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Original Contribution

Signaling pathways activated by the phytochemical nordihydroguaiaretic acid contribute to a Keap1-independent regulation of Nrf2 stability: Role of glycogen synthase kinase-3

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ABSTRACT

Defense against oxidative stress is executed by an antioxidant program that is tightly controlled by the transcription factor Nrf2. The stability of Nrf2 involves the interaction of two degradation domains, designated Neh2 and Neh6, with the E3 ubiquitin ligase adaptors, Keap1 and β-TrCP, respectively. The regulation of Nrf2 through the Neh6 degron remains largely unexplored but requires GSK-3 to form a phosphodegron. In this study, the cancer-chemopreventive agent nordihydroguaiaretic acid (NDGA) increased the level of Nrf2 protein and expression of heme oxygenase-1 (HO-1) in kidney-derived LLC-PK1 and HEK293T cells and in wild-type mouse embryo fibroblasts (MEFs). However, NDGA did not induce HO-1 in Nrf2$^{-/-}$ MEFs, indicating that Nrf2 is required for induction. The relevance of the Nrf2/HO-1 axis to antioxidant protection was further demonstrated by the finding that the HO-1 inhibitor stannous-mesoporphyrin abolished protection against hydrogen peroxide conferred by NDGA. NDGA increased Nrf2 and HO-1 protein levels in Keap1$^{-/-}$ MEFs, implying that Keap1-independent mechanisms regulate Nrf2 stability. Mutants of the Neh2 or Nrh6 domain and chimeric proteins comprising cyan fluorescent protein fused to Neh2 and green fluorescent protein fused to Neh6 exhibited longer half-lives in the presence of NDGA, demonstrating that NDGA targets both the Neh2 and the Neh6 degrons. In common with other chemopreventive agents, NDGA activated the ERK1/2, p38, JNK, and PI3K pathways. By using selective kinase inhibitors we found that PI3K, JNK, and p38 were responsible for the stabilization of Nrf2 and induction of HO-1 by NDGA. To explain how NDGA might up-regulate Nrf2 in a Keap1-independent manner we explored the participation of GSK-3β because it controls the Neh6 phosphodegron. Importantly, NDGA caused inhibitory phosphorylation of GSK-3β at Ser9 and at Thr390, and this was associated with a substantial reduction in Neh6 phosphorylation. Our study demonstrates that NDGA activates Nrf2 through multiple signaling cascades and identifies GSK-3β as an integrator of these signaling pathways and a gatekeeper of Nrf2 stability at the level of the Neh6 phosphodegron.

Transcription factor Nrf2 is a cap'n'collar (CNC) basic-region leucine zipper (bZIP) protein that has emerged in recent years as a master regulator of cellular redox homeostasis [1]. It controls the expression of more than 100 genes that contain antioxidant response elements (AREs) in their promoter regulatory regions [2], including those encoding antioxidant proteins such as heme oxygenase-1 (HO-1) [3]. Collectively, these enzymes participate in scavenging reactive oxygen species (ROS) and maintaining redox homeostasis. It is becoming increasingly evident that Nrf2 contributes to many physiological processes, including detoxification, biotransformation, inflammation, cytoprotection, proliferation, aging, etc., and that consequently its activity must be tightly controlled for optimal response to endogenous and exogenous agents.

Nrf2 is controlled primarily at the level of protein stability, because of the presence of at least two degradation domains (degrons) within its Neh2 and Neh6 domains [4,5]. The Neh2 domain (residues 1–90) contains a redox-sensitive degron that under unstressed conditions interacts with the redox sensor Keap1 to allow ubiquitination and subsequent degradation of Nrf2 [6–9]. The interaction between the Neh2 domain and Keap1 occurs via two motifs in the CNC–bZIP protein, a high-affinity ETGE motif and a low-affinity DLG motif, each interacting with a separate Kelch-repeat domain present in the

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Keap1 homodimer [10,11]. In addition to its interaction with Nrf2, Keap1 also binds Cullin-3 (Cul3), which forms a core E3 ubiquitin ligase complex through an association with ring-box 1 protein (Rbx1, also called Roc1) [6–9]. The Keap1–Cul3–Rbx1 complex ubiquitinates Nrf2 and targets it for proteasomal degradation only under normal redox conditions. However, upon exposure to oxidants or electrophiles, at least Cys151, Cys273, and Cys288 in Keap1 become modified, leading to a disturbance in the interaction between Nrf2 and Keap1 [12–14]. Failure of Keap1 to dock properly to the Neh2 domain permits Nrf2 to escape ubiquitination by Cul3–Rbx1 [13–15]. Thus, stress-related modification of Keap1 results in Nrf2 stabilization, accumulation of the transcription factor in the nucleus, and upregulation of ARE-driven genes. Regulation of Nrf2 stability through an interaction between its Neh2 domain and Keap1 seems to be the main redox-sensitive mechanism that accounts for cellular adaptation to oxidative stress [16].

The Neh6 domain (residues 300–378) contains a degron that includes a DSG motif embedded in a cluster of serine residues. This region is a docking site for the adaptor protein β-TrCP, which mediates the ubiquitination of Nrf2 by a Cullin1–Rbx1 complex [5]. Although very little is known about the regulation of Nrf2 by β-TrCP-dependent ubiquitination, evidence from other well-established β-TrCP substrates, such as Snail, β-catenin, Gli2 and Gli3, Xom, Cdc25a, FGD1 and FGD3, Mcl-1, securin, prolactin receptor, and the phosphatase PHLPP1 [17], suggests that the association between Nrf2 and β-TrCP is likely to be facilitated by phosphorylation of serine residues within and around the region in Nrf2 to which β-TrCP binds. Indeed, we have reported that, as for other β-TrCP substrates, the Neh6 degron is phosphorylated at least in part by glycogen synthase kinase-3 (GSK-3) [5]. Although the regulation of Nrf2 by Keap1 is now well established, very little is known about the regulation of Nrf2 by β-TrCP. Moreover, it is not known whether either endogenous signals or xenobiotics can antagonize the Neh6 degron.

Xenobiotic compounds that activate Nrf2 are diverse and share few common properties except for their ability to modify sulfhydryl groups of cysteines by oxidation or adduct formation. These classes include allyl sulfides, dithiolethiones, flavonoids, isothiocyanates, polyphenols, terpenoids, etc. [18,19]. Therefore much attention has been devoted to studying how these compounds alter the interaction between Keap1 and Neh2, which leads to Nrf2 stabilization [20]. On the other hand, numerous studies have consistently demonstrated that many xenobiotics, including chemopreventive agents, stimulate cell signaling cascades that participate in Nrf2 regulation. Although most compounds activate more than one kinase cascade they seem to show some preference. For instance, carnosol, *tert*-butylhydroquinone (tBHQ), triterpenoids, and statins may use mostly Akt signaling [21–25]; curcumin, α-lipoic acid, quercetin, and hydroxychalcones may use stress-related MAP kinases p38, JNK, or ERK [23,25-27]; most compounds, including some of those mentioned above and resveratrol, brazilin, or epigallocatechin-3-gallate seem to require a combination of Akt and ERK [28–30], and kahweol requires both Akt and p38 [31].

Whereas stimulation of the above signaling pathways by xenobiotics has been thoroughly studied, little effort has been made to determine the cross talk among them or to identify a common downstream effector, such as GSK-3, that might integrate these pathways at the level of Nrf2. Therefore, it is still not clear how these compounds might signal to up-regulate Nrf2 aside from the Keap1 interaction. To address this question, in this study, we have analyzed the activation of Nrf2 and the cytoprotective response elicited by induction of HO-1 in response to the dicatechol nordihydroguaiaretic acid (NDGA). As will be shown in this study, NDGA targets a variety of signaling pathways that include PI3K/Akt and the MAP kinases ERK1/2, p38, and JNK. In addition, this compound has been reported to inhibit receptor tyrosine kinase signaling by growth factors such as IGF-1 [32] and it has been used as an inhibitor of lipooxygenase. Consequently, NDGA provides a complex but integrative model to study how xenobiotics might activate various signaling pathways that converge at the up-regulation of Nrf2.

NDGA, more rigorously termed 4-[4-(3,4-dihydroxyphenyl)-2,3-dimethylbutyl] benzene-1,2-diol, is a phenolic lignan containing two *o*-catechols at the ends of a short alkane chain (Supplementary Fig. 1A). It was originally isolated from *Larrea tridentata* and infusions from this plant have been used in popular medicine for multiple diseases [33]. NDGA is a bifunctional antioxidant, i.e., it has intrinsic ROS-scavenging activity and indirect antioxidant properties due to the induction of expression of antioxidant genes [34]. As an antioxidant molecule, NDGA is a potent in vitro scavenger of peroxynitrite, singlet oxygen, hydroxyl radical, superoxide anion, and hypochlorous acid [35,36]. In addition to its intrinsic antioxidant nature, we have previously shown that NDGA induces the expression of the antioxidant enzyme HO-1 through Nrf2 in primary cultures of cerebellar granule neurons [37].

In this study, we have explored how various signaling pathways elicited by the model chemopreventive agent NDGA might lead to Nrf2 stabilization. Our findings indicate that NDGA targets both the Neh2 and the Neh6 degrons to increase Nrf2 stability and that multiple kinase cascades confer Nrf2 stability by preventing GSK-3-mediated degradation of Nrf2 through the Neh6 degron.

Material and methods

Cell culture and reagents

Porcine LLC-PK1 cells, human HEK293T cells, and mouse embryo fibroblasts (MEFs) were cultured in Dulbecco's modified Eagle's medium (DMEM; high glucose concentration) supplemented with 10% fetal bovine serum, 0.33% sodium bicarbonate, and antibiotics (100 units/ml penicillin, 0.1 mg/ml streptomycin) under a humidified atmosphere of 5% CO_2 at 37 °C. Keap1$^{-/-}$ and Keap1$^{+/+}$ MEFs were kindly provided by Dr. Ken Itoh (Center for Advanced Medical Research, Hirosaki University Graduate School of Medicine, Hirosaki, Japan). All tissue culture reagents were from GIBCO (Invitrogen Corp., Carlsbad, CA, USA). NDGA, bovine serum albumin (BSA), ethylene glycol tetraacetic acid (EGTA), 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (Hepes), dithiothreitol (DTT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), phenylmethylsulfonyl fluoride (PMSF), Fluoromount aqueous mounting medium, poly-L-lysine, paraformaldehyde, Hoechst 33258, fluorescein diacetate (FDA), and protein kinase inhibitors LY294002, PD98059, SB203580, SP600125, and SB216763 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dihydroethidium (DHE), 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA), and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were from Invitrogen. Stannous mesoporphyrin (SnMP) was from Frontier Scientific (Logan, UT, USA). H_2O_2 and isopropanol were from J.T. Baker (Mallinckrodt Baker S.A. de C.V., Xalostoc, Edo. de México, México). Biodyne-B nylon membranes were from Thermo Fisher Scientific (Waltham, MA, USA). Kinase inhibitors and NDGA were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in cell culture was less than 0.2%.

Plasmids

For luciferase assays we used an expression vector for *Renilla* (Promega, Madison, WI, USA) and ARE-Luc (a gift from Dr. J. Alam, Department of Molecular Genetics, Ochsner Clinic Foundation, New Orleans, LA, USA).Vector pCFP-Neh2 (2–101) was generated by PCR amplification using the following primers: forward, 5′-TAAC*AAGCTT*TGGATTTGATTGACATC-3′, and reverse, 5′-TAAC*AAGCTT*CTACATACAGTCTTCAAA-3′. The pcDNA3.1/V5HisB-mNrf2 plasmid was used as template. HindIII sites in italic were used to introduce PCR products into pCFP-CI (Clontech). For vector pEGFP-Neh6 (306–483) the primers