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Sulforaphane induces autophagy through ERK activation in neuronal cells

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ABSTRACT

Sulforaphane (SFN), an activator of nuclear factor E2-related factor 2 (Nrf2), has been reported to induce autophagy in several cells. However, little is known about its signaling mechanism of autophagic induction. Here, we provide evidence that SFN induces autophagy with increased levels of LC3-II through extracellular signal-regulated kinase (ERK) activation in neuronal cells. Pretreatment with NAC (N-acetyl-L-cysteine), a well-known antioxidant, completely blocked the SFN-induced increase in LC3-II levels and activation of ERK. Knockdown or overexpression of Nrf2 did not affect autophagy. Together, the results suggest that SFN-mediated generation of reactive oxygen species (ROS) induces autophagy via ERK activation, independent of Nrf2 activity in neuronal cells.

1. Introduction

Sulforaphane (1-isothiocyanoate-4-[methylsulfinyl]-butane, SFN) is an organic isothiocyanate that is formed from glucoraphanin, by myrosinase when certain cruciferous vegetables are damaged (or chewed) allowing the enzyme to mix with its substrate [1]. SFN is a potent inducer of the phase II antioxidant enzymes such as glutathione transferase, heme oxygenase-1 (HO-1) and NAD(P)H:quinine reductase [2,3]. The expression of the phase II enzymes is under the control of antioxidant response element (ARE) in their promoter regions. The activation of AREs is modulated by nuclear factor E2-related factor 2 (Nrf2). Nrf2 is sequestrated by its cytoplasmic partner, Kelch-like ECH-associated protein 1 (Keap1), which promotes its ubiquitination and degradation through the proteasome [4,5]. Modification of two crucial cysteine residues (C273 and C288) on Keap1 protein by oxidation, alkylation or arylation stemming from oxidative stress results in dissociation of the Nrf2/Keap1 complex. Nrf2 dissociated moves into the nucleus and binds to ARE sequences, thus increasing the transcription of the phase II antioxidant enzymes as well as other Nrf2-responsive genes [5,6]. SFN was reported to block Nrf2 ubiquitination by altering Keap1 conformation via the reaction with thiols of Keap1 cysteines [7]. In addition to the induction of the phase II detoxifying enzymes, SFN activates heat shock response and enhances proteasome activity through the induction of heat shock protein 27 (HSP27) expression [8], and reduces the lipopolysaccharide-induced secretion of pro-inflammatory cytokines [9,10]. Also, SFN induces cell cycle arrest and apoptosis in many types of cancer cells, thus inhibiting tumor growth in vivo [11,12].

Autophagy is an evolutionally conserved pathway involved in the organized elimination of proteins, organelles and invading microbes by lysosomes. The autophagic process starts with the formation of an autophagosome, which enclosed within a

Abbreviations: SFN, sulforaphane; Nrf2, nuclear factor E2-related factor 2; Keap1, Kelch-like ECH-associated protein 1; ARE, antioxidant response element; LC-3, microtubule-associated protein 1 light chain 3; AV, autophagic vesicle; ERK, extracellular signal-regulated kinase; JNK, c-jun N-terminal kinase; SAPK, stress-activated protein kinase; MEK, mitogen protein kinase kinase; HO-1, heme oxygenase-1; NAC, N-acetyl-L-cysteine; DCF-DA, 6-carboxy-2′,7′-dichlorodihydrofluorescien diacetate

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double-membrane that entraps cytoplasmic components. Then, autophagosomes fuse with lysosomes to generate autolysosomes, where cytoplasmic molecules and organelles are degraded by protein hydrolyases, including cathepsin D, and recycled [13,14]. Since altered or damaged proteins and organelles cannot be reduced by cell division in differentiated neurons, autophagy is essential for cellular quality control in neurons. Thus, dysfunction of autophagy has been implicated in a number of neurodegenerative diseases, Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) [15,16].

Reactive oxygen species (ROS) play crucial roles as signaling messengers in cell survival, death and immunity under physiological conditions [17]. ROS can also act as signals for the activation of autophagy [18–21]. SFN has been known to mediate antioxidative and antiproliferative response as well as cell death via generating ROS in epithelial or cancer cells [22–24]. A recent study suggested that SFN-induced ROS play a crucial role in inducing autophagy in pancreatic cancer cells [25]. Administration of SFN enhances autophagic activity in neurons [26] and brain [27]. Although the study revealed that SFN activates autophagy, little is known about the signal mechanism of their action, especially in neuronal cells.

In present study, we provide evidences that SFN induces autophagy via ROS-mediated extracellular signal-regulated kinase (ERK) activation in neuronal cells, and it is independent of Nrf2 activity.

2. Materials and methods

2.1. Antibodies and reagents

Anti-phospho-specific mTOR, mTOR, phospho-specific stress-activated protein kinase/c-jun N-terminal kinase (SAPK/JNK), SAPK/JNK, phospho-specific p38, p38, Myc (9B11) and Nrf2 antibodies were obtained from Cell Signaling Technology. Anti-phospho-specific ERK and ERK antibodies were purchased from Santa Cruz. Anti-LC3 antibody was obtained from MBL Medical & Biological Laboratories. Anti-β-actin antibody was purchased from Millipore. Myc-Nrf2 and GFP-LC3 plasmids were used previously [28]. SFN was purchased from Calbiochem. U0126 was obtained from Biomol. PD184352 was kindly provided by R. Marquez (University of Dundee, UK). N-acetyl-L-cysteine (NAC), 6-carboxy-2′,7′-dichlorodihydrofluorescien diacetate (DCF-DA), resazurin and chloroquine were purchased from Sigma.

2.2. Cell culture

Immortalized mouse CN1.4 cortical neurons and human SH-SY5Y neuronal cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 10 units/ml penicillin and 100 units/ml streptomycin in a humidified atmosphere containing 5% CO_2. CN1.4 cortical neurons and SH-SY5Y cells were maintained at 33 °C and 37 °C, respectively.

2.3. Immunoblotting

Cells were washed once with PBS and lysed with modified RIPA buffer (10 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1 mM EGTA, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS) containing 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and 10 μg/ml each of aprotinin, leupeptin and pepstatin. Proteins were extracted on ice with periodic vortexing for 30–40 min, and lysates were cleared by centrifugation at 10000×*g* for 10 min at 4 °C, and the supernatants were used for immunoblotting following boiling in 1× SDS-sample loading buffer for 5 min. For analysis protein samples (40 μg) were separated on 4–12% gradient SDS–polyacrylamide gels (Invitrogen) followed by transfer to nitrocellulose membranes (GE Healthcare) and immunoblotting with the indicated antibodies. Blots were developed with chemiluminescence (GE Healthcare). All protein concentrations were determined using the BCA method (Sigma).

2.4. Electron microscopy (EM)

Immortalized mouse CN1.4 cortical neurons were treated with 10 μM SFN for 24 h. Cells were fixed overnight in a mixture of cold 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and 2% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.2) and embedded with epoxy resin. The epoxy resin-mixed samples were loaded into capsules and polymerized at 60 °C for 48 h. Thin sections were sliced on an ultramicrotome (UC7, Leica) and collected on a copper grid. Appropriate areas for thin sectioning were cut at 70 nm and stained with saturated 2% uranyl acetate and 2% lead citrate before examination on a transmission electron microscope (Libra 120, Carl Zeiss) at 120 kV.

2.5. ROS measurement

Immortalized mouse CN1.4 cortical neurons were treated with SFN for 8 h. Then, the cells were treated with 10 μM of 6-carboxy-2′,7′-dichlorodihydrofluorescien diacetate (DCF-DA, Sigma) in culture media without serum for 30 min. The cells were washed three times with PBS followed by fixation with 4% PFA. The fluorescence was observed using a fluorescence microscope (Carl Ziess). For the analysis of flow cytometry, the cells were trypsinized and resuspended in PBS. The intracellular fluorescence was detected at the channel for FITC (BD FACSVerse Instrument).

2.6. siRNA transfection

For knock-down of endogenous mouse Nrf2, mouse Nrf2 specific siRNA or scrambled RNA as a control was mixed with Oligofectamine (Invitrogen) in serum-free Opti-MEM medium for 15 min. The mixture was loaded onto CN1.4 cortical cells following exchange of the culture medium for serum-free DMEM medium. After 4 h, FBS was added up to 5% and maintained at 33 °C in a humidified atmosphere containing 5% CO_2 for 36 h. The On-TARGET plus smart pool mouse Nrf2 specific siRNA was purchased from Thermo Scientific.

3. Results

3.1. SFN induces autophagy in neuronal cells

To examine whether SFN could induce autophagy in neuronal cells, immortalized mouse CN1.4 cortical cells were treated with SFN (10 μM) for 24 h. The level of LC3-II, a well-known marker of autophagy, was analyzed. As shown in Fig. 1A and B, the level of LC3-II in cells treated with SFN was significantly increased compared to control cells not treated. An increase in the number of autophagic vesicles (AV) in the cells treated with SFN was also observed (Figs. 1C–E and 2). The increase of LC3-II could be due to an interruption in autophagosome–lysosome fusion, raising the lysosomal pH or inhibiting lysosome-mediated proteolysis [29]. To further clarify whether the increased level of LC3-II by SFN treatment was caused by interrupting the autophagosome-lysosome fusion or not, we treated neuronal cells with SFN followed by treatment with chloroquine (CQ), a lysosomal inhibitor for 6 h. Expectedly, LC3-II was accumulated in cells treated with CQ, and the level of accumulated LC3-II was more increased in cells treated with SFN along with CQ than those treated with SFN or CQ alone (Fig. 3). Together, these results suggest that SFN induces autophagy via increasing autophagic flux in neuronal cells.