J. Biochem. 2010;147(1):73–81 doi:10.1093/jb/mvp149

JB THE JOURNAL OF BIOCHEMISTRY

Role of Nrf2 and p62/ZIP in the neurite outgrowth by carnosic acid in PC12h cells

Received August 22, 2009; accepted August 31, 2009; published online September 17, 2009

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Neurotrophins such as NGF promote neuronal survival and differentiation *via* the cell surface TrkA neurotrophin receptor. Compounds with neurotrophic actions that are low in molecular weight and can permeate the blood–brain barrier are promising therapeutic agents against neurodegenerative diseases such as Alzheimer's disease. Carnosic acid (CA), an electrophilic compound in rosemary, activates antioxidant responsive element (ARE)-mediated transcription *via* activation of Nrf2. In the present study, we discovered that CA strongly promotes neurite outgrowth of PC12h cells. NGF as well as CA activated Nrf2, whereas CA and NGF-mediated neuronal differentiation was suppressed by Nrf2 knockdown. On the other hand, CA activated TrkA-downstream kinase Erk1/2 independently of Nrf2. CA-induced p62/ZIP expression in an Nrf2-dependent manner, while the CA-induced neural differentiation was suppressed by p62/ZIP knockdown. Furthermore, CA-induced ARE activation was attenuated both by p62/ZIP knockdown and a Trk signal inhibitor. These results suggest that the CA induction of p62/ZIP by Nrf2 enhances TrkA signaling which subsequently potentiates Nrf2 pathway. This is the first demonstration that activation of the Nrf2-p62/ZIP pathway by a low-molecular natural electrophilic compound plays important roles in TrkA-mediated neural differentiation and may represent the common molecular mechanism for neurotrophic activities of electrophilic compounds.

Keywords: carnosic acid/Nrf2/p62/PC12/rosemary.

Abbreviations: ARE, antioxidant responsive element; CA, carnosic acid; Egr-1, early growth response protein-1; Erk1/2, extracellular signal-regulated kinase1/2; Keap1, Kelch-like ECH-associated protein 1; MafK, musculoaponeurotic fibrosarcoma oncogene homolog K; Nrf2, NF-E2-related factor 2; PI3K, phosphatidylinositol 3-kinase; Trk, tropomyosin-related kinase; NF, neurofilament; NGF, nerve growth factor; miR, micro RNA; ZIP, PKC-ζ interacting protein.

Neurotrophins such as NGF, BDNF and NT3 promote neuronal growth, differentiation and plasticity, mainly through the Trk family (A, B and C) of cell surface receptors. The neurotrophins bind to Trk receptors and activate various kinase cascades such as Erk1/2, PI3K and PKCs (*1*). Trk is internalized upon ligand-induced phosphorylation to form signaling vesicles that are critical for neurotrophin-induced neuronal differentiation and survival (*2–4*). Upon neurotrophin such as NGF binding to the TrkA, TRAF6 induces the K63-mediated polyubiquitination of TrkA required for receptor signaling (*4*). p62/ZIP/A170/SQSTM1 (hereafter called p62/ZIP for simplicity) interacts with TrkA and enhances TRAF6-mediated receptor internalization (*5–7*). Accordingly, it has been shown in PC12 cells and HiB5 neuronal progenitor cells that an increase in p62/ZIP levels leads to an enhancement of NGF activity (*4*, *5*, *8*). Thus, the p62/ZIP level is critical for neuronal differentiation, but how p62/ZIP expression is actually regulated in neurons remains largely unknown.

Electrophilic compounds, such as carnosic acid (CA) reportedly activate various biological actions including, anti-tumor, anti-inflammatory and neuroprotective effects through *S*-alkylation on specific cysteine residues of Keap1 resulting in Nrf2 stabilization (*9*, *10*). In homeostatic condition, Keap1 represses Nrf2 either by sequestration of Nrf2 in the cytosol or ubiquitin-mediated proteasomal degradation. Upon activation, Nrf2 is liberated from Keap1 and heterodimerizes with one of the small Maf (sMaf) proteins to bind to antioxidant responsive element (ARE) (*11–13*). The binding of Nrf2/sMaf complex to ARE sequences enhances induction of a set of genes called 'phase 2 enzymes' including drug detoxifying enzymes such as glutathione-*S*-transferase (GST), NAD(P)H oxidoreductase 1 (NQO1) and UDP-glucuronosyltransferase (UGT), and antioxidant proteins such as heme oxygenase-1 (HO-1) and γ-glutamate cysteine ligase (GCL) (*14*). The induction of these enzymes provides efficient neuroprotection *via* modulation of the intracellular redox balance both *in vitro* and *in vivo*.

Cyclopentenone prostaglandins possess electrophilic α, β-unsaturated carbonyls in their cyclopentenone rings and act as endogenous signaling molecules *via* *S*-alkylation (*15*, *16*). We previously demonstrated that the synthetic derivatives of cyclopentenone prostaglandins, called neurite outgrowth-promoting

prostaglandins (NEPPs), promote the neurite outgrowth in the presence of NGF (*17*, *18*). Several other electrophilic compounds, such as flavonoids (*19*), curcumin (*20*) and epigallocatechin gallate (*21*) also possess neurite outgrowth-promoting activities in combination with NGF. Because these chemicals are also activators of Nrf2, it occurred to us that the Keap1/Nrf2 pathway mediates the common actions of electrophiles that synergizes with NGF. In order to investigate the intracellular mechanism, we employed rat pheochromecytoma PC12h cells, in which NGF induces neurite outgrowth and neuron-like differentiation *via* TrkA signaling pathway (*22*). Among ARE activating natural electrophilic compounds, we focused on CA, a major ingredient of an herb rosemary (*Rosmarinus offcinalis* L.) that is famous for its memory enhancing effect (*23*).

Materials and methods

Reagents

CA was prepared from rosemary (*24*). Rat nerve growth factor-β (NGF-β) was purchased from R&D systems. LY294002, U0126, K252a and MG132 were purchased from Calbiochem. Other materials were obtained from Sigma. These drugs were dissolved in DMSO and used at a final vehicle concentration of less than 0.2%.

Cell culture and transfections

Rat pheochromocytoma subclone PC12h cells were a kind gift from Prof. Hatanaka (*22*). PC12h cells were seeded on normal plastic plates (Sumilon) and grown in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 8% heat-inactivated fetal bovine serum (FBS), 8% heat-inactivated horse serum (HS), 100 μg/ml streptomycin and 100 U/ml penicillin (Gibco) in a 5% CO_2 incubator at 37°C. Cells were maintained overnight on collagen type I coated plates (Iwaki) in DMEM supplemented with 1% FBS and 1% HS (low-sera medium) at a density of 1×10^5 cells per well or 5×10^5 cells per well on 24- or 6-well plates, respectively. Transfection was performed with TransFast reagent (Promega) or Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Differentiation analysis

For neuronal differentiation, PC12h cells (1×10^5 cells per well) were plated on collagen type I coated plates in low-sera medium. After 48 h, CA or NGF-β was added to the medium and a minimum of 200 cells were examined for each different subject group with the criteria that cells having at least one neurite longer than the diameter of the cell body were considered as differentiated cells. The cells were analyzed by phase-contrast microscopy (Eclipse TE-2000E, NIKON).

Plasmid construction

pGL3-hNQO1 promoter-luciferase construct was generated as follows. The human NQO1 promoter region, ranging from the transcriptional start site to the upstream ARE recognition motif, was amplified by PCR using the following primers: 5′-GGA CGC GTG CAG TCA CAG TGA CTC AGC AGA-3′ for wild type forward; 5′-GGA CGC GTG CAG TCA aAG TGc CTa AGC AGA ATC-3′ for mutant ARE forward; 5′-GGA AGC TTT TTA TAT ATC CTG TCC GGC CCG-3′ for both wild type and mutant reverse primers. The PCR products were digested with *Mlu*I and *Hind*III restriction enzymes and cloned into pGL3-Basic vector (Promega).

In order to make pEF-p62/ZIP, cDNA for p62/ZIP was obtained from PC12h cells. Insert DNA was amplified by PCR using the following primers: forward 5′-CCG CCA TGG CTT CGC TCA CGG TGA AG-3′ and reverse 5′-CAA TGG TGG AGG GTG CTT TGA ATA C-3′. The amplified product was cloned into pEF6/V5-His TOPO vector (Invitrogen). For generation of the miRNA p62/ZIP knockdown expression vector (pcDNA-p62miR1/2), the BLOCK-iT Pol II RNAi Expression Vector kit (Invitrogen) was used. The following single-stranded DNAs were annealed to generate double-stranded oligos: 5′-TGC TGT TTA GAA GAC AAA TGC GTC CAG TTT TGG CCA CTG ACT GAC TGG ACG CAT GTC TTC TAA A-3′ (top strand for ZIPmiR) and 5′-CCT GAT AAT TCT TGG TGT AGG AGG TCA GTC AGT GGC CAA AAC CTC CTA CAG ACC AAG AAT TAT C-3′ (bottom strand for ZIPmiR). The double-stranded oligo was cloned into pcDNA6.2-GW-miR vector according to the manufacturer's instructions. ZIPmiR expression vector or empty vector was transfected into PC12h cells with Lipofectamine 2000 to established stable transformants. Two weeks after blastcidine selection (30 μg/ml), ZIPmiR-overexpressing cells were obtained.

Luciferase assay

PC12h cells were seeded onto 24-well collagen coated plates in low-sera medium the day before transfection. pGL3-hNQO1 promoter-luciferase, pSV-β GAL (Promega) and other constructs were co-transfected into cells using TransFast. Cells were maintained in low-sera medium for 24 h before addition of CA or other reagents. Luciferase activity was assayed with the Luciferase Assay System (Promega) according to the manufacturer's instructions. Transfection efficiency was normalized to that of β-galactosidase activity.

Small interfering RNA

Synthetic 25-nt stealth siRNA was purchased from Invitrogen. The sequence of our prepared siRNA for rat Nrf2 was 5′-GGG CUG UGA UCU GUC CCU GUG UAA A-3′; 5′-UUU ACA CAG GGA CAG AUC ACA GCC C-3′. Non-specific control stealth siRNA (NC) was also purchased from Invitrogen, and were used as controls. Transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Quantitative PCR

Cells were plated on 6-well plates and total cellular RNA was extracted using TRIzol reagent (Invitrogen). Equal amounts (1 μg) of RNA from the different treatments were reverse-transcribed using a Transcription First Strand cDNA Synthesis kit (Roche). Amplification of cDNA was performed with 50 pmol of synthetic gene-specific primers in 20 μl of PCR reaction buffer (iQ SYBR Green Supermix, Bio-Rad). The following gene-specific primers were employed: neurofilament-M (forward, 5′-AGG AGA AAG GGG TGG TCA CT-3′; reverse, 5′-CCT CAA AGG TCT CCT CAT GC-3′); egr-1 (forward, 5′-CCT GTG ACA TTT GTG GGA GA-3′; reverse, 5′-GTG CAG GAG ACG GGT AGG TA-3′); mafK (forward, 5′-AAG TGA GTT TTC TGT CTT GTT-3′; reverse, 5′-CAA AGA TAC AAA AGC AGT CAC GG-3′) (*25*); p62/zip (forward, 5′-TAG TCT GCG GTT ATG GCT-3′; reverse, 5′-TGC GGA AGA TGT CAT C-3′) or (forward, 5′-TTG GCC ACC TCT CTG ATA GC-3′; reverse, 5′-GGT TTG CTG ACT TCC GAA G-3′); cyclophilin A (forward, 5′-ACA GGT CCT GGC ATC TTG TC-3′; reverse, 5′-TTC ACC TTC CCA AAG ACC AC-3′); β-actin (forward, 5′-GTC GTA CCA CTG GCA TTG TG-3′; reverse, 5′-CTC TCA GCT GTG GTG GTG AA-3′). Amplification was conducted in a Bio-Rad Chromo 4 Real Time-PCR analysis system. PCR conditions were as follows: 94°C for 1 min, then 40 cycles at 94°C for 15 s, 60°C for 1 min, and 72°C for 1 min. The expression level of each gene was estimated in duplicate samples and normalized to that of cyclophilin A.

Immunoblot analysis

PC12h cells were rinsed once with ice-cold phosphate buffered saline (−) and lysed in ice-cold buffer containing 150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 0.25% deoxycholic acid, 1 mM EDTA, 1% Nonidet P-40, 50 mM NaF, 1 mM Na_3VO_4, 50 μM MG132, and 1× Complete protease inhibitor cocktail (Roche Diagnostics). Lysates were sonicated for 10 s on ice and centrifuged for 10 min at 10 000 rpm at 4°C to remove debris. Protein was quantified by BCA assay (Pierce). Equivalent amounts of protein were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) followed by transfer to Hybond-P PVDF membrane (Amersham) using an iBlot system (Invitrogen). Blots were analyzed with the appropriate antibodies: anti-Erk1/2, anti-phospho-Thr^{202}/Tyr^{204} Erk1/2, anti-Akt, anti-phospho-Ser^{473} Akt (Cell Signaling), anti-β-actin (Sigma), anti-p62 (BioMol), anti-neurofilament-M and anti-neurofilament-H (Zymed), anti-Nrf2 (*26*). Appropriate peroxidase-conjugated secondary antibodies and ECL reagents (Amersham) were used to detect the proteins of interest by enhanced chemiluminescence.

Statistics

Student's *t*-test was used to assess differences between groups. A *P*-value <0.03 was considered significant. Unless otherwise indicated, experiments were performed at least three times. Values in graphs correspond to the mean ± SD.

Results

Carnosic acid (CA) promotes neurite outgrowth in PC12h cells

We discovered that CA, a major ingredient of rosemary, markedly induced neurite outgrowth in rat pheochromecytoma PC12h cells in a concentration-dependent manner from 5 to 20 μM (Fig. 1). Following studies were conducted with 15 μM of CA except the cases specified, since 20 μM of CA seemed to be slightly cytotoxic on this condition. The effect of 15 μM CA on neurite outgrowth was largely comparable to that of NGF (3–10 ng/ml) (Fig. 1). Neurite outgrowth was also promoted by carnosol and luteolin, other ingredients of rosemary, albeit both with weaker effects than CA (data not shown).

CA activates Nrf2 signaling pathway and Nrf2-mediated neurite outgrowth of PC12h cells

We recently demonstrated that CA activates Nrf2 *via* *S*-alkylation of Keap1 and protects cells from oxidative stress (*10*). Therefore, we examined the effect of CA on NQO1 promoter-mediated reporter gene expression that contains a functional ARE in PC12h cells. As shown in Fig. 2A, CA induced reporter gene expression in a concentration-dependent manner from 5 to 20 μM several folds higher than did sulforaphane and other ingredients of rosemary such as carnosol and luteolin (data not shown). In addition, CA strongly activated wild-type reporter expression, but not the ARE-mutated NQO1 promoter-luciferase expression (Fig. 2B). These results indicated that CA induced ARE-mediated gene expression in PC12h cells. Interestingly, NGF also potently activated ARE-mediated gene expression but weaker than CA (Fig. 2A and B).

Neurite outgrowth and neural differentiation are tightly correlated in PC12 cells (*27–29*). As shown in Fig. 2C and D, both CA and NGF increased Nrf2 as well as neural differentiation markers NF-M and NF-H in PC12h cells although CA more strongly enhanced Nrf2 expression than NGF. NF-M protein level and neurite outgrowth were significantly increased 6 h after CA or NGF treatment (Figs 2C, D and 3A). Since CA-induced Nrf2 and NF-M accumulations occurred coincidently in a time-course, we hypothesized that Nrf2 may mediate neural differentiation of PC12h. In order to examine the role of Nrf2 in neural differentiation in PC12h cells, we examined whether Nrf2 knockdown affects CA- or NGF-induced differentiation. As expected, both CA- and NGF-induced neurite outgrowth and the expressions

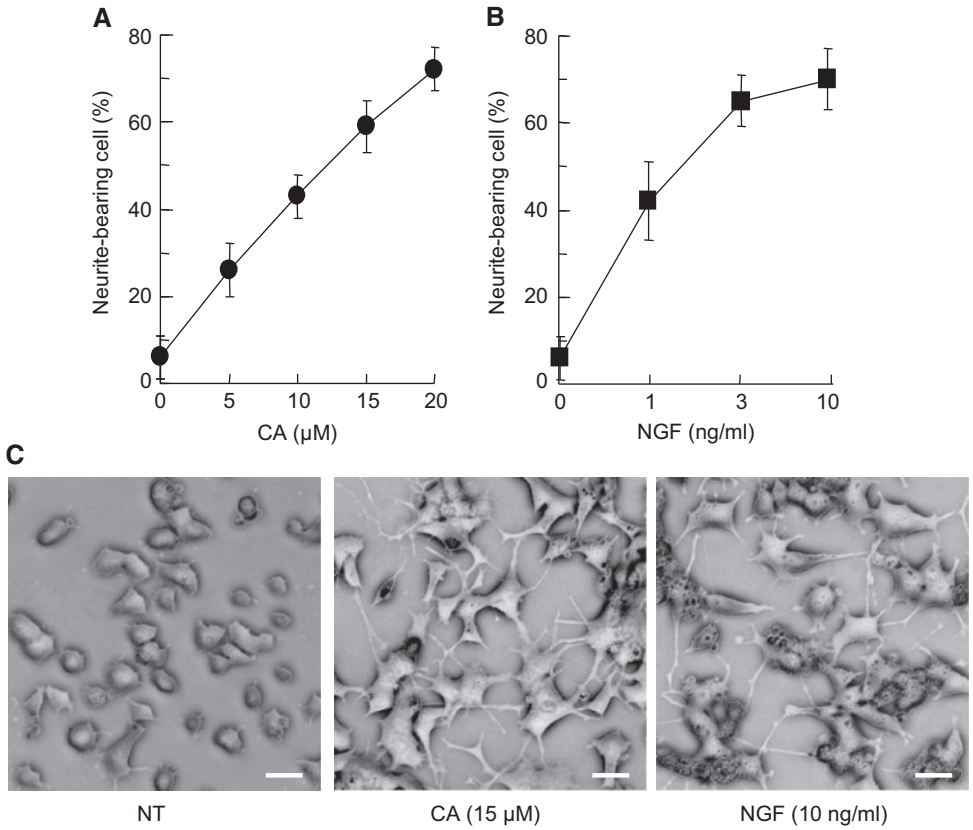

Fig. 1 CA promotes neurite outgrowth in PC12h cells. PC12h cells were seeded onto 24-well collagen-coated plates at a density of 1×10^5 cells per well in DMEM supplemented with 1% FBS and 1% HS (low-sera medium) and cultured for 2 days. Then cells were treated with CA or rat NGF-β for 24 h at the indicated concentrations, followed by observation of the cell morphologies by microscopy. Scale bar, 25 μm. The cells that have neurites after treatments are counted and the means of three independent experiments are shown with SEM. (A and B). (C) Representative images of treated cells. NT: no treatment.

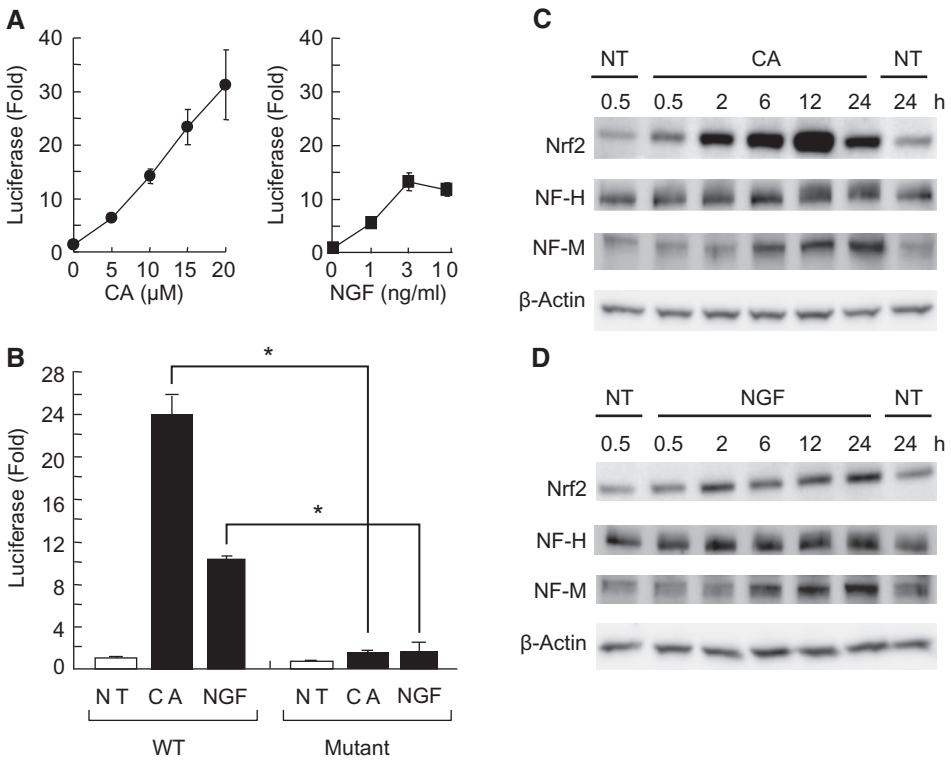

Fig. 2 CA and NGF activate Nrf2-ARE and increase neurofilaments. (A) Cells were treated with CA or NGF at the indicated concentrations for 24 h and then performed luciferase assay. (B) CA (15 μM) or NGF (10 ng/ml) was added to the PC12h cells transfected with wild-type NQO1 promoter-luciferase or mutant ARE promoter-luciferase construct. Cells were treated with CA or NGF for 24 h prior to luciferase assay. (C and D) PC12h cells were treated with 15 μM of CA (C) or 10 ng/ml of NGF (D) for 0.5–24 h, and total cell lysates were then prepared. The lysates were subjected to WB analysis using anti-Nrf2, anti-NF-H, anti-NF-M or anti-β-actin. NT: non-treatment. $*p < 0.03$.

of NF-M and NF-H as well as MAP2, another neural differentiation marker, were attenuated by Nrf2 knockdown (Fig. 3). Thus, these findings indicated that Nrf2 plays an important role in neurite outgrowth and differentiation of PC12h cells.

CA activates multiple kinase pathways including Erk1/2 and PI3K

It is well known that binding of neurotrophins such as NGF to TrkA receptor activates various signaling pathway including Erk1/2 and PI3K, which are essential for differentiation of PC12h cells. Therefore, we next examined whether CA activates the kinases downstream of NGF. As shown in Fig. 4A, CA induced the phosphorylation of Erk1/2 and Akt, a downstream target of PI3K. CA-induced phosphorylations of Akt and Erk1/2 peaked much later than NGF. Consistent with the activation of the kinases, promoting effect of CA and NGF on neural differentiation was attenuated by PI3K inhibitor LY294002 or MEK1/2 inhibitor U0126 (data not shown).

Next, we examined how CA activates Erk1/2 and PI3K. As shown in Fig. 4B, CA-induced expression of NF-M was inhibited by K252a. However, CA-induced Erk1/2 phosphorylation was not inhibited by K252a, but PI3K activation was partially inhibited by K252a. Similarly, the induction of Erk1/2 phosphorylation was not affected by the knockdown of Nrf2, but CA-induced PI3K activation was decreased by Nrf2 knockdown. These results indicate that Erk1/2 activation by CA is independent of TrkA and Nrf2 signaling pathway, but CA-induced PI3K activation at least in part needs Nrf2 and TrkA signaling.

CA-induced Nrf2 accumulation is attenuated by the inhibitors of MEK1/2 and PI3K

It is interesting to note that the peak time of kinase phosphorylation coincided with the peak of Nrf2 accumulation (Figs 2C and 4A). Previously, it was reported that several electrophilic compounds activate Nrf2 *via* Erk1/2 and PI3K-Akt pathway (*30*, *31*). Therefore, we examined the effect of the kinase inhibitors on the CA-induced Nrf2 accumulation in PC12h cells. As shown in Fig. 5A, CA-induced Nrf2 accumulation is inhibited both by the inhibitors of P13K and Erk1/2. Consistent with this, CA-induced ARE activation was strongly inhibited by LY294002. Inhibition of upstream kinase of Erk1/2 by U0126 also suppressed CA-induced ARE activation, although this inhibitory effect was partial (Fig. 5B).

CA provokes a differentiation program distinct from NGF

Previous studies demonstrated that NGF provokes a characteristic transcriptional program in PC12 cells that commit the cells to differentiation (*32*). We therefore compared CA-induced gene expression profiles with those of NGF. For this purpose, we performed quantitative PCR analysis of the NGF responsive genes nf-M, egr-1, p62/zip and mafK (Fig. 6).

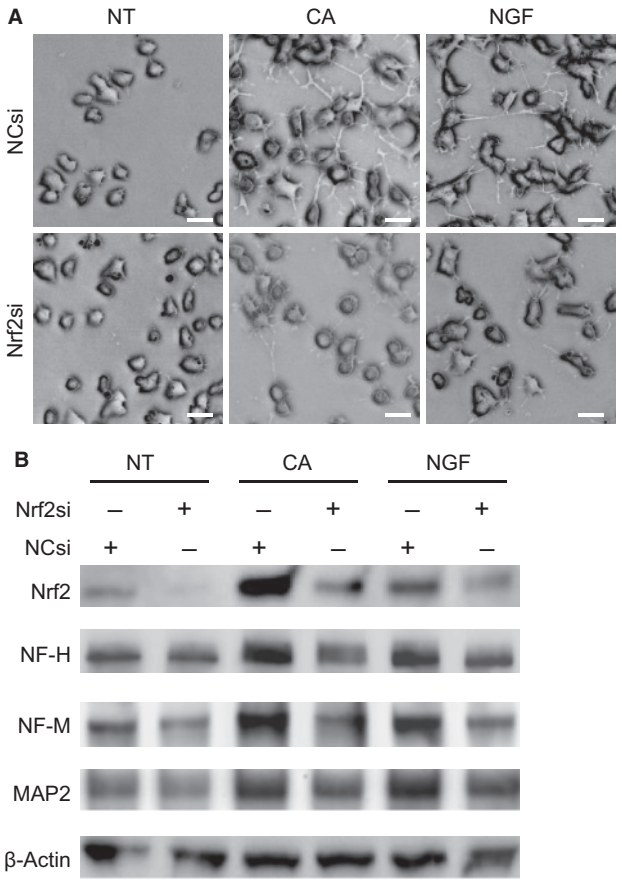

Fig. 3 Nrf2 mediates neural differentiation in PC12h cells. PC12h cells were transfected with Nrf2 siRNA (Nrf2si) or negative control siRNA (NCsi) and then cells were treated with 15 μM of CA or 10 ng/ml of NGF. After 6 h cell morphologies were observed under microscopy; scale bar: 25 μm (A), and the whole cell lysates were subjected to WB analysis with anti-Nrf2, anti-NF-H, anti-NF-M, anti-MAP2 or anti-β-actin antibody (B).

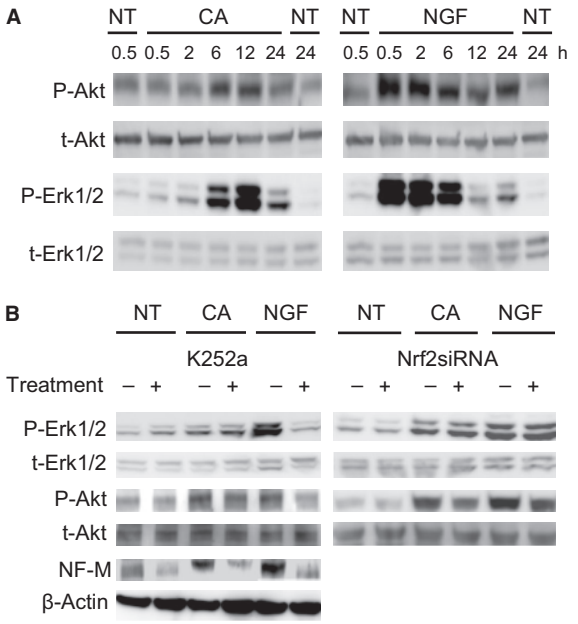

Fig. 4 CA activates kinase pathways including PI3K and Erk1/2. (A) PC12h cells were treated with 15 μM of CA or 10 ng/ml of NGF for 0.5–24 h, and whole cell lysates were prepared. The lysates were subjected to WB analysis using anti-phosphorylated Akt (P-Akt), anti-total Akt (t-Akt), anti-phosphorylated Erk1/2 (P-Erk1/2) or anti-total Erk1/2 (t-Erk1/2) antibodies. (B) PC12h cells were treated with 150 nM of K252a for 1 h (left panel) or Nrf2 siRNA for 24 h (right panel). Subsequently, the cells were treated with 15 μM of CA or 10 ng/ml of NGF for 6 h and the lysates were analyzed by WB analysis.

MafK as well as p62/ZIP has been previously shown to be critical for NGF-induced differentiation of PC12 cells (*5*, *8*, *25*). The mRNA level of nf-M was induced by CA and NGF treatment by 12–14-fold. The nf-M gene expression level correlated with the degree of neurite outgrowth and peaked 24 h after CA and NGF treatment (Fig. 6A). egr-1 mRNA level was induced almost immediately, with NGF being a superior inducer to CA (Fig. 6B). In contrast, the extent of mafK and p62/zip induction by CA was much greater than NGF (Fig. 6C and D). These results imply that CA provokes overlapping, but distinct differentiation programs compared to that by NGF.

p62/ZIP is essential for CA-mediated PC12h differentiation

Since p62/ZIP plays crucial roles in NGF-induced neuronal differentiation, we wanted to assess the roles of p62/ZIP in CA-induced differentiation of PC12h cells. As shown in Fig. 7A, Nrf2 siRNA attenuated CA- and NGF-induced p62/ZIP in PC12h cells.

Next, we established stable transformant cells carrying knockdown vector specific for p62/ZIP (ZIPmiR).

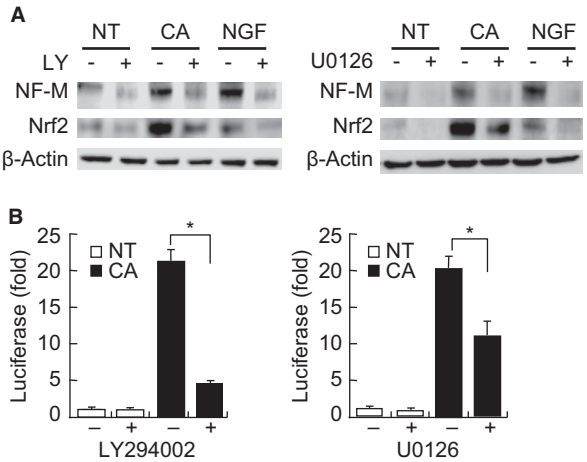

Fig. 5 CA activates Nrf2-ARE pathway *via* PI3K and Erk1/2. (A) PC12h cells were treated with 30 μM of LY294002 (left panel) or 10 μM of U0126 (right panel) for 1 h and then the cells were untreated (NT) or treated with 15 μM of CA or 10 ng/ml of NGF for 6 h. The whole cell lysates were analyzed by WB analysis using anti-NF-M, anti-Nrf2 or anti-β-actin antibodies. (B) hNQO1 promoter-luciferase construct were transfected into PC12h cells. After 24 h LY294002 (30 μM) or U0126 (10 μM) were added to the cells 1 h prior to addition of CA. Cells were treated with 15 μM of CA for 24 h and then luciferase assay was performed. $*p < 0.03$.

As expected, p62/ZIP knockdown in PC12h cells showed reduced p62/ZIP protein levels (Fig. 7B). In ZIPmiR transformant, CA- or NGF-induced NF-M level was also reduced and the cells remained round

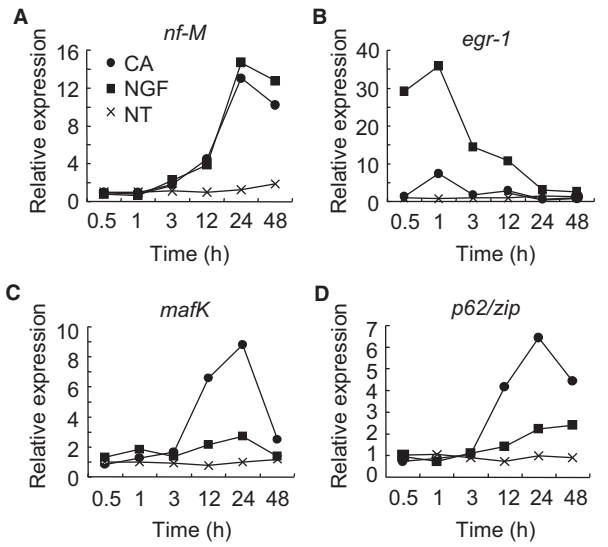

Fig. 6 CA transcriptionally regulates a similar, but distinct differentiation program from NGF. Fifteen micromolar CA or 10 ng/ml of NGF was added to the cells and then RNA was extracted at the times indicated. The mRNA levels of neurofilament-M (nf-M) (A), egr-1 (B), mafK (C) and p62/zip (D) were examined using quantitative PCR. The value of NT at 0.5 h was arbitrarily set as 1 and the relative values were presented.

in shape. Furthermore, they developed almost no neurites even in the presence of CA or NGF (Fig. 7C). These findings indicated that p62/ZIP was essential for CA-mediated PC12h differentiation.

Nrf2 and p62/ZIP form positive feedback loops

During the course of this study, we happened to find that the overexpression of p62/ZIP by transient transfection activates the ARE-mediated transcription (Fig. 8A). Since p62/ZIP expression is regulated by Nrf2/ARE (Fig. 7A), it is likely that the activation of ARE by p62/ZIP establishes a positive feedback loop between p62/ZIP and Nrf2/ARE. Consistent with this hypothesis, CA- and NGF-mediated ARE-luciferase activities were reduced by cotransfection of p62/ZIPmiR knockdown vector (Fig. 8B). The activity of p62/ZIP to enhance ARE reporter gene expression was inhibited by LY294002, suggesting that p62/ZIP-induced ARE transactivation was dependent on PI3K (Fig. 8C). The ARE activation by NGF was completely abrogated by K252a, a Trk inhibitor, indicating that NGF-induced transactivation of ARE was dependent on TrkA. CA-induced ARE luciferase expression was partially attenuated by K252a (Fig. 8D). These results indicate that TrkA signals also contribute to the strong ARE induction by CA.

Discussion

Previously we demonstrated that CA induces cytoprotective genes through Nrf2 signaling pathway and protects neural cells from oxidative cell damage. In this study, we discovered that CA promotes neural differentiation of PC12h cells. Because Nrf2 was strongly activated by CA, we focused on the role of Nrf2. Interestingly, transfection of Nrf2 siRNA inhibited both CA- and NGF-mediated neural differentiation of PC12h cells. These results revealed a novel role of Nrf2 as an essential mediator of neural differentiation. We previously demonstrated that CA activates Nrf2 *via* covalent modification of Keap1 (*10*). In this study, CA-activated Nrf2 then induced p62/ZIP expression that is essential for neural differentiation of PC12h cells (Fig. 7) (*8*). Our results indicated that p62/ZIP plays indispensable roles in downstream of Nrf2 in CA-mediated neuronal differentiation.

We summarized the possible mechanism for the CA-induced neural differentiation in Fig. 9. CA-induced neural differentiation is mediated by two different mechanisms. One is Nrf2-mediated induction of pro-neural differentiation genes such as p62/ZIP, and the other is Nrf2-independent activation of Erk1/2 and PI3K. Nrf2 accumulation and the induction of p62/ZIP mRNA showed their peaks in the relatively late phase of neural differentiation (i.e. 12 and 24 h after CA treatment, respectively) (see Figs 2C and 6D). However, Nrf2 also plays an important role both in neurite outgrowth and the expression of neuronal differentiation markers in the early phase of neural differentiation (i.e. 6 h after CA treatment, Fig. 3A and B). We surmise that this Nrf2-dependent neural differentiation is mediated by p62/ZIP-mediated enhancement of TrkA signaling, because p62/ZIP is known to activate TrkA signaling by enhancing K63-polyubiquitination of TrkA (*4*). In accordance with this, the CA-activated neural differentiation is inhibited not only by the knockdown of Nrf2, p62/ZIP, but also by TrkA inhibitor K252a in the early phase of differentiation (Fig. 4B and 7B). We speculate that the later induction of Nrf2 target genes including p62/ZIP may also play an important role in the neural differentiation although there is no direct evidence at present. In parallel with this Nrf2-dependent pathway, Nrf2-independent activation of Erk1/2 and PI3K plays an essential role in CA-mediated neural differentiation because both Erk1/2 and PI3K inhibition prevented neural differentiation. Interestingly, Nrf2 overexpression in PC12h cells promotes neural differentiation in the absence of CA (Supplementary Figure S1). The mechanisms of Nrf2-promoted neural differentiation are under investigation in our laboratory including the identification of Nrf2-regulated pro-neural differentiation genes.

It is quite intriguing how CA activates Erk1/2 and PI3K in an Nrf2-independent manner. It was reported that certain phosphatases, especially protein tyrosine phosphatases such as PTP1B, have reactive cysteines and their activities are inhibited by modification of their cysteine residues by oxidative stress (*33*). Actually, it was reported that the inactivation of PTB1B by 6-methylsulfinylhexyl isothiocyanate (6-HITC), an electrophile contained in the Japanese horseradish, enhances TrkA phosphorylaiton and NGF signaling (*34*). In the case of CA, we surmise that the inactivation of the phosphatase is more likely than the direct activation of the kinase by CA, because the activation of Erk1/2 and PI3K peaked in the relatively late phase (i.e. 12 h after CA treatment).

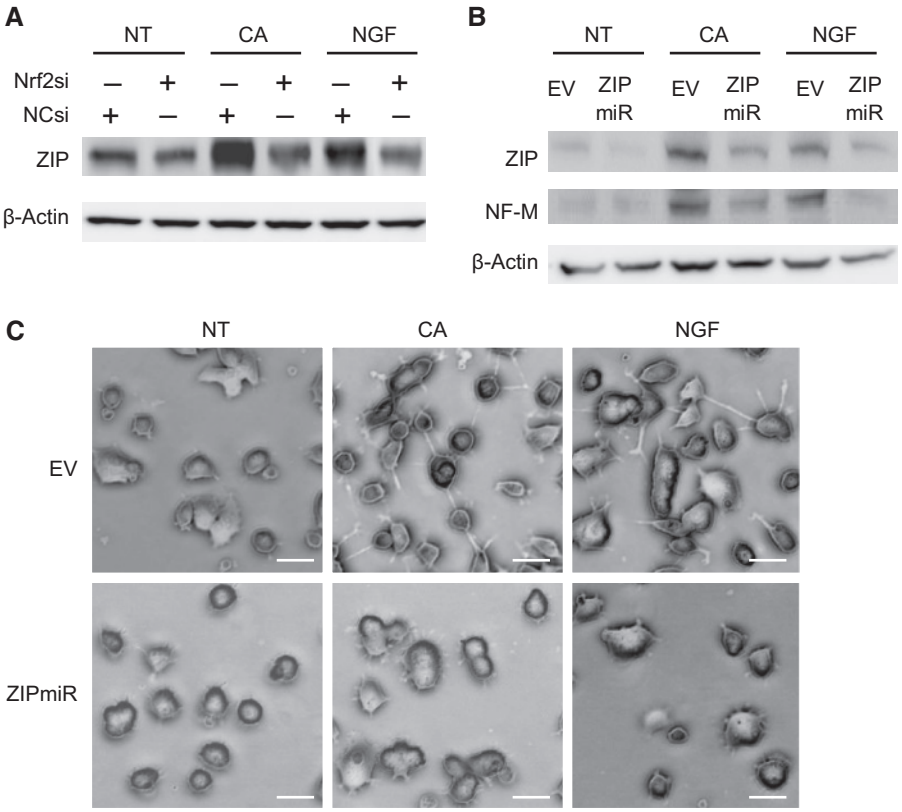

Fig. 7 p62/ZIP is essential for CA-mediated neuronal differentiation. (A) PC12h cells were transfected with Nrf2 siRNA (Nrf2si) or negative control siRNA (NCsi) and then cells were treated with 15 μM of CA or 10 ng/ml of NGF for 6 h. The lysates were subjected to WB analysis with anti-p62/ZIP or anti-β-actin antibody. (B and C) ZIPmiR or empty vector (EV) stable transformant of PC12h cells were treated with 15 μM of CA or 10 ng/ml of NGF for 6 h and then the morphologies were observed under microscopy. Scale bar, 25 μm. p62/ZIP and NF-M protein levels were examined by WB analysis.

The elucidation of CA-targeted phosphatases or proteins, except for Keap1, is under investigation.

As shown in Figs. 2C and 4A, the accumulation of Nrf2 showed the sharp peak at 12 h after CA treatment, which coincided with the peak of Erk1/2 and PI3K activation. Interestingly, CA-induced Nrf2 accumulation and ARE reporter expression was inhibited by PI3K inhibitor LY294002 and MEK1/2 inhibitor U0126 (Fig. 5). Thus, we surmise that CA synergistically activates Nrf2 both by Keap1 oxidative modification and the activation of Erk1/2 and PI3K, although it is not clear at present whether these kinases directly phosphorylate Nrf2 or not. In addition, Nrf2 activity is also boosted by the positive feedback mechanism by p62/ZIP that depends on PI3K activity (Fig. 8C). Liu *et al*. (*35*) also found from a systematic screen for ARE activators that p62/ZIP enhances Nrf2 nuclear accumulation in a PI3K-, PKC- and MEK1-dependent manner in IMR-32 neuroblastoma cells. The precise mechanism of p62/ZIP-mediated Nrf2 activation in PC12h cells is under investigation.

Although we previously demonstrated that CA promotes NGF expression in T98G glioma cells (*24*), CA also increased NGF mRNA level in PC12h cells (data not shown). Furthermore, CA-induced transactivation of ARE was partially inhibited by K252a, suggesting that TrkA phosphorylation was induced by CA (Fig. 8D). CA-induced neural differentiation was inhibited by K252a, suggesting necessity of a small amount of NGF and/or TrkA activity in our experimental condition (Fig. 4B). Therefore, NGF induced by CA probably contributes to promotion of neuronal differentiation of PC12h cells to some extent. But further studies are required to clarify this point.

Previously, it was demonstrated that NGF transcriptionally regulates a set of genes that may commit PC12 cells to a neural differentiation programme (*32*). These include egr-1 at an immediate early time point and mafK at a later stage. A previous report demonstrated that interfering the expression of MafK by miRNA suppressed NGF-induced neurite outgrowth both in PC12 cells and immature telencephalic neurons. Furthermore, overexpression of MafK was sufficient to enhance neuronal differentiation in the telencephalic neurons (*25*). We demonstrated that CA induced the expressions of both p62/zip and mafK to a much greater intensity compared to NGF (Fig. 6). Therefore, it is plausible that the strong neural differentiation activity of CA may also involve its potent induction of mafK as well as p62/zip. Whether or not Nrf2-ARE pathway mediates mafK induction by CA remains to be clarified. On the other hand, we demonstrated that NGF activates Nrf2-ARE *via* TrkA (Fig. 8D). Therefore, the NGF-mediated induction of p62/ZIP also utilizes Nrf2 pathway. The mechanism by which Nrf2 regulates neuronal differentiation and the

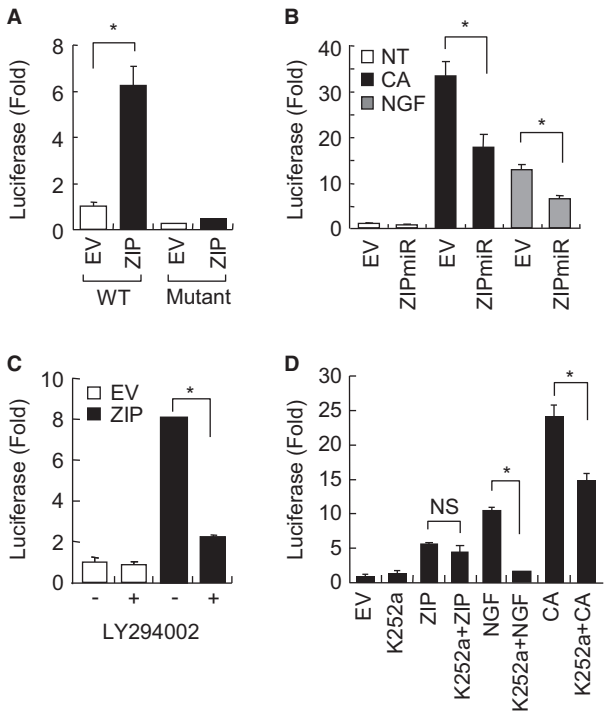

Fig. 8 Role of p62/ZIP in the CA-induced ARE activation. (A) hNQO1 promoter-luciferase construct (wild-type or ARE mutant) and the expression vectors (pEF-p62/ZIP or pEF-EV) were co-transfected into PC12h cells. Luciferase assay was performed 24 h later. (B) hNQO1promoter-luciferase vector and the knockdown vector (pcDNA-ZIPmiR or pcDNA-EV) were co-transfected. After 24 h cells were treated with 15 μM of CA or 10 ng/ml of NGF for 24 h and then luciferase assay was performed. (C and D) hNQO1promoter-luciferase vector and the expression vector (pEF-p62/ZIP or pEF-EV) were co-transfected into the cells. After 24 h, the cells were treated with 15 μM of CA or 10 ng/ml of NGF in the presence or absence of 30 μM LY294002 or 150 nM K252a for 24 h and then luciferase assay was performed. ZIP: ZIP expression vector, ZIPmiR: ZIP knock down vector, EV: empty vector, NS: not significant. $*p < 0.03$.

physiological functions of Nrf2 downstream of NGF requires further clarification.

To date, several electrophilic compounds have been reported to promote neuronal differentiation (*17–21*). In this study, we have demonstrated that Nrf2 plays pivotal roles in CA-mediated neural differentiation in PC12h cells suggesting that Nrf2 is a common target of electrophile-induced neuronal differentiation mechanism. Considering the profound cytoprotective activities of Nrf2 in brain (*36*), CA must be a potent therapeutic agent against a wide range of neurodegenerative disorders, such as Alzheimer's disease, both through its cytoprotective and neurotrophic activities.

Supplementary data

Supplementary data are available at *JB* online.

Acknowledgements

The authors thank Dr Tania O'Connor for their help and critical reading of the manuscript.

Funding

Grant from Ministry of Education, Science, Sports and Technology of Japan and grant for Priority Research Designated by the President of Hirosaki University (to K.I.).

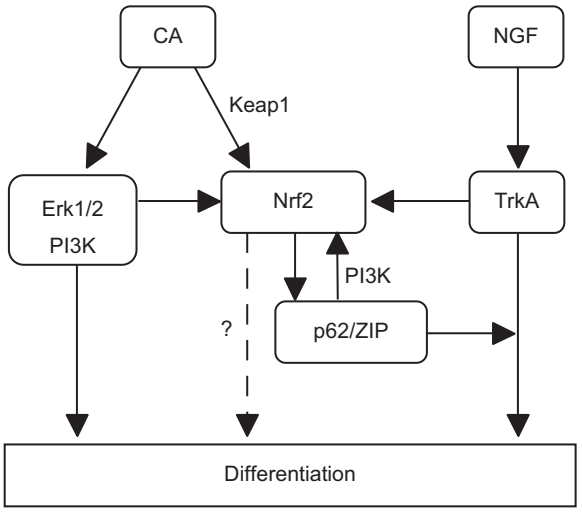

Fig. 9 CA enhances neural differentiation both by Nrf2-dependent and -independent mechanisms. CA activates Nrf2 *via* the direct modification of Keap1. CA also activates Erk1/2 and PI3K by yet unidentified mechanisms. The activation of theses kinases leads to the enhanced Nrf2 accumulation. Activated Nrf2 induce p62/ZIP that enhances TrkA signaling and facilitates neural differentiation. p62/ZIP in turn activates Nrf2 pathway, establishing the positive feed-back loop. NGF also activates Nrf2 *via* TrkA in an Erk1/2- and PI3K-dependent manner. Nrf2 contributes both to the CA- and to the NGF-induced neural differentiation *via* the induction of p62/ZIP. See also DISCUSSION section.

Conflict of interest

None declared.

References

1. Arevalo, J.C. and Wu, S.H. (2006) Neurotrophin signaling: many exciting surprises! *Cell Mol. Life Sci.* **63**, 1523–1537
2. Zhang, Y., Moheban, D.B., Conway, B.R., Bhattacharyya, A., and Segal, R.A. (2000) Cell surface Trk receptors mediate NGF-induced survival while internalized receptors regulate NGF-induced differentiation. *J Neurosci.* **20**, 5671–5678
3. Gong, J., Xu, J., Bezanilla, M., van Huizen, R., Derin, R., and Li, M. (1999) Differential stimulation of PKC phosphorylation of potassium channels by ZIP1 and ZIP2. *Science* **285**, 1565–1569
4. Geetha, T., Jiang, J., and Wooten, M.W. (2005) Lysine 63 polyubiquitination of the nerve growth factor receptor TrkA directs internalization and signaling. *Mol Cell* **20**, 301–312
5. Samuels, I.S., Seibenhener, M.L., Neidigh, K.B., and Wooten, M.W. (2001) Nerve growth factor stimulates the interaction of ZIP/p62 with atypical protein kinase C and targets endosomal localization: evidence for regulation of nerve growth factor-induced differentiation. *J. Cell Biochem.* **82**, 452–466
6. Geetha, T. and Wooten, M.W. (2003) Association of the atypical protein kinase C-interacting protein p62/ZIP with nerve growth factor receptor TrkA regulates receptor trafficking and Erk5 signaling. *J. Biol. Chem.* **278**, 4730–4739
7. Wooten, M.W., Seibenhener, M.L., Mamidipudi, V., Diaz-Meco, M.T., Barker, P.A., and Moscat, J. (2001) The atypical protein kinase C-interacting protein p62 is a scaffold for NF-kappaB activation by nerve growth factor. *J. Biol. Chem.* **276**, 7709–7712

8. Joung, I., Kim, H.J., and Kwon, Y.K. (2005) p62 modulates Akt activity via association with PKCzeta in neuronal survival and differentiation. *Biochem. Biophys. Res. Commun.* **334**, 654–660
9. Satoh, T. and Lipton, S.A. (2007) Redox regulation of neuronal survival mediated by electrophilic compounds. *Trends Neurosci.* **30**, 37–45
10. Satoh, T., Kosaka, K., Itoh, K., Kobayashi, A., Yamamoto, M., Shimojo, Y., Kitajima, C., Cui, J., Kamins, J., Okamoto, S.I., Izumi, M., Shirasawa, T., and Lipton, S.A. (2008) Carnosic acid, a catechol-type electrophilic compound, protects neurons both in vitro and in vivo through activation of the Keap1/Nrf2 pathway *via* *S*-alkylation of target cysteines on Keap1. *J. Neurochem.* **104**, 1116–1131
11. Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J.D., and Yamamoto, M. (1999) Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* **13**, 76–86
12. Kobayashi, M. and Yamamoto, M. (2005) Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. *Antioxid. Redox Signal.* **7**, 385–394
13. Itoh, K., Tong, K.I., and Yamamoto, M. (2004) Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles. *Free Radic. Biol. Med.* **36**, 1208–1213
14. Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M., and Nabeshima, Y. (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem. Biophys. Res. Commun.* **236**, 313–322
15. Satoh, T., Furuta, K., Suzuki, M., and Watanabe, Y. (1999) Prostaglandin J2 and its metabolites promote neurite outgrowth induced by nerve growth factor in PC12 cells. *Biochem. Biophys. Res. Commun.* **258**, 50–53
16. Itoh, K., Mochizuki, M., Ishii, Y., Ishii, T., Shibata, T., Kawamoto, Y., Kelly, V., Sekizawa, K., Uchida, K., and Yamamoto, M. (2004) Transcription factor Nrf2 regulates inflammation by mediating the effect of 15-deoxy-Delta (12,14)-prostaglandin j(2). *Mol. Cell Biol.* **24**, 36–45
17. Satoh, T., Furuta, K., Tomokiyo, K., Namura, S., Nakatsuka, D., Sugie, Y., Ishikawa, Y., Hatanaka, H., Suzuki, M., and Watanabe, Y. (2001) Neurotrophic actions of novel compounds designed from cyclopentenone prostaglandins. *J. Neurochem.* **77**, 50–62
18. Satoh, T., Okamoto, S.I., Cui, J., Watanabe, Y., Furuta, K., Suzuki, M., Tohyama, K., and Lipton, S.A. (2006) Activation of the Keap1/Nrf2 pathway for neuroprotection by electrophilic [correction of electrophillic] phase II inducers. *Proc. Natl Acad. Sci. USA* **103**, 768–773
19. Sagara, Y., Vanhnasy, J., and Maher, P. (2004) Induction of PC12 cell differentiation by flavonoids is dependent upon extracellular signal-regulated kinase activation. *J. Neurochem.* **90**, 1144–1155
20. Dikshit, P., Goswami, A., Mishra, A., Chatterjee, M., and Jana, N.R. (2006) Curcumin induces stress response, neurite outgrowth and prevent NF-kappaB activation by inhibiting the proteasome function. *Neurotox Res.* **9**, 29–37
21. Reznichenko, L., Amit, T., Youdim, M.B., and Mandel, S. (2005) Green tea polyphenol (-)-epigallocatechin-3-gallate induces neurorescue of long-term serum-deprived PC12 cells and promotes neurite outgrowth. *J. Neurochem.* **93**, 1157–1167
22. Hatanaka, H. (1981) Nerve growth factor-mediated stimulation of tyrosine hydroxylase activity in a clonal rat pheochromocytoma cell line. *Brain Res.* **222**, 225–233
23. Moss, M., Cook, J., Wesnes, K., and Duckett, P. (2003) Aromas of rosemary and lavender essential oils differentially affect cognition and mood in healthy adults. *Int. J. Neurosci.* **113**, 15–38
24. Kosaka, K. and Yokoi, T. (2003) Carnosic acid, a component of rosemary (Rosmarinus officinalis L.), promotes synthesis of nerve growth factor in T98G human glioblastoma cells. *Biol. Pharm. Bull.* **26**, 1620–1622
25. Torocsik, B., Angelastro, J.M., and Greene, L.A. (2002) The basic region and leucine zipper transcription factor MafK is a new nerve growth factor-responsive immediate early gene that regulates neurite outgrowth. *J. Neurosci.* **22**, 8971–8980
26. Ishii, T., Itoh, K., Takahashi, S., Sato, H., Yanagawa, T., Katoh, Y., Bannai, S., and Yamamoto, M. (2000) Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. *J. Biol. Chem.* **275**, 16023–16029
27. Zopf, D., Dineva, B., Betz, H., and Gundelfinger, E.D. (1990) Isolation of the chicken middle-molecular weight neurofilament (NF-M) gene and characterization of its promoter. *Nucleic Acids Res.* **18**, 521–529
28. Suzuki, A. and Tsutomi, Y. (1998) Bcl-2 accelerates the neuronal differentiation: new evidence approaching to the biofunction of bcl-2 in the neuronal system. *Brain Res.* **801**, 59–66
29. Fischer, I., Richter-Landsberg, C., and Safaei, R. (1991) Regulation of microtubule associated protein 2 (MAP2) expression by nerve growth factor in PC12 cells. *Exp. Cell Res.* **194**, 195–201
30. Martin, D., Rojo, A.I., Salinas, M., Diaz, R., Gallardo, G., Alam, J., De Galarreta, C.M., and Cuadrado, A. (2004) Regulation of heme oxygenase-1 expression through the phosphatidylinositol 3-kinase/Akt pathway and the Nrf2 transcription factor in response to the antioxidant phytochemical carnosol. *J. Biol. Chem.* **279**, 8919–8929
31. Nakaso, K., Yano, H., Fukuhara, Y., Takeshima, T., Wada-Isoe, K., and Nakashima, K. (2003) PI3K is a key molecule in the Nrf2-mediated regulation of antioxidative proteins by hemin in human neuroblastoma cells. *FEBS Lett.* **546**, 181–184
32. Greene, L.A. and Angelastro, J.M. (2005) You can't go home again: transcriptionally driven alteration of cell signaling by NGF. *Neurochem. Res.* **30**, 1347–1352
33. Groen, A., Lemeer, S., van der Wijk, T., Overvoorde, J., Heck, A.J., Ostman, A., Barford, D., Slijper, M., and den Hertog, J. (2005) Differential oxidation of protein-tyrosine phosphatases. *J. Biol. Chem.* **280**, 10298–10304
34. Shibata, T., Nakahara, H., Kita, N., Matsubara, Y., Han, C., Morimitsu, Y., Iwamoto, N., Kumagai, Y., Nishida, M., Kurose, H., Aoki, N., Ojika, M., and Uchida, K. (2008) A food-derived synergist of NGF signaling: identification of protein tyrosine phosphatase 1B as a key regulator of NGF receptor-initiated signal transduction. *J. Neurochem.* **107**, 1248–1260
35. Liu, Y., Kern, J.T., Walker, J.R., Johnson, J.A., Schultz, P.G., and Luesch, H. (2007) A genomic screen for activators of the antioxidant response element. *Proc. Natl Acad. Sci.USA* **104**, 5205–5210
36. van Muiswinkel, F.L. and Kuiperij, H.B. (2005) The Nrf2-ARE Signaling pathway: promising drug target to combat oxidative stress in neurodegenerative disorders. *Curr. Drug Targets CNS Neurol. Disord.* **4**, 267–281