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Cytoprotective effects of 12-oxo phytodienoic acid, a plant-derived oxylipin jasmonate, on oxidative stress-induced toxicity in human neuroblastoma SH-SY5Y cells

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ABSTRACT

Background: Jasmonates are plant lipid-derived oxylipins that act as key signaling compounds when plants are under oxidative stress, but little is known about their functions in mammalian cells. Here we investigated whether jasmonates could protect human neuroblastoma SH-SY5Y cells against oxidative stress-induced toxicity.
Methods: The cells were pretreated with individual jasmonates for 24 h and exposed to hydrogen peroxide (H_2O_2) for 24 h. Before the resulting cytotoxicity, intracellular reactive oxygen species (ROS) levels, and mitochondrial membrane potential were measured. We also measured intracellular glutathione (GSH) levels and investigated changes in the signaling cascade mediated by nuclear factor erythroid 2-related factor 2 (Nrf2) in cells treated with 12-oxo phytodienoic acid (OPDA).
Results: Among the jasmonates, only OPDA suppressed H_2O_2-induced cytotoxicity. OPDA pretreatment also inhibited the H_2O_2-induced ROS increase and mitochondrial membrane potential decrease. In addition, OPDA induced the nuclear translocation of Nrf2 and increased intracellular GSH level and the expression of the Nrf2-regulated phase II antioxidant enzymes heme oxygenase-1, NADPH quinone oxidoreductase 1, and glutathione reductase. Finally, the cytoprotective effects of OPDA were reduced by siRNA-induced knockdown of Nrf2.
Conclusions: These results demonstrated that among jasmonates, only OPDA suppressed oxidative stress-induced death of human neuroblastoma cells, which occurred via activation of the Nrf2 pathway.
General significance: Plant-derived oxylipin OPDA may have the potential to provide protection against oxidative stress-related diseases.

1. Introduction

In plants, jasmonates such as 12-oxo phytodienoic acid (OPDA), jasmonic acid (JA), methyl jasmonate (MeJA), jasmonoyl isoleucine (JA-Ile), and 12-hydroxy-jasmonic acid (12-OH-JA) are ubiquitously occurring signaling compounds formed in response to biotic and abiotic stresses [1]. Jasmonates are synthesized from linolenic acid via an octadecanoid pathway consisting of several enzymatic steps (Fig. 1). They are therefore regarded as plant-derived oxylipins similar to mammalian oxylipins such as prostaglandins and leukotrienes [2]. Biosynthesis of jasmonates is initiated by the release of α-linolenic acid from the galactolipids of plastid membranes by a galactolipase. α-Linolenic acid is then converted to OPDA via the three enzymes 13-lipoxygenase, 13-allene oxide synthase, and allene oxide cyclase [1]. A cyclopentenone ring of OPDA is subsequently reduced by the peroxisome-localized enzyme 12-oxo-phytodienoic acid reductase 3. The reaction product then undergoes three rounds of β-oxidation in the peroxisome, generating JA. MeJA is formed by methylation of JA by jasmonate methyl transferase. JA is converted by the amino acid conjugate synthetase to produce JA-Ile. 12-OH-JA was reported to be biosynthesized from JA by hydroxylation and an inactivation of JA signaling by this hydroxylation was shown [1,3]. Jasmonates function as important regulators in plant immunity, germination, and development

Abbreviations: OPDA, 12-oxo phytodienoic acid; JA, jasmonic acid; MeJA, methyl jasmonate; JA-Ile, jasmonoyl isoleucine; 12-OH-JA, 12-hydroxy-jasmonic acid; ROS, reactive oxygen species; GSH, glutathione; GCL, glutamate cysteine ligase; GCLC, glutamate cysteine ligase catalytic subunit; GCLM, glutamate cysteine ligase modulatory subunit; Nrf2, nuclear factor erythroid 2-related factor 2; Keap1, Kelch-like ECH-associated protein 1; HO-1, heme oxygenase-1; NQO1, NAD(P)H quinone oxidoreductase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; ARE, antioxidant response element; siRNA, small interfering RNA

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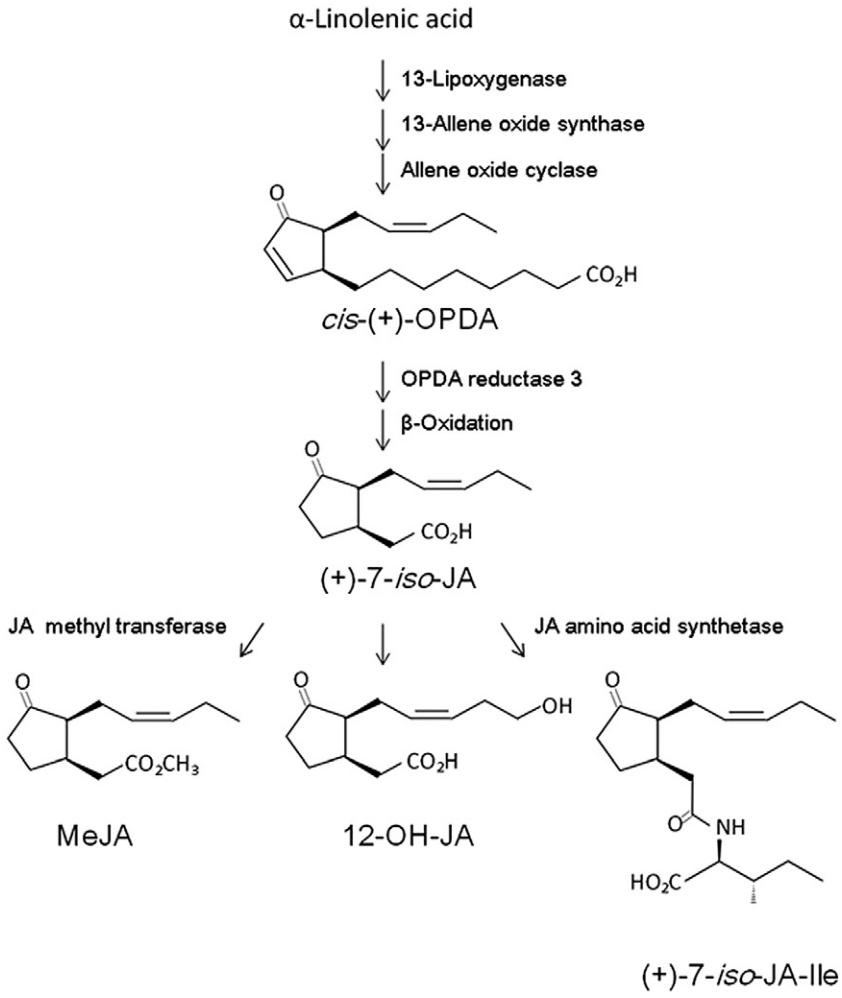

Fig. 1. Structure and biosynthesis of jasmonates. OPDA, 12-oxophytodienoic acid; JA, jasmonic acid; MeJA, methyl jasmonate; 12-OH-JA, 12-hydroxy-jasmonic acid; JA-Ile, jasmonoyl isoleucine.

[1,4]. Jasmonates exist universally in plants but not in animal systems and are found in relatively high amounts in fruits and vegetables such as tomato, apple, and sweet cherry and rice [5–7]. Jasmonates play an important role in plant defense against oxidative stress caused by wounding or ozone exposure by regulating gene expression or secondary metabolism [8,9]. Among jasmonates, OPDA is a type of reactive electrophile species (RES) [10]. In plants, RES contribute to protection of cells under wounding stress by increasing the expression of genes related to detoxification functions and chaperones [10].

Neuronal cells are thought to be more sensitive to oxidative stress than cells in other tissues [11], and oxidative stress is implicated in human neurological diseases including Parkinson's disease [12]. Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) cause neuronal damage [13,14]. To prevent oxidative stress, several non-enzymatic and enzymatic factors that prevent ROS formation or scavenge radicals are present in the body, such as glutathione (GSH) [15,16] and glutamylcysteine ligase (GCL), which is the rate-limiting enzyme for GSH synthesis [17]. Induction of GCL expression is associated with an increase in nuclear factor erythroid 2-related factor 2 (Nrf2), which regulates the transcription of phase II and other enzymes that reduce oxidative stress [18]. Under normal conditions, Nrf2 is degraded through the ubiquitin-proteasome pathway by binding to Kelch-like ECH-associated protein 1 (Keap1) because Keap1 act as an adaptor molecule for the E3 ubiquitin ligase [19]. Upon stimulation with oxidative stress or chemopreventive compounds, Nrf2 dissociates from Keap1 and translocates to the nucleus where it binds to the *cis*-acting antioxidant response element (ARE) in the promoter regions of genes encoding antioxidant proteins; Nrf2 binding thereby activates the expression of these genes [19,20]. During the cellular response to oxidative stress, Nrf2 regulates the transcription of downstream genes such as γ-GCL, heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1), and glutathione reductase [21–24]. Therefore Nrf2 activation is associated with neuroprotective effects [25–29].

Jasmonates are structurally similar to mammalian oxylipins including prostaglandins, which are also synthesized from membrane-derived fatty acids. It has been reported that prostaglandins regulate inflammatory responses and vascular homeostasis [30–32], and some reports have suggested that jasmonates have anti-inflammatory activity in mammalian cells [33,34]. Recently the anti-cancer properties of jasmonates have been intensively studied [35–37]. In fact, plants with high content of JA such as *Viscum album* are of pharmaceutical interest in order to prepare anticancer drugs [38]. However, little is known about jasmonate effects against oxidative stress in neuronal cells.

The aim of our study was to investigate whether jasmonates affect oxidative stress-induced cell damage and cell death in human neuroblastoma SH-SY5Y cells. We found that one of the jasmonates, OPDA, suppressed H_2O_2-induced oxidative damage and cell death. The effect of OPDA was due to Nrf2 activation and subsequent expression of antioxidants and phase II detoxification enzymes, which resulted in inhibition of ROS production and reduced oxidative damage.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (1:1 (*v*/*v*), DMEM/F-12), 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA), Lipofectamine 2000, and Lipofectamine RNAiMAX were obtained from Life Technologies. Fetal bovine serum was obtained from Cell Culture Bioscience. *cis*-OPDA, (±)-JA, and (±)-MeJA were obtained from Cayman Chemical. (-)-JA-Ile and (±)-12-OH-JA were purchased from OlchemIm Ltd. (+)-7-*iso*-JA-Ile was synthesized as indicated in Chen et al. [39]. H_2O_2 and the LDH-Cytotoxic Test were obtained from Wako Pure Chemicals. GSH-Glo Glutathione Assay was obtained from Promega. Antibodies against Nrf2 and GCLM were obtained from Santa Cruz Biotechnology. Anti-lamin was obtained from Cell Signaling. Anti-HO-1 was obtained from Biomol. Antibodies against GCLC, β-actin, NQO1, and glutathione reductase were obtained from Abcam. Horseradish peroxidase-conjugated secondary antibodies and ECL Prime Western Blotting Detection Reagent were from GE Healthcare. NE-PER Nuclear and Cytoplasmic Extraction Reagent and Micro BCA Protein Assay kit were from Pierce. The mitochondria-selective dye JC-1 was obtained from Immunochemistry Technologies. Protease inhibitor cocktail was purchased from Sigma. RNeasy Mini kit and All Stars Negative Control siRNA were obtained from Qiagen. ReverTra Ace was from TOYOBO.

2.2. Cell culture

Human neuroblastoma SH-SY5Y cells were obtained from DS Pharma Biomedical. Cells were grown in DMEM/F-12 containing 10% fetal bovine serum. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. Cells were plated at appropriate density according to each experimental scale. Cells were treated with various concentrations of jasmonates for various times. Control cells were treated with medium lacking jasmonates. For oxidative stress treatment, cells were pretreated with each of several jasmonates for 24 h. After the medium was changed to that without jasmonates, the cells were exposed to 200 μM H_2O_2 for various times. Control cells were treated with the medium without jasmonates and H_2O_2.

2.3. Cytotoxicity assay

Cells dying by apoptosis or necrosis release lactate dehydrogenase (LDH) into the culture medium [40]. The amount of LDH in the medium was assayed with a LDH-cytotoxic test. As a positive control, cells were completely lysed using 2% (*v*/*v*) Triton X-100 to give maximum LDH release. We measured LDH level at 24 h after H_2O_2 treatment because LDH was not detected within the first 8 h of treatment.

2.4. Measurement of intracellular ROS

ROS were measured using the oxidation-sensitive fluorescent probe, CM-H2DCFDA. SH-SY5Y cells were incubated with CM-H2DCFDA (10 μM) for 30 min at 37 °C in the dark. Then the cells were harvested and analyzed on a flow cytometer.

2.5. Measurement of mitochondrial membrane potential

Changes in mitochondrial membrane potential were analyzed using JC-1, a lipophilic cation that selectively enters mitochondria and reversibly changes color from green to red depending on the membrane potential. In healthy cells with high mitochondrial membrane potential, JC-1 forms red fluorescent complexes that are detected in FL2 (488 nm excitation; 590 nm emission); in damaged cells with low mitochondrial membrane potential, JC-1 exists in monomeric form with green fluorescence that is detected in FL1 (488 nm excitation; 525 nm emission) [41]. SH-SY5Y cells were harvested and incubated with JC-1 for 15 min at 37 °C. Cells were then washed twice with phosphate-buffered saline and analyzed using a flow cytometer (excitation wavelength, 488 nm). We created log FL1 (*x*-axis) versus log FL2 (*y*-axis) dot plots and set two regions, 1 and 2, which correspond to the same areas in all dot plots. Cells were then counted in region 1 and in region 2.

2.6. Immunoblotting

Cells were solubilized using NE-PER Nuclear and Cytoplasmic Extraction Reagent or RIPA buffer with 1% protease inhibitor cocktail for nuclear protein extraction or whole-cell protein extraction, respectively. Protein concentrations were determined using the Micro BCA Protein Assay kit. The proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subsequently transferred to a polyvinylidene difluoride membrane. The membrane was blocked with polyvinylidene difluoride blocking reagent and incubated overnight at 4 °C with a primary antibody against Nrf2, lamin, GCLM, GCLC, HO-1, NQO1, glutathione reductase, or β-actin. The membrane was washed in phosphate-buffered saline containing 0.1% (*v*/*v*) Tween-20 and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody. Chemiluminescence signals generated by ECL Prime Western Blotting Detection Reagent were detected with the Image Quant LAS 3000 CCD camera system (Fujifilm).

2.7. Quantitative real-time PCR analysis

Total RNA was extracted with the RNeasy system, which includes a DNase step, and cDNA was synthesized using random hexamer primers and ReverTra Ace reverse transcriptase. Quantitative real-time PCR was

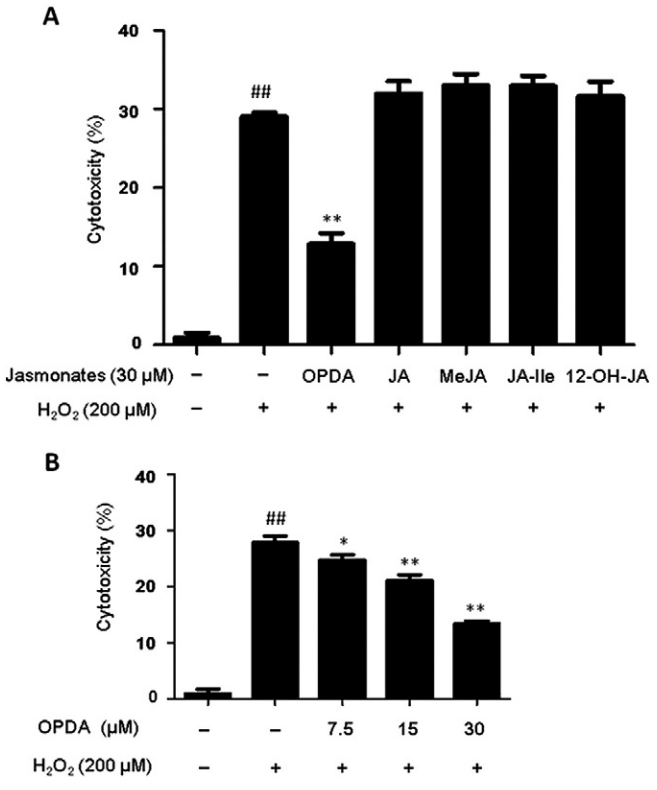

Fig. 2. OPDA decreases H_2O_2-induced cytotoxicity. SH-SY5Y cells were pretreated with (A) 30 μM OPDA, JA, MeJA, JA-Ile, or 12-OH-JA for 24 h and (B) 7.5, 15, or 30 μM OPDA for 24 h. After the medium was changed, the cells were exposed to 200 μM H_2O_2 for 24 h. Cytotoxicity was assessed by LDH assay. Values indicate the mean ± S.E.M. from three separate experiments. ##$p < 0.01$ versus control; *$p < 0.05$; **$p < 0.01$ versus H_2O_2 alone. OPDA, 12-oxophytodienoic acid; JA, jasmonic acid; MeJA, methyl jasmonate; 12-OH-JA, 12-hydroxy-jasmonic acid; JA-Ile, jasmonoyl isoleucine; LDH, lactate dehydrogenase.

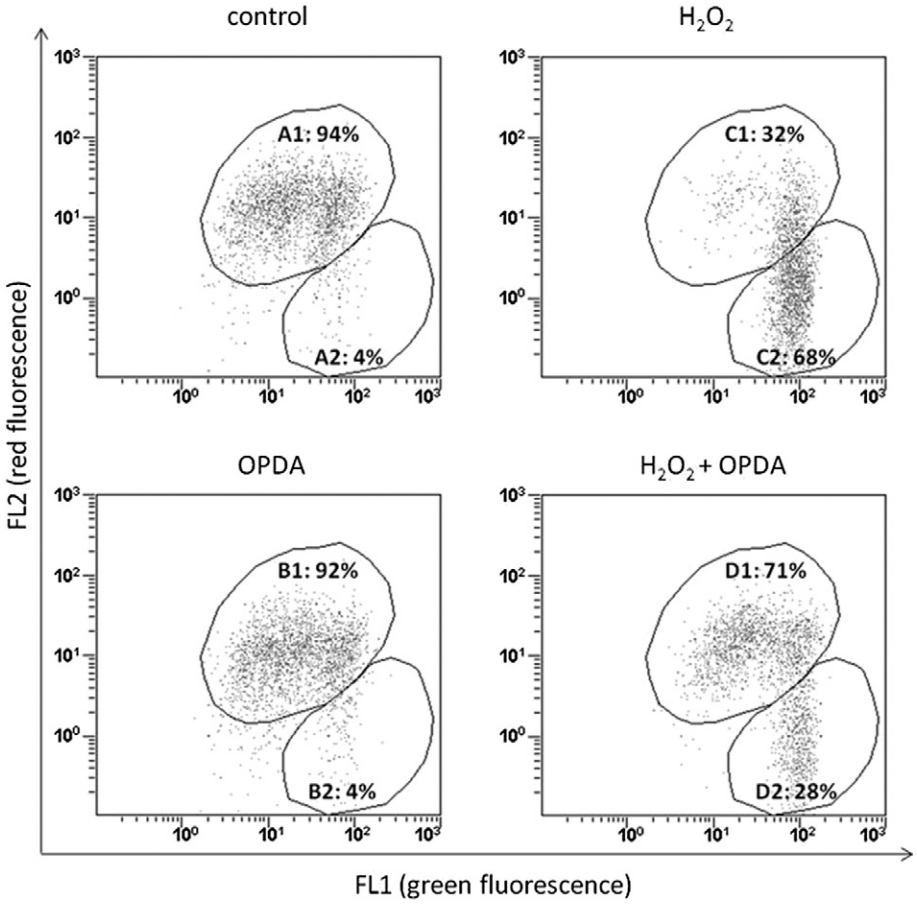

Fig. 3. OPDA reduces the H_2O_2-induced depolarization of the mitochondrial membrane potential. SH-SY5Y cells were preincubated with 30 μM OPDA for 24 h followed by treatment with 200 μM H_2O_2 for 24 h. The cells were then labeled with JC-1 and analyzed by flow cytometry. Dot plots show the number of cells emitting red fluorescence (FL2) versus green fluorescence (FL1). OPDA, 12-oxophytodienoic acid.

performed using the 7500HT Fast Real-Time PCR system (Applied Biosystems). The data presented are normalized to GAPDH transcripts. The following primers and probes were used (5′ to 3′). Nrf2 primers: Nrf2 forward (fwd), TACTCCCAGGTTGCCCACA; Nrf2 reverse (rev), CATCTACAAACGGGAATGTCTGC; Nrf2 probe (FAM-TAMRA), TCAGAT GCTTTGTACTTTGATGACTGCATGC. HO-1 primers: HO-1 fwd, CTTCTT CACCTTCCCCAACATT; HO-1 rev, GCAGTCTTGGCCTCTTCTATCAC; HO-1 probe, AGATGACTCCCGCAGTCAGGCA. NQO1 primers: NQO1 fwd, CCTGGAAGGATGGAAGAAACG; NQO1 rev, AGAATCCTGCCTGGAAGTTT AGG; NQO1 probe, ACACCACTGTATTTTGCTCCAAGCAGCC. GCLC primers: GCLC fwd, TCTCTAATAAAGAGATGAGCAACATGC; GCLC rev, TTGACGATAGATAAAGAGATCTACGAA; GCLC probe, CAGGAGATGATC AATGCCTTCCTGCAAC. GCLM primers: GCLM fwd, GTTCAGTCCTTGGA GTTGCACA; GCLM rev, CCCAGTAAGGCTGTAAATGCTC; GCLM probe, GCTGGATTCTGTGATCATTGCTTCACC. Glutathione reductase primers: glutathione reductase fwd, AGTGATCCCAAGCCCACAATAG; glutathione reductase rev, CCATCGCTGGTTATTCCTAAGC; glutathione reductase probe, AAAAAGTACACCGCCCCACACATCCT.

2.8. Measurement of GSH level

Intracellular GSH level was measured using GSH-Glo Glutathione Assay and normalized to total cell protein.

2.9. ARE assay

For the stable ARE-driven reporter system, the pGL4 vector (Promega), containing a hygromycin selectable marker, was used to generate ARE reporter plasmids. Briefly, a DNA fragment containing three copies of the ARE (TTTCTGCTGCGTCATGTTTG) sequence was cloned upstream of the promoter-luciferase transcriptional unit of vector pGL4, resulting in pGL4-3 × ARE. Plasmid pGL4-3 × ARE was stably transfected into SH-SY5Y cells using Lipofectamine 2000, and transfected cells were selected using 75 μg/ml hygromycin. Luciferase activity was measured using the Bright-Glo Luciferase Assay System.

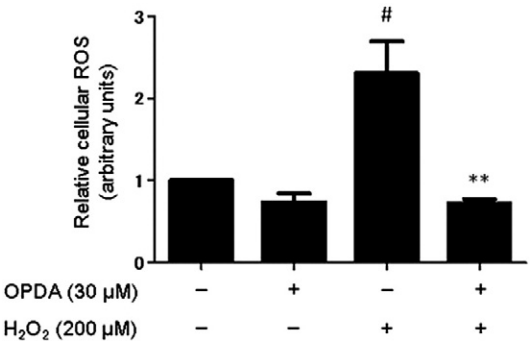

Fig. 4. OPDA attenuates H_2O_2-induced cytotoxicity. SH-SY5Y cells were preincubated with 30 μM OPDA for 24 h followed by treatment with 200 μM H_2O_2 for 2 h. The cells were labeled with the fluorescent probe CM-H2DCFDA, and intracellular ROS accumulation was determined by measuring fluorescence intensity using a flow cytometer. Values (mean ± S.E.M., $n = 3$) are expressed as the fold induction relative to untreated cells. #$p < 0.05$ versus control; **$p < 0.01$ versus H_2O_2 alone. OPDA, 12-oxophytodienoic acid; ROS, reactive oxygen species.

2.10. Nrf2 knockdown by siRNA transfection

SH-SY5Y cells were transiently transfected with Nrf2 siRNA (5′-CCCATTGATGTTTCTGATCTA-3′) or All Stars Negative Control siRNA using Lipofectamine RNAiMAX.

3. Results

3.1. OPDA decreases H_2O_2-induced cytotoxicity

We first examined the role of OPDA, JA, MeJA, JA-Ile, and 12-OH-JA in oxidative stress-induced cytotoxicity in human neuroblastoma SH-SY5Y cells. Cells were pretreated with jasmonates for 24 h and exposed to H_2O_2 for an additional 24 h (Fig. 2 and supplemental Fig. 1). H_2O_2 treatment significantly increased LDH activity in the cell culture medium, indicating that cell death occurred. Only pretreatment with OPDA significantly reduced H_2O_2-induced cell death in a concentration-dependent manner (Fig. 2A and B), whereas all other jasmonates showed no cytoprotective effects. These results indicated that, among jasmonates, OPDA has unique properties that protect SH-SY5Y cells against oxidative stress.

3.2. OPDA suppresses the H_2O_2-induced reduction of the mitochondrial membrane potential

Cell death is accompanied by the collapse of the mitochondrial membrane potential. Thus, we used JC-1 dye to detect the mitochondrial membrane potential of SH-SY5Y cells with or without OPDA pretreatment followed by H_2O_2 exposure. In healthy cells with high mitochondrial membrane potential, JC-1 forms complexes that release red fluorescence (Fig. 3, regions A1, B1, C1, and D1). In dying cells with low mitochondrial membrane potential, JC-1 exists as a monomer and releases green fluorescence (Fig. 3, regions A2, B2, C2, and D2). Treatment of SH-SY5Y cells with H_2O_2 resulted in a significant change in the fluorescence of cells having a high (Fig. 3, region A1 versus C1) or low mitochondrial membrane potential (Fig. 3, region A2 versus

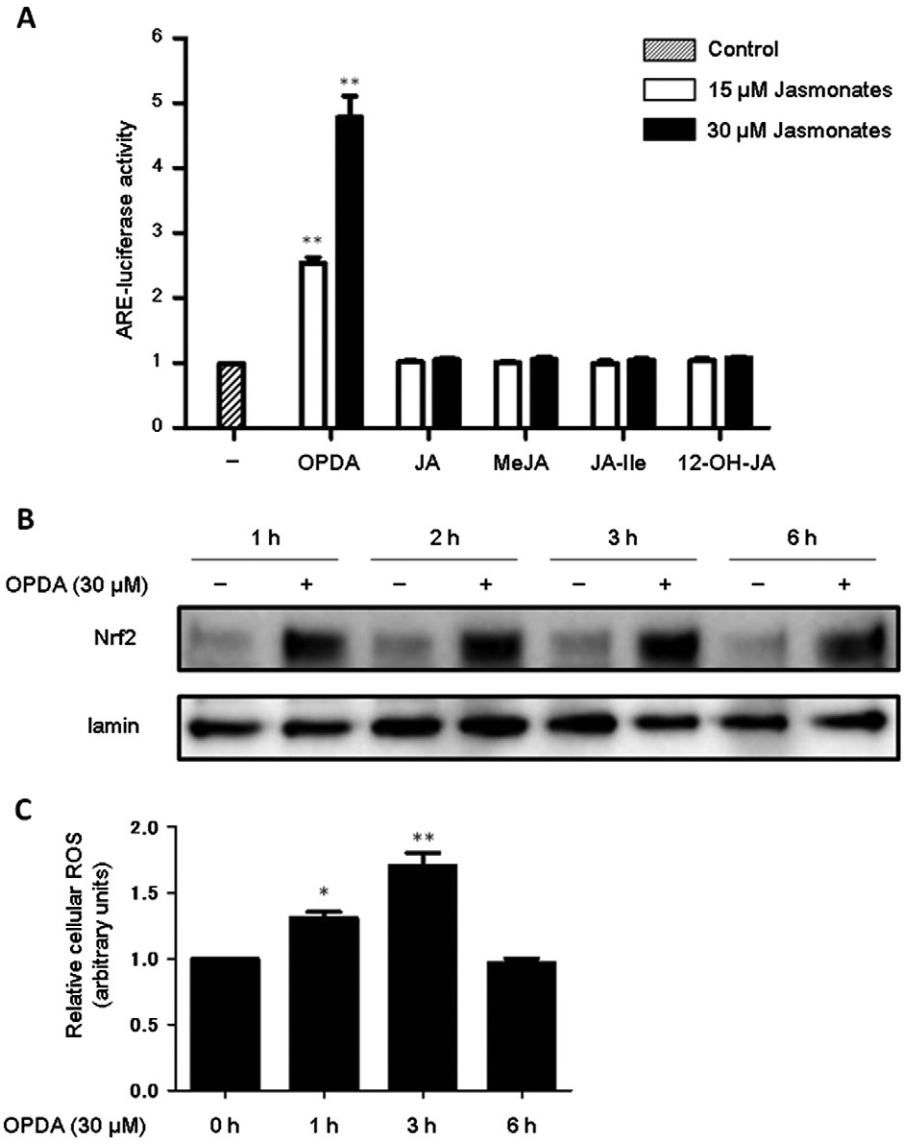

Fig. 5. OPDA induces the Nrf2 signaling pathway. (A) SH-SY5Y cells transfected with the ARE reporter plasmid were treated with 15 or 30 μM OPDA, JA, MeJA, JA-Ile, or 12-OH-JA for 24 h. Luciferase activities were then determined. Values (mean ± S.E.M., $n = 3$) are expressed as the fold induction relative to untreated cells as a control. $^{**}p < 0.01$ versus control. (B) Cells were treated with 30 μM OPDA for 1, 2, 3, or 6 h. Nuclear Nrf2 was determined by immunoblotting. Lamin was used as a loading control. (C) Cells were treated with 30 μM OPDA for 1, 3, or 6 h. Intracellular ROS level was determined by measuring the fluorescence of cells labeled with CM-H2DCFDA. Values (mean ± S.E.M., $n = 3$) are expressed as the fold induction relative to untreated cells at each time point. $^{*}p < 0.05$, $^{**}p < 0.01$ versus control. OPDA, 12-oxophytodienoic acid; JA, jasmonic acid; MeJA, methyl jasmonate; 12-OH-JA, 12-hydroxy-jasmonic acid; JA-Ile, jasmonoyl isoleucine; ARE, antioxidant response element; Nrf2, nuclear factor erythroid 2-related factor 2.

C2). In control cells, 94% had a high mitochondrial membrane potential (Fig. 3, region A1), but upon incubation with H_2O_2 this was reduced to 32% (Fig. 3, region C1). Notably, pretreatment with OPDA did not affect the percentage of cells having a high or low membrane potential (Fig. 3, regions A1 versus B1, and A2 versus B2). However, pretreatment with OPDA significantly countered the deleterious effects of H_2O_2 on the membrane potential (Fig. 3, regions C1 versus D1, and C2 versus D2). These results suggested that OPDA exerts its cytoprotective effects by preserving mitochondrial function.

3.3. OPDA attenuates H_2O_2-induced oxidative stress

To determine whether OPDA affects the H_2O_2-induced death of SH-SY5Y cells, we measured cellular ROS levels using the fluorescent dye CM-H2DCFDA. Exposure of SH-SY5Y cells to H_2O_2 at 200 μM for 2 h caused an increase in intracellular ROS levels (Fig. 4). However, pretreatment of the cells with 30 μM OPDA for 24 h attenuated the H_2O_2-induced ROS production, suggesting that OPDA pretreatment induced a cellular antioxidative response.

3.4. OPDA activates the Nrf2 pathway

Because Nrf2 signaling regulates cellular antioxidant response by promoting ARE-dependent gene expression [19], we examined whether OPDA protected cells from intracellular oxidative stress by activating the Nrf2 signaling pathway. First, we determined whether jasmonates in general enhanced ARE activity using a luciferase reporter assay. Whereas JA, MeJA, JA-Ile, and 12-OH-JA did not enhance ARE activity, OPDA did increase ARE activity (Fig. 5A).

We then examined the effect of OPDA on the intracellular localization of Nrf2 in SH-SY5Y cells. OPDA increased Nrf2 nuclear translocation after only 1 h of OPDA treatment (Fig. 5B). These findings suggested that

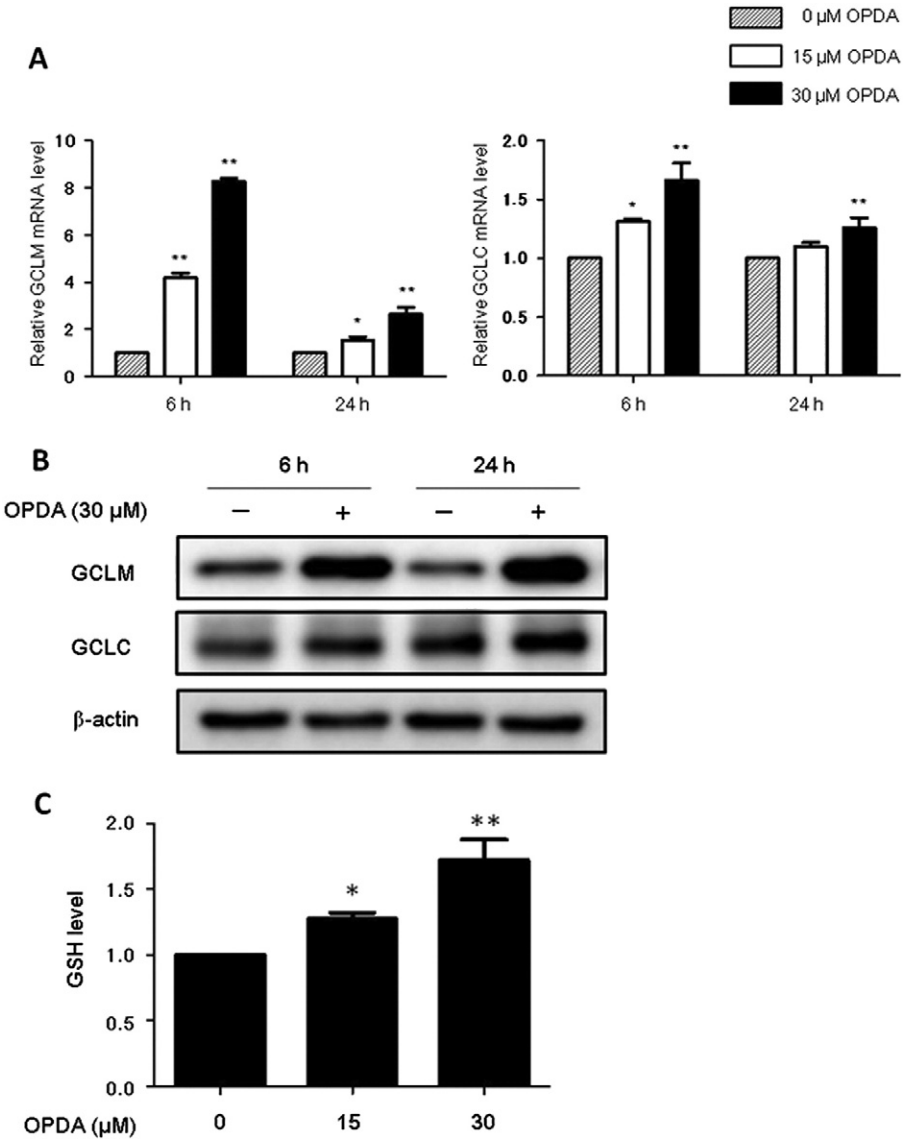

Fig. 6. OPDA increases the expression of antioxidant enzymes. (A) SH-SY5Y cells were treated with 15 or 30 μM OPDA for 6 or 24 h. GCLM and GCLC mRNA levels were determined by quantitative real-time PCR. Values (mean ± S.E.M., $n = 3$) are expressed as the fold induction relative to untreated cells. $^*p < 0.05$, $^{**}p < 0.01$ versus control. (B) Cells were treated with 30 μM OPDA for 6 or 24 h. GCLM and GCLC protein levels were determined by immunoblotting. β-actin was used as a loading control. A representative blot from three independent experiments is shown. β-actin was used as a loading control. (C) Cells were treated with 15 or 30 μM OPDA for 24 h. Total GSH level was measured. Values (mean ± S.E.M., $n = 3$) are expressed as the fold induction relative to untreated cells. $^*p < 0.05$, $^{**}p < 0.01$ versus control. (D) Cells were treated with 15 or 30 μM OPDA for 6 or 24 h. Levels of mRNAs encoding HO1, NQO1, and glutathione reductase were determined by quantitative real-time PCR. Values (mean ± S.E.M., $n = 3$) are expressed as the fold induction relative to untreated cells. $^*p < 0.05$, $^{**}p < 0.01$ versus control. (E) Cells were treated with 30 μM OPDA for 6 or 24 h. Levels of the proteins HO1, NQO1, and glutathione reductase were determined by immunoblotting. β-actin was used as a loading control. A representative blot from three independent experiments is shown. OPDA, 12-oxophytodienoic acid; GCLC, glutamate cysteine ligase catalytic subunit; GCLM, glutamate cysteine ligase modulatory subunit; HO-1, heme oxygenase-1; NQO1, NAD(P)H quinone oxidoreductase 1.

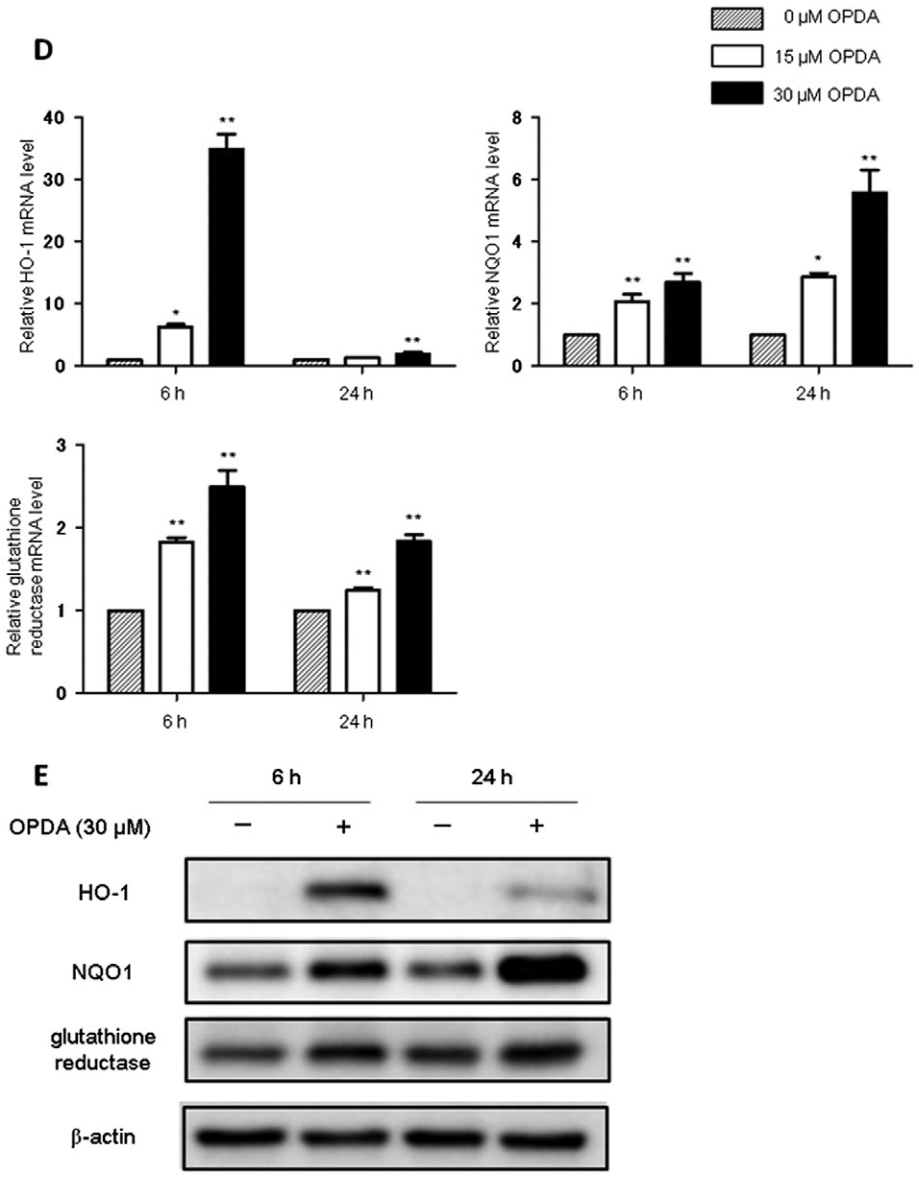

Fig. 6 (*continued*).

OPDA promptly induces Nrf2 signaling. Modification of Keap1 cysteine residues by ROS and electrophiles changes the conformation of Keap1 [19,20,42,43] and thus induces Nrf2 signaling cascade. Here we investigated whether OPDA could promote the production of intracellular ROS. Intracellular ROS level increased slightly at 1–3 h after treatment with OPDA and then decreased to the basal level at 6 h (Fig. 5C), suggesting that transient ROS production could be involved in Nrf2 pathway activation.

3.5. OPDA increased the expression of antioxidant enzymes

Activation of Nrf2 signaling leads to up-regulation of genes important for cellular antioxidant defense activity [19]. One of these Nrf2 targets is GCL, which consists of modulatory (GCLM) and catalytic (GCLC) subunits. In SH-SY5Y cells, we investigated whether mRNA and protein levels of these GCL subunits were altered by OPDA. OPDA treatment significantly induced both mRNA and protein levels of GCLM (Fig. 6A), and although the GCLC mRNA level increased, the protein level did not change (Fig. 6B). Because OPDA changed the mRNA and protein levels of GCLM, we also measured intracellular GSH levels at 24 h after OPDA treatment. OPDA induced a dose-dependent increase in GSH level (Fig. 6C). In addition to measuring GSH synthesis, we examined the expression of other phase II antioxidant/detoxification enzymes such as HO-1, NQO1, and glutathione reductase in SH-SY5Y cells after treatment with OPDA for 6 and 24 h. OPDA increased both mRNA and protein levels of HO-1, NQO1, and glutathione reductase after 6 and 24 h (Fig. 6D and E), suggesting that OPDA initiates the cellular antioxidative defense system via activation of Nrf2 signaling.

3.6. The Nrf2 pathway is involved in the cytoprotective effect of OPDA

To confirm the contribution of Nrf2 signaling to the suppressive effects of OPDA on H_2O_2-induced cytotoxicity, we transiently transfected SH-SY5Y cells with Nrf2 siRNA and evaluated protein expression levels of Nrf2 and Nrf2 target genes and H_2O_2-induced cell death. Nrf2 siRNA reduced the OPDA-induced nuclear translocation of Nrf2 (Fig. 7A). We also investigated whether Nrf2 siRNA transfection suppressed OPDA-induced up-regulation of GCLM, HO-1, NQO1, and glutathione reductase. OPDA-induced expression of these genes was blocked by Nrf2 siRNA (Fig. 7B). We further examined whether the cytoprotective

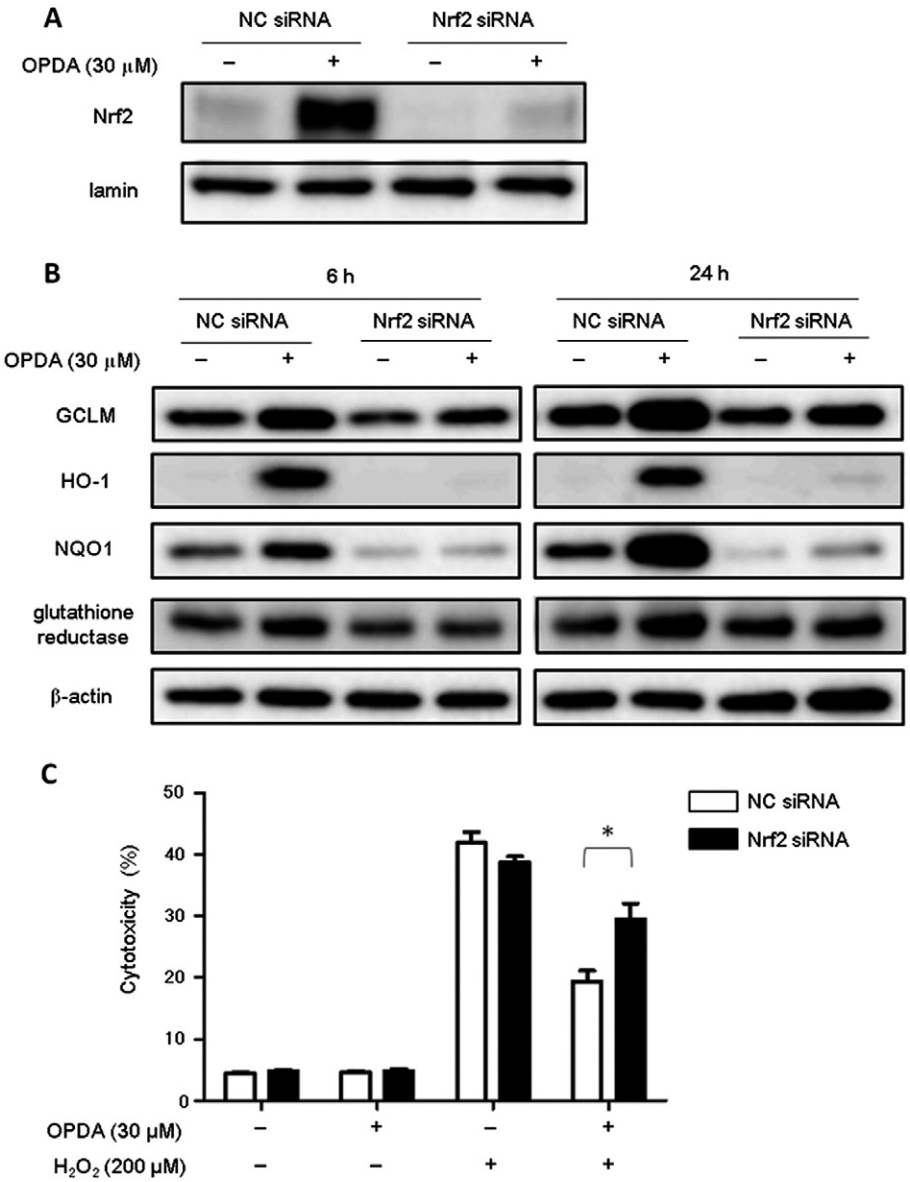

Fig. 7. Antioxidant protection of neuronal SH-SY5Y cells by OPDA is Nrf2 dependent. (A) SH-SY5Y cells were transiently transfected with negative control (NC) or Nrf2 siRNAs for 24 h, followed by treatment with 30 μM OPDA for 24 h. Nuclear Nrf2 was detected by immunoblotting. Lamin was used as a loading control. (B) Cells were transiently transfected with NC or Nrf2 siRNA for 24 h and were then treated with 30 μM OPDA for an additional 6 or 24 h. Levels of the proteins GCLM, HO1, NQO1, and glutathione reductase were measured by immunoblotting. β-actin was used as a loading control. A representative blot from three independent experiments is shown. (C) Cells were transiently transfected with NC or Nrf2 siRNAs for 24 h, followed by treatment with 30 μM OPDA for 24 h. After the medium was changed, cells were exposed to H_2O_2 for an additional 24 h. Cytotoxicity was assessed by LDH assay. Values indicate the mean ± S.E.M. from three separate experiments. $^*p < 0.05$ versus H_2O_2-treated cells transfected with control siRNA. OPDA, 12-oxophytodienoic acid; Nrf2, nuclear factor erythroid 2-related factor 2; GCLM, glutamate cysteine ligase modulatory subunit; HO-1, heme oxygenase-1; NQO1, NAD(P)H quinone oxidoreductase 1.

effects of OPDA were reduced by siRNA knockdown of Nrf2. OPDA-induced cytoprotection against H_2O_2 was suppressed by Nrf2 knockdown in SH-SY5Y cells (Fig. 7C). These data suggested that Nrf2 activation and the subsequent upregulation of genes encoding antioxidative enzymes are crucial for the cytoprotective properties of OPDA.

4. Discussion

Our results are the first to demonstrate that the plant-derived oxylipin OPDA has cytoprotective and antioxidative effects via activation of Nrf2 signaling in human neuroblastoma SH-SY5Y cells. Among the tested jasmonates, only OPDA induced Nrf2/ARE activation and showed subsequent cytoprotective effects. Electrophiles such as prostaglandin J_2, epigallocatechin-3-gallate and sulforaphane induce Nrf2/ARE activation by inhibiting Keap1-mediated Nrf2 degradation, which protect cells from subsequent oxidative stress [44–46]. This inhibition requires posttranslational modification of Keap1 by electrophiles or ROS [19,20]. It is reported that H_2O_2 treatment, which induces ROS production, results in transient Nrf2 activation [47]. Because OPDA induced transient production of ROS and it can act as an electrophile, a potential modification of Keap1 may be related to Nrf2/ARE activation. As shown in Fig. 5B and C, Nrf2 activation continued after ROS production ceased, and thus direct modification of Keap1 by OPDA—acting as an electrophile—might be responsible for Nrf2 activation. This is consistent with the result that none of the other jasmonates, which are not electrophiles, could activate Nrf2/ARE signaling. Although the precise mechanisms of OPDA action remain to be elucidated, our results demonstrate that Nrf2 activation may be associated with the cytoprotective properties of OPDA.

Activation of Nrf2 in vivo is linked to the cytoprotective effects seen in neuronal cells in model of Parkinson's disease and of brain ischemia [48–50]. In contrast, Nrf2 deficiency exacerbates neuronal damage in

Parkinson's disease and ischemic injury models [51,52]. As shown in Figs. 6 and 7, Nrf2 activation by OPDA induced the expression of the antioxidative enzymes HO-1, NQO1, and glutathione reductase and the subsequent generation of intracellular GSH. GSH is the most abundant non-protein thiol in mammalian cells and acts as a major reducing agent that tightly regulates redox status [53] and is an important molecule for protecting against neurodegenerative diseases [54]. The sulfhydryl form GSH can reduce H_2O_2 via glutathione peroxidase to form disulfide-linked GSH. GSH can be regenerated from disulfide-linked GSH by glutathione reductase with NADPH [53,55]. Hence, glutathione reductase is an important enzyme for maintaining a redox balance in cells. HO-1 serves as one of the defense systems against oxidative damage by converting toxic heme into carbon monoxide, iron, and biliverdin [56]. Biliverdin is subsequently converted to bilirubin through the action of biliverdin reductase, and this molecule serves as a potent antioxidant [56]. Up-regulation of HO-1 expression by chemical treatment or adenovirus-mediated overexpression in brain is therapeutic in models of neurodegenerative diseases [57,58]. NQO1 is a flavoprotein that catalyzes the two-electron reduction of quinones to protect against ROS generation [59] and is believed to prevent severe oxidative damage in various neuronal disorders [60,61]. Because OPDA induced the expression of various Nrf2 related antioxidative enzymes in human neuroblastoma cells, we expect that it could be therapeutically useful to protect against oxidative stress-related neuronal diseases. We applied 24 h for pretreatment with OPDA because the cytoprotective effects against H_2O_2 were observed after 24 h but not 6 h, suggesting that longer pretreatment is required to accumulate suitable levels of antioxidants and cytoprotective proteins. Consistently, GSH increased significantly after 24 h of OPDA treatment but not 6 h.

OPDA induced an Nrf2-dependent antioxidative response, which may have contributed to the observed decrease in H_2O_2-induced ROS levels. Intracellular ROS accumulation induces mitochondrial membrane depolarization, which is linked to mitochondrial dysfunction in neurodegenerative diseases [62–64]. Antioxidants, however, prevent the loss of mitochondrial membrane potential [65]. We demonstrated that OPDA treatment suppressed the H_2O_2-induced reduction in mitochondrial membrane potential, which may be due to the ability of OPDA to inhibit H_2O_2-induced ROS accumulation. Preservation of mitochondrial function may be related to cytoprotective effects of OPDA.

We previously reported that the OPDA signaling pathway functions independently of JA/MeJA signaling and is required for the wounding response in Arabidopsis [8]. Moreover, OPDA, but not JA, regulates cellular redox homeostasis by binding to cyclophilin 20-3 in response to cellular stress in plants [66]. Our present study demonstrates that only OPDA, among all jasmonates tested, has a cytoprotective effect through Nrf2 activation in mammalian cells. Although further studies are needed to explore the cytoprotective effects of OPDA in other cell types, it is interesting that OPDA has a unique cytoprotective function in mammals as well as in plants. A variety of plant-derived nutraceuticals have demonstrated cytoprotective activity against oxidative stress through Nrf2 activation in SH-SY5Y or PC12 cells [67–72]. Additionally, the potential therapeutic value of each of these compounds in neurogenerative diseases has been discussed [73]. OPDA is ubiquitous in higher plants such as Arabidopsis, tomato, maize, and soybean [5,74–76]. Although further investigations about the absorption, metabolism, and ability of OPDA to cross the blood–brain barrier are needed, intake of natural foods containing OPDA could potentially help to prevent oxidative stress-mediated neurodegenerative diseases. It also has been reported that JA and MeJA have anti-cancer activities [36–38]. Therefore, OPDA, in addition to other jasmonates reported so far, may also be of pharmaceutical or medicinal interests as a plant-derived oxylipin.

5. Conclusions

Our findings indicate that OPDA rescues neuronal cells from H_2O_2-induced death by increasing cellular antioxidative defenses, as demonstrated by its ability to regulate ROS production and mitochondrial membrane potential via activation of the Nrf2 pathway. Therefore OPDA has the potential to provide protection against oxidative stress-related diseases.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.09.003.

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