transfection. Accordingly, Neuro-2a cells were kept for 2 or 3 days after transfection with HO-1 siRNA and then used for live imaging analysis to examine the effects of HO-1 gene knockdown in DHEP-induced apoptosis. Live imaging analysis were carried out using 100 μM of DEHP, because ECFP/Venus emission in SCAT3-expressing Neuro-2a cells was significantly increased by exposure to DEHP at 100 μM but not at 1 or 10 μM (Fig. 3).

The morphology and fluorescent intensities of ECFP

and Venus were not drastically changed with time in SCAT-3 expressing Neuro-2a cells, which were transfected with control siRNA or HO-1 siRNA and were not exposed to DEHP (Fig. 6A and B; Supplementary material: movies 5 and 6). However, in SCAT3-expressing Neuro-2a cells transfected with control siRNA, the morphology and fluorescence intensities of ECFP and Venus of the cells were altered with time after DEHP exposure (Fig. 6C; Supplementary material: movie 7). The shrinkage of cells and apoptotic body formation were observed in the group of SCAT3-expressing and control siRNA-transfected Neuro-2a cells with time after DEHP exposure. The fluorescence intensity of ECFP was increased and that of Venus was decreased with time after DEHP exposure, and the signals of ECFP and Venus were drastically reduced until the end of live imaging. On the other hand, the morphology and fluorescent signal intensities of ECFP and Venus did not change with DEHP exposure,

Fig. 3. Effects of DEHP on apoptosis of SCAT3-expressing Neuro-2a cells. (A) Temporal changes in the ECFP/Venus emission ratio of four different groups of DEHP exposure (0, 1, 10, or 100 μ M). The ratio at each time point was calibrated with the ratio at the same time in the control group, which was set at 1. (B) The means of the ECFP/Venus emission ratio calculated for 1.5-8 hr, 8.5-16 hr, or 16.5-24 hr after initiation of DEHP exposure. The values were the mean $\pm$ S.E.M. of four independent experiments. * $p < 0.05$ vs. control group.

Fig. 4. Effects of DEHP on the mRNA level of hemeoxygenase-1 (HO-1) in Neuro-2a cells. Cells were exposed to DEHP (0, 1, 10, or 100 μ M) for 24 hr. The mRNA levels of HO-1 were normalized by dividing them with the mRNA level of GAPDH. The obtained values are presented as the percent of the value in the control. Values are the means $\pm$ S.E.M. of six independent experiments. * $p < 0.05$ vs. control group (0 μ M DEHP).

Fig. 5. Effects of HO-1 siRNA on the mRNA level of HO-1 in Neuro-2a cells. Cells were transfected with 10 μ M of control siRNA or HO-1 siRNA and were cultured for 2, 3, or 4 days. The mRNA level of HO-1 was normalized to the mRNA level of GAPDH and was presented as the percent of the value in the intact group, which was not transfected with any siRNA. Values are the means $\pm$ S.E.M. of three independent experiments. * $p < 0.05$ vs. intact group.

Fig. 6. Time-lapse images of SCAT3-expressing Neuro-2a cells, which were captured using transmitted light (TL), emissions of Venus and ECFP, and images of Venus and ECFP were merged after the original fluorescent colors were converted into pseudo colors (Venus: yellow-green to green; ECFP: cyan blue to red). Digital images were obtained from SCAT3-expressing Neuro-2a cells pre-treated with control siRNA or HO-1 siRNA, which were exposed to DEHP at a concentration of 0 μ M (A and B) or 100 μ M (C and D) for 24 hr. Scale bars indicate 20 μ m.

when SCAT3-expressing Neuro-2a cells were transfected with HO-1 siRNA (Fig. 6D; Supplementary material: movie 8).

A two-way ANOVA for repeated measures indicated that the temporal pattern of the ECFP/Venus emission ratio of SCAT3-expressing Neuro-2a cell significantly differed among groups with or without DEHP exposure and HO-1 siRNA transfection ($F_{3,564} = 138.92$, $p < 0.01$). The pattern of ECFP/Venus emission ratio of SCAT3-expressing Neuro-2a cell transfected with control siRNA was increased with time by DEHP exposure (Fig. 7A). However, the ratio of SCAT3-expressing Neuro-2a cells transfected with HO-1 siRNA was not changed with time by DEHP exposure.

There was no significant difference in the mean of ECFP/Venus emission ratio during 1.5-8 hr after DEHP exposure (Fig. 7B). However, significant differences among experimental groups were found in the mean of

ECFP/Venus emission ratio of SCAT3-expressing Neuro-2a cells during 8.5-16 hr ($F_{3,12} = 5.41$, $p < 0.05$) and 16.5-24 hr ($F_{3,12} = 5.87$, $p < 0.05$) after DHEP exposure. The mean values of SCAT3-expressing Neuro-2a cells, which were transfected with control siRNA, were significantly increased by DEHP during 8.5-16 hr and 16.5-24 hr after DHEP exposure. However, in SCAT3-expressing and HO-1 siRNA-transfected Neuro-2a cells, DEHP did not increase the mean of ECFP/Venus emission ratio.

DISCUSSION

It has been demonstrated that DEHP exhibits developmental neurotoxicity (Testa *et al.*, 2012; Cho *et al.*, 2010; Masuo *et al.*, 2004), although the mechanisms, by which DEHP adversely affects the developing brain, are not well understood. DEHP are also detected in the blood of umbilical cord of newborns (Latini *et al.*, 2003), indicat-

Fig. 7. Effects of HO-1 gene knockdown on DEHP-induced apoptosis in SCAT3-expressing Neuro-2a cells. (A) Temporal changes in the ECFP/Venus emission ratio of SCAT3-expressing Neuro-2a cells. The ratio at each time point was calibrated with the ratio at the same time point in the group treated with control siRNA and 0 μ M DEHP, which was set at 1. (B) The mean of ECFP/Venus emission ratio of SCAT3-expressing Neuro-2a cells transfected with control siRNA or HO-1 siRNA for 1.5-8 hr, 8.5-16 hr, or 16.5-24 hr after initiation of DEHP exposure (0 or 100 μ M). Values (mean $\pm$ S.E.M. of four independent experiments) with difference characters were significantly ($p < 0.05$) different from each other of the same time.

ing that DEHP can pass through the placenta and enter the body of fetuses. Newborns who had contact with plastic medical devices containing DEHP are exposed to the chemical at higher levels, because significantly higher concentrations of DEHP metabolites are detected in the urine samples (Koch *et al.*, 2006). These indicate the risk of DEHP exposure affecting the brain of fetuses and infants. DEHP may directly affect neurons of the developing brain, and then neurons exposed to DEHP may die from apoptosis, because DEHP and its metabolites MEHP activate caspase-3 and induce apoptosis in cultured neuronal cells (Lin *et al.*, 2011; Lim *et al.*, 2009).

Recently, DEHP at a concentration of 40-80 μ M, but not at a concentration of 0.1-10 μ M, was reported to decrease the cell viability and to induce apoptosis in Neuro-2a cells (Lin *et al.*, 2011). In the present study, we showed a decrease in the cell viability of Neuro-2a cells in response to DEHP exposure at a dose of 100 μ M and confirmed that the viability of Neuro-2a cells was not changed by DEHP exposure at doses less than 10 μ M. We next performed live imaging of SCAT3-expressing Neuro-2a cells to evaluate DEHP neurotoxicity and to determine the mode of toxic action of DEHP. SCAT3 is an indicator having caspase-3 cleavage sequence between two fluorescent proteins ECFP and Venus (Takemoto *et al.*, 2003). The usefulness of FRET technology using SCAT3 for monitoring caspase-3 activation in living cells has been well documented (Takemoto *et al.*, 2007; Kuranaga *et al.*, 2006; Kanuka *et al.*, 2005; Takemoto *et al.*, 2003). In addition, we previously showed that live imaging analysis of SCAT3-expressing cultured cells enable screening of the cytotoxicity of chemicals within a short time and with high accuracy compared to other *in vitro* method for testing cell viability and/or cell death (Koike-Kuroda *et al.*, 2010). In the results of our present study, the morphology of SCAT3-expressing Neuro-2a cells and the ECFP/Venus emission ratio indicating the activity of caspase-3 were not changed until 12 hr after exposure to DEHP at any dose was started. However, the temporal pattern of ECFP/Venus emission ratio was started to increase significantly by 100 μ M DEHP, but not at 1 and 10 μ M, at 15 hr after initiation of DEHP, although the significant increase in the mean of the ECFP/Venus emission ratio was only detected during 16.5 and 24 hr after cells were exposed to 100 μ M of DEHP. Moreover, apoptotic morphological changes such as shrinkage of the cells and apoptotic body formation were observed with time, especially started from 15 hr after DEHP exposure at 100 μ M. These results suggest that the initiation of apoptosis via caspase-3 activation was started at 15 hr after DEHP exposure. At the end of live imaging, the flu-

orescent intensities of both ECFP and Venus were drastically decreased in Neuro-2a cells exposed to 100 μ M of DEHP. We previously reported that the fluorescent intensities of ECFP and Venus were decreased after increasing the ECFP/Venus emission ratio in PC12 cells, when the cell showed abnormal morphology by exposure to sodium arsenite (Koike-Kuroda *et al.*, 2010). It is possible that such changes in the fluorescent signals of SCAT3 are due to degradation of SCAT3 proteins accompanied with apoptotic cell death, although it remains to be elucidated. Taken together, the present study showed that exposure to high-dose DEHP at 100 μ M, but not at lower doses less than 10 μ M, stimulates the activity of caspase-3 in Neuro-2a cells within 24 hr after exposure, followed by death of cell until 24 hr after exposure. A report has demonstrated that caspase-3 activity in Neuro-2a cells is stimulated by 48-hr exposure to DEHP at 40 or 80 μ M but not at less than 10 μ M (Lin *et al.*, 2011). The results of our present study support the data of this report and suggest that live imaging of SCAT3-expressing cells can detect the significant effect of DEHP in the activity of caspase-3 with shorter period. Moreover, our study showed that only high-dose DEHP exposure reduced the cell viability and induced apoptosis in Neuro-2a cells, which is consistent and supports our previous *in vivo* study in which only high level of DEHP induces a neuroinflammatory response in the hypothalamus of mice (Win-Shwe *et al.*, 2013).

HO-1 is known to be an oxidative stress marker that responds to pathological stimulation, including toxin exposure. For example, the expression of HO-1 in the brain and *in vitro* cultured neuronal cells is induced by sodium arsenite (Imuta *et al.*, 2007; Kietzmann *et al.*, 2003; Elbirt *et al.*, 1998), cadmium (Alam *et al.*, 2000), and lipopolysaccharides (Hambleton *et al.*, 1996; Han *et al.*, 1994). In addition, we recently reported that DEHP exposure increases the mRNA level of HO-1 in the hypothalamus of ovalbumin-immunized allergic mice (Win-Shwe *et al.*, 2013). DEHP is known to decrease the cellular levels of antioxidants (Manojkumar *et al.*, 1998) and increases lipid peroxidation (Santhosh *et al.*, 1998). Taken together, it is presumable that DEHP exposure elicits oxidative stress with inducing HO-1 expression. Several lines of evidence indicate that HO-1 has dual effects under oxidative stress (Suttner and Dennery, 1999; Suttner *et al.*, 1999; da Silva *et al.*, 1996). It has been well demonstrated that HO-1 has cytoprotective and anti-inflammatory actions in a variety of tissues and cells (Ryter *et al.*, 2007; Grosser *et al.*, 2003; Otterbein *et al.*, 1995). On the other hand, it is also known that HO-1 mediates adverse effects of oxidative stress. Accumu-

lation of free iron that is produced by HO-1 is associated with induction of apoptosis via caspase-3 activation in neurons (Hsieh *et al.*, 2008a, 2008b). In the present study, we found that high-dose DEHP exposure significantly increased the mRNA level of HO-1 in Neuro-2a cells. It has been demonstrated that DEHP activates caspase-3 and induced caspase-3-dependent apoptosis in Neuro-2 cells (Lin *et al.*, 2011). In this context, it raises the possibility that HO-1, the expression of which is induced by DEHP, is involved in the mechanisms responsible for DEHP-induced apoptosis. To evaluate this possibility, we further performed live imaging analysis of SCAT3-expressing Neuro-2a cells and examined the effects of HO-1 gene knockdown in DEHP-induced apoptosis. As the results of analysis, shrinkage of cells and apoptotic body formation were found and the ECFP/Venus emission ratio, an indicator of enzymatic activity of caspase-3, was increased with time after SCAT3-expressing Neuro-2a cells were exposed to DEHP without HO-1 siRNA treatment. This result again indicates that DEHP activates the enzymatic activity of caspase-3, followed by induction of apoptosis in Neuro-2a cells. On the other hand, when Neuro-2a cells were treated with HO-1 siRNA to reduce the expression level of HO-1 before DEHP exposure was started, such changes in the morphology and indicator of caspase-3 activity were not observed. These results indicate that HO-1 mediates the toxic actions of DEHP to activate caspase-3 and that knockdown of the HO-1 gene can protect neuronal cells against DEHP-induced apoptosis.

The data in this study from monitoring of apoptotic cell death of SCAT3-expressing Neuro-2a cells showed that the mean ECFP/Venus emission ratio was significantly increased by 100 μ M DEHP at 16.5-24 hr after DEHP exposure, although the temporal pattern of ECFP/Venus emission ratio in the DEHP (100 μ M)-exposed group was started to at 12 hr after initiation of exposure. However, in the experiments with siRNA treatment, the temporal pattern of ECFP/Venus emission ratio tended to increase early by DEHP exposure, and the significant effect DEHP on the mean ECFP/Venus emission ratio was detected at 8.5-16 hr and 16.5-24 hr after initiation of DEHP exposure. Possible explanation for the observed difference in the results is the different experimental condition. In the experiments of siRNA treatments, we carried out repeated transfection to Neuro-2a cells with SCAT3 and siRNA, while only one transfection procedure for SCAT3 was done in the first experiment. The repeated transfection procedures with different transfection reagents may induce the changes in the cell viability of Neuro-2a cells. Moreover, the cytotoxicity of transfection reagent and siRNA itself should also be considered in this con-

text because we maintained Neuro-2a cells in the medium containing the transfection complex for 2 days to achieve the sufficient suppression of the target gene. Studies have shown that siRNA treatment can generate the measurable changes in cellular function and viability in a target-independent manner (Fedorov *et al.*, 2006; Lin *et al.*, 2005). These results indicate that differences in the experimental condition may produce the different sensitivities of cells to toxic chemicals.

It has recently been demonstrated that DEHP increases the production of reactive oxygen species (ROS), a biomarker of oxidative stress, in primary neurons and astrocytes (Wu *et al.*, 2013). This suggests a possibility that oxidative stress-mediated signaling pathways appear to be involved to some extent in the upregulation of HO-1 expression in DEHP-induced neurotoxicity. In fact, the increased ROS production is reportedly known to induce HO-1 expression through activation of nuclear-factor-E2-related factor-2 (Nrf-2), a transcriptional factor, in culture retinal neurons (Koriyama *et al.*, 2013). Moreover, activation of Nrf-2 in combination with activating Peroxisome proliferator-activated receptor- γ (PPAR γ), a ligand-activated transcription factor, is known to contribute the induction of HO-1 expression under oxidative stress condition (Kang *et al.*, 2005). Several studies revealed that PPAR γ increases the expression of HO-1 in human hepatocytes (Wang *et al.*, 2011), human vascular endothelial and smooth muscle cells (Krönke *et al.*, 2007), and rat motor neurons (Park *et al.*, 2007). Analysis of the human HO-1 promoter indicates that HO-1 is a direct PPAR-target gene, the transcription of which is regulated by PPAR α and PPAR γ via PPAR-responsive elements (Krönke *et al.*, 2007). These studies indicate that HO-1 is transcriptionally regulated by PPARs especially by PPAR γ . Interestingly, DEHP exposure stimulates the induction of PPAR γ expression in the testes of rats (Ryu *et al.*, 2007) and Neuro-2a cells (Lin *et al.*, 2011). Furthermore, cleavage of pro-caspase-3 for activating caspase-3 are caused by DEHP exposure in Neuro-2a cells, and this cleavage occurring with DEHP exposure is suppressed by a PPAR γ antagonist and enhanced by a PPAR γ agonist (Lin *et al.*, 2011), suggesting that PPAR γ mediates the pro-apoptotic actions of DEHP. Taking this evidence together, the mechanisms of DEHP-induced apoptosis in Neuro-2a cells can be speculated that DEHP firstly activates transcriptional factors such as Nrf-2 and PPAR γ , followed by induction of the transcription of target gene including HO-1, and HO-1 then activates caspase-3 and induces apoptosis in Neuro-2a cells. Thus, HO-1 could be a downstream molecule of transcription factors Nrf-2 and PPAR γ in the signal cascade of DEHP-induced apoptosis. HO-1 cata-

lyzes the degradation of heme to biliverdin, iron, and CO. Accumulation of free iron and CO produced by HO-1 in astrocytes and neurons causes intracellular oxidative stress and mitochondrial damage, which appears to contribute the pathogenesis of neurodegenerative diseases (Justicia *et al.*, 2008; Schipper *et al.*, 2000, 1998). One report showed that increased formation of iron by HO-1 is followed by activation of caspase-3 and induction of apoptosis (Hsieh *et al.*, 2008a). Hence, intercellular free iron that is excessively produced by HO-1 may be partially involved in the downstream cascade of DEHP-induced apoptosis, although further studies are needed to clarify it.

In conclusion, we investigated the effects of DEHP exposure on apoptosis and clarified the role of HO-1 in DEHP-induced apoptosis of Neuro-2a cells. The present study demonstrated that high-dose of DEHP exposure induced the neurotoxic effects in neurons. We found that the expression of HO-1 in Neuro-2a cells was significantly induced by high-dose DEHP. Live imaging analysis of Neuro-2a cells confirmed that high-dose DEHP significantly activates the enzymatic activity of caspase-3, followed by induction of apoptotic cell death in Neuro-2a cells. These results suggest raising the awareness of DEHP usage such as neonatal exchange transfusion with PVC-made catheters that can cause high-level exposure to DEHP. In this study, we also found that the toxic effect of DEHP on apoptosis of Neuro-2a cells was nullified by knockdown of the HO-1 gene. This indicates that HO-1 is involved in DEHP-induced apoptosis through activation of caspase-3 in Neuro-2a cells. This is the first study showing the involvement of HO-1 in the mechanism of DEHP-induced neurotoxicity and apoptosis. Therefore, HO-1 could be a potential therapeutic target for treatment of DEHP-induced neurotoxicity and its related neurological disorders.

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