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Cytoprotective Effects of Docosyl Cafferate against tBHP-Induced Oxidative Stress in SH-SY5Y Human Neuroblastoma Cells

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Abstract

Neuronal cell death is a common characteristic feature of a variety of neurodegenerative disorders including Alzheimer's disease and Parkinson's disease. However, there have been no effective drugs to successfully prevent neuronal death in those diseases. In the present study, docosyl cafferate (DC), a derivative of caffeic acid, was isolated from *Rhus verniciflua* and its protective effects on tBHP-induced neuronal cell death were examined in SH-SY5Y human neuroblastoma cells. Pretreatment of DC significantly attenuated tBHP-induced neuronal cell death in a concentration-dependent manner. DC also significantly suppressed tBHP-induced caspase-3 activation. In addition, DC restored tBHP-induced depletion of intracellular Bcl-2, an anti-apoptotic member of the Bcl-2 family. Furthermore, DC significantly suppressed tBHP-induced degradation of IKB, which retains NF-kB in the cytoplasm, resulting in the suppression of nuclear translocation of NF-kB and its subsequent activation. Taken together, the results clearly demonstrate that DC exerts its neuroprotective activity against tBHP-induced oxidative stress through the suppression of nuclear translocation of NF-kB.

Key Words: Docosyl cafferate (DC), SH-SY5Y human neuroblastoma cells, tBHP, Apoptosis, NF-kB

INTRODUCTION

Oxidative stress has been implicated in diverse neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease (PD), and many inflammatory conditions (Simonian and Coyle, 1996; Liu *et al.*, 2002). Many physiological and environmental toxicants exert oxidative stress to cell by overproducing reactive oxygen species (ROS), such superoxide anions and hydroxyl radicals (Halliwell, 1989; Nohl *et al.*, 1996). An increasing evidence suggests that neuronal apoptosis is involved in the pathological processes of neurodegenerative disorders such as AD and PD (Friedlander, 2003). Therefore, novel pharmacological agents that can suppress oxidative stress-induced neuronal damages might provide a promising therapeutic strategy for numerous neuronal death-related CNS pathologies.

Rhus verniciflua of the Anacardiaceae family, commonly known as the lacquer tree, has been reported to possess a wide range of biological activities such as antioxidant (Kim *et al.*, 1997; Kim and Choi, 2002), anti-microbial, anti-cancer (Park *et al.*, 2004; Jeong and Park, 2008), and anti-platelet properties (Jeon *et al.*, 2006). However, the exact chemical components of *Rhus verniciflua* have not been fully elucidated. Recently, it has been reported that stigmasterols isolated from *Rhus verniciflua* exhibit neuroprotective activity in a mouse model of kainic acid-induced excitotoxicity (Byun *et al.*, 2010).

To further provide biologically active compounds from *Rhus verniciflua*, docosyl cafferate (DC) was purified from the stem bark of *Rhus verniciflua*. DC is an ester derivative of caffeic acid that has been reported to possess a wide variety of biological activities such as antioxidant and anti-inflammatory properties (Kim and Kim, 2000) and neuroprotective effect (Sul *et al.*, 2009). Ester derivatives of caffeic acid have also been reported to possess numerous biological activities (Kim, 2010). For example, caffeic acid phenylethyl ester (CAPE)

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has been reported to possess protective activities against glutamate-induced neurotoxicity (Wei *et al.*, 2008) and cerebral ischemia (Tsai *et al.*, 2006). Although DC was previously identified in the root of *Sohora subprostrata* (Komatsu *et al.*, 1970), its biological activity has never been demonstrated.

In the present study, we investigated the neuroprotective activity of DC, isolated from the stem bark of *Rhus verniciflua*, and a possible underlying mechanism by which DC exerts its neuroprotective action in tBHP-induced neuronal cell death in SH-SY5Y human neuroblastoma cells.

MATERIALS AND METHODS

Chemicals

Docosyl cafferate (DC, CAS No. 50432-89-8) (Fig. 1) was isolated from *Rhus verniciflua* and dissolved in 99% ethanol. Briefly, the dried stem bark of *Rhus verniciflua* was extracted with methanol and then partitioned with n-hexane, dichlormethane, ethyl acetate, and butanol, successively. DC was isolated from a dichlormethane fraction using silica gel column chromatography. The chemical structure of the compound was determined by means of 1H-NMR (600 MHz) and EI-MS and identified to DC by comparison with spectral data from the literature (Iinuma et al., 1992). tert-Butyl hydroperoxide (tBHP, Sigma Chemical Co. MO, USA), a prototypical organic oxidant that has been extensively used in the study of oxidant-induced cell death (Liu *et al.*, 1998; Amoroso *et al.*, 1999), was used to induce oxidative stress in SH-SY5Y cells in the present study.

Cell culture and drug treatment

Human neuroblastoma SH-SY5Y cells obtained from American Type Culture Collection (Rockville, USA) were cultured in DMEM medium supplemented with 10% heat-inactivated fetal calf serum, 20 mM glutamine, 10 U/ml penicillin, and 100 μg/ml streptomycin and maintained at 37°C in a humidified atmosphere with 5% CO_2. DC was added to the cells 6 hr prior to the incubation with tBHP for 12 hr. All experiments were carried out on sub-confluent cultures.

Determination of cell viability

SH-SY5Y cells were placed into 12-well culture plates and were incubated at 37°C under a 5% CO_2/95% humidified air incubator for 24 hr. Cells were then incubated DC for 6 hr prior to treatment with tBHP for another 12 hr. Cell viability was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, which yields a blue formazan product in living cells, but not in dead cells or their lytic debris. MTT dissolved in phosphate-buffered saline was added at the end of incubation to a final concentration of 0.5 mg/ml, and then incubated at 37°C for 2 hr, the resultant formazan product was extracted for 4 hr with extraction solution (20% SDS, 50% DMF and 2% acetic acid) and detected by a UV-VIS spectrometer (Perkin Elmer Co.) at 570 nm.

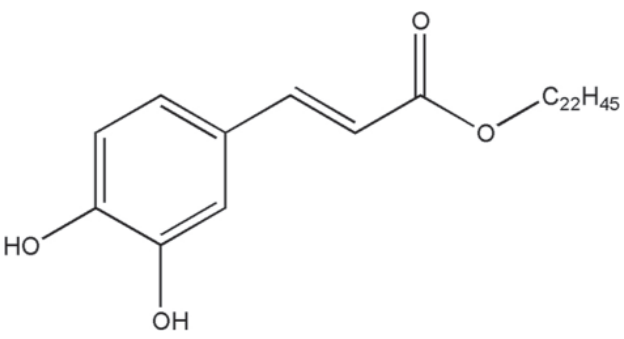

Fig. 1. Chemical structure of docosyl cafferate (DC).

Western blot analysis

SH-SY5Y cells were washed with PBS two times and lysed with lysis buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, 0.5% sodium deoxycholate, 1 mM phenyl methylsulfonyl fluoride]. Equal amounts of protein were separated on 10% SDS-polyacrylamide gel. Proteins were transferred to Hypond PVDF membrane (Amersham Biosciences, Piscataway, NJ, USA). The membranes were blocked in 5% skim milk in TBST for 1 hr at room temperature and sequentially incubated with an appropriate antibody; anti-cleaved caspase-3 (Sigma, Saint Louis, MO. USA), anti-cleaved PARP (Sigma), anti-Bcl2 (Sigma), anti-IkB-α (Santa Cruz Biotechnology Inc., Eugene, OR, USA), or β-actin (Sigma). After thoroughly washing with TBST, the membranes were then washed three times with TBST and incubated with HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) or HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) for 2 hr at room temperature. The blots were developed by the enhanced chemiluminescence detection kit (ECL Amersham Biosciences, Piscataway, NJ, USA). The immunoblots were quantified by measuring the density of each band using densitometry with ImageQuant™ software.

Immunocytochemistry

The effect of DC on the nuclear translocation of p65 subunit of NF-kB was examined by immunofluorescence assay using confocal microscopy. SH-SY5Y cells were cultured in sterile coverslips and pretreated with DC for 1 hr and stimulated with LPS. At 30 min after the tBHP treatment, the cells were fixed in 4% paraformaldehyde for 20 min at room temperature. The fixed cells were then permeabilized with 0.1% Triton X-100 in PBS and blocked with 3% BSA. Afterwards, the cells were sequentially incubated with rabbit p65 antibody (Santa Cruz Biotechnology Inc., Eugene, OR, USA) at room temperature and Alexa 546-labeled goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) at room temperature for 1 hr. Nuclei were stained with 0.5 μg/ml of propidium iodide (PI) staining solution for 10 min at room temperature. After washing with PBS, the sample were mounted and observed by means of confocal microscopy.

Statistical analysis

All values shown in the figures are expressed as the mean ± SD obtained from at least three independent experiments. Statistical analysis was carried out by one-way analysis of variance (ANOVA) with Tukey's post-hoc test using SPSS software 12K (SPSS, Chicago, IL, USA). A value of $p<0.05$ was considered as statistically significant.

RESULTS

DC attenuates tBHP-induced neuronal cell death in SH-SY5Y human neuroblastoma cells

In the present study, neuroprotective effects of DC were examined with tBHP-induced oxidative stress model using SH-SY5Y human neuroblastoma cells. To obtain appropriate concentration of tBHP, which exhibits approximately 50% of cell death, SH-SY5Y cells were treated with indicated concentrations of tBHP for 12 hr, and cell viability was determined using MTT assay. Concentration-dependent cell death was observed with tBHP treatment (Fig. 2A) and 250 μM tBHP, which exhibited approximately 50% cell death, was chosen in the following experiments.

To examine possible protective effects of DC on neuronal cell viability, SH-SY5Y cells were pretreated with DC for 6 hr prior to tBHP treatment. As shown in Fig. 2B, DC significantly attenuated tBHP-induced cell death in a concentration-dependent manner (Fig. 2B). To examine whether tBHP-induced cell death in SH-SY5Y cells was mediated through apoptosis, activation of caspase-3, which is the effector caspase in the apoptotic cascade, was measured. Treatment of the cells with tBHP resulted in activation of casapse-3 and pretreatment of DC significantly attenuated tBHP-induced caspase-3 activation in a concentration-dependent manner (Fig. 3). These results demonstrate that DC possesses inhibitory effects on tBHP-induced apoptotic cell death in SH-SY5Y cells.

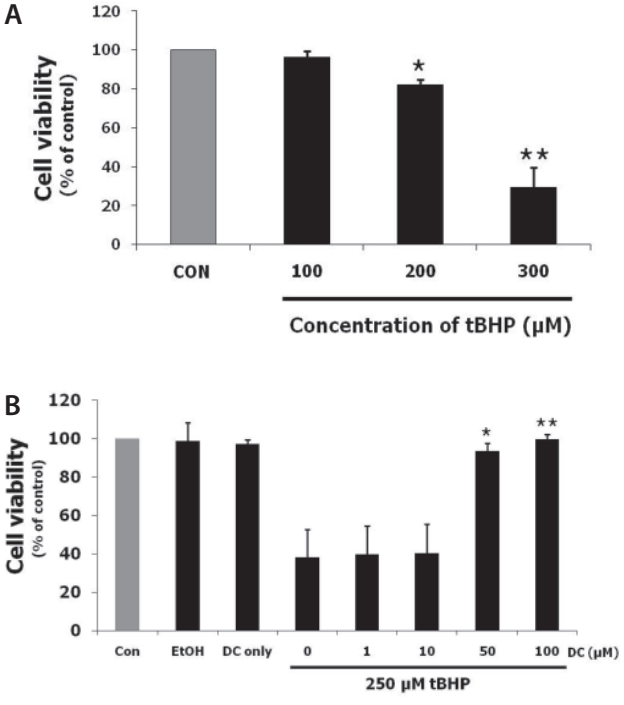

Fig. 2. Concentration-dependent cytotoxicity of tBHP (A) and protective effect of DC (B) on tBHP-induced neuronal cell death in SH-SY5Y human neuroblastoma cells. (A) After 12 hr of incubation with tBHP, neuronal cell viability at indicated concentrations of tBHP was measured using MTT assay. Concentration-dependent cell death was observed and 250 μM concentration, which exhibited approximately 50% cell death, was chosen in the following experiments. (B) tBHP-induced neuronal death was significantly attenuated with DC treatment in a concentration-dependent manner. No noticeable cell death was observed with DC-only treatment. Data represent three independent experiments and are expressed as mean ± SD. *p<0.05 and **p<0.01 indicate statistically significant difference with tBHP alone.

DC inhibits tBHP-induced degradation of Bcl-2 in SH-SY5Y human neuroblastoma cells

Given the fact that Bcl-2 family members play an important role in regulating cytochrome c release and caspase-3

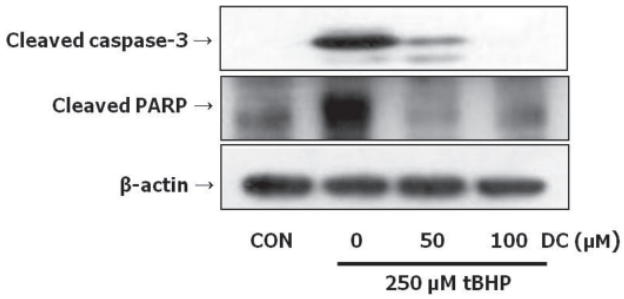

Fig. 3. Attenuation of tBHP-induced apoptosis by DC in SH-SY5Y human neuroblastoma cells. Cells were pretreated with indicated concentration of DC for 6 hr and then incubated with tBHP for 12 hr. Cell lysates were subjected to SDS/PAGE and immunoblotted with caspase-3 or PARP antibodies. Actin antibody was used as a control for equal loading. tBHP-induced apoptotic neuronal death was significantly attenuated with DC treatment in a concentration-dependent manner.

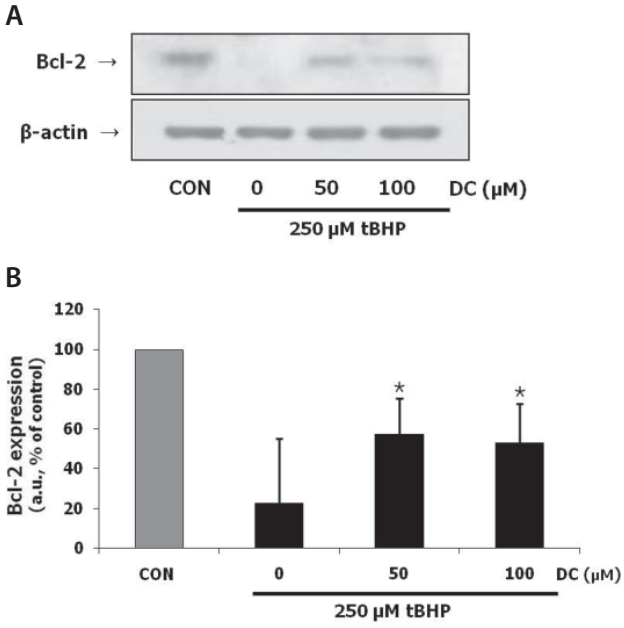

Fig. 4. Effects of DC on the intracellular level of Bcl-2 in tBHP-exposed SH-SY5Y human neuroblastoma cells. Intracellular level of Bcl-2 was determined with immunoblotting analysis: a representatative immunoblot (A) and quantitative analysis (B) of immunoblots. β-Actin was used as an internal control. Quantitative data represent three independent experiments and are expressed as mean ± SD. *p<0.05 indicates statistically significant difference with tBHP alone.

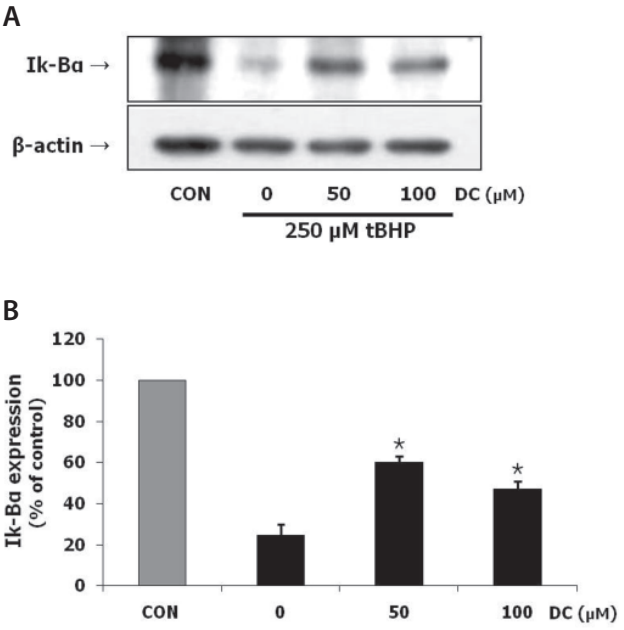

Fig. 5. Inhibitory effects of DC on tBHP-induced IkB-α degradation in SH-SY5Y human neuroblastoma cells. Intracellular level of IKB-α was determined with immunoblotting analysis: a representatative immunoblot (A) and quantitative analysis (B) of immunoblots IKB-α. β-Actin was used as an internal control. Quantitative data represent three independent experiments and are expressed as mean ± SD. *p<0.05 indicates statistically significant difference with tBHP alone.

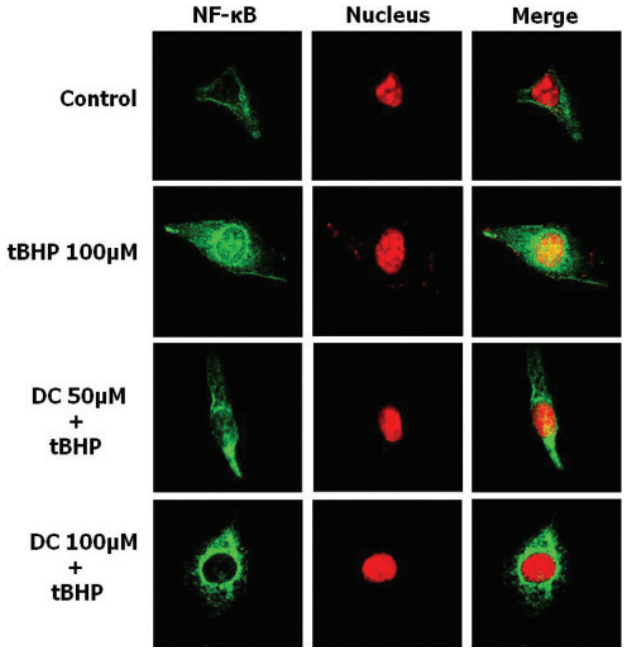

Fig. 6. Blockade of nuclear translocation of p65 subunit of NF-kB by DC in tBHP-induced SH-SY5Y human neuroblastoma cells. Localization of NF-kB p65 subunit was determined using a p65 antibody and an Alexa 546-labeled goat anti-rabbit IgG antibody. Nuclei were visualized by propidium iodide (PI) staining. In basal condition, immunostaining of p65 subunit was diffuse throughout the cytoplasm. However, tBHP stimulation resulted in the apparent translocation of p65 subunit into the nucleus. Pretreatment of DC appear to attenuate tBHP-induced nuclear translocation of p65 subunit.

activation. The change in intracellular level of Bcl-2, an anti-apoptotic protein, was examined upon tBHP stimulation (Fig. 4). Treatment of tBHP resulted in the apparent depletion of intracellular Bcl-2. However, pretreatment of DC significantly suppressed tBHP-induced depletion of Bcl-2, albeit not concentration-dependent (Fig. 4).

DC suppresses tBHP-induced degradation of IKB and nuclear translocation of NF-kB

In an effort to examine a possible underlying mechanism by which DC exerts neuroprotection in SH-SY5Y cells, the level of nuclear translocation of NF-kB was examined given the fact that nuclear NF-kB is essential for its proapoptotic roles in various cell culture models (Bian *et al.*, 2001; Wang *et al.*, 2002). In the present study, oxidative stress with tBHP resulted in a significantly decreased intracellular level of IKB in SH-SY5Y cells (Fig. 5). However, pretreatment of DC exhibited a significant attenuation of tHBP-induced degradation of IKB (Fig. 5). To further determine whether the change in the level of IKB affects the nuclear translocation of NF-kB, the level of nuclear NF-kB was examined using confocal microscopy. Immunostaining of p65 subunit of NF-kB was present predominantly in the cytoplasm in basal condition (Fig. 6). tBHP stimulation resulted in apparent nuclear translocation of p65 subunit of NF-kB in SH-SY5Y cells. However, pretreatment of DC appeared to attenuate the tBHP-induced nuclear translocation of p65 subunit of NF-kB in a concentration-dependent manner (Fig. 6).

DISCUSSION

Reactive oxygen species (ROS) may disturb the inherent cellular antioxidant defense system, resulting in damage to biological macromolecules such as nucleic acids, proteins, carbohydrates, and lipids. Such forms of damage have been implicated in several neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and ischemia (Hyslop *et al.*, 1995; Chandra *et al.*, 2000). Therefore, the protection of neuronal cells from reactive oxygen species can be beneficial for the prevention and treatment of these diseases. In the present study, we demonstrated that DC isolated from the stem bark of *Rhus verniciflua* possesses neuroprotective activity in tBHP-induced neuronal cell death in SH-SY5Y human neuroblastoma cells. DC significantly suppressed tBHP-induced apoptosis and Bcl-2 depletion. Furthermore, DC significantly attenuated tBHP-induced IKB degradation and subsequent nuclear translocation of NF-kB.

Numerous ester derivatives of caffeic acid such as CAPE, chlorogenic acid, pedicularioside A, forsythoside B, and echinachoside have been reported to exhibit biological activities such as antioxidant and anti-inflammatory properties, inhibition of caspase-3, and maintenance of mitochondrial function (Deng *et al.*, 2004; Jiang *et al*., 2010; Lapchak, 2007; Li *et al.*, 2008; Wei *et al.*, 2008). Although ester derivatives of caffeic acid with high molecular weight aliphatic alcohol such as DC and 1-eicosanoyl cafferate have been isolated from *sophora*

subprostrata (Komatsu *et al.*, 1970) and *Echinosophora koreensis* (Kang and Kim, 1987), respectively, their biological activities have not been examined. It is presumed that high molecular fatty alcohols in their chemical structures may provide the compounds with lipid solubility, facilitating intracellular penetration through the plasma membrane. In the present study, DC exhibited a significant neuroprotective activity in tBHP-induced neuronal cell death in a micromolar range, suggesting that DC might efficiently enter the cells due to its high lipid solubility. Although DC was not a novel compound, the present study was the first demonstration that DC possesses neuroprotective activity in an oxidative stress-induced neuronal cell death model.

The inappropriate regulation of NF-kB and its downstream genes has been associated with various pathological conditions (Karin *et al.*, 2001; Li and Verma, 2002). Aberrant NF-kB activation was observed in CA1 hippocampal neurons following transient global forebrain ischemia in rats (Clemens *et al.*, 1997; Cho *et al.*, 2008). In addition, it has been reported that NF-kB plays a pro-appototic role in doxorubicin-induced apoptosis (Wang *et al.*, 2002; Ashikawa *et al.*, 2004). In accordance with these reports, the present study showed that pretreatment of DC significantly attenuated tBHP-induced nuclear translocation of NF-kB. However, it has been also suggest that NF-kB might be anti-apoptotic (Romano *et al.*, 2004) and that activation of NF-kB can provide neuroprotection against amyloid β-induced toxicity (Barger *et al.*, 1995) and oxidative stress (Goodman and Mattson, 1996; Mattson *et al.*, 1997), suggesting a diverse function of NF-kB in the nervous system depending on the cellular context.

In conclusion, the present study demonstrates that DC exerts the neuroprotective activity by inhibiting nuclear translocation of NF-kB against tBHP-induced oxidative stress in SH-SY5Y human neuroblastoma cells. The present study suggests that DC might be a valuable therapeutic agent in the treatment of neuronal cell death-related brain pathologies such as neurodegenerative diseases.

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