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Research Paper

Neuroprotection against 6-OHDA toxicity in PC12 cells and mice through the Nrf2 pathway by a sesquiterpenoid from *Tussilago farfara*

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ABSTRACT

Oxidative stress plays a key role in neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. Therefore, the nuclear factor-E2-related factor 2 (Nrf2), a key regulator of the antioxidative response, is considered to be important as a therapeutic target for neurodegenerative diseases. We investigated the underlying mechanism of Nrf2-mediated neuroprotective effects against oxidative stress in the PC12 cell line by 7β-(3-ethyl-*cis*-crotonoyloxy)-1α-(2-methylbutyryloxy)-3,14-dehydro-*Z*-notonipetranone (ECN), one of the sesquiterpenoids in Farfarae Flos. Pretreatment of PC12 cells with ECN had a protective effect against hydrogen peroxide (H_2O_2)- or 6-hydroxydopamine (6-OHDA)-induced cytotoxicity. ECN upregulated the ARE-luciferase activity and induced the mRNA expression of Nrf2 and antioxidant enzyme heme oxygenase-1 (HO-1). Knockdown of Nrf2 by small, interfering RNA (siRNA) abrogated the upregulation of HO-1, indicating that ECN had induced HO-1 *via* the Nrf2 pathway. Pretreatment with the thiol reducing agents, *N*-acetylcysteine (NAC) or dithiothreitol (DTT), attenuated Nrf2 activation and HO-1 expression. However, the non-thiol reducing antioxidant, Trolox, failed to inhibit HO-1 induction by ECN. These results suggest that ECN may directly interact with Kelch-like ECH-associated protein 1 (Keap1) and modify critical cysteine thiols present in the proteins responsible for Nrf2-mediated upregulation of HO-1. In a 6-OHDA-induced mouse model of PD, administration of ECN ameliorated motor impairments and dopaminergic neuronal damage. Taken together, ECN exerts neuroprotective effects by activating the Nrf2/HO-1 signaling pathway in both PC12 cells and mice. Thus, ECN, as an Nrf2 activator, could be an attractive therapeutic candidate for the neuroprotection or treatment of neurodegenerative diseases.

1. Introduction

Neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS), are characterized by the progressive dysfunction and loss of structure or function of neurons in the central nervous system [1]. Although the exact cause of each disease remains unclear, many lines of evidence suggest that oxidative stress play a key role in the pathogenesis of neurodegenerative diseases [1,2]. In the brain, reactive oxygen species (ROS), which cause oxidative stress, are mainly generated by dopamine metabolism, mitochondrial dysfunction, aging, and neuroinflammation. Several proteins related to the pathogenesis of AD and PD, such as amyloid-β peptide (Aβ), amyloid precursor protein (APP), α-synuclein, parkin, PTEN-induced kinase1 (PINK1), DJ-1, and leucine-rich repeat kinase (LRRK2) are also associated with oxidative stress and mitochondrial dysfunction [3,4]. In this regard, modulation of oxidative stress can be an effective pharmacological strategy for the prevention or treatment of neurodegenerative disorders.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is an essential transcription factor that regulates antioxidant defense genes in maintaining cellular homeostasis. Under normal conditions, Nrf2 remains inactive in the cytoplasm, by forming a complex with its inhibitory protein Kelch-like ECH-associated protein 1 (Keap1), which promotes ubiquitination and the eventual degradation of Nrf2. Under stress conditions, Nrf2 is released from Keap1, translocates to the nucleus and binds to the antioxidant response element (ARE) in the promoter region

Abbreviations: 6-OHDA, 6-hydroxydopamine; Act. D, actinomycin D; AD, Alzheimer's disease; APO, apomorphine; ARE, antioxidant response element; CHX, cycloheximide; DAT, dopamine transporter; DTT, dithiothreitol; ECN, 7β-(3-ethyl-*cis*-crotonoyloxy)-1α-(2-methylbutyryloxy)-3,14-dehydro-*Z*-notonipetranone; ERK1/2, extracellular signal regulated protein kinase 1/2; HO-1, heme oxygenase-1; H_2O_2, hydrogen peroxide; Keap1, Kelch-like ECH-associated protein 1; LPS, lipopolysaccharide; MAPKs, mitogen-activated protein kinases; NAC, *N*-acetylcysteine; Nrf2, nuclear factor-E2-related factor 2; PD, Parkinson's disease; PI3K, phosphoinositide 3-kinase; SN, substantia nigra; SnPP, tin protoporphyrin IX; ST, striatum

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of several cytoprotective genes, such as heme oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (NQO1) [5,6]. The Nrf2 signaling pathway has been reported to be closely related to neurodegenerative diseases [5]. Nrf2 activation in astrocyte mediates neuroprotection against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity PD model in mice [7], and electrophilic compounds show protective effects both *in vitro* and *in vivo* against neuronal degeneration by activating the Keap1/Nrf2/HO-1 pathway [8]. Upregulation of HO-1, one of the target genes induced by Nrf2, plays a key role in neurodegenerative damage associated with AD and PD [9]. Therefore, the Nrf2/HO-1 pathway is a beneficial therapeutic target in the protection or treatment of neurodegenerative diseases.

Phytochemicals are potent antioxidants and act as activators of Nrf2 inducing phase II detoxification enzymes. Terpenoids, including mono-, sesqui-, di-, and triterpenoids, induce Nrf2 through the Michael reaction of reactive cysteine residues on the Keap1 protein. Because of this common feature, various terpenoids have been reported to possess protective effects [10,11]. A previous study revealed that several sesquiterpenoids isolated from the buds of *Tussilago farfara*, including 7β-(3-ethyl-*cis*-crotonoyloxy)-1α-(2-methylbutyryloxy)-3,14-dehydro-*Z*-notonipetranone (ECN), have anti-inflammatory actions in activated microglia and cytoprotective effects against LPS-induced neuronal cell death [12]. However, the Nrf2-mediated neuroprotective properties of ECN against oxidative stress and *in vivo* studies on the 6-OHDA-induced neurotoxicity in mice have yet to be elucidated. The objective of this study was thus to investigate cytoprotective activities against cell damage induced by oxidative stress and underlying molecular mechanisms of ECN. The potency of ECN to activate Nrf2 and induce HO-1 was also identified. In addition, we aimed to determine whether ECN exerted any protective effects in an animal experimental model of neurodegeneration.

2. Materials and methods

2.1. Materials and reagents

ECN was isolated from dried buds of *Tussilago farfara* and identified, as previously reported by our group [13]. Fetal bovine serum (FBS), penicillin, and streptomycin were purchased from GenDepot (Barker, TX, USA). Horse serum (HS) was the product of GIBCO BRL (Grand Island, NY, USA). Ham's F-12K, Dulbecco's phosphate buffered saline, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 6-hydroxydopamine (6-OHDA), cycloheximide, actinomycin D, 2′,7′-dichlorofluorescein diacetate (DCF-DA), dithiothreitol (DTT), *N*-acetyl-L-cysteine (NAC), LY294002, U0126, SB203580, SP600125, protease inhibitor cocktail, paraformaldehyde (PFA), diaminobenzidine (DAB), sucrose, apomorphine (APO), and tribromoethanol (TBE) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2) solution (30% purified) was purchased from Merck (Darmstadt, Germany). The primary antibodies for Nrf2, HO-1, p-Akt, Akt, and β-actin, as well as all secondary antibodies and tin protoporphyrin IX (SnPP), were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary antibodies for Keap1 and PCNA were supplied by Genetex (Irvine, CA, USA). Rabbit anti-tyrosine hydroxylase (TH), rabbit anti-dopamine transporter (DAT) and polyvinylidene fluoride were obtained from Millipore (Marlborough, MA, USA). Trolox was provided by Cayman Chemical (Ann Arbor, MI, USA). The ARE-binding site-luciferase construct was a generous gift from Prof. Young-Joon Surh (Seoul National University, Seoul, Korea). All other chemicals were purchased from Sigma-Aldrich Co. unless otherwise specified.

2.2. Cell culture

PC12 rat pheochromocytoma cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). PC12 cells were cultured in Ham's F-12K medium, supplemented with 15% HS, 2.5% FBS. Cells were maintained in the presence of 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

2.3. Measurement of cell viability

The cytoprotective effect of ECN on H_2O_2- or 6-OHDA-induced PC12 cells was measured with an MTT-based colorimetric assay. In brief, cells were treated with indicated concentrations of ECN before exposure to H_2O_2 (500 μM) or 6-OHDA (250 μM) for 24 h. MTT solution was added at the end of the treatment to the cell culture media at 0.5 mg/ml final concentration and incubated for 2 h at 37 °C in the dark. The absorbance at 595 nm was determined with an EMax® microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.4. Western blot analysis

Total cell lysates were prepared using the lysis buffer previously described [14]. To prepare cytosolic and nuclear extracts, cells were collected and washed with PBS. Cells were resuspended in the lysis buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, and a protease inhibitor cocktail), incubated on ice for 15 min, and then 10% of NP-40 was added. The mixture was vortexed for 10 s and centrifuged at 15,000 rpm for 5 min, with this supernatant containing the cytoplasmic fraction. The nuclear pellets were resuspended in nuclear extraction buffer (20 mM HEPES [pH 7.9], 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and a protease inhibitor cocktail) for 1 h on ice with vortexing at 10 min intervals and then centrifuged at 15,000 rpm for 10 min. The protein concentration was quantified using the Bradford protein assay (Bio-Rad Laboratories, Richmond, CA, USA). Equal amounts of protein (30 μg) were loaded on 8% of SDS polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked by 5% bovine serum albumin in T-BST buffer (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.6) and incubated with primary antibodies overnight at 4 °C. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The immunoblots were detected with EZ-Western detection kit (DoGEN, Seoul, Korea). The values above the figures represent the relative density of the bands normalized to that for β-actin or PCNA.

2.5. Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was isolated using the Trizol reagent kit (Invitrogen, Carlsbad, CA, USA). Both the quantity and purity of RNA were measured using the Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Total RNA (1 μg) was synthesized into cDNA using the amfiRivert Platinum cDNA Synthesis Master Mix (GenDepot, Barker, TX, USA), in accordance with the manufacturer's instructions. PCR amplification of Nrf2, HO-1, and β-actin genes was performed using forward and reverse primers and a SYBR Green working solution (iTaq™ Universal SYBR Green Supermix, Bio-Rad, Hercules, CA, USA) with an Applied Biosystems 7300 real-time PCR system and software (Applied Biosystems, Carlsbad, CA, USA). The following primers were used: Nrf2, 5′-CTC GCT GGA AAA AGA AGT G-3′ (sense) and 5′-CCG TCC AGG AGT TCA GAG G-3′ (antisense); HO-1, 5′-CAC GCA TAT ACC CGC TAC CT-3′ (sense) and 5′-CCA GAG TGT TCA TTC GAG A-3′ (antisense).

2.6. ARE-luciferase assay

PC12 cells were co-transfected with 0.5 μg of pRL-CMV control vector (Promega, Madison, WI, USA) and 5 μg of ARE-luciferase