A Thesis for the Degree of Master of Science

Sulforaphane increases BDNF expression via HDAC inhibition in primary cortical neurons and 3xTg-AD mice

일차신경세포와 치매동물 모델에서 설포라판의 HDAC 활성 저해를 통한 BDNF의 증가 및 작용기작 규명

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석사학위논문

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일차신경세포와 치매동물 모델에서 설포라판의 **HDAC** 활성 저해를 통한 **BDNF**의 증가 및 작용기작 규명

지도교수 이기원
이 논문을 석사학위 논문으로 제출함

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김지성

김지성의 학위논문을 인준함
2014 년 8 월

위 원 장 문태화 (인)

부위원장 이기원 (인)

위 원 서진호 (인)

Abstract

Brains of patients with Alzheimer's disease (AD) contain abnormally low levels of brain-derived neurotrophic factor (BDNF), a neurotrophin that regulates learning and memory and promotes the survival of injured neurons. As recent studies indicate that sulforaphane improves learning and memory in animal models, I hypothesized that sulforaphane influences synaptic activity by regulating BDNF levels. I found that sulforaphane treatment increased BDNF levels in mouse primary cortical neurons and restored frontal cortex levels of BDNF in a triple-transgenic mouse model of AD. Also, sulforaphane inhibited histone deacetylase (HDAC) and increased acetylation of histone 3 and 4, suggesting that sulforaphane regulates BDNF expression via HDAC inhibition. Furthermore, sulforaphane increased levels of several BDNF pathway components, including tyrosine kinase receptor B (TrkB), cAMP-responsive element-binding protein (CREB), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), extracellular signal-regulated kinase (ERK), and Akt, as well as neuronal and synaptic molecules such as microtubule-associated protein 2 (MAP2), synaptophysin, and postsynaptic density protein-95 (PSD-95). These findings suggest that sulforaphane could be used to prevent or treat AD.

Key Words: sulforaphane ; brain-derived neurotrophic factor ; histone deacetylase ; Alzheimer's disease ;

Student ID: 2012-23372

Contents

I. Introduction

Neurotrophins such as nerve growth factor, brain-derived neurotrophic factor (BDNF), neurotrophin-3, and neurotrophin-4 are a small family of secreted proteins that critically regulate the survival and differentiation of specific subpopulations of neurons [1]. BDNF regulates synaptogenesis and neuronal plasticity and supports the function of several brain areas and neuronal subsystems [1-3]. It is also involved in the induction and maintenance of long-term potentiation (LTP) and memory formation [4]. Patients with Alzheimer's disease (AD) show abnormal neurotrophin signaling, leading to the suggestion that neurotrophins could prevent or delay AD-related neuronal and synaptic loss [5]. In particular, several studies show that BDNF expression is down-regulated in human AD brains [6-8] and AD mouse models [7]. Moreover, viral transduction or direct infusion of BDNF protein in the entorhinal cortex reverses neuronal atrophy and synaptic loss in aged primates and rats as well as in a mouse model of AD [9].

Emerging evidence demonstrates that chromatin remodeling via histone acetylation regulates BDNF transcription and that BDNF acetylation is associated with synaptic plasticity [10]. Acetylation of histones at lysine residues by histone acetyltransferase relaxes chromatin structure, allowing

recruitment and initiation of transcriptional machinery, whereas deacetylation of histones by histone deacetylase (HDAC) is generally associated with transcriptional repression [11]. Interventions that promote acetylation (e.g., HDAC inhibitors such as sodium butyrate and valproic acid) increase BDNF levels during memory formation *in vivo* [12] and enhance quantal neurotransmitter release and dendritic spine density in hippocampal slices [13].

Sulforaphane (1-isothiocyanato-4-methylsulfinylbutane, Figure 1A) is an isothiocyante found in cruciferous vegetables (e.g., broccoli, cabbage, watercress, Brussels sprouts) [14]. Recent studies show that sulforaphane inhibits HDAC activity and increases global histone acetylation in cell and animal cancer models (for review, see [15]). Also, *in vitro* and *in vivo* studies demonstrate that the neuroprotective effects of sulforaphane are accompanied by activation of the transcription factor NF-E2-related factor 2 (Nrf2) and up-regulation of its target genes [16]. Although sulforaphane is known to exhibit neurotrophic factor-like activity, how sulforaphane affects a diverse array of neuronal functions remains unclear.

Here, I investigated whether sulforaphane regulates BDNF expression via chromatin remodeling (i.e., HDAC activity and histone acetylation) in primary mouse cortical neurons and the frontal cortex of triple-transgenic AD model mice (3×Tg-AD) that exhibit both amyloid-β

(Aβ)- and tau-dependent pathology [17]. I also examined the effect of sulforaphane on several components of the BDNF pathway, including tyrosine kinase receptor B (TrkB), cAMP response element-binding protein (CREB), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), extracellular signal-regulated kinase (ERK), and Akt, as well as neuronal and synaptic markers such as microtubule-associated protein 2 (MAP2), synaptophysin, and postsynaptic density protein-95 (PSD-95). I report, for the first time, that sulforaphane inhibits HDAC, increases histone acetylation, and elevates BDNF levels in primary cortical neurons and the frontal cortex of AD model mice. These results suggest that sulforaphane could be used as a neuroprotective agent against AD.

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Ⅱ. Materials and Methods

2.1. Matherials

Sulforaphane was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Fetal bovine serum (FBS) was purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), Neurobasal medium, Ca^{2+}/Mg^{2+}-free Hank's Balanced Salt Solution (HBSS), L-glutamine, B-27 supplement, and penicillin/streptomycin were purchased from Gibco BRL (Carlsbad, CA).

2.2. Cell culture

Primary cortical neurons were prepared from ICR mice at embryonic day 15 (E15). Cerebral cortices from E15 embryos were dissected and placed in ice-cold Ca^{2+}/Mg^{2+}-free HBSS (4 ml/embryo). The tissue was centrifuged (300 × g, 2 min), dissociated with 0.05 % trypsin in HBSS for 10 min at 37 ℃, re-suspended in minimal essential medium (10 %

FBS (Fetal bovine serum), 10 % heat-inactivated horse serum, 2 mM L-glutamine, and 1 % penicillin/streptomycin), and filtered through a 70-μm cell strainer twice. Cells were placed on 0.2-mg/ml poly-D-lysine-coated plates and incubated in a 37 ℃ humidified atmosphere. Cells were allowed to adhere to the plates for 45 min before culture media was changed to Neurobasal medium supplemented with B27, 1 % L-glutamine, and 1 % penicillin/streptomycin. Neurites sprouting from neuronal cell bodies were observed starting on the third day after initial plating. Neurobasal medium was changed every other day. Primary neurons were treated with 10 or 20 μM sulforaphane, and phosphate-buffered saline (PBS) was used as vehicle.

2.3. 3xTg-AD mice

Mice harboring the presenilin 1 (PS1, M146V), Swedish mutant amyloid precursor protein (APPswe, KM670/671NL), and tau (P301L) transgenes driven by the Thy1.2 promoter [17], as well as non-transgenic

mice in the same C57BL6/129 background, were graciously provided by Dr. Frank LaFerla (University of California Irvine, Irvine, CA). Female mice (12 months of age) were housed individually in ventilated cages under specific pathogen-free conditions at 21 ± 2 ℃ with a 12-h light-dark cycle (light period starting at 8 AM) and free access to food and water. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Seoul National University. Primers used for polymerase chain reaction (PCR) genotyping were: PS1, 5′-CAC ACG CAA CTC TGA CAT GCA CAG GC-3´ and 5′-AGG CAG GAA GAT CAC GTG TTC AAG TAC-3´; APP, 5′-GCT TGC ACC AGT TCT GGA TGG-3′ and 5′-GAG GTA TTC AGT CAT GTG CT-3′; Tau, 5′ GAG GTA TTC AGT CAT GTG CT-3′ and 5′-TTC AAA GTT CAC CTG ATA GT-3′.

2.4. Treatment schedules

To test the effects of sulforaphane, mice were randomly assigned

to four groups (n = 6 per group): (1) non-transgenic mice treated with vehicle, (2) 3×Tg-AD mice treated with vehicle, (3) 3×Tg-AD mice treated with 10 mg/kg/day sulforaphane (100 μl/day), or (4) 3×Tg-AD mice treated with 50 mg/kg/day sulforaphane (100 μl/day). Sulforaphane was dissolved in PBS and administered by oral gavage (p.o.) 6 days a week for 8 weeks. Brains were then removed, and frontal cortices were dissected on ice and frozen at -80 ℃ until analysis.

2.5. Western blot analysis

Cells and tissue were homogenized in radioimmunoprecipitation assay buffer (Cell Signaling Technology, Beverly, MA), and protein concentration was determined using Protein Assay Reagent (Bio-Rad, Hercules, CA). Protein was separated by 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). Membranes were blocked in 5 % fat-free dry milk or 5 % bovine serum albumin in

tris-buffered saline and Tween-20 and then incubated with primary antibodies against BDNF (1:1000, Santa Cruz), acetyl-histone 3 (Ac-H3, 1:1000, Millipore), acetyl-histone 4 (Ac-H4, 1:1000, Cell Signaling), histone 3 (H3, 1:1000, Cell Signaling), histone 4 (H4, 1:1000, Cell Signaling), HDAC2 (1:1000, Cell Signaling), TrkB (80E3) (1:1000, Cell Signaling), phosphorylated (p)-CREB (Ser133, D1G6) (1:1000, Cell Signaling), CREB (48H2) (1:1000, Cell Signaling), p-CaMKII (1:1000, Cell Signaling), CaMKII (1:1000, Cell Signaling), p-ERK (E-4) (1:1000, Santa Cruz), ERK (1:1000, Santa Cruz), p-Akt (Ser473) (1:1000, Cell Signaling), Akt (1:1000, Cell Signaling), MAP2 (1:1000, Millipore), synaptophysin (1:1000, Millipore), PSD-95 (1:1000, Cell Signaling), and β-actin (1:5000, Sigma-Aldrich). After incubation with horseradish-peroxidase-conjugated secondary antibodies, protein bands were detected using an enhanced chemiluminescence detection kit (GE Healthcare, St. Giles, UK).

2.6. Real-time (RT)-PCR

Total RNA was extracted from cells and tissue using RNAiso reagent (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. RNA was quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA (1 μg/μl) served as a template for the synthesis of cDNA using a PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio). cDNAs were amplified using TaKaRa Ex Taq™ DNA polymerase and the following PCR primers (Bioneer, Daejeon, Korea) : *Bdnf*, 5'-TTG TTT TGT GCC GTT TAC CA-3' and 5'-GGT AAG AGA GCC AGC CAC TG-3'; *β-actin*, 5'-TGT CCA CCT TCC AGC AGA TG-3' and 5'-AGC TCA GTA ACA GTC CGC CT-3'. For quantitative RT-PCR, the iQ™ SYBR® Green Supermix and CFX Connect™ Real-Time PCR Detection System (Bio-Rad) were used. PCR amplification of *Bdnf* and *β-actin* consisted of 40 cycles of 30 s at 95 ℃, 10 s at 95 ℃, 10 s at 60 ℃, and 30 s at 72 ℃.

2.7. Chromatin immunoprecipitation (ChIP)

The Chromatin Immunoprecipitation (ChIP) Assay Kit (Upstate Biotechnology) was used to assess ChIP assay. Primary cultured neurons cultured on 10-well dishes were crosslinked with formaldehyde (1 % final concentration) for 10 minutes at 37 ℃. After samples were washed with PBS, 200 µl lysis buffer was added to cells, incubated for 10 minutes on ice. The protein concentration of lysates was determined using a protein assay kit (Bio-Rad, Hercules, CA, USA) and 500 µg protein was sonicated to generate 200-1000 bp chromatin fragments. The sonicated cell supernatant was diluted 1:10 with ChIP dilution buffer (Upstate Biotechnology) and 5 µl of anti-AcH3 antibody (Millipore), 5 µl of anti-AcH4 antibody (Millipore) was added and were rotated overnight at 4 ℃. After they were pulled down with protein A Agarose/Salmon sperm DNA (50 % Slurry) (Upstate Biotechnology), washed 5 times with Low Salt Immune Complex Wash Buffer, High Salt Immune Complex Wash Buffer, LiCl Immune Complex wash Buffer, TE Buffer (Upstate Biotechnology).

Chromatin complexes were eluted with elution buffer (1 % SDS, 0.1M $NaHCO_3$) and cross-linkes were reversed by adding 20 µl 5 M Nacl for 4 hours at 65 ℃. The DNA was digested with 10 µl of 0.5 M EDTA, 20 µl 1 M Tris-HCl (pH 6.5) and 2 µl of 10 mg/ml proteinas K for one hour at 45 ℃. DNA was recovered by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. Real-time PCR was performed using iQ™ SYBR® Green Supermix and CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The primer (Bioneer, Daejeon, Korea) sequences used were as follows : *Bdnf-pI* 5'-TGA TCA TCA CTC ACG ACC ACG-3' and 5'-CAG CCT CTC TGA GCC AGT TAC G-3'; *Bdnf-pII* 5'-CGG AGA GCA GAG TCC ATT CAG-3' and 5'-CCA GTA TAC CAA CCC GGA GC-3'; *Bdnf-pIV* 5'-GCG CGG AAT TCT GAT TCT GGT AAT-3' and 5'-GAG AGG GCT CCA CGC TGC CTT GAC G-3'.

2.8. HDAC activity

The Color-de-Lys™ HDAC colorimetric activity assay kit (Enzo Life Sciences, Farmingdale, NY) was used to assess HDAC activity. Color de Lys™ substrate, which comprises an acetylated lysine side chain, was incubated with homogenates (25 μg protein) from cells and tissue for 90 min at 37 ℃ to initiate deacetylation of the substrate. Color de Lys™ developer was then added, and the mixture was incubated for another 15 min at 37 ℃ to stop the reaction and cause an increase in yellow color intensity. Colorimetric readout at 405 nm was performed using a VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, CA).

2.9. Immunofluorescence staining

Primary neurons (1.0×10^6 cells) were seeded on chamber slides coated with poly-D-lysine. Cells were washed with PBS, fixed with 4 % paraformaldehyde (Duksan, Ansan, Korea) for 20 min, and blocked with permeabilization buffer containing 0.5 % Triton X-100 (Calbiochem, La Jolla, CA) and 10 % goat serum (Vector, Burlingame, CA) in PBS for 1 h

at room temperature. Cells were rinsed three times with PBS and incubated with anti-MAP2 (1:200) overnight at 4 ℃. Fluorescein-conjugated anti-mouse IgG (Vector) was used as a secondary antibody at a dilution of 1:200 in permeabilization buffer for 1 h at room temperature. 4', 6-diamidino-2-phenylindole (DAPI, 1.5 μg/ml, Vector) was used to stain nuclear DNA of primary neurons for 30 min at room temperature. All images were taken using the confocal scanning module of the LSM 700 CLSM (Carl Zeiss, Jena, Germany) and analyzed using Zen 2009 software (Carl Zeiss).

2.10. Statistical analysis

Statistical analyses were performed using SPSS 19.0 for Windows (SPSS Inc., Chicago, IL). Data were expressed as mean ± standard error of the mean (SEM) and analyzed using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference tests.

Ⅲ. Results

3.1. Sulforaphane increases BDNF levels in primary cortical neurons and the frontal cortex of 3xTg-AD mice

I first examined the effect of sulforaphane on BDNF levels in primary cortical neurons, which fully develop morphologically and become completely functional in culture [18]. I found that sulforaphane treatment significantly increased levels of BDNF protein (Figure 1B) and mRNA (Figure 1C) in primary cortical neurons. Next, I examined the effect of sulforaphane on BDNF levels in the frontal cortex of 3×Tg-AD mice. Levels of BDNF protein in 3×Tg-AD mice were significantly lower than those in non-transgenic mice, but sulforaphane treatment restored levels of BDNF protein in the frontal cortex of 3×Tg-AD mice (Figure 1D).

3.2 Sulforaphane increases levels of TrkB, p-CREB, p-CaMKII, p-

ERK, and p-Akt in primary cortical neurons and the frontal cortex of 3xTg-AD mice

Sulforaphane treatment significantly increased levels of TrkB, p-CREB, p-CaMKII, p-ERK, and p-Akt in primary cortical neurons (Figure 2A). Frontal cortex levels of TrkB and p-Akt did not differ between non-transgenic and 3×Tg-AD mice, but sulforaphane treatment increased TrkB and p-Akt levels in 3×Tg-AD mice (Figure 2B). Frontal cortex levels of p-CREB, p-CaMKII, and p-ERK were significantly lower in 3×Tg-AD mice than in non-transgenic mice, but sulforaphane treatment increased p-CREB, p-CaMKII, and pERK levels in 3×Tg-AD mice (Figure 2B).

3.3 Sulforaphane increases acetylation of H3 and H4 in primary cortical neurons and the frontal cortex of 3xTg-AD mice

Sulforaphane treatment increased acetylation of H3 and H4 in primary cortical neurons (Figure 3A). Although there was no difference in levels of acetylated (Ac)-H3 and Ac-H4 in the frontal cortex of non-

transgenic and 3×Tg-AD mice, sulforaphane treatment significantly increased levels of Ac-H3 and Ac-H4 in 3×Tg-AD mice (Figure 3B).

3.4 Sulforaphane increases acetylation of H3 and H4 at Bdnf promoter regions in primary cortical neurons

After validation of Bdnf promoter I, II and IV immunoprecipitation with Ac-H3 and Ac-H4 antibodies in primary cortical neurons, I found Ac-H3 and Ac-H4 levels at these promoters after treating neurons with sulforaphane, that potently induced Bdnf mRNA levels (Figure 4A and 4B).

3.5 Sulforaphane decreases HDAC activity in primary cortical neurons and the frontal cortex of 3xTg-AD mice

Sulforaphane treatment significantly inhibited HDAC activity (Figure 5A) and reduced levels of HDAC2 in primary cortical neurons (Figure 5B). HDAC activity in the frontal cortex of 3×Tg-AD mice was

significantly lower than that of non-transgenic mice, and sulforaphane treatment further reduced HDAC activity in 3×Tg-AD mice (Figure 5C). Sulforaphane treatment also significantly reduced levels of HDAC2 in 3×Tg-AD mice (Figure 5D).

3.6 Sulforaphane increases levels of MAP2, synaptophysin, and PSD-95 in primary cortical neurons and the frontal cortex of 3xTg-AD mice

To identify morphological changes in primary cortical neurons after sulforaphane treatment, I assessed the shape of neurons and nuclei after MAP2 and DAPI immunofluorescent staining. Sulforaphane treatment increased the number of intact nuclei and neurites (Figure 6A). Sulforaphane treatment also significantly increased levels of MAP2, synaptophysin, and PSD-95 in primary cortical neurons (Figure 6B). Frontal cortex levels of MAP2 were significantly lower in 3×Tg-AD mice than in non-transgenic mice, but sulforaphane treatment restored levels of

MAP2 in 3×Tg-AD mice (Figure 6C). Although frontal cortex levels of synaptophysin and PSD-95 did not differ between non-transgenic and 3×Tg-AD mice, sulforaphane treatment significantly increased synaptophysin and PSD-95 levels in 3×Tg-AD mice (Figure 6C).

Fig. 1

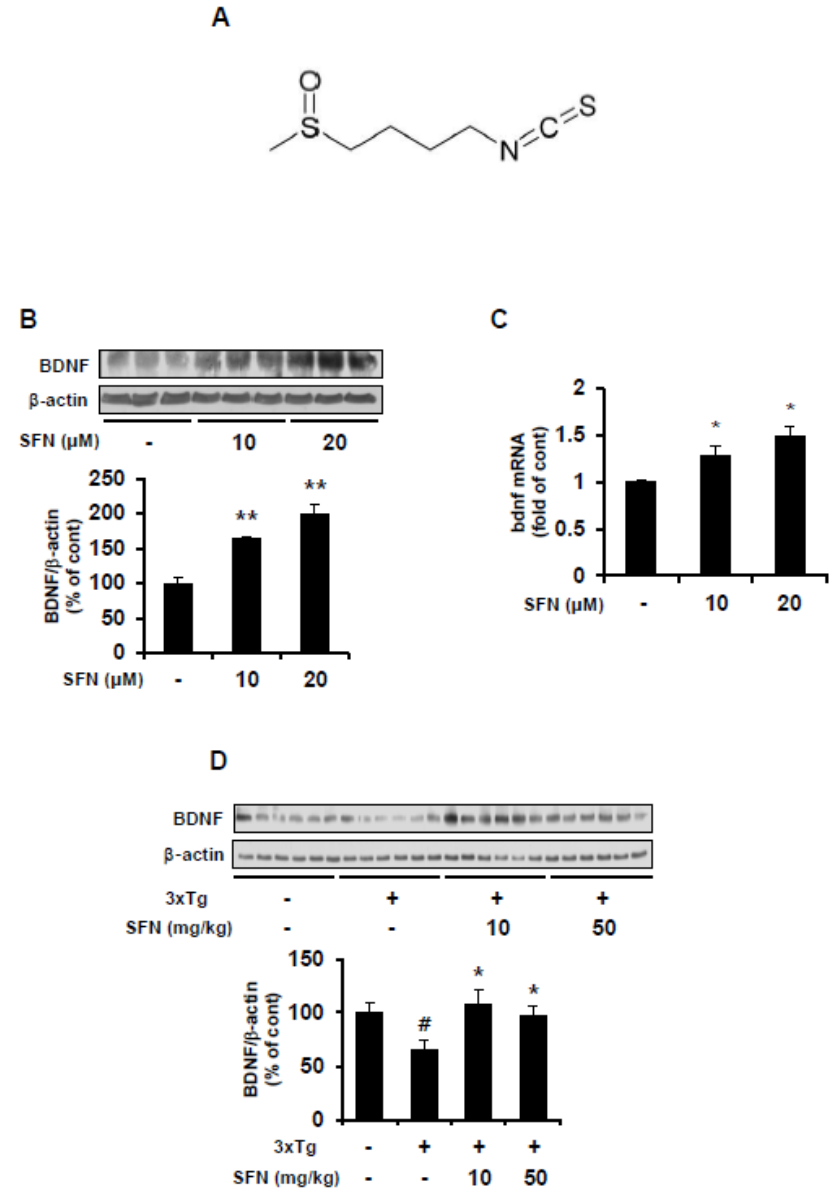

A
O
S
N=C=S
B
BDNF
β-actin
SFN (μM) - 10 20
BDNF/β-actin (% of cont)
250
200
150
100
50
0
**
**
SFN (μM) - 10 20
C
bdnf mRNA (fold of cont)
2
1.5
1
0.5
0
*
*
SFN (μM) - 10 20
D
BDNF
β-actin
3xTg - + + +
SFN (mg/kg) - - 10 50
BDNF/β-actin (% of cont)
150
100
50
0
#
*
*
3xTg - + + +
SFN (mg/kg) - - 10 50

Figure 1. Effect of sulforaphane on BDNF levels in primary cortical neurons and the frontal cortex of 3xTg-AD mice.

Cells were treated with sulforaphane at 10 μM or 20 μM for 3 h to test Bdnf mRNA and 6 h to test BDNF protein. 3xTg mouse were administered by oral gavage with sulforaphane at 10 mg/kg/day or 50 mg/kg/day for 8 weeks. (A) Chemical structure of sulforaphane. (B) Representative western blot gel and densitometry analysis of BDNF protein levels. β-actin was used as a loading control. (C) qRT-PCR analysis of *Bdnf* mRNA. $n = 3$; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. vehicle control. (D) Representative western blot gel and densitometry analysis of BDNF protein levels. β-actin was used as a loading control. $n = 6$; $\#p < 0.05$ vs. non-transgenic mice treated with vehicle, $^{*}p < 0.05$ vs. 3×Tg-AD mice treated with vehicle.

Fig. 2

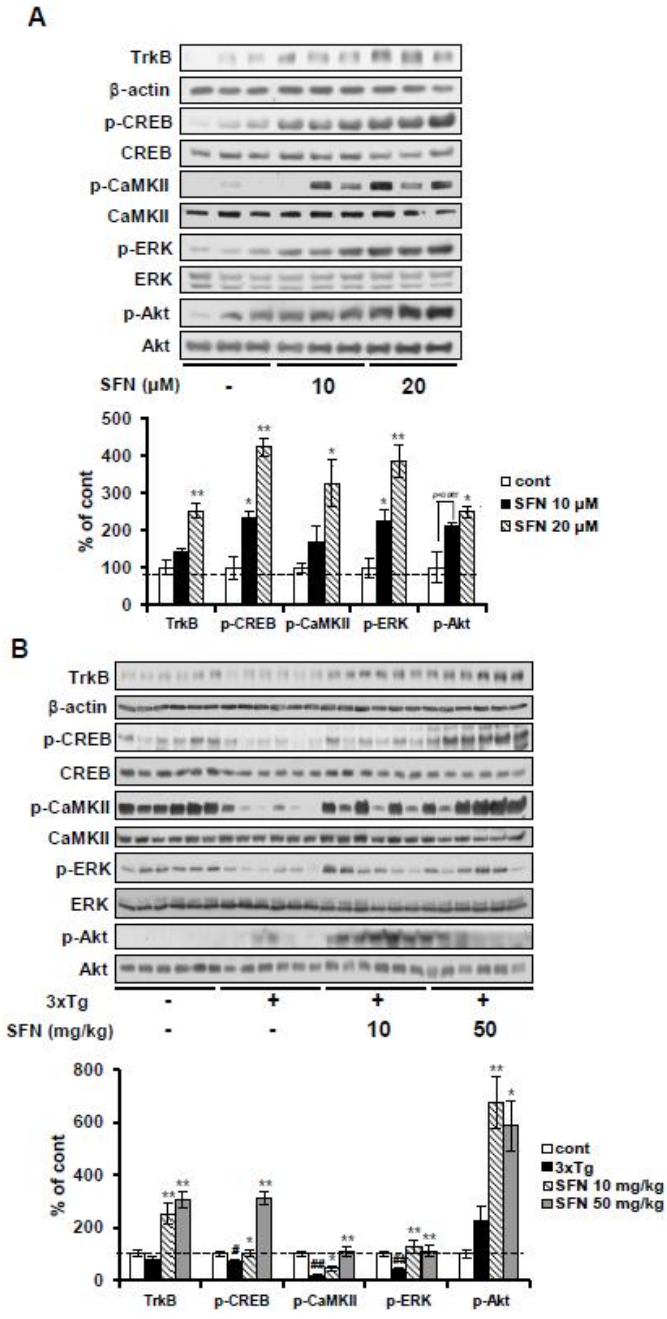

A
TrkB
β-actin
p-CREB
CREB
p-CaMKII
CaMKII
p-ERK
ERK
p-Akt
Akt
SFN (μM) - 10 20
% of cont
cont
SFN 10 μM
SFN 20 μM
TrkB p-CREB p-CaMKII p-ERK p-Akt
B
TrkB
β-actin
p-CREB
CREB
p-CaMKII
CaMKII
p-ERK
ERK
p-Akt
Akt
3xTg - + + +
SFN (mg/kg) - - 10 50
% of cont
cont
3xTg
SFN 10 mg/kg
SFN 50 mg/kg
TrkB p-CREB p-CaMKII p-ERK p-Akt

Figure 2. Effect of sulforaphane on TrkB, p-CREB, p-ERK, and p-Akt levels in primary cortical neurons and the frontal cortex of 3xTg-AD mice.

Cells were treated with sulforaphane at 10 μM or 20 μM for 6 h and 3xTg mouse were administered by oral gavage with sulforaphane at 10 mg/kg/day or 50 mg/kg/day for 8 weeks. (A) Representative western blot gel and densitometry analysis of TrkB/β-actin, p-CREB and CREB, CaMKII/CaMKII, p-ERK/ERK, and p-Akt/Akt protein levels. $n = 3$; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. vehicle control. (B) Representative western blot gel and densitometry analysis of TrkB/β-actin, p-CREB/CREB, p-CaMKII/CaMKII, p-ERK/ERK, and p-Akt/Akt protein levels. $n = 6$; $\#p < 0.05$ and $\#\#p < 0.01$ vs. non-transgenic mice treated with vehicle; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. 3×Tg-AD mice treated with vehicle.

Fig. 3

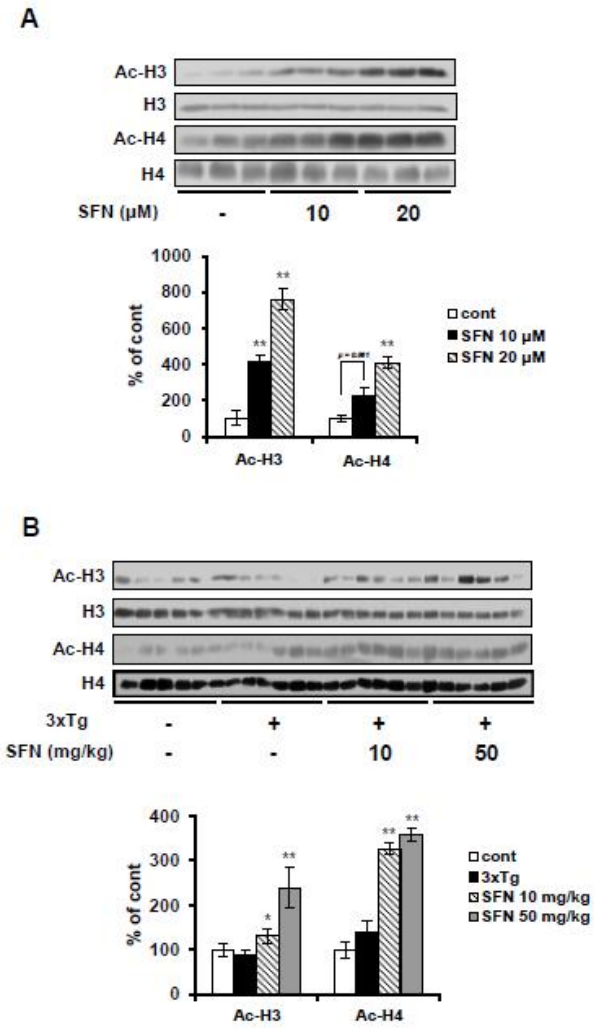
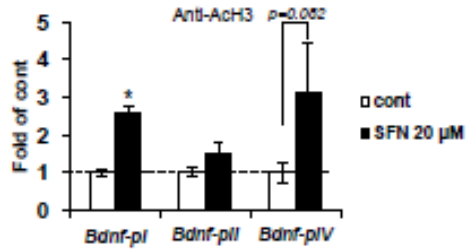

A
Ac-H3
H3
Ac-H4
H4
SFN (μM) - 10 20
1000
800
600
400
200
0
% of cont
cont
SFN 10 μM
SFN 20 μM
Ac-H3
Ac-H4
B
Ac-H3
H3
Ac-H4
H4
3xTg - + + +
SFN (mg/kg) - - 10 50
400
300
200
100
0
% of cont
cont
3xTg
SFN 10 mg/kg
SFN 50 mg/kg
Ac-H3
Ac-H4

Figure 3. Effect of sulforaphane on acetylation of H3 and H4 in primary cortical neurons and the frontal cortex of 3xTg-AD mice.

Cells were treated with sulforaphane at 10 μM or 20 μM for 3 h and 3xTg mouse were administered by oral gavage with sulforaphane at 10 mg/kg/day or 50 mg/kg/day for 8 weeks. (A) Representative western blot gel and densitometry analysis of Ac-H3/H3 and Ac-H4/H4 protein levels. $n = 3$; $^{**}p < 0.01$ vs. vehicle control. (B) Representative western blot gel and densitometry analysis of Ac-H3/H3 and Ac-H4/H4 protein levels. $n = 6$; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. 3×Tg-AD mice treated with vehicle.

Fig. 4

A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SFN 20 µM
Bdnf-pI
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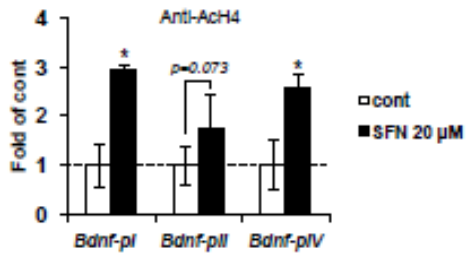
Bdnf-pIV

B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4
3
2
1
0
*
p=0.073
*
cont
SFN 20 µM
Bdnf-pI
Bdnf-pII
Bdnf-pIV

Figure 4. Effect of sulforaphane in histone acetylation in specific Bdnf promoter regions in primary cortical neurons.

Cells were treated with sulforaphane at 10 μM or 20 μM for 3 h and 3xTg mouse were administered by oral gavage with sulforaphane at 10 mg/kg/day or 50 mg/kg/day for 8 weeks. (A-B) Representative Bdnf promoter I, II and IV chromatin immunoprecipitation analysis. Fragmented chromatin was immunoprecipitated with antibody recognizing Ac-H3 or Ac-H4 and quantified with real-time PCR. $n = 3$; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. vehicle control.

Fig. 5

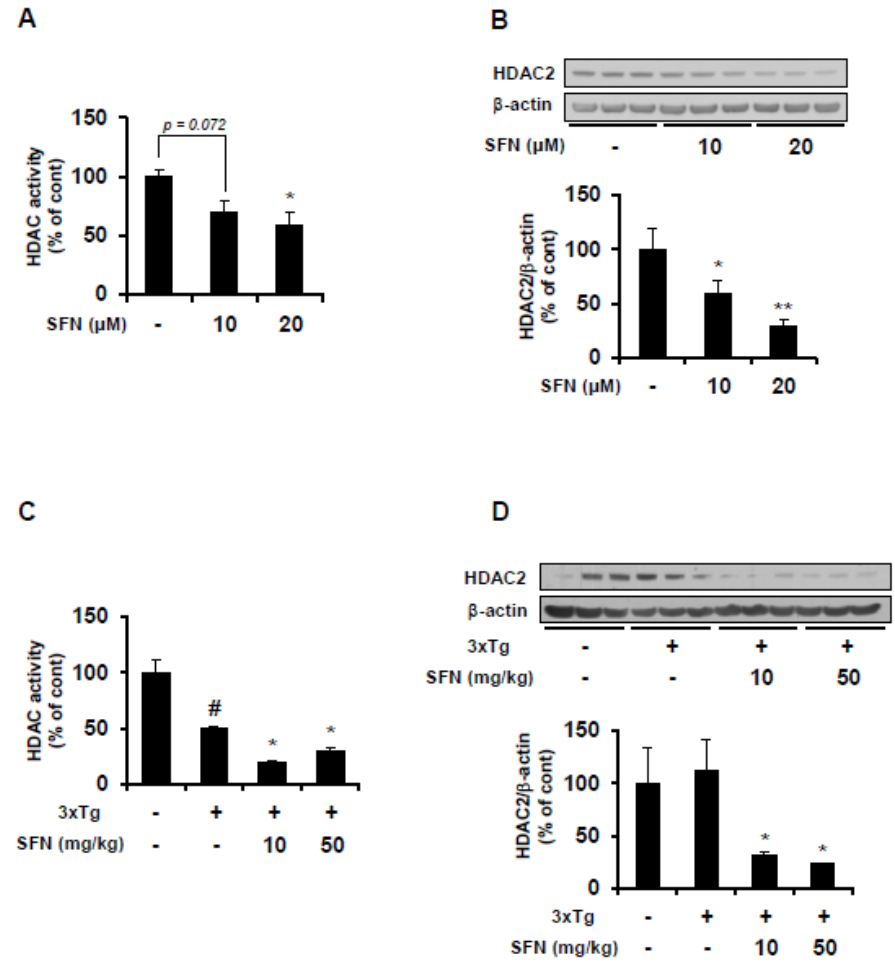
A
HDAC activity (% of cont)
150
100
50
0
p = 0.072
*
SFN (μM) - 10 20
B
HDAC2
β-actin
SFN (μM) - 10 20
HDAC2/β-actin (% of cont)
150
100
50
0
*
**
SFN (μM) - 10 20
C
HDAC activity (% of cont)
150
100
50
0
#
*
*
3xTg - + + +
SFN (mg/kg) - - 10 50
D
HDAC2
β-actin
3xTg - + + +
SFN (mg/kg) - - 10 50
HDAC2/β-actin (% of cont)
150
100
50
0
*
*
3xTg - + + +
SFN (mg/kg) - - 10 50

Figure 5. Effect of sulforaphane on HDAC in primary cortical neurons and the frontal cortex of 3xTg-AD mice.

Cells were treated with sulforaphane at 10 μM or 20 μM for 3 h and 3xTg mouse were administered by oral gavage with sulforaphane at 10 mg/kg/day or 50 mg/kg/day for 8 weeks. (A) HDAC activity. (B) Representative western blot gel and densitometry analysis of HDAC2 protein levels. β-actin was used as a loading control. $n = 3$; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. vehicle control. (C) HDAC activity. (D) Representative western blot gel and densitometry analysis of HDAC2 protein levels. β-actin was used as a loading control. $n = 6$; $\#p < 0.05$ vs. non-transgenic mice treated with vehicle; $^{*}p < 0.05$ vs. 3×Tg-AD mice treated with vehicle.

Fig. 6

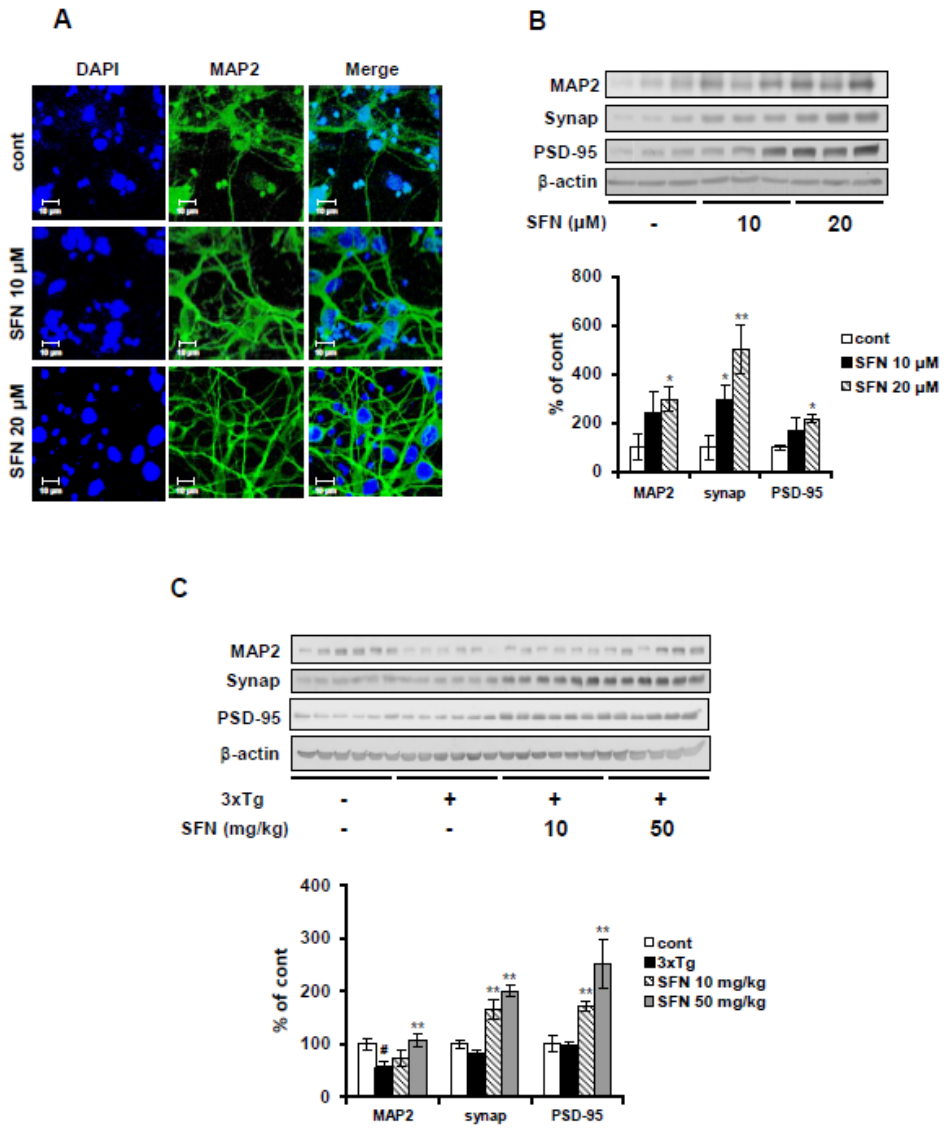
A
DAPI
MAP2
Merge
cont
SFN 10 μM
SFN 20 μM
B
MAP2
Synap
PSD-95
β-actin
SFN (μM) - 10 20
800
600
400
200
0
% of cont
cont
SFN 10 μM
SFN 20 μM
MAP2
synap
PSD-95
C
MAP2
Synap
PSD-95
β-actin
3xTg - + + +
SFN (mg/kg) - - 10 50
400
300
200
100
0
% of cont
cont
3xTg
SFN 10 mg/kg
SFN 50 mg/kg
MAP2
synap
PSD-95

Figure 6. Effect of sulforaphane on MAP2, synaptophysin (Synap), and PSD-95 levels in primary cortical neurons and the frontal cortex of 3xTg-AD mice.

Cells were treated with sulforaphane at 10 μM or 20 μM for 6 h and 3xTg mouse were administered by oral gavage with sulforaphane at 10 mg/kg/day or 50 mg/kg/day for 8 weeks. (A) Neurons were immunostained for nuclei (DAPI) and MAP2. (B) Representative western blot gel and densitometry analysis of MAP2, synaptophysin (Synap), and PSD-9 protein levels. β-actin was used as a loading control. $n = 3$; $*p < 0.05$ and $**p < 0.01$ vs. vehicle control. (C) Representative western blot gel and densitometry analysis of MAP2, synaptophysin (Synap), and PSD-95 protein levels. β-actin was used as a loading control. $n = 6$; $\#p < 0.05$ vs. non-transgenic mice treated with vehicle; $*p < 0.05$ and $**p < 0.01$ vs. 3×Tg-AD mice treated with vehicle.

Fig. 7

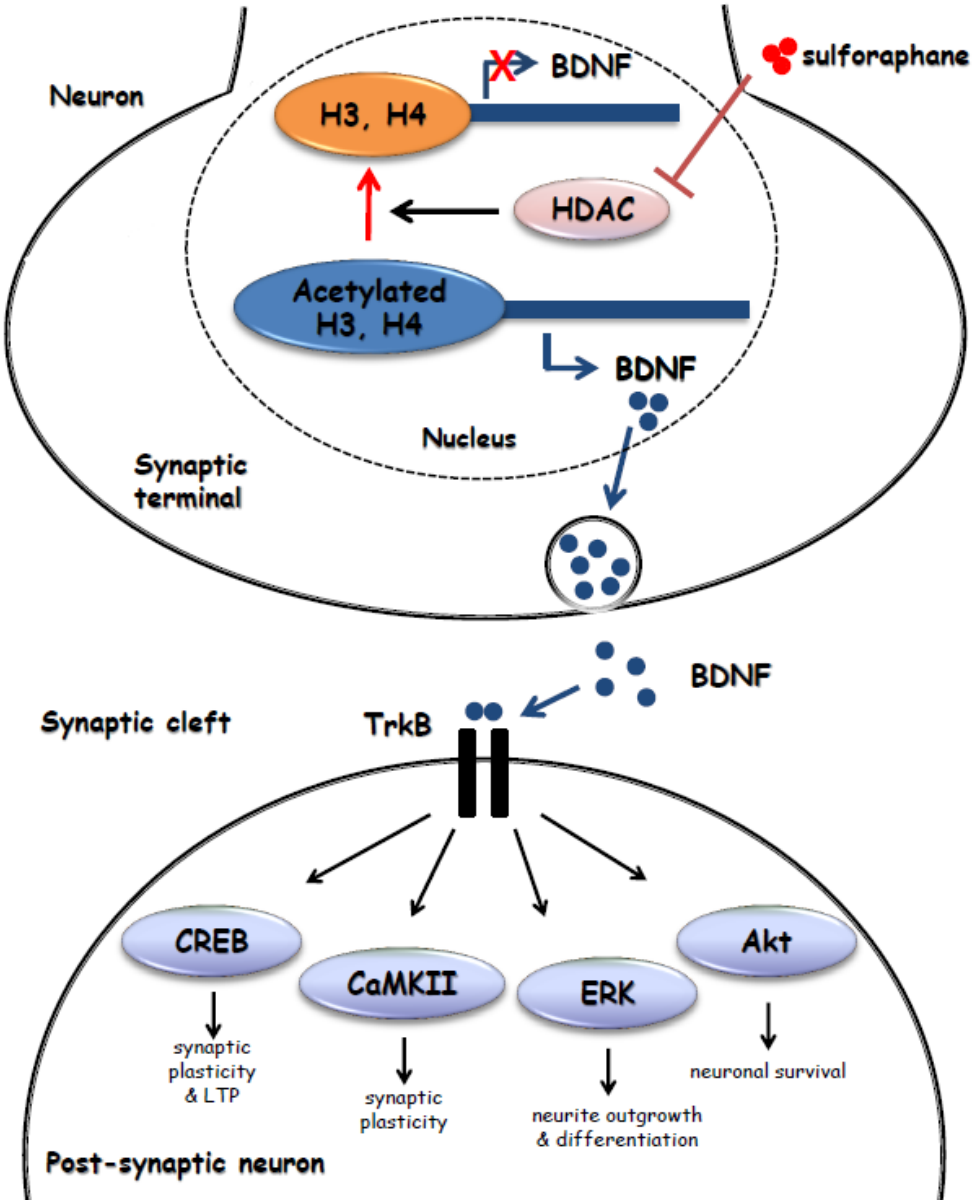
Neuron
BDNF
sulforaphane
H3, H4
HDAC
Acetylated H3, H4
BDNF
Nucleus
Synaptic terminal
BDNF
Synaptic cleft
TrkB
CREB
CaMKII
ERK
Akt
synaptic plasticity & LTP
synaptic plasticity
neurite outgrowth & differentiation
neuronal survival
Post-synaptic neuron

Figure 7. Proposed model for sulforaphane-mediated increase in neuronal BDNF expression and regulation of neurotrophic pathways.

Sulforaphane inhibits HDAC activity and induces changes in histone-tail acetylation, thereby increasing BDNF levels and enhancing the activity of BDNF-TrkB signaling pathways. As a consequence, ERK facilitates neuronal differentiation and growth, Akt promotes survival of neurons, and CaMKII and CREB induce synaptic plasticity and LTP. Thus, an increased level of BDNF may be responsible, at least in part, for the ability of sulforaphane to enhance neuronal function.

Ⅳ. Disscussion

Sulforaphane protects against traumatic brain injury-induced impairments in Morris water maze performance [19] and ameliorates Aβ-induced decline in Y-maze and passive avoidance test performance [20]. Sulforaphane treatment also protects neurons against the effects of 5-*S*-cysteinyl-dopamine and Aβ [21, 22]. These previous studies, however, have assumed that the neuroprotective effects of sulforaphane are mediated by activation of Nrf2 and up-regulation of its target genes without providing definitive evidence. Thus, the molecular actions underlying the protective effects of sulforaphane on cognitive function warrant clearer elucidation. The present investigation provides possible insight into this question by showing, for the first time, that sulforaphane induces the production of BDNF via chromatin remodeling in neurons.

I previously observed that sulforaphane increases the production of BDNF in both neurons that contain Nrf2 and those that lack Nrf2 (data not shown), suggesting that sulforaphane-induced production of BDNF is independent of Nrf2 activation or up-regulation of its target genes. Thus, I hypothesized that sulforaphane-induced BDNF production may involve HDAC-related epigenetic mechanisms, as emerging evidence demonstrates that histone acetylation regulates BDNF transcription [12, 13]. Here, I

showed that sulforaphane inhibits HDAC activity and decreases the level of HDAC2, which are known to negatively regulate memory formation and synaptic plasticity [23, 24], and increases H3 and H4 acetylation in mouse primary cortical neurons. This sulforaphane-mediated histone acetylation, which was associated with HDAC inhibition and BDNF production, was also found in the frontal cortex of 3×Tg-AD mice.

The mercapturic acid pathway, a glutathione-dependent pathway for the detoxification of several compounds present in cells, sequentially converts sulforaphane to sulforaphane-glutathione, sulforaphane-cysteine, and sulforaphane-N-acetylcysteine [25]. It has been suggested that sulforaphane-cysteine forms from sulforaphane-glutathione or after the deacetylation of sulforaphane-N-acetylcysteine by HDAC, leading to competitive HDAC inhibition [25]. Specifically, the sulforaphane-cysteine α-amino group forms H-bonds with buried His residues, whereas the sulforaphane-cysteine carboxylate group forms a bidentate ligand with the active site zinc atom within the HDAC pocket [26]. Molecular modeling studies with sulforaphane-cysteine provide firm support for complex formation with HDAC [27]. *In vitro*, sulforaphane-cysteine markedly inhibits HDAC activity in a cell-free system when tested at a range of concentrations (3-15 μM), whereas the sulforaphane parent compound has no effect on HDAC unless incubated with cells to permit metabolism [27].

Based on these studies, it has been proposed that sulforaphane-cysteine may be the ‘ultimate’ HDAC inhibitor generated from the parent compound via the mercapturic acid pathway [26].

Our present findings show that sulforaphane-dependent increases in histone acetylation elevate not only BDNF levels but also levels of key signaling molecules involved in synaptic plasticity, including TrkB, CREB, CaMKII, ERK, and Akt (Figure 2). BDNF and its receptor TrkB modulate synaptic transmission during LTP and learning [28]. In an animal model of AD, reduced TrkB signaling aggravates memory impairment, whereas increased TrkB signaling ameliorates memory impairment [29]. The interaction between TrkB and BDNF activates major intracellular signaling pathways [28] such as the Ras–mitogen-activated protein kinase (MAPK) signaling cascade, which promotes neuronal differentiation and growth through ERK, and the phosphatidylinositol 3-kinase (PI3K) cascade, which promotes neuronal survival through Akt [28]. The interaction between TrkB and BDNF also results in the generation of inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$), which promotes the release of Ca^{2+} from internal stores and subsequent activation of CaMKII and CREB, thereby inducing long-lasting synaptic changes and LTP [28]. Levels of MAP2, a dendritic marker and structural protein present in neurons, are correlated with cognitive decline in different types of transgenic mice [30, 31]. I found that sulforaphane

treatment increased levels of MAP2 as well as both pre-synaptic (i.e., synaptophysin) and post-synaptic (i.e., PSD-95) proteins. Such sulforaphane-dependent increases in synaptic proteins and key signaling molecules involved in synaptic plasticity may act to protect neurons and ameliorate declines in cognitive function.

Some studies report that Aβ reduces BDNF levels [32-34] and inhibits proteolytic maturation of proBDNF, the precursor form of BDNF [35]. On the other hand, tau pathology is not associated with down-regulation of BDNF mRNA or protein levels in mice, suggesting that the altered BDNF homeostasis observed in brains of AD patients might be ascribed to Aβ rather than tau pathology [36]. In 3×Tg-AD mice, the genetic knockdown of BDNF does not alter Aβ or tau pathology [37]. However, BDNF gene delivery in amyloid-transgenic mice restores learning and memory function independently of changes in amyloid plaque load [9]. Similarly, lentiviral delivery of BDNF into the entorhinal cortex of J20 AD model mice reverses synaptic loss and restores cognition but does not alter Aβ load [9]. These observations suggest that targeting BDNF signaling has clear promise for treating synaptic and cognitive deficits in AD, but such treatment may not modify AD-related neuropathology.

In summary, I provide evidence that sulforaphane engages epigenetic control mechanisms and serves as a HDAC inhibitor. The permissive chromatin state induced by sulforaphane elevated levels of BDNF as well as synaptic proteins and signaling molecules involved in synaptic plasticity. These findings add to accumulating evidence that sulforaphane is a neuroprotective agent that could ameliorate the decline of cognitive function in AD patients.

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Ⅵ. 초록

알츠하이머 치매를 앓고 있는 환자들의 뇌에서는 학습과 기억형성에 관여하는 뇌유래신경영양인자 (brain-derived neurotrophic factor) 가 정상인들에 비해 많이 감소된다는 사실이 보고되었다. 최근 여러 선행연구들에서 설포라판이 학습과 기억력에 도움이 된다는 보고가 있으나, 그 구체적인 작용기작에 대해서는 명확하지 않다. 따라서 본 연구에서는 설포라판이 뇌유래신경영양인자의 발현을 늘려준다는 사실과 그로 인한 기억력 개선 효능 및 그 작용기전을 규명하였다. 설포라판이 일차신경세포와 치매동물 모델에서 감소하는 뇌유래신경영양인자를 늘리는 것을 단백질 발현량 분석실험 및 mRNA 분석실험을 통해 확인하였다. 또한 설포라판이 히스톤 탈아세틸화효소 (histone deacetylase) 의 활성을 저해하고 그로 인해 히스톤 3, 4 단백질의 아세틸화를 증가시켰다. 그리고 히스톤 단백질의 아세틸화로 인해 염색질 재형성이 일어나 뇌유래신경영양인자의 발현량이 증가하는 것을 면역침강

정제법을 통해 확인함으로써 설포라판이 히스톤 탈아세틸화효소의 활성 저해를 통한 염색질 재형성으로 뇌유래신경영양인자의 발현이 증가된다는 것을 분자생물학적 관점에서 증명하였다. 뿐만 아니라 설포라판이 여러 뇌유래신경영양인자의 신호전달 단백질들, TrkB, CREB, CaMKII, ERK, Akt의 인산화를 증가시키는 것을 확인할 수 있었고 MAP2, synaptophysin, PSD-95와 같은 기억력 형성에 있어 필요한 신경세포 및 시냅스 단백질들의 발현량 역시 증가시키는 것을 확인하였다. 결론적으로, 위 연구결과를 통해 설포라판이 알츠하이머 치매를 예방 또는 치료할 수 있는 치료제로써의 가능성을 제시하였다.

주요어 : 설포라판; 뇌유래신경영양인자 (brain-derevied neurotrophic factor); 히스톤 탈아세틸화효소 (histone deacetylase); 알츠하이머 치매