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ROLE OF NRF2 IN THE NEUROTROPHIC ACTION OF THE ELECTROPHILES

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Abstract Electrophilic compounds, such as curcumin and neurite outgrowth-promoting prostaglandins (NEPPs), have been known to enhance neurite outgrowth in the presence of small amounts of nerve growth factor (NGF) in PC12 cells. However, the redox-sensitive molecular target for enhanced neurite outgrowth is largely unknown. NGF exerts its function by binding to the cell surface tyrosine kinase receptor, TrkA. Recently, Shibata *et al.* reported that PTP1B catalyses the dephosphorylation of TrkA. They also demonstrated that an electrophile from Japanese horseradish Wasabi, 6-methylsulfinylhexyl isothiocyanate (6-HITC), inactivates PTP1B causing sustained phosphorylation and activation of TrkA and promoting neural differentiation of PC12 cells. Further, we recently discovered that carnosic acid (CA), a catechol-type electrophilic compound that is a major ingredient in the herb rosemary, strongly promotes neurite outgrowth of PC12h cells by activating the redox-sensitive transcription factor, Nrf2. Activation of Nrf2 by CA caused a marked induction of p62/ZIP, an important signaling scaffold protein for NGF signaling. Unexpectedly, NGF also activates Nrf2, which is essential for NGF-mediated neural differentiation. Thus, electrophiles may enhance neural differentiation by both TrkA-dependent and -independent mechanisms.

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Introduction

Neurotrophins, such as NGF, BDNF and NT3, promote neuronal growth, differentiation and plasticity, mainly through the Trk family of cell surface receptors. NGF binds to the TrkA-p75^{NTR} complex and activates neurite extension *via* both genomic and non-genomic mechanisms[1,2)]. Through the activation of the cell surface tyrosine kinase receptor, TrkA, NGF activates various kinases, including Erk1/2, PI3K and PKCs[3)]. Recent studies have shown that receptor internalization is also important for NGF signaling[4)]. Shortly after NGF binds to the TrkA-p75^{NTR} receptor complex, the E3 ubiquitin ligase, TRAF6 and the E2 ubiquitin conjugating enzyme H7 (UbcH7) are recruited to p75^{NTR} followed by p62/ZIP/A170/SQSTM1 (hereafter called p62/ZIP for simplicity). p62/ZIP interacts with TrkA, serving as a bridge between p75^{NTR} and TrkA. Thereby, p62/ZIP poly-ubiquitinates TrkA and enhances receptor internalization[5)]. The internalized receptors activate downstream kinases such as Erk5[6)]. Accordingly, it has been shown in PC12 cells and HiB5 neuronal progenitor cells that an increase in p62/ZIP levels leads to enhanced NGF activity[5,7,8)].

Redox regulation of PC12 neural differentiation

PC12 cells respond to NGF with marked neurite extension[1)]. Therefore, PC12 cells have been utilized as a model system in the study of NGF-induced neural differentiation and its signaling pathways. It was previously reported that NGF-induced neural differentiation of PC12 cells is modulated by redox status. Katoh et al. reported that hyperoxia induced neural

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differentiation of PC12 cells *via* sustained activation of MAP kinase[9]. Kamata et al. reported that the treatment of PC12 cells with buthionine sulfoximine (BSO) enhanced NGF-induced neural differentiation, whereas the treatment with N-acetyl cysteine (NAC) attenuated PC12 cell differentiation[10]. They speculated that the cellular redox status affects TrkA activity through the modulation of protein tyrosine phosphatases (PTPs). Cellular redox status may affect PTPs, such as PTP1B because reactive cysteines within their catalytic centers are susceptible to oxidative modification. Shibata et al. recently reported that PTP1B specifically dephosphorylates TrkA[11]. Furthermore, they showed that an electrophile from Japanese horseradish wasabi, 6-methylsulfinylhexyl isothiocyanate (6-HITC), inactivates PTP1B in a redox-dependent manner, leading to sustained phosphorylation and activation of TrkA and the promotion of neurite outgrowth of PC12 cells[11]. Therefore, PTP1B inactivation is one of the targets of redox signaling that enhances neural differentiation.

Electrophiles as a modulator of redox-sensitive neural differentiation

Cyclopentenone prostaglandins (cyPGs) possess electrophilic α, β-unsaturated carbonyls in their cyclopentenone rings and therefore act as endogenous signaling molecules *via* the S-alkylation of proteins[12,13]. We previously demonstrated that synthetic derivatives of cyPGs, called neurite outgrowth-promoting prostaglandins (NEPPs), promote neurite outgrowth in the presence of NGF[14]. Several other electrophilic compounds, such as flavonoids[15], curcumin[16] and epigallocatechin gallate[17], also possess neurite outgrowth-promoting activities when administrated in combination with NGF. In the case of NEPPs, an electrophilic dienone structure has been shown to be important for the neurite outgrowth-promoting activity[14]. These observations indicate that electrophiles possess modulatory effects on neural differentiation.

Nrf2 is emerging as a major electrophile-responsive transcription factor

A *cis*-acting sequence, called the antioxidant

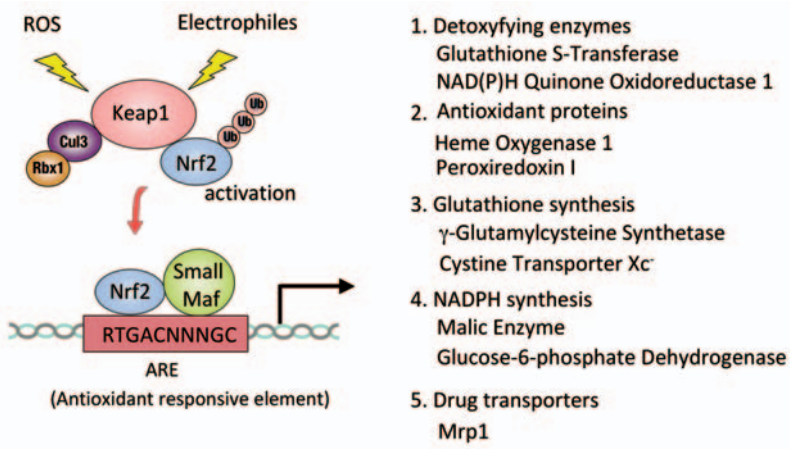

Figure 1 The Keap1/Nrf2 pathway confers protection against oxidative stress and toxic electrophiles *via* the coordinate induction of cytoprotective phase 2 genes.

Under non-stressed conditions, Nrf2 activity is repressed by the ubiquitin-proteasome pathway and Keap1 acts as an adaptor molecule for the Cul3 ubiquitin ligase complex. Electrophiles, or reactive oxygen species (ROS), modify Keap1 and inhibit Keap1's ability to repress Nrf2, resulting in Nrf2 activation and target gene transcription. See text for the details.

or electrophile responsive element (ARE/EpRE), is the regulatory component responsible for the induction of a battery of cytoprotective genes in response to oxidative or electrophilic stress[18] (Figure 1). ARE-regulated genes include phase 2 detoxifying enzymes, such as glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase 1 (NQO1), and antioxidant proteins, such as heme oxygenase 1 (HO-1) and glutathione synthetic enzymes. Using Nrf2 knockout mice, we previously demonstrated that Nrf2 coordinately regulates ARE-mediated gene expression in the liver, intestine and also in macrophages[19,20]. During homeostasis, Keap1 (Kelch-like ECH associating protein 1), a cytosolic actin-binding protein, constitutively represses Nrf2 activity by the ubiquitin-proteasome system. Upon oxidative or xenobiotic stress, Nrf2 is released from Keap1-mediated repression and rapidly accumulates in the nucleus. Recently, we demonstrated that the Nrf2 pathway is activated by endogenous 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) during carrageenan-induced pleurisy[21], indicating that cyclopentenone-type PGs may act as physiological regulators of Nrf2. Therefore, we reasoned that the Keap1/Nrf2 pathway may mediate the common actions of electrophiles that synergize with NGF.

Carnosic acid protected neural cells from oxidative stress in an Nrf2-dependent manner

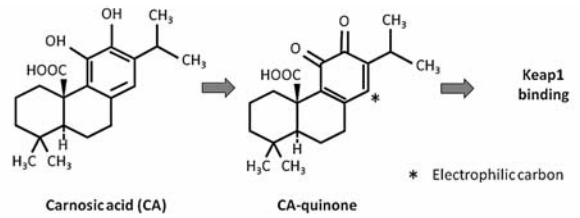

Figure 2 CA is converted to CA-quinone and acts as an electrophile.
Carnosic acid (CA) is a major ingredient of the herb rosemary. CA has a catechol-like structure and is converted to CA-quinone in cells.

Carnosic acid (CA) is a major ingredient of the herb rosemary, which is known for its memory enhancing effects[22]. CA has a catechol-like structure and is easily converted to CA-quinone[23] (Figure 2). NMR analysis showed that CA-quinone possesses a reactive electrophilic carbon in the presence of glutathione. Also, we have shown that biotin-CA binds to Keap1 by immunoprecipitation assay from PC12h cell lysates[23]. CA protected PC12h cells from glutamate-induced excitotoxicity by activating the Keap1-Nrf2 pathway[23]. PC12h cells that have been stably transfected with Nrf2 are constitutively resistant to glutamate-induced excitotoxicity, irrespective of CA. Likewise, cells transfected with a dominant negative Nrf2, named D5D, are sensitive to glutamate-induced toxicity. Furthermore, CA protected neurons from glutamate-induced oxidative stress in the cerebrocortical cultures obtained from day 17 mouse embryos[23]. These observations indicated that CA decreases both excitotoxicity- and oxidative stress-induced neural cell damage through an Nrf2-dependent pathway.

CA enhances neural differentiation via Nrf2-p62/ZIP pathway

We examined the roles of Nrf2 in the neural differentiation of PC12h cells. Nrf2 knock-down significantly attenuated both CA- and NGF-induced neurite extension and neural differentiation, based on expression of the neural differentiation markers NF-H, NF-M and MAP2[24]. Unexpectedly, NGF also activated Nrf2 and NGF-mediated neural differentiation was attenuated by Nrf2 knockdown. Furthermore, cells stably overexpressing Nrf2 exhibited marked neurite outgrowth compared to the control cells when placed in differentiation medium for 72 hours. In contrast, cells transfected with Keap1 or Nrf2 dominant negative mutants exhibited the attenuation of the neurite outgrowth. These results unequivocally demonstrate that Nrf2

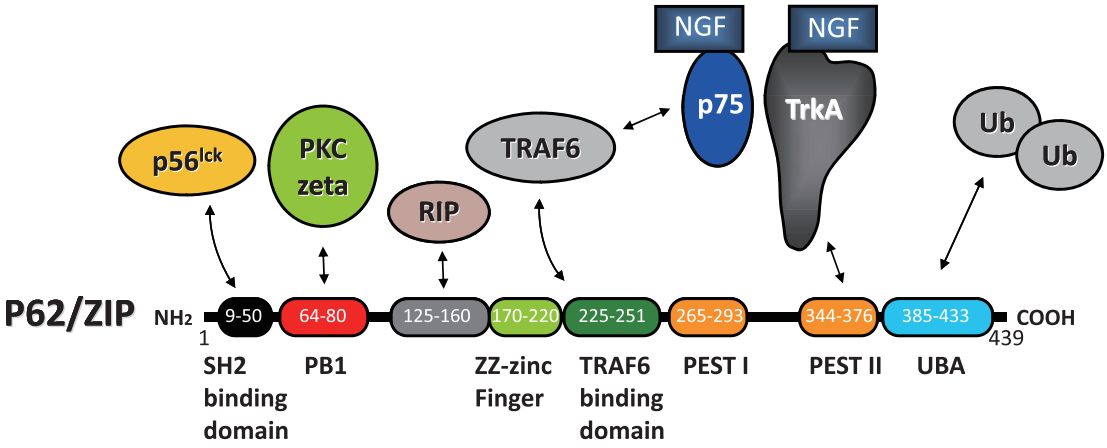

Figure 3 Domain structure of p62/ZIP.
At its N terminus, p62/ZIP has a PB1 (Phox and Bem1p-1) domain that binds to the homotypic region of atypical PKCs, followed by a ZZ finger that binds to the tumor necrosis factor α (TNF-α) signaling adaptor, receptor interacting protein (RIP). Following these domains, there is a short stretch of amino acids that includes the TBS sequence for binding to TRAF6. The C-terminal part of p62/ZIP harbors a UBA (ubiquitin-associated) domain.

plays an important role in both CA- and NGF-induced neural differentiation.

p62/ZIP is a multi-domain scaffold protein that possesses PB1 and UBA domains and a TRAF6 binding sequence (Figure 3). P62/ZIP is essential for various functions, including osteoclastogenesis, inflammation and obesity, as well as neurotrophin biology[25]. Because p62/ZIP expression is known to be regulated by Nrf2[20] and p62/ZIP regulates neuronal differentiation in PC12 cells[7,8], we hypothesized that p62/ZIP acts downstream of Nrf2 in the CA-induced neural differentiation. Indeed, the knockdown of p62/ZIP by microRNA markedly attenuated CA-induced neural differentiation. Consistent with this observation, a strong induction of p62/ZIP was observed 12 hours after CA exposure and this induction was dependent on Nrf2.

Role of kinases in CA-induced neural differentiation

NGF is known to activate various downstream kinases, including PKC, PI3K, Erk1/2 and Erk5[1]. Moreover, in PC12 cells it is known that PI3K is important for survival whereas MAPK is important for their differentiation[26]. Therefore, we examined CA-mediated kinase activation, focusing specifically on PI3K and Erk1/2. Western blot analysis demonstrated that CA induced phosphorylation of Akt and Erk1/2[24]. However, CA-induced phosphorylation of Akt and Erk1/2 was delayed, exhibiting peak kinase activation 12 hours after treatment, as compared to NGF-mediated activation, which peaked at 30 minutes post-treatment. Intriguingly, the time course of CA-induced Akt and Erk1/2 activation was similar to that of Nrf2 activation by CA, which led us to hypothesize that CA-induced Nrf2 accumulation is downstream of Erk1/2 and PI3K. Consistent with this expectation, inhibitors of both Erk1/2 and PI3K attenuated Nrf2 accumulation. A role for Erk1/2 and PI3K in CA-induced Nrf2 activation was also demonstrated by the previous observation that Erk1/2 and PI3K are important for Nrf2 activation by 15d-PGJ_2 in PC12 cells[27].

Then we examined how CA activates these kinases. Firstly, we examined the role of TrkA kinase using its inhibitor K252a. K252a almost completely abrogated the phosphorylation of Erk1/2 by NGF, but had no effect on CA-induced phosphorylation of Erk1/2. K252a

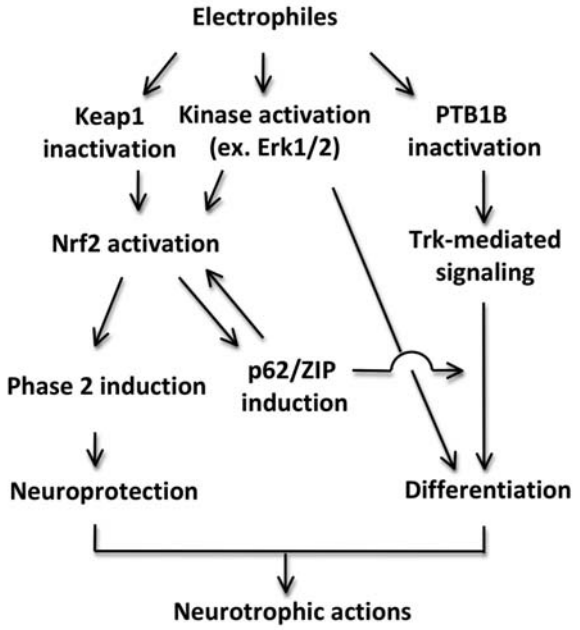

Figure 4 Electrophiles exerts neurotrophic actions both by Nrf2-dependent and -independent mechanisms.

Nrf2 exerts its neuroprotective function through the induction of phase 2 cytoprotective gene expression. Nrf2 also contributes to neural differentiation by the induction of p62/ZIP. Electrophiles such as CA, activate ERK1/2 in addition to inactivating Keap1, which contributes both to the Nrf2 activation and the neural ditterentiation. On the other hand, certain electrophiles (i.e., 6-HITC) may inactivate PTP1B and cause sustained phosphorylation and activation of TrkA. These mechanisms may contribute to the neurotrophic action of electrophiles.

partially abolished the activation of PI3K by CA. Moreover, Nrf2 siRNA did not affect the Erk1/2 phosphorylation, but partially diminished the activation of PI3K both by CA and NGF. These results unequivocally demonstrate that CA activates Erk1/2 *via* a TrkA- and Nrf2-independent mechanisms.

p62/ZIP-mediated positive feedback mechanism

During the course of the study, we happened to find that an overexpression of p62/ZIP enhances ARE activity. Likewise, knockdown of p62/ZIP significantly diminished ARE-reporter gene activity following induction by CA or NGF[24]. This indicates the existence of a positive feedback mechanism in which p62/ZIP, induced by Nrf2, in turn activates the Nrf2-ARE system (Figure 4). Actually, Liu et al. also found that overexpression of p62/ZIP enhances Nrf2 nuclear accumulation in PI3K-, PKC- and MEK1-dependent manners using a systematic screen for ARE activators in IMR-32 neuroblastoma cells[28]. The *in vivo* physiological significance of a Nrf2-p62/ZIP axis is an interesting issue to be explored in the future.

Future perspectives

Electrophilic compounds enhance neurite outgrowth in PC12 cells when a small amount of NGF is present. Recent studies have demonstrated that these effects could be mediated through PTP1B- and Nrf2-dependent mechanisms (Figure 4). In support for the latter, Zhao et al. recently reported the contribution of Nrf2 signaling pathway to the TPA- and RA-induced neural differentiation in human SH-SY5Y cells[29]. The SH-SY5Y cell line is a neuroblastoma cell line that expresses TrkA. Therefore, it is possible that the Nrf2 pathway somehow modulates the TrkA pathway in SH-SY5Y cells, although they did not examine p62/ZIP regulation by Nrf2 in their study. Zhao et al. also reported that neurite outgrowth was retarded in neurons derived from Nrf2 knockout mice when compared to that of wild-type mice. Neurons that express the TrkA receptor exist in the hippocampus and basal forebrain, including cholinergic neurons. Therefore, Nrf2 may play an important role in memory or learning[30], and the dysregulation of Nrf2 in basal forebrain cholinergic neurons may be involved in Alzheimer's disease[31]. Thus, the role of Nrf2 in normal brain functions and in neurological diseases remains to be explored.

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