Naringenin protects against 6-OHDA-induced neurotoxicity via activation of the Nrf2/ARE signaling pathway

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There is increasing evidence that oxidative stress is critically involved in the pathogenesis of Parkinson’s disease (PD), suggesting that pharmacological targeting of the antioxidant machinery may have therapeutic value. Naringenin, a natural flavonoid compound, has been reported to possess neuroprotective effect against PD related pathology; however the mechanisms underlying its beneficial effects are poorly defined. Thus, the purpose of the present study was to investigate the potential neuroprotective role of naringenin and to delineate its mechanism of action against 6-hydroxydopamine (6-OHDA)-induced neurotoxicity in models of PD both in vitro and in vivo. Naringenin treatment resulted in an increase in nuclear factor E2-related factor 2 (Nrf2) protein levels and subsequent activation of antioxidant response element (ARE) pathway genes in SH-SY5Y cells and in mice. Exposure of SH-SY5Y cells to naringenin provided protection against 6-OHDA-induced oxidative insults that was dependent on Nrf2, since treatment with Nrf2 siRNA failed to block against 6-OHDA neurotoxicity or induce Nrf2-dependent cytoprotective genes in SH-SY5Y cells. In mice, oral administration of naringenin resulted in significant protection against 6-OHDA-induced nigrostriatal dopaminergic neurodegeneration and oxidative damage. Our results indicate that activation of Nrf2/ARE signaling by naringenin is strongly associated with its neuroprotective effects against 6-OHDA neurotoxicity and suggest that targeting the Nrf2/ARE pathway may be a promising approach for therapeutic intervention in PD.

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1. Introduction

Parkinson’s disease (PD) is a neurodegenerative disorder that is characterized by progressive loss of dopaminergic neurons in substantia nigra pars compacta (SNc) that underlie the characteristic motor symptoms. Because there is currently no therapy that delays the neurodegenerative process, development of drugs that will modify the course of PD is crucial (Savitt et al., 2006). Oxidative stress has been proposed to play a major role in the pathogenesis of PD (Hwang, 2013). An extremely promising pathway that confers protection to a variety of oxidative stress-related neurodegenerative insults is the nuclear factor erythroid-2 related factor 2 (Nrf2)/antioxidant response element (ARE) pathway (Calkins et al., 2009; Cuadrado et al., 2009; de Vries et al., 2008). The transcription factor Nrf2 is a key regulator in the coordinated induction of several cytoprotective genes, including those encoding for endogenous antioxidant such as hemeoxygenase (HO-1), glutathione cysteine ligase regulatory subunit (GCLC) and glutathione cysteine ligase modulatory subunit (GCLM).

Several important findings connect Nrf2 with PD. First, Nrf2 activity declines with age, and aging is the main risk factor for PD (Sykiotis and Bohmann, 2010). Second, in postmortem PD brain, nuclear localization of Nrf2 occurs, showing that it had been induced but to levels that was insufficient to halt neurodegeneration (Ramsey et al., 2007), because under normal conditions, Nrf2 remains in the cytosol of nigral dopaminergic neurons.
Third, genetic studies report a protective haplotype of the Nrf2 gene that can delay PD onset by as much as five years (von Otter et al., 2010). Fourth, Nrf2 knockout mice are more susceptible to 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), toxins that are particularly toxic to SN neurons, whereas Nrf2 activation provides a neuroprotective response (Chen et al., 2009; Jazwa et al., 2011; Kaidery et al., 2013). These data provide compelling evidence that activation of Nrf2 offers a new attractive therapeutic strategy for the treatment of PD.

Flavonoids are potent antioxidants and free radical scavengers that have little toxicity even with extended use, suggesting that they may be appropriate as a long term therapy for PD. Importantly, epidemiological studies link the intake of flavonoid-containing dietary sources with a reduced risk of PD (Albarracin et al., 2012; Gao et al., 2012; Prasain et al., 2010). Naringenin (Fig. 1A), the predominant flavonoid found in grapefruit, provides neuroprotection in both PD and Alzheimer’s disease models (Heo et al., 2004; Zbarsky et al., 2005). However, the molecular targets of naringenin are currently unknown. As several other antioxidants have broad spectrum of protective properties, including the transcription factor Nrf2, we hypothesize that naringenin might have similar actions. In this paper, we reported the identification of naringenin as a novel Nrf2 activator and provide neuroprotection against 6-OHDA-mediated dopaminergic neuronal damage through activation of the Nrf2/ARE signaling pathway.

2. Materials and methods

2.1. Cell culture and 6-OHDA exposure

Human neuroblastoma SH-SY5Y cells were maintained in DMEM-F12 (Invitrogen) that was supplemented with 10% fetal calf serum. The neurotoxin 6-OHDA HBr (Sigma) was dissolved in 0.02% ascorbic acid and prepared fresh for each experiment. Cultures were exposed to 100 μM 6-OHDA for 24 h before being harvested for various assays.

2.2. Luciferase assay

The MDA-MB-231-ARE-Luc stable cell line has been previously described and was used for initial screening for Nrf2 activators (Yu et al., 2008). Cells were seeded the day before and treated with different doses of naringenin for 16 h. Cells were lysed and assayed for luciferase activity.

2.3. Cell death/viability assessment

Cell viability was measured by the MTT assay. Briefly, the cells were pre-treated with vehicle alone or naringenin (20, 40 and 80 μM) for 2 h, and then were co-treated with 100 μM 6-OHDA for 24 h in the continued presence of vehicle or naringenin. After incubation, cells were treated with 5 mg/ml MTT for 4 h at 37 °C, and then the media were carefully removed. The formazan crystals that had formed after cell exposure to MTT were dissolved in 150 μL of DMSO and the absorbance was measured at 570 nm on a plate reader (Thermo Scientific Varioskan Flash). Controls utilized the same concentration of medium with DMSO alone.

2.4. Western blot analysis

Protein extracts were prepared from SH-SYSY cells and mouse striatum by homogenization of tissues in RIPA buffer with added protease inhibitors at 4 °C. Protein concentrations were determined by the bicinchoninic acid (BCA) method (Thermo/Pierce). The primary antibodies used in this study were: Nrf2, HO-1, ECM1, GCLC, Lamin A, and β-actin (Santa Cruz Biotechnology), cleaved caspase-3, p-JNK, total JNK, p-p38 and total p38 (Cell signaling technology). Densitometry analyses were performed using ImageJ software.

2.5. Reduced glutathione levels

Cells were treated with 40 μM and 80 μM of naringenin for 24 h. Glutathione (GSH) levels were determined by using Glutathione Quantification Kit. 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and GSH reacted to generate 2-nitro-5-thiobenzoic acid and glutathione disulfide (GSSG) (Rahman et al., 2007). Since 2-nitro-5-thiobenzoic acid is a yellow colored product, GSH concentration in the sample was determined by reading 412 nm absorbance with a multi-well plate reader.

2.6. ROS determination

Levels of oxidative stress were determined by measuring intracellular reactive oxygen species (ROS) generation as detected using the 2’,7’-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescence assay. Briefly, cells were seeded in 96-well plates at the density of 7 × 10^4 per well for overnight incubation. After treatment with various concentrations of test samples, cells were incubated in serum-free medium containing 25 μM DCFH-DA at 37 °C for 30 min. ROS generation was measured by the fluorescence intensity of DCF at 525 nm after excitation at 488 nm on a fluorescence plate reader (Thermo Scientific Varioskan Flash).

2.7. Animal studies

All animal experiments were approved by Institutional Animal Care and Use Committee of Shandong University. Ten-week-old male C57BL/6 mice were randomly assigned to 3 experimental groups: a control group that was treated with vehicle only, a 6-OHDA group, or a 6-OHDA + naringenin 70 mg/kg group. Mice were given 70 mg/kg naringenin or vehicle alone by oral gavage for 4 days prior to lesioning. On the 4th day of treatment, at one hour after final dosing, mice were placed in a stereotaxic device under 1.5% pentobarbital sodium anesthesia and given 6 μg of 6-OHDA (in 2 μL of normal saline with 0.02% ascorbic acid) or saline alone into two different sites of the striatum separately. The stereotaxic coordinates, measured in millimeters, from Bregma were: anterior/posterior = ±1.0, medial/lateral = ±2.1, and dorsal/ventral = −2.9 as well as anterior/posterior = ±0.3, medial/lateral = ±2.3, and dorsal/ventral = −2.9. Mice were sacrificed at different time points (between 1 day and 21 days) following 6-OHDA injection, and tissues were collected for biochemical or histological assessment.

2.7.1. Behavioral testing

Behavior testing was performed using previously published protocols (Signore et al., 2006). Apomorphine-induced rotations were monitored over 3 weeks’ time, starting 1 week post 6-OHDA lesioning. Mice given a subcutaneous injection of apomorphine (0.1 mg/kg in normal saline), were placed individually in plastic beakers (diameter: 13 cm), and videotaped from above for 30 min. Quantitative

Fig. 1. Identification of naringenin as a potent Nrf2 activator. (A) Chemical structure of naringenin. (B) Naringenin induced ARE-dependent luciferase activity. MDA-MB-231-ARE-Luc cells were seeded in 96-well plates; cells were treated with several doses of naringenin for 16 h before analysis of luciferase activity. Data are expressed as mean ± SD. *p < 0.05, **p < 0.01 vs. control.
analyses of completed (360°) left and right rotations were made off-line by an investigator blinded to the experimental conditions.

2.7.2. Determination of DA, DOPAC and HVA by HPLC-mass spectrometry
The striata of mice were removed at 3 weeks following 6-OHDA administration. Dopamine (DA) and its metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured using a highly sensitive high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method. Briefly, striata were individually weighed and homogenized in ice-cold 0.5 M formic acid with the concentration of 5 mg tissue. Lysates were centrifuged at 15,000 × g for 30 min, 4 °C. The supernatant was separated and analyzed according to established protocols (Zhu et al., 2011).

2.7.3. Immunohistochemistry
Animals were anesthetized with sodium pentobarbital at 3 weeks after 6-OHDA administration, transcardially perfused with 0.9% normal saline, followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were dissected out, postfixed in 4% paraformaldehyde for 24 h, and cryopreserved in 30% sucrose for 48 h. Frozen brains were then coronally sectioned at 25 μm thickness on a cryomicrotome and sections were mounted on slides. Using an antibody against tyrosine hydroxylase (TH) (Chemicon), DA neurons were identified with biotinylated secondary antibody and streptavidin ABC solution (Vector Laboratories). Immunostaining was visualized after diaminobenzidine staining (Vector Laboratories) using bright field microscopy (Olympus).

2.8. Statistical analysis
All data are expressed as the mean ± SD. Statistical significance between multiple groups was examined by one-way ANOVA followed by Duncan’s multiple range test. p < 0.05 was considered statistically significant.

3. Results
3.1. Naringenin is a potent Nrf2 activator
We took advantage of a cell model that stably expresses an ARE luciferase reporter gene in MDA-MB-231 cell, to measure the downstream effects of naringenin on ARE evaluated by 96 well high-throughput screening. As shown in Fig. 1B, naringenin induced transcription of the ARE-dependent luciferase gene in a dose-dependent manner, clearly demonstrating its ability to stimulate Nrf2 transcription.

3.2. Naringenin activates the Nrf2/ARE pathway in dopaminergic SH-SY5Y cells
As our studies shown above, using MDA-MB-231 reporter cells showed that the activity of the ARE-dependent reporter genes was correlated with the levels of Nrf2 protein, we next tested the ability of naringenin to induce the Nrf2 pathway in SH-SY5Y cells using dose response and time course studies. Cells were treated either with 0, 20, 40 or 80 μM of naringenin for 8 h or with 80 μM of naringenin for 0, 2, 4 and 8 h. Control groups were treated with DMSO vehicle alone after which all cells were processed by sub-cellular fractionation. When we evaluated nuclear extracts by immunoblot we noted that nuclear Nrf2 had been significantly increased by treatment with naringenin in a dose and time-dependent manner (Fig. 2A and B). Next, we determined whether naringenin-induced nuclear upregulation of Nrf2 resulted in increase in protein levels of ARE downstream genes. Consistent with the upregulation of nuclear Nrf2 protein levels, the protein levels of HO-1, GCLC and GCLM were also significantly increased at 24 h after treatment with different doses of naringenin (Fig. 2C and D). Since naringenin significantly changed levels of key proteins that regulate GSH metabolism, we also measured cellular levels of GSH at 24 h after naringenin treatment. We noted that naringenin produced a dose-dependent increase in GSH levels (Fig. 2E). Taken together, these data suggest that naringenin stimulates nuclear translocation of Nrf2 in vitro, which then up regulates downstream ARE genes, leading to dose-dependent increase in GSH, a potent cellular antioxidant.

3.3. Naringenin attenuates 6-OHDA neurotoxicity in SH-SY5Y cells
Activation of the Nrf2/ARE pathway is known to confer resistance of cells to oxidative stress (Cuadrado et al., 2009). To test this in SH-SY5 cells, we first treated cells with 100 μM of 6-OHDA for 24 h then measured the effects by performing MTT or lactic dehydrogenase (LDH) release assays. Compared to vehicle treated
control cells that never received naringenin, pretreatment of cells with the compound significantly reduced 6-OHDA-associated cell death as well as LDH release in a dose-dependent manner (Fig. 3A and B). To determine whether naringenin may also prevent the production of ROS after 6-OHDA, the accumulation of ROS was measured in SH-SYSY cells by the fluorescent probe DCFH-DA. As shown in Fig. 3C, naringenin effectively reduced 6-OHDA-induced intracellular ROS in a concentration-dependent manner.

Previous studies have shown that the kinases c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK), two well-characterized oxidative stress-responsive pro-death signaling pathways appear to participate in dopaminergic neuronal cell death in PD models (Klintworth et al., 2007; Tobón-Velasco et al., 2013). Therefore, we also examined the ability of naringenin to inhibit 6-OHDA-induced JNK/p38 apoptotic pathways. Our finding revealed that naringenin treatment inhibited the phosphorylation of both JNK and p38 and subsequently inhibited the activation of caspase-3 after 6-OHDA exposure (Fig. 3D and E).

3.4. Naringenin protects SH-SYSY cells against 6-OHDA neurotoxicity via Nrf2/ARE signaling pathway

To determine if the specificity of the apparent neuroprotective effects of was occurring by Nrf2 activation, we next utilized Nrf2 knockdown in SH-SYSY cells. We transfected the cells with either control (si-Control) or Nrf2-specific small interfering RNAs (si-Nrf2) (Santa Cruz Biotechnology) for 48 h, followed by treatment with 80 μM of naringenin for an additional 8 h or 24 h. The efficiency of the Nrf2 siRNA in knocking down Nrf2 was confirmed by Western blot analysis. As shown in Fig. 4A and B, the Nrf2 siRNA treatment significantly decreased the levels of Nrf2 protein in nuclear extracts in SH-SYSY cells treated with naringenin, and furthermore, we found that the knockdown also reduced upregulation of its downstream targets, HO-1, GCLC, and GCLM. We subsequently exposed si-Control or si-Nrf2-treated cells to 80 μM naringenin for 2 h and then subjected the cells to 6-OHDA for another 8 h or 24 h.

The results demonstrated that in si-Control conditions, naringenin still conferred cytoprotective responses in 6-OHDA-challenged SH-SYSY cells, similar to previous data in untransfected cells, but when Nrf2 was silenced, there was less JNK/p38 activation and also less cytoprotection against 6-OHDA (Fig. 4C and D) suggesting that Nrf2 activation is required for naringenin-dependent protection against oxidative stress.

3.5. Naringenin up regulates protein levels of Nrf2/ARE genes in vivo

Because we found that naringenin has the ability to activate the neuroprotective Nrf2/ARE pathway in vitro, we next tested the ability of naringenin to activate Nrf2/ARE signaling in mice. It has been reported that naringenin exhibited high permeability across the in vitro and in situ blood brain barrier models and reached different brain areas after its administration (Youdim et al., 2003). Ten-week-old C57BL/6 mice were given 70 mg/kg of naringenin by oral gavage once daily for 4 consecutive days. Control animals received vehicle alone at the same frequency and volume as naringenin. Brains were removed at the indicated time points after the final dosing and different brain were collected and evaluated by western blot analysis. As shown in Fig. 5A–D, striatal protein levels of Nrf2 were up regulated at 2 h and reached peak at ~8 h, while downstream ARE genes such as GCLC, GCLM, and HO-1 were also significantly up regulated in a time-dependent manner after the final dose of naringenin. We also tested the protein levels of Nrf2 and its downstream ARE genes in different regions of the brain. Immunoblot (Fig. 5E and G) and densitometry analysis (Fig. 5F and H) confirmed significant increases in Nrf2 protein levels as well as of downstream ARE genes in both striatum and SNc, whereas the same proteins were less affected in cortex and hippocampus. Collectively, these data provide compelling in vivo evidence that naringenin can activate the Nrf2-mediated ARE gene transcription to provide neuroprotective effects.
3.6. Naringenin protects against 6-OHDA-associated striatal oxidative stress and subsequent apoptotic signaling cascades in mouse striatum

We measured the levels of ROS and GSH at 1 day and 7 days after 6-OHDA lesions in order to verify our in vitro findings regarding the neuroprotective effects of naringenin and to test if the in vivo activation of Nrf2/ARE signaling by naringenin was able to protect against 6-OHDA neurotoxicity. As an index of ROS formation, we utilized the fluorescent probe DCFH-DA to measure levels of ROS in the striatum of 6-OHDA-treated animals. As shown in Fig. 6A, 6-OHDA stimulated a significant increase in ROS formation at 1 day and 7 days after the toxin injection. In turn, naringenin pretreatment significantly decreased the 6-OHDA-induced ROS formation. When we measured the levels of GSH at those same time points; naringenin pretreatment consistently protected against the 6-OHDA-associated oxidative stress by maintaining GSH levels at close to baseline values for all the time points evaluated (Fig. 6B). Altogether, these results strongly suggest that the pretreatment with naringenin protected mice against 6-OHDA-associated ROS damage in striatum.

In view of the importance of the JNK and p38 signaling pathways for 6-OHDA-induced DA cell death and our observations that naringenin provided neuroprotection in vitro, we further investigated the role of this oxidative stress-induced apoptotic signaling cascades in vivo after 6-OHDA in mice. Consistent with our in vitro results (see Fig. 3D above), 6-OHDA increased p-JNK and p-p38 protein levels in striatum of the mice 7 days after lesioning. Naringenin pretreatment significantly inhibited 6-OHDA-associated activation of JNK and p38 signaling pathways (Fig. 6C–E).

3.7. Naringenin protects against DA neurodegeneration in 6-OHDA-lesioned mice

To further determine if the protection exerted by naringenin against the 6-OHDA helped preserve the integrity of the...
nigrostriatal tract, brains were collected at 21 days after 6-OHDA lesioning. Immunohistochemical staining demonstrated that the 6-OHDA-induced loss of TH-positive neurons in the striatum and SNc were remarkably attenuated by naringenin treatment (Fig. 7A). These results were also supported by immunoblots of striatal extracts evaluated using an anti-TH antibody, which show higher TH protein levels in naringenin treated mice (Fig. 7B).

Striatal dopamine (DA) analysis is an important way to measure dopaminergic neuron degeneration in PD models. Three weeks after 6-OHDA administration, we measured striatal levels of DA, DOPAC and HVA using highly sensitive HPLC-MS/MS analysis. Consistent with a loss of striatal dopaminergic terminals, we also noted a profound reduction in striatal DA and its metabolites after 6-OHDA lesioning that was attenuated by naringenin treatment, which produced a significant elevation in striatal DA and the metabolites DOPAC and HVA at 3 weeks post 6-OHDA lesioning (Fig. 7C).

In order to correlate early biochemical changes in the striatum with long-term motor alterations induced by 6-OHDA, we also measured apomorphine-induced rotation at 7, 14 and 21 days after 6-OHDA lesioning. Apomorphine-induced asymmetrical rotations contralateral to the 6-OHDA injection site were significantly reduced by naringenin treatment as compared to mice lesioned with 6-OHDA (Fig. 7D). Together, the data provide further evidence for naringenin as an activator of the Nrf2/ARE pathway with great potential as a novel therapy to protect the nigrostriatal pathway from oxidative damage that can lead to PD motor problems.

4. Discussion

Here we reported on mechanisms underlying the protective effects of naringenin in vitro and in vivo. The findings support an ROS scavenging effect as noted by others (Zharsky et al., 2005), that occurs by stimulating Nrf2 transcription and its downstream
antioxidant pathways. This is supported by several lines of evidence. We showed that naringenin activated Nrf2 in vitro and in vivo (Figs. 2 and 5) and protected SH-SY5Y cells against 6-OHDA toxicity (Fig. 3). Our in vivo studies in 6-OHDA-lesioned mice demonstrated that naringenin ameliorated dopaminergic neurodegeneration, an effect that in cells could be blocked by knockdown using Nrf2 siRNA. These data imply that naringenin mediates neuroprotection by activating Nrf2 transcription. These results further suggest that activating the Nrf2/ARE pathway in vivo using naringenin produces neuroprotection against ROS, modeled here using 6-OHDA. Our data further imply that targeting the Nrf2/ARE pathway is a promising approach for therapeutic intervention in PD, which may be especially useful for cases that are diagnosed early in the disease process before too much loss of SNc neurons has occurred.

Others have shown that pharmacological agents that activate Nrf2 may be able to block changes associated with PD in different model systems (Jazwa et al., 2011; Kaidery et al., 2013; Tobón-Velasco et al., 2012; Yang et al., 2009). While our data are consistent with previous reports, we showed that an orally bioavailable natural product that can be given long term not only activates Nrf2/ARE pathways, but does so in a manner to turn on neuroprotection by the potent antioxidant GSH. This may be particularly useful for cases that are diagnosed early in the disease process before too much loss of SNc neurons has occurred.

As 6-OHDA administration leads to a marked increase in oxidative damage that results in nigrostriatal neurodegeneration, it is important to note that naringenin significantly blocked ROS accumulation, providing robust evidence that naringenin induces Nrf2 activation in a manner to block neurotoxicity in humans. This is also supported by data showing that naringin (naringenin-7-rhamnosidoglucoside) can block oxidative stress in the 3-nitropropionic acid-induced neurodegeneration model (Gopinath and Sudhandiran, 2012). Additional support comes from studies showing that 6-OHDA induces activation of JNK and p38 MAPK, signaling pathways upstream of mitochondrial-associated apoptosis that are critical in mediating dopaminergic cell death (Klintworth et al., 2007; Tobón-Velasco et al., 2013), further supporting the therapeutic potential of naringenin. In our study, 6-OHDA up-regulated JNK and p38 MAPK in cells and mice, while naringenin treatment blocked this activation, providing further support for naringenin’s neuroprotective effects occurring by Nrf2 activation that blocks ROS-induced activation of the JNK/p38 apoptotic pathway.

In summary, we have demonstrated a neuroprotective mechanism for naringenin against PD associated neurodegeneration of the nigrostriatal pathway. As naringenin is a natural compound that can be increased in the diet to levels that can protect this pathway, it may be beneficial to use naringenin together with standard

Fig. 6. Naringenin reduces 6-OHDA-induced striatal oxidative stress and subsequent apoptotic signaling cascades in mice. Mice were given naringenin by oral gavage for 4 days, and then received striatal infusion of 6-OHDA. At 1 day and 7 days after 6-OHDA infusion, striatum were collected for measurement of ROS (A) and GSH levels (B). (C) Immunoblot analysis of striatal extracts for JNK/p38 apoptotic signaling proteins at 7 days after 6-OHDA infusion. Quantitative data were illustrated in (D) and (E). All data are presented as the mean ± SD of triplicate independent experiments. **p < 0.01 vs. control, *p < 0.05, ***p < 0.01 vs. 6-OHDA group.
dopamine replacement therapies as a more potent neuroprotective therapy. The results of this study also provide compelling support for finding more potent pharmacological agents to activate Nrf2/ARE pathway to add to the arsenal for treating neurodegenerative diseases, especially PD at a time when the aging population is increasing worldwide.

Conflict of interest

The authors declare no conflict of interests.

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