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Activation of Nrf2-antioxidant signaling attenuates NFκB-inflammatory response and elicits apoptosis[☆]

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ARTICLE INFO

Article history:

Received 28 May 2008

Accepted 15 July 2008

Keywords:

Nrf2

NFκB

DSS

AOM

Apoptosis

ABSTRACT

Oxidative stress has been implicated in the etiology of neurodegenerative disease, cancer and aging. Indeed, accumulation of reactive oxygen and nitrogen species generated by inflammatory cells that created oxidative stress is thought to be one of the major factor by which chronic inflammation contributes to neoplastic transformation as well as many other diseases. We have recently reported that mice lacking nuclear factor-erythroid 2-related factor 2 (Nrf2) are more susceptible to dextran sulfate sodium (DSS)-induced colitis and colorectal carcinogenesis. Nrf2 is a basic leucine zipper redox-sensitive transcriptional factor that plays a center role in ARE (antioxidant response element)-mediated induction of phase II detoxifying and antioxidant enzymes. We found that increased susceptibility of Nrf2 deficient mice to DSS-induced colitis and colorectal cancer was associated with decreased expression of antioxidant/phase II detoxifying enzymes in parallel with upregulation of pro-inflammatory cytokines/biomarkers. These findings suggest that Nrf2 may play an important role in defense against oxidative stress possibly by activation of cellular antioxidant machinery as well as suppression of pro-inflammatory signaling pathways. In addition, in vivo and in vitro data generated from our laboratory suggest that many dietary compounds can differentially regulate Nrf2-mediated antioxidant/anti-inflammatory signaling pathways as the first line defense or induce apoptosis once the cells have been damaged. In this review, we will summarize our thoughts on the potential cross-talks between Nrf2 and NFκB pathways. Although the mechanisms involved in the cross-talk between these signaling pathways are still illusive, targeting Nrf2-antioxidative stress signaling is an ideal strategy to prevent or treat oxidative stress-related diseases.

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1. Introduction

Nrf2 is an important cytoprotective transcription factor. When challenged by oxidants or electrophiles, Nrf2 induces the transcription of diverse antioxidant enzymes, phase II detoxification enzymes, phase III efflux transporters. In combine, these cytoprotective genes can rapidly and coordinately neutralize, detoxify and remove those invading

xenobiotics. Accumulating evidences show that Nrf2 is also the promising target for cancer chemoprevention.

2. Nrf2 signaling (I): the hinge and latch model

At unstressed condition, Nrf2 is sequestered in the cytoplasm by a cytosolic repressor Keap1 (Kelch-like ECH-associated

[☆] Supported by NIH Grants CA-073674, CA-094828 and CA-118947.

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0006-2952/\$ – see front matter. Published by Elsevier Inc.

doi:10.1016/j.bcp.2008.07.017

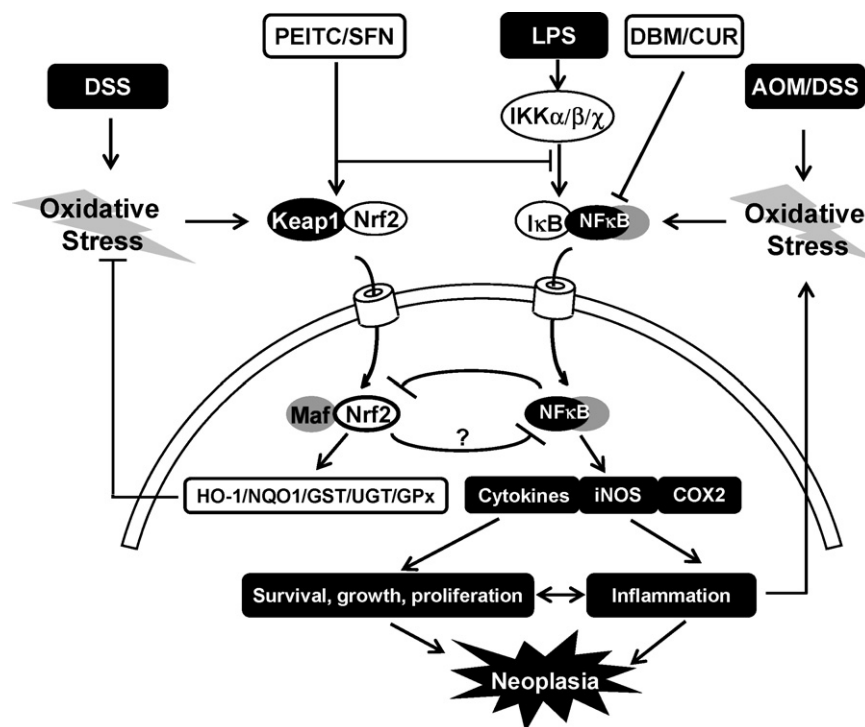


Fig. 1 – Chronic colon colitis model. AOM/DSS treatments induce colon colitis via activating NFκB-mediated pro-inflammatory pathway. Prolonged colon colitis promotes neoplastic transformation. Pretreatment with chemopreventive compounds such as PEITC, SFN, DBM and CUR alone or in combine can considerably ameliorate colon carcinogenesis either by the induction of Nrf2-mediated antioxidant response to attenuate oxidative stress or by suppressing NFκB-mediated inflammatory response.

protein 1) [1]. Recently, Keap1 is also characterized as a Cullin 3-dependent ubiquitination substrate adaptor protein [2–5]. So Nrf2 is not only physically sequestered in the cytoplasm, but also constantly degraded. At the oxidative condition, Nrf2 is released from Keap1 repression, translocates to the nucleus, forms heterodimer with small Maf (musculoponeurotic fibrosarcoma) proteins, recognizes and binds to a cis-acting enhancer called antioxidant response element (ARE), and eventually recruits the whole transcription machinery including the RNA polymerase II to transcribe phase II/III genes [1].

In an old model of Nrf2 signaling [1], Keap1 is depicted as the primary redox sensor. The redox signals are subsequently relayed to Nrf2 in the process of Nrf2 releasing. In this model, there are some tacit assumptions. First, oxidants can induce Nrf2 releasing from Keap1. Second, free Nrf2 automatically translocates into the nucleus. The intensity of oxidative signals is encoded by the amount of Nrf2 proteins released from Keap1. New experimental observation in Keap1/Nrf2 studies paints a quite different picture.

Recently, two Keap1 binding motifs, i.e., an ETGE motif [6] and a DLG motif [7] have been characterized in the Neh2 domain of Nrf2. In an isothermal calorimetric measurement, two phases of Nrf2/Keap1 binding, i.e., a high affinity phase I binding, mediated by the ETGE motif and a low affinity phase II binding mediated by the DLG motif, has been distinguished [8]. Accordingly, an elegant “Hinge and Latch” model is proposed by Yamamoto’s group [9] to vividly illustrate how Keap1 functions as a substrate adaptor protein. At unstressed

condition, Keap1 exists in homodimer mediated by its BTB (Broad complex, Tramtrack and Bric a brac) domain. Nrf2 first forms high affinity binding with Keap1 via the ETGE motif. This strong binding functions as the “hinge”. Nrf2 can also form weak binding with Keap1 via the DLG motif, as the “latch”. Linking the DLG and the ETGE motifs is an alpha helix. Six out of seven lysine residues of this alpha helix locate at the same side. Recently, the crystal structure of the Nrf2-binding Kelch domain of Keap1 has been resolved [10]. The geometric distance between the two Kelch domains matches perfectly with the length of this alpha helix [10]. So when the “latch” is in position, the alpha helix is straightened up to maximally expose those lysine residues, presumably as ubiquitination acceptors. At the oxidative condition however, an intermolecular disulfide bond is formed between Cys273 and Cys 288 of Keap1 dimer. Consequently, the profound conformation change in Keap1 dimer disrupts the latch-binding. As a result, the randomly floating alpha helix will not be in appropriate orientation for effective ubiquitination. The Nrf2 ubiquitination is impeded. The oxidants however, may fail to disrupt Keap1/Nrf2 binding mediated by the ETGE motif. Recently, Mesecar’s group reported that overdose sulforaphane (SFN) treatments failed to disrupt Keap1/Neh2 binding, no matter the treatment of SFN was on Keap1 first, on Neh2 first or on Keap1/Neh2 complex [11]. So oxidants uncouple Keap1-mediated repressions: whereas oxidants indeed impede Nrf2 degradation, oxidants fail to disrupt Keap1/Nrf2 binding [12]. The observation that oxidants failed to disrupt Keap1/

Nrf2 binding put a big question mark on this old Nrf2 signaling model.

3. Nrf2 signaling (II): graded nuclear translocation of Nrf2

The second question is whether free Nrf2 automatically translocates into the nucleus? The answer is a definite no. Recently we characterized in Nrf2 a nuclear export signal (NES) in the leucine zipper domain (NES_{Zip}) [13] and another NES motif located in the Neh5 transactivation domain (NES_{TA}) [14]. In addition, we also identified a bipartite nuclear localization signal (NLS) in the basic region (bNLS) [13]. It is the combined activities of multiple NLS/NES motifs that determine the subcellular localization of Nrf2. When full length wild type Nrf2 was expressed, a mixed distribution pattern was observed [14]. So the jointed nuclear exporting activities mediated by NES_{TA} and NES_{Zip} motifs can counter balance the nuclear importing activity mediated by the bNLS motif. When the NES_{TA} motif was disabled by L183A mutation, the distribution of mutant was converted to a nuclear distribution [14]. When the NES_{Zip} motif was disabled by L544A mutation, the distribution of mutant was also converted into a nuclear distribution [14]. We naturally ask a question: does the disabling of NES_{TA} or NES_{Zip} motif occurs at physiological condition? Embedded in the NES_{TA} motif there is a cysteine residue (Cys 183). Our tandem mass spectrometry analysis showed that this cysteine residue is a redox-reactive cysteine (Li et al., unpublished data). Sulfhydryl modification at this cysteine residue may generate spatial hindrance to nuclear exporting protein CRM1 (chromosome region maintenance 1). In other word, this NES_{TA} motif is a conditional NES that can be switched off by oxidants. The disabling of this NES_{TA} motif triggers Nrf2 nuclear translocation.

We further examined the kinetics of Nrf2 nuclear translocation mediated by the NES_{TA} motif. We treated EGFP-NES_{TA} expressing cells with different dosages of sulforaphane (SFN). We used two parameters to describe nuclear translocation: the maximal nuclear accumulation (A_{max}) and half time to achieve maximal nuclear accumulation (t_{1/2}). The A_{max} values were positively correlated with SFN concentrations. The t_{1/2} values were inversely correlated with SFN concentrations [14]. In other words, the higher the concentration of oxidants, more Nrf2 proteins translocate into the nucleus at higher speed. This graded nuclear translocation of Nrf2 showed that Nrf2 cannot only transmit the presence of oxidative stress, but also the intensity of oxidative stress.

Based on those observations, we propose a new Nrf2 signaling model. At unstressed condition, Nrf2 is constantly degraded via Keap1-mediated ubiquitination. Nrf2 degradation may be counter balanced by constitutive Nrf2 translation. As a consequence, very small pool size of free Nrf2 proteins exists. At the oxidative condition, Keap1-mediated ubiquitination is impeded. Nrf2 translation is elevated as reported by Chen's group at Arizona [15]. As a result, the pool of free Nrf2 proteins expands. But the influx of Nrf2 is still determined by the intensity of oxidative stress. In this new model, Nrf2 is the primary redox sensor. Keap1 still modulates the redox-sensitivity of Nrf2 by controlling the availability of free Nrf2

proteins. This new model may compromise discrepancies that cannot be explained by the old model.

4. Nrf2-mediated cancer chemoprevention

Nrf2 is an important anti-neoplastic factor and the key target of cancer chemoprevention. We compared the anti-neoplastic effect between Nrf2 wild type (WT) and knockout (KO) mice in 7,12-dimethylbenz(a)anthracene (DMBA)-12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin carcinogenesis model [16]. Topical application of a single dose DMBA (200 nmol) followed by TPA treatment (8 nmol, twice a week) for 25 weeks elicited significantly higher tumor incidence in Nrf2 KO mice in comparison with WT mice [16]. Pretreatment of sulforaphane (daily topical application of 100 nmol) for 2 weeks significantly inhibited carcinogenesis in WT mice ($p < 0.05$) but not in KO mice ($p > 0.05$) [16]. It is also very noteworthy that in tumor samples from both Nrf2 WT and KO mice, the expression of Nrf2 and Nrf2 regulated gene heme oxygenase 1 (HO-1) was shut off [16]. The mechanism(s) of Nrf2 shut off is/are currently intensively investigated.

5. Chronic inflammation promotes neoplastic transformation

Accumulated evidence showed that chronic inflammation promotes neoplastic transformation. Recently we developed a chronic inflammation mice model. We used dextran sulfate sodium (DSS) to induce colonic colitis [17]. Feeding of 1% DSS in drinking water for 1 week induce colonic colitis. In comparison with Nrf2 WT mice, the colonic colitis observed in Nrf2 KO mice appeared to be more severe, including lost of colonic crypt, massive infiltration of inflammatory cells and anal bleeding. In addition, immunocytochemical staining of nitrotyrosine, a biomarker of inflammation, was more intense in Nrf2 KO mice. One reason why DSS-induced colonic colitis is more severe in Nrf2 KO mice is the lower induction of phase II protective gene, such as HO-1, GST (Glutathione-S-Transferase), UDP-glucuronosyltransferase (UGT) 1A1, in Nrf2 KO mice. Concomitantly, more intense induction of inflammatory biomarkers, such as cytokine interleukin (IL)-1 β , IL-6 and TNF α (tumor necrosis factor- α) as well as pro-inflammatory enzymes inducible nitric oxide synthetase (iNOS) and cyclooxygenase 2 (COX2), was observed in Nrf2 KO mice in comparison with WT mice. Since these pro-inflammatory biomarkers are all effector genes regulated by the NF κ B pathway, these observation is consistent with previous observation of anti-inflammatory function of Nrf2. Ablation of Nrf2 seems to accelerate NF κ B-mediated pro-inflammatory reaction.

Based on DSS-induced colonic colitis model, we went further to test the role of Nrf2 in an azoxymethane (AOM)-DSS induced colonic carcinogenesis mice model (Khor et al., Cancer Prevent Res. in press). Mice were subcutaneously injected with a single dose of AOM (10 mg/kg body weight). Two weeks after the AOM injection, the mice were fed with 1% DSS in drinking water for 1 week. Tumorigenesis was examined after 20 weeks. The tumor incidence was signifi-

cantly higher in Nrf2 KO mice (92.9%) in comparison with WT mice (53.3%). In addition, 80% of tumors are adenocarcinomas in Nrf2 KO mice in comparison with 29% in WT mice. The higher tumor incidence in Nrf2 KO mice was paralleled with severe colonic colitis including prolapsed rectum and anal bleeding. The nitrotyrosine staining was more intense in colon samples from Nrf2 KO mice in comparison with the WT samples. Induction of HO-1, UGT1A1 and NQO1 (NAD(P)H:quinone oxidoreductase 1) was diminished in Nrf2 KO mice. The induction of arachidonic acid (AA) metabolites, which is involved in pro-inflammatory response, is more intense in KO mice. The induction of COX2 and 5-lipoxygenase (5-LOX) as well as prostaglandin E2 (PGE2) and leukotriene B4 (LTB4), the major metabolites of COX2 and 5-LOX pathways, were significantly higher in NO mice than in the WT mice. The present study highlighted the important role of Nrf2 in the protection against colonic inflammation as well as carcinogenesis.

6. Potential cross-talk between Nrf2 and NFκB pathways

These data obtained from animal studies underlined the possibility that Nrf2-mediated anti-tumorigenic effect may be achieved by activation of antioxidant machinery as well as suppression of pro-inflammatory pathways mediated by NFκB signaling. Previously, we observed that lipopolysaccharide (LPS) induced NFκB activation could be attenuated by diverse Nrf2 activators, such as phenethyl isothiocyanate (PEITC), SFN and curcumin (CUR) [18]. Dietary administrations of SFN and dibenzoylmethane (DBM) alone or in combination could significantly inhibited the development of intestinal adenomas in ApcMin mice in parallel with decreased PGE2, LTB4 and COX2 activities [19]. Furthermore, PEITC and SFN treatment was found to be able to inhibit IKK/IκB phosphorylation and p65 NFκB subunit nuclear translocation, consequently alleviating NFκB signaling [20]. Our data are summarized in Fig. 1. However, definite evidence showing that Nrf2 can directly inhibit NFκB signaling is still wanting. On the contrary, it was reported recently that NFκB could directly repress Nrf2 signaling at the transcription level. NFκB competes against Nrf2 for transcription co-activator CREB binding protein (CBP) [21]. In addition, NFκB recruits histone deacetylase 3 (HDAC3) to cause local hypoacetylation to hamper Nrf2 signaling [21]. Further studies are needed to unravel complicate interplay between Nrf2 and NFκB pathways.

7. PEITC and curcumin treatments induce apoptotic death

Previously, cancer chemopreventive compound PEITC was reported to be able to delete preneoplastic cells through induction of apoptosis [22,23]. Treatment of tumeric curcumin was also found to be able to induce apoptosis in prostate cancer cells [24]. Indeed, we observed that treatment of PEITC and curcumin could inhibit the growth of human PC-3 prostate xenografts in nude mice [25]. The growth inhibitory effect may, at least partially, be attributed to induced

apoptosis. Using an *in situ* apoptosis detection kit (Chemicon), we detected significantly higher percentage of apoptotic cells in mice samples treated with PEITC alone (5 μmol), curcumin alone (6 μmol), or PEITC (2.5 μmol) and curcumin (3 μmol) in combine. Consistent with the observed induced apoptosis, significantly high induction of cleaved caspase 3 and poly(ADP-ribose)polymerase (PARP) were observed in tumor samples [25].

Therefore treatments with cancer chemopreventive compounds can not only block the initiation of carcinogenesis, but also suppress the promotion and progression of carcinogenesis, at least partially by induction of apoptosis.

8. Perspectives

Previous MS-MS studies have identified reactive cysteine residues in Keap1, now Nrf2 also seems to possess reactive cysteine(s). What is the functional significance? The existence of reactive cysteines in Nrf2, especially those cysteines located in functional motifs of Nrf2, implies that Nrf2 *per se* is a redox-sensitive probe. It awaits further experimental examination of the relative sensitivity and functional roles of those cysteine residues, both in Keap1 and in Nrf2.

Acknowledgements

We thank Dr. Jefferson Chan for his general gift of Nrf2 knockout mice. We thank all collaborators in Drs. Allan Conney and C.S. Yang's laboratory. We thank Drs. Hong Li and Tong Liu for their assistance in MS-MS analysis. We thank all current and former members of Dr. Kong's laboratory for their contribution and comment.

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