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Review Article

Keap1-Nrf2/ARE signaling pathway- A promising therapeutic pathway for diseases: A Review

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ABSTRACT
Oxidative stress is the major causes of development and progression of many diseases. Nrf2 is a transcriptional factor that regulates the stress response by binding with ARE in nucleus and causes induction of antioxidant genes and phase 2 detoxifying enzymes. In normal conditions Nrf2 inhibited by Keap 1 and during redox imbalance or oxidative stress, Nrf2 gets activated. During such condition Nrf2 activity eventually gets increased and causes the expression of antioxidant genes like HO-1 (Heme oxygenase-1), NQO-1 (NADPH quinone oxidoreductase 1), glutathione peroxidase. Thus, Nrf2-ARE pathway plays important role in diseases like liver disorders, respiratory diseases, inflammatory diseases, neurodegenerative diseases etc.

Keywords: Nrf2, Keap 1, liver, arthritis, neurodegenerative disease.

INTRODUCTION

Endogenous and exogenous metabolic processes takes place in the body leads to generation of reactive oxygen species.[1]Uncontrolled reactive species production leads to oxidative stress, which compromises cellular functioning and causes ROS/RNS-mediated damage to cellular macromolecules such nucleic acids, proteins, and lipids, and moreover in cell death. In order to maintain cellular redox equilibrium, Nrf2 defends against oxidative stress.[2,3]The Keap1-Nrf2-AREpathway is the most important protective mechanism against oxidative and/or electrophilic stresses., which is also closely linked to inflammatory diseases, cancer, neurodegenerative diseases,cardiovascular diseases, and ageing.[4]

History

In 1970, Wattenberg and his co-workers demonstrated that phenolic antioxidants used in food additives, inhibit tumour development in mice following exposure to carcinogens.[5] When antioxidants are given before a carcinogen exposure, prevention occured. Later, evidence suggests that phenolic antioxidants causes expression of phase II enzymes, which promotes the metabolism and deposition of free radicals. Later found that BHA (butylated hydroanisole) significantly boosts the transcriptional expression of GSTs and detoxifying enzymes in the liver and intestine of mouse and rat.[6,7]

Talalay and colleagues showed through the examination of diphenols and diamines found these are redox labile substances and act as monofunctional inducers, showing that redox chemistry is involved in the phase II enzyme induction process.[8,9] In 1990, Pickett and groups discovered a regulatory element distinct in the promoter region of rat Gst-Ya and named it as antioxidant responsive element (ARE) that is responsive to t-butylhydroquinone (t-BHQ) and β-naphthoflavone. The responsive element was determined by point-mutation analysis and found to be of core sequence RGTGACNNNGC, where R and N represents purine any base respectively.[10,11]Identical responsive components were also discovered in mice and humans , known as an electrophile responsive element in mice (EpRE).[12]

Talalay and his companions referred ARE-regulated and electrophile-inducible cytoprotective genes- phase 2 enzymes.[13]They examined the structural and functional relationship of phase 2 inducers. They used a reporter construct in which the mouse Gst-Ya ARE is connected to a growth hormone reporter gene in order to systemically investigate the inducer property.[14]They measured the concentration of NQO1(NADPH quinone oxidoreductase 1) for evaluating inducer potency.

Structure

Nrf2 is a protein , composed of 589 or 605 amino acids and its expected molecular weight is 66 kDa, it is detected on SDS-polyacrylamide gel electrophoresis. [15] Seven functional Nrf2-derived proteins with CNC homology (Neh) domains make up Nrf2. The CNC-bZIP motif found in the Neh1 domain enables Nrf2 to bind to the ARE. [16]

The N- terminal of Nrf2 has Neh2 domain, that functions as a regulatory domain. [17]Neh2 has two binding sites that control the stability and integrity of Nrf2. And it has 7 lysine residues essential for conjugation with ubiquitin. [18,19]ETGE and DLG motifs are two conserved binding sites interact with Nrf2 inhibitor, keap1. [20] The Nrf2 transactivation is mediated by Neh3, Neh4, and Neh5 domains by interacting with coactivators and Neh6 domain maintain its stability. [21]

Keap1 is a major regulator of Nrf2 activity and it is an essential Nrf2 regulator and a sensor of ARE inducers and it prevents Nrf2 transactivation. [22]The 624 amino acids found in both mouse and human Keap1 proteins. Three protein-interacting domains in Keap1 are BTB (Broad-complex, Tram track, and Bric a brac) domain, DGR (Double Glycine Repeat), and IVR (Intervening Region) domain. The N-terminal has BTB domain that associates with the Keap1 and cause dimerization and its binding to Cul3, a scaffold protein of Nrf2 ubiquitin E3 ligase. [23,24] The DGR domain must be present in the C terminal for Keap1 to attach to Neh2 domain of Nrf2. [25] The IVR domain, which is located between BTB and DGR, contains cysteine residues, the most of which are susceptible to oxidation by oxidants and electrophiles. [26,27]

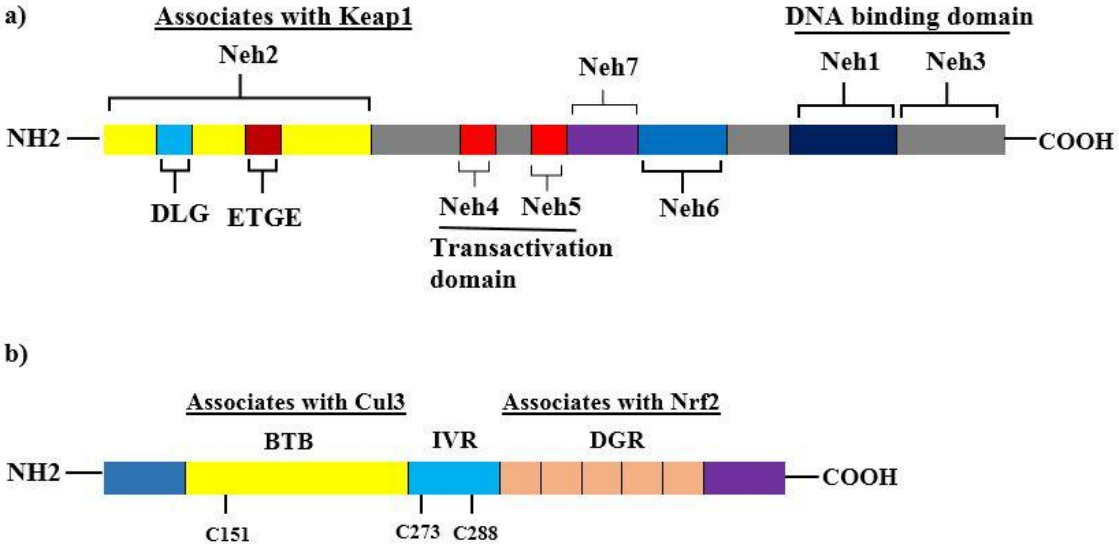

Figure 1: Structure a) Nrf2 b) Keap1 [15]

Keap1-Nrf2/Are Signaling Pathway

In Keap1-Nrf2 signaling pathway, Keap 1 act as sensor, Nrf2 as controller and ARE as responders. The CNC family contains regulatory proteins like Nrf2, which is a transcription factor with a highly conserved basic leucine zipper structure (bZip).[28]

Keap1 is the repressor of Nrf2, it has BTB domain and DGR domain which binds with CuI3-Rbx1 and Nrf2 respectively. [29] The BTB region of Keap1 functions as a sensor to detect electrophilic agent and oxidative stress.[30,31]Keap1 represses Nrf2 by keap1 homodimer attracted to the Nrf2 DLG- ETGE motifs, and the Cul3-Rbx1-E3 ligase complex ubiquitinates and degrades the lysine-rich - helical conformation between EGTE and DLG. Keap1 maintains Nrf2 in the cytoplasmby causing its ubiquitination and 26 S proteasome mediated degradation.[32] In the presence of oxidative stress, cysteine residues present in the Keap1 changes and which causes Nrf2 to stabilise and accumulate in the nucleus. [33]The main repressor protein Keap1 undergoes modification by oxidation and undergo conformational changes, which are the classical mechanism of Nrf2 activation. Nrf2 is stabilised as a result of Cul3-Rbx1 releasing it from ubiquitination. Nrf2 consists of Nrf2-ECH homologies on which Neh 1 and Neh2 domains of Nrf2 heterodimerize with smallMaf and Jun proteins and then bind with ARE for

transcription. [34] Glycogen synthase kinase 3 (GSK-3) mediates phosphorylation, it generates motif for the E3 ligase adapter (E3 ubiquitin protein ligase), resulting in degradation of Nrf2 via ubiquitin-dependent proteasome. Acetylation of Nrf2 by p300/cAMP response element-binding protein-binding protein (CBP) can modulate it and the acetylation causes it to adhere to the ARE and activate gene transcription, whereas deacetylation causes it to be released, which causes transcription to end and nuclear export.[35,36]

The Keap1-Nrf2/ARE signaling pathway modulation mechanisms can essentially be classified into two categories: Keap1-dependent and Keap1-independent methods. (i)Hinge &latch model (ii) Keap1 ubiquitination (iii) Keap1-CUl 3 dissociation modelmodel are three models for Keap1-dependent regulation mechanisms.The hinge &latch model indicates that the DLG, ETGE motifs in the Neh2 region of Nrf2 have varying binding affinities to Keap1. [37]

In normal state, homologous Keap1 dimers link with ETGE and DLG to form a closed Keap1-Nrf2 conformation, which causes Nrf2 to be degraded by the proteasome.[38]Cysteine residues of Keap1 contain essential sulfhydryl groups undergo oxidation or production of adducts when encounter with substances that alter redox balance. Positions 151, 273 and 288 of cysteines appear to be particularly susceptible to redox alterations and interactions with electrophilic substances.[39,40]Meanwhile, the free Keap1 dimer prepared for attaching to the freshly produced Nrf2 for the subsequent cycle. Low-affinity DLG (latch) is dissociated under oxidative stress, whereas high-affinity ETGE (hinge) interacts with Keap1 homopolymers to create an open Keap1-Nrf2 conformation.[41]In order to prevent the newly produced Nrf2 from being identified by ubiquitin ligase and degraded by the proteasome, the Keap1 binding site is occupied. Cumulative Nrf2 triggers the expression of the target gene by being transduced into the nucleus. Only the closed conformation is suitable for the ubiquitination of Nrf2 by Keap1. This Keap1-Nrf2 complex can flip between the two different states. In Keap1 ubiquitination model: Keap1 undergoes a cysteine alteration that causes Nrf2 ubiquitin conjugate to shift to Keap1.In the Keap1 dissociation hypothesis, inducers like tert-butylhydroperoxide (t- BHP) can interfere with Keap1 and CUL3's connection, reducing Nrf2 ubiquitination. [42] Keap1 plays two roles in Keap1-dependent regulation is to sense stimulation from oxidants or electrophiles through its several cysteine residues, and the other is to prevent Nrf2 from being ubiquitinated when the body is under oxidative stress by altering Keap1's conformation. [43]

With other transcription factors that include a leucine zipper, such as small Maf proteins, Nrf2 must form heterodimers in order to exhibit the transcriptional activity. The battery of antioxidant genes are induced to express, which leads to the recovery of redox balance. [44]Numerous experimental findings validate this hypothesis suggesting that electrophilic treatments which target the Keap1/Nrf2 pathway may operate to boost Nrf2 transcriptional activity.

Protein kinases play important role in Nrf2-Keap1 independent pathway. [45] The stability of Nrf2 can be enhanced by phosphorylation at a certain amino acid residue and thereby its transactivation. PKC, PI3K, c-Jun N-terminal kinase (JNK), and other kinases can phosphorylate Nrf2, which favourably regulates the Nrf2 pathway. However, p38 MAPK negatively regulates the Nrf2 pathway. [46,47] Independent way of Nrf2 regulation include self-regulation, and post transcriptional regulation. Nrf2 binds with promoter site and increase regulation. DNA and histone modification, Serine phosphorylation and ubiquitination can regulate Nrf2. [48]

Nrf2 phosphorylation, which also leads in the association between Nrf2 and ARE in the nucleus, facilitates the transcription of numerous genes that produce antioxidants, including HO-1 and NQO1. As a consequence, it is believed that activating the Nrf2-ARE pathway will be effective against variety of diseases. [49]

Role of the Nrf2 pathway in liver diseases

The liver is a versatile organ that regulates metabolic equilibrium and detoxification. [50] Uncontrolled generation of reactive speciesdue to liver injuries results in the degradation of cells and themacromolecules. It also leads to the production of pro-inflammatory genes. [51]It has been suggested that anti-inflammatory and antioxidant therapy can be effective in the management of liver disorders. Researchers have found a link between Nrf2 activity dysregulation and the emergence of inflammatory diseases. Nrf2 is the primary regulator of cellular defence by modulating anti-inflammatory,antioxidant, and cytoprotective effects. [52]A key defence mechanism used by cells and organisms to combat oxidative stress is the Keap1-Nrf2 pathway. The Nrf2 performs a variety of functions in the body's defence against

oxidative stress, including regulating cell metabolism, detoxification, and cell proliferation, all of which are crucial in the pathological process of a number of ailments. [53]Both parenchymal hepatocytes and non-parenchymal cells like Kupffer cells, exhibit Nrf2 activation. [54] Moreover, the liver also expresses a large number of Nrf2 target genes. Through the induction of this target genes, Nrf2 playsimportant role in hepatic fibrosis, inflammation, hepatocarcinogenesis, and regeneration. According to research, Nrf2 activation reduces acute liver damage. [55]In a study using a mouse model of acute liver injury caused by cadmium, Wu et al. compared the levels of serum ALT, LDH, and necrosis in Nrf2-null and Nrf2-enhanced mice. They discovered that Nrf2-enhanced mice seemed to have lower ALT and LDH levels as well as less morphological changes. Only Nrf2-enhanced mice showed increased expression of cytoprotective genes, such as glutathione peroxidase, glutamate-cysteine ligase, glutamate cysteine synthetase. Sulfiredoxin 1 activates Nrf2, which affects the expression of genes involved in antioxidant protection to reduce oxidative stress and subsequently liver damage. [56]

Role of Nrf2-ARE pathway in rheumatoid arthritis

Rheumatoid arthritisis an autoimmune, inflammatorydisease that attacks cells itself and cause pain in joints. Results of various *in vitro* ,*in vivo* studies shown that Nrf2 has prominent role in inflammation. [57]Inflammatory mediators, such as cytokines, macrophages, lymphocytes, neutrophils, and become activated and are delivered to the inflamed area, causing synovium fibroblasts to hyper proliferate, swell, and cause pain, which leads to the damage of cartilage and bone. Radicals produced by oxidation may play a significant role in inflammation, which increases levels of protein, causes lipid peroxidation and leads to DNA damage. [58]The Nrf2 controls the redox activity may contribute tothe activation of the NLRP3 inflammasome, member of NLR family and it regulate the tissue repair process. It is then activated by Nrf2 in order to activate theNLRP3 inflammasome, which controlsa number of activities including antioxidant, tissue repair and homeostasis. [59,60]Auto inflammatory and autoimmune disorders both exhibit increased NLRP3 inflammasome activity, and Nrf2-ARE pathway is suggested as an important way for the therapeutic regulation of NLRP3-related diseases. [61]

Nrf2 activation supports the resolution of inflammation, through the increase of prostaglandin (PG) D synthase expression in macrophages, which results in the synthesis of PGD2, PGJ2 . In order to reduce the inflammation, PGJ2 stimulates Nrf2, which causes the induction of HO-1 and CD36 in macrophages. [62]Interleukins and pro-inflammatory mediators activate COX-2, which leads to inflammation. COX 2 promotes the binding of several transcription factors to NF-kb in order to maintain its transcriptional activity and also enhance its expression. Through direct interference with c-JNK, Nrf2 inhibits and decreases COX-2 expression. [63,64]

The dual role of cytokines include pro-inflammatory effects and anti-inflammatory effects. Pro-inflammatory cytokines, such as interleukins IL-2, IL-6, and IL-12, TNF-α, are generated whenever there is an increased level of oxidative stress. They target cartilages and synovial membranes, increasing oxidative stress.[65] These can also result in the development and formation of ROS, which stimulates and activates nuclear factor kB, reducing the amount of these inflammatory cytokines produced and formed. The inflammation caused by cytokines can be minimized using the Nrf2 activators. Rheumatoid arthritis patients show increased Nrf2/HO-1 gene expression. Nrf2 regulates both the adaptive and innate immune responses in addition to playing a significant role in the maintenance of inflammation. [66]

Role of Nrf2 in respiratory diseases

The Keap1-Nrf2 pathway is a major antioxidant protection pathway that defends the lung against oxidative stress. Oxidative stress have a great impact on number of respiratory related diseases, including asthma, lung cancer, chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS) [67] Since airways serve as the site of gas exchange, are continually in contact with the environment and subject to a variety of airborne pollutants, like inhaled oxidants. The airways are situated in highly oxidative microenvironments and as a result the redox balance in the airways is readily affected. [68]Antioxidant substances are seen in lung tissues and epithelial lining fluid, protects the lungs from oxidative stress. GSH and antioxidants are depleted in susceptible individuals of respiratory infections. [69] Numerous phase 2 detoxifying enzymes, including HO-1, are regulated by the transcription factor Nrf2, demonstrating that it is

a crucial regulator of anti-oxidative responses that preserves cellular homeostasis and minimizes oxidative damage.[70]

Asthma is chronic inflammatory disorder characterized by airway obstruction. Various studies shown that pathogenesis of asthma involves oxidative stress. Patients with asthma may struggle more than healthy individuals to manage oxidant burden, which may be directly related to decreased Nrf2 activity. [71,72]Additionally, it was shown that Nrf2controls the growth of airway smooth muscle cells, which act abnormally in asthma, and the antioxidant response. [73] Epithelial tight connections are disrupted so that airborne chemicals can more easily enter into the airway wall and interact with immunological and inflammatory cells and further impairing the barrier function and raising the risk of respiratory virus infection. [74]Nrf2 pathways enhances epithelial barrier integrity and reduces the incidence of asthmatic episodes. Studies shows that Nrf2 deficiency increases ovalbumin (asthma inducer) induced oxidative stress in mice. It also increased levels of neutrophils and eosinophils, which in turn causes more oxidative damage to lung tissues. [75]

Nrf2 has two roles in lung cancer according to target action, activator protects the cell and inhibitor prevent cancer cells from proliferating. It also disrupts the initiation of cancer cell proliferation and remove ROS, DNA damaging substances, carcinogens.[76] Satoh et al. discovered that after 8 weeks of treatment, Nrf2-deficient mice exposed to the carcinogen show a relative increase in the number of tumour foci, and by 16 weeks of treatment, they exhibit less malignancy.[77]

Increased prevalence and severity of emphysema occurred due to disruption of Nrf2 in mice. Diet with Nrf2 activator reduces the oxidative stress, cell apoptosis and alveolar damage due to cigarette smoke exposure. These findings imply that Nrf2 participates in a pathogenic process that leads in lung emphysema led by cigarette smoke exposure, and that this pathogenic process can be stopped by Nrf2 activator.[78]

Role of Nrf2 in neurodegenerative diseases

Nrf2 activation combats various pathogenic processes involved in neurodegenerative diseases by the enhancement of antioxidant defences, maintenance of protein homeostasis, enhancement of mitochondrial function, and suppression of inflammation.[79] The main risk factor for neurological disorders is ageing. Environmental and genetic factors also influence neurodegenerative disorders like Parkinson's disease, Alzheimer's disease, Huntington's disease etc. These disorders occurred due to similar pathogenic mechanisms like increased level of ROS, disruption in protein metabolism, mitochondrial dysfunction, neuro-inflammation.[80]Various studies have shown that inducing Nrf2-dependent antioxidant activities improves neurological phenotypes in disease models. [81] Nrf2 regulates the production of a series of antioxidant enzymes and proteins that have cytoprotective effects.

Alzheimer's disease has increased oxidative stress, mitochondrial dysfunction, accumulation of amyloid -β plaque, APP mutation, aggregation of tau protein. Lipton et al. shown that carnosic acid, Nrf2 activator inhibited the loss of dendritic spines in rat neurons exposed to neurotoxic Aβ. [82] *In vivo* study in mice injected with human amyloid precursor protein experienced better learning and memory. [83]A histological study of the hippocampus revealed that carnosic acid lowered phospho-tau staining, astrogliosis, and the amount of Aβ plaques while increasing dendritic and synaptic markers.

Parkinson's disease is a progressive neurodegenerative disease characterized by tremor, rigidity, motor instability and akinesia. Dopaminergic neuron loss in the e substantia nigra is the primary pathogenic feature for the development of disease. [84]Recent studies have focused on the link between Nrf2 signaling and Parkinson’s disease, and as a result, Nrf2 activation has emerged as a prospective therapeutic target for drugs intended to retard or inhibit neuronal cell death in Parkinson's disease. [85,86]Nrf2 deficient mice injected with MPTP shown decreased dopamine level and on Nrf2 activator treatment reverses the effect. [87]The transplantation of astrocytes overexpressing Nrf2 in the striatum provided additional *in vivo* proof of Nrf2-mediated neuroprotection. Mice that received astrocytes that overexpressed Nrf2 became more and more resistant to the toxicity caused by 6-hydroxydopamine (6-OHDA).Some preliminary *in vitro* data strongly support that Nrf2 inducers can slow the progression of PD. Also, tert-butylhydroquinone (t-BHQ), a Nrf2 inducer provided protection from neurotoxicant 6-OHDA. [88,89]

Huntington's disease (HD) is a progressive neurological disorder with a unique phenotype, such as chorea and dystonia, incoordination, cognitive loss, and behavioural issues. [90]Expansion of CAG repeats in Huntingtin gene causes this disorder. Neuronal degeneration in

neostriatal and cortex region causes motor impairement and loss of memory.[91]In post-mortem HD brain, mitochondrial complex defects were seen in the striatum. Nrf2 activator protects both *in vitro* and *in vivo* models.[92]The oral administration of dimethyl fumarate (DMF), a Nrf2-ARE activator, to HD mice models shows elevated neuronal Nrf2 and improvement in motor function, and the protection of neurons in the striatum and motor cortex.[93]

CONCLUSION

In this review, we addressed the potential therapeutic benefits of Nrf2 in various ailments and provided a short summary of the history, structure and Nrf2 pathway. The transcriptional control of ARE related genes in relation to oxidative stress has advanced significantly. In order to keep up cellular redox homeostasis and inhibit the pathogenesis of numerous inflammatory and related diseases, it is crucial that these genes are to be expressed. Nrf2 activators target keap1-Nrf2 interactions and degrades keap1, which regulate the signaling pathway. It has become the focus of recent studies on molecular mechanism of pathways. Pharmaceutical companies are in urge to develop drugs that target Keap 1 and causes activation of Nrf2.

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