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REVIEW

Targeting Nrf-2 is a promising intervention approach for the prevention of ethanol-induced liver disease

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Abstract

Alcoholic liver disease (ALD) remains to be a worldwide health problem. It is generally accepted that oxidative stress plays critical roles in the pathogenesis of ALD, and antioxidant therapy represents a logical strategy for the prevention and treatment of ALD. Nuclear factor erythroid-derived 2-like 2 (NFE2L2 or Nrf-2) is essential for the antioxidant responsive element (ARE)-mediated induction of endogenous antioxidant enzymes such as heme oxygenase 1 (HO-1) and glutamate–cysteine ligase [GCL, the rate-limiting enzyme in the synthesis of glutathione (GSH)]. Activation of Nrf-2 pathway by genetic manipulation or pharmacological agents has been demonstrated to provide protection against ALD, which suggests that targeting Nrf-2 may be a promising approach for the prevention and treatment of ALD. Herein, we review the relevant literature about the potential hepatoprotective roles of Nrf-2 activation against ALD.

Keywords Alcoholic liver disease · Nuclear factor erythroid-derived 2-like 2 (Nrf-2) · Oxidative stress · p62 · Autophagy

Introduction

Excessive ethanol consumption can cause a progressively aggravated liver disease, namely alcoholic liver disease (ALD). ALD presents as a broad spectrum of disorders, ranging from simple fatty liver (steatosis) to alcoholic hepatitis (AH), alcoholic fibrosis (AF), alcoholic cirrhosis (AC), and the superimposed hepatocellular carcinoma (HCC) [1]. ALD is highly prevalent and is listed among the top 20 causes of death worldwide [2]. In Europe, it is estimated that more than 2,370,000 years of life are lost from liver diseases before the age of 50, and about 60–80% of these deaths are ethanol related [3]. In the USA, ethanol abuse is also the leading cause of death from liver diseases [4]. Although the number of newly hepatitis B virus (HBV)-infected patients in China is significantly declined due to the establishment of the expanded program on immunization in 1992, the number of ALD patients is rising at an alarming rate with the prevalence of ALD ranging from 2.3 to 6.1% in different local areas [5]. Thus, ALD has been becoming a worldwide health problem. Unfortunately, in contrast to the steady progress in the clarification of the pathogenic mechanisms of ALD, no significant advance has been made in the management of ALD, especially in the field of long-term treatments [6]. Currently, abstinence remains to be the cornerstone of ALD treatment which is largely dependent on patient's willingness and compliance [7]. Due to the increasing prevalence of ALD and the lack of effective therapeutic agents, there is an urgent need to develop effective and safe pharmacological interventions for patients with ALD [8].

Nuclear factor erythroid-derived 2-like 2 (NFE2L2 or Nrf-2) is a transcription factor that plays a key role in the activation of cellular antioxidant enzymes in response to oxidative stress. Nrf-2 belongs to the Cap"n"Collar (CNC)-bZip (basic leucine zipper) family, which includes Nrf-1, Nrf-2, Nrf-3, and p45 NFE2 [9, 10]. After heterodimerization with one of three small Maf proteins, the indispensable partners of CNC-bZip transcription factors, Nrf-2 binds to the Maf recognition element-related sequence, namely antioxidant or electrophile response element (ARE/EpRE) [11, 12]. ARE has been identified in the transcriptional regulatory regions of antioxidant and xenobiotic-metabolizing enzyme genes including glutamate–cysteine ligase (GCL), the rate-limiting

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enzyme in the synthesis of glutathione (GSH), heme oxygenase-1 (HO-1), glutathione S-transferase (GST) family, and NAD(P)H quinone oxidoreductase 1 (NQO-1). Nrf-2 has been suggested to be a potential target for new therapeutics in various liver diseases [13, 14]. Activation of Nrf-2 signaling by pharmacological agents is considered as a promising strategy for protecting against liver injury induced by various chemicals including ethanol [15].

Critical roles of oxidative stress in the pathogenesis of ALD

ALD is a multifactorial disease involving complicated mechanisms, among which oxidative stress has been demonstrated to play critical roles [16]. As early as 1964, the pioneer study by Diluzio demonstrated that simultaneous supplementation of antioxidants could prevent acute ethanol-induced fatty liver, proposing the possible relationship between ethanol-induced steatosis and oxidative stress [17]. Using electron spin resonance (ESR) spectroscopy technique, increasing production of several reactive oxygen species (ROS) including nitroxyl radical, hydroxyl radicals, and hydroxyethyl free radicals has been detected [18, 19]. In addition, a variety of studies have provided evidence that both chronic and binge drinking could result in the enhancement of lipid peroxidation (shown as the elevation of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) levels) and protein carbonyl formation, and the impairment of the hepatic antioxidant defense system including reduced GSH level and superoxide dismutase (SOD) activity [16, 20]. Interestingly, ethanol-induced liver injury could be significantly attenuated by various kinds of antioxidants including N-acetyl cysteine (NAC), resveratrol, silymarin, quercetin, and organosulfur compounds derived from garlic [21–26]. Furthermore, overexpression of the Cu/Zn-SOD or Mn-SOD gene with adenovirus suppressed alcohol-induced early liver injury in rats [27, 28], while ethanol-induced liver damage was aggravated in Cu/Zn-SOD-deficient mice and glutathione peroxidase (GPX)/catalase double-knockout mice [29–31]. Lastly, cytochrome P4502E1 (CYP2E1) and NADPH oxidase (NOX) have been demonstrated to be the two major sources of ethanol-generated ROS in ALD models [24, 32, 33]. Collectively, these results provide solid evidence for the causal roles of oxidative stress in the pathogenesis of ALD.

The canonical and non-canonical activation of Nrf-2/Keap-1 pathway

Under basal or unstressed conditions, Nrf-2 is kept in the cytoplasm by a cluster of proteins including Kelch like-ECH-associated protein 1 (Keap-1) and Cullin 3 (Cul3). Keap-1 contains three major domains: a N-terminal BTB (broad complex, tram track, and bric-a-brac) domain, a linker region, and a C-terminal Kelch domain. The Kelch domain contains six conserved Kelch repeat sequences and binds to the Neh2 domain of Nrf-2, while the BTB domain is responsible for the homodimerization of the Keap-1 protein. The linker region is a cysteine-rich domain which is demonstrated to be indispensable for the activity of Keap-1 [10, 34, 35]. Nrf-2 has six Neh (Nrf-2-ECH homology) domains (Neh1–Neh6). The Neh2 domain has two different motifs (the ETGE and the DLG motifs) which can bind Keap-1, resulting in an Nrf-2–Keap-1 complex of 1:2 stoichiometry with two binding sites [36, 37]. Cul3 ubiquitinates Nrf-2, while Keap-1 is a substrate adaptor protein for the Cul3 E3 ubiquitin (Ub) ligase complex that facilitates the reaction. The ubiquitinated Nrf-2 then transports to the proteasome for degradation, ensuring the low basal levels of Nrf-2 [38].

Two activation models of Nrf-2, the canonical activation and non-canonical activation, have been proposed (Fig. 1). Upon attack by ROS or electrophiles, the sensory cysteines of Keap-1 can be modified, resulting in a conformational change which could prevent Nrf-2 from ubiquitination. The newly synthesized Nrf-2 escapes from Keap-1-mediated repression and translocates to the nucleus, and then binds to small Maf protein and turns on the transcription of ARE-controlled genes to maintain cellular redox homeostasis. This mechanism of Keap-1 cysteine-dependent Nrf-2 activation is termed canonical activation [10, 38–40]. The non-canonical activation of Nrf-2 is mediated by the interactions between Keap-1 and p62 (also known as sequestosome-1, SQSTM1), which localizes to sites of autophagosome formation and acts as a receptor for ubiquitinated proteins and organelles in autophagy [41]. p62 is considered to be a predictor of autophagy flux, as the protein level of p62 is usually inversely correlated with autophagy activity [42]. p62 is a multifunctional protein, which contains more than ten domains and putative binding sites including a Keap-1-interacting region in the C terminus [41]. Keap-1 can interact with p62 via the STGE motif, leading to the stabilization, nucleus translocation and activation of Nrf-2, namely the non-canonical activation of Nrf-2 [43, 44]. Although the STGE motif in the Keap-1-interacting region of p62 has lower affinity for Keap-1 compared with the Nrf-2 ETGE motif; however, the affinity could markedly increase by some post-translational modification of p62 [45, 46]. For example, it has been demonstrated that the phosphorylation of serine 349 in the STGE motif of p62 by mammalian target of rapamycin (mTOR) and phosphorylation of serine 24 in the PB1 domain of p62 could increase its affinity to Keap-1 [47, 48]. Interestingly, the expression of p62 is regulated in an Nrf-2-dependent manner during oxidative stress, thus forming a positive feedback loop [46]. However, it should be noted that the activation of Nrf-2 by p62-mediated Keap-1 dissociation may be associated with some negative effects such as the tumorigenesis and the resistance to chemotherapy [49,

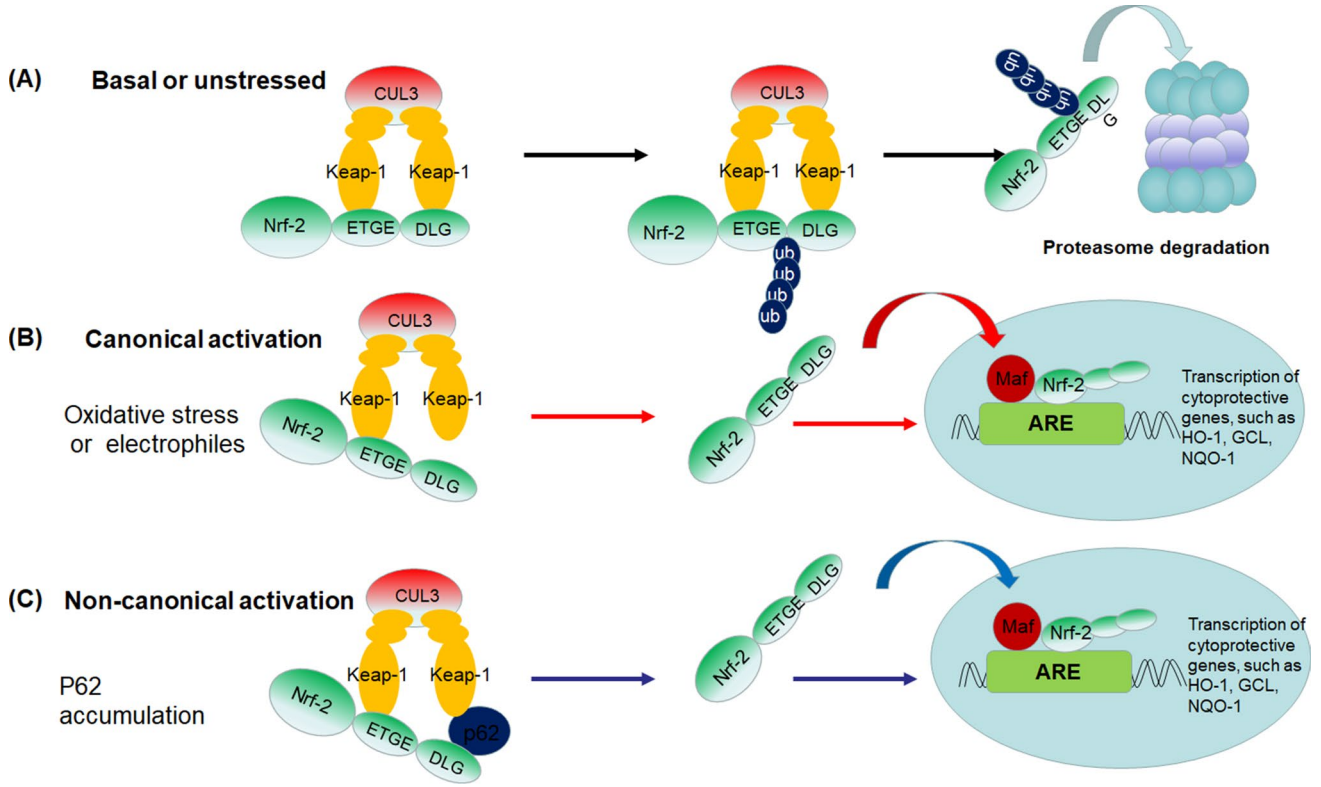

Fig. 1 The canonical and non-canonical models for the activation of Nrf-2/Keap-1 system. **a** Under basal or unstressed conditions, Keap-1 dimer binds Nrf-2 via the DLG and ETGE motifs of Nedh2 domain, leading to the ubiquitination of the lysine residues located between the ETGE and DLG motifs and subsequent degradation of Nrf-2 by proteasome. **b** The canonical activation of Nrf-2. Oxidants or electrophiles can modify cysteine residues of Keap-1, resulting in a conformational change in Keap-1 leading to detachment of the weaker binding DLG motif and the termination of Nrf-2 ubiquitination. Nrf-2 then translocates to nucleus and activates the transcription of a battery of antioxidant enzymes and phase II detoxifying enzymes. **c** The non-canonical activation of Nrf-2. p62 can compete with Nrf-2 to bind Keap-1, resulting in the liberation of Nrf-2 from Keap-1-dependent ubiquitination and degradation in the cytoplasm

50]. More recently, Hu and colleagues demonstrated that inhibitor of apoptosis stimulating protein of p53 (iASPP) could compete with Keap-1 for Nrf-2 binding, leading to decreased Nrf-2 ubiquitination and increased Nrf-2 accumulation and antioxidative transactivation [51]. Results of this study expand our understanding of the antioxidant Nrf-2/Keap-1 pathway, which is needed to be further studied.

In addition to the above two activation models, a number of studies have provided evidence that Nrf-2 activity could be modulated by several putative kinases including protein kinase C (PKC), mitogen-activated protein kinase (MAPK), and Fyn kinase [52]. It has been demonstrated that PKC could phosphorylate Nrf-2 at serine 40 which is a critical signaling event leading to ARE-mediated cellular antioxidant response [53, 54]. p38MAPK could directly phosphorylate the recombinant GST-tagged Nrf-2 protein, promoting the interaction between recombinant protein and endogenous Keap-1 in vitro [55], whereas results of another study suggested that Nrf-2 phosphorylation by MAPKs might have minimal effects on Nrf-2 stability or its subcellular localization [56]. In addition, Jain and colleagues demonstrated that Fyn kinase could phosphorylate Nrf-2 protein at tyrosine 568 by glycogen synthase kinase-3β (GSK-3β) and promote its nuclear export and degradation, thereby contributing to the suppression of ARE-mediated gene expression [57, 58].

The alteration of Nrf-2 activity in ALD models

Nrf-2 is theoretically to be activated after ethanol exposure via the canonical activation mechanism, as ethanol could induce ROS production. Indeed, a couple of studies have suggested that ethanol exposure led to the activation of Nrf-2 pathway, which might act as a compensatory or adaptive mechanism to suppress ethanol-induced oxidative injury [20]. For example, Gong et al. found that the mRNA and protein levels of Nrf-2 significantly increased in the liver of liquid diet (ethanol accounting for 35% of the total calories)-fed male C57BL/6 mice and in the isolated hepatocytes of ethanol-containing liquid diet-fed rats, which was thought to be mediated by CYP2E1-generated ROS [59]. The study by Bardag-Gorce et al. also reported that Nrf-2 mRNA level in ethanol-fed rats was significantly increased compared with

that of dextrose-fed animals. However, the authors found that proteasome inhibitor (PS-341) could protect against ethanol-induced liver injury in rats via regulating the ARE by activating transcription factor 4 (ATF-4), but not Nrf-2, as the combination of proteasome inhibitor and ethanol led to a significant decrease of Nrf-2 expression [60]. Yeligar et al. demonstrated that ethanol exposure by intragastric infusion (9–16 g/kg body weight/day) augmented Nrf-2-mediated transcription of HO-1 in rat Kupffer cells (KCs) [61]. Similarly, short-term ethanol treatment resulted in the induction of HO-1 and NQO-1 in liver tissues of mice [62]. Results of these studies are consistent with the canonical activation theory: ethanol metabolism-associated ROS may lead to the modulation of cysteine residues of Keap-1, resulting in the nucleus translocation and activation of Nrf-2; the activated Nrf-2 then upregulates important antioxidant genes and detoxification enzymes, helping to maintain cellular protective pathways [59].

In contrast to the results of above studies, some studies reported that Nrf-2 expression was not altered [63, 64] or even decreased in the livers of ethanol-exposed animals/hepatocytes [65–67]. At present, it remains unclear why Nrf-2 responded differently in different studies. Anyway, the difference in the experimental animals (such as the species, ages, and sexes), mode of ethanol delivery, and diet composition may be responsible for these contradictory results. For example, the liquid diet-induced chronic mice model was used in the study by Gong et al. [59], while binge drinking-induced acute ALD mice models were used in the study by Choi et al. and the study by Zhou et al. [63, 65]. Thus, it may be speculated that Nrf-2 activation in ethanol-treated mice is time dependent. Although the same strains of rats (Sprague–Dawley rats) were used in the studies by Lu et al. and in the study by Gong et al.; however, the former study used gavage model (56%, v/v, 10 ml/kg body weight, once daily) for 9 weeks [66–68], while the study by Gong et al. used a liquid diet feeding model for 2 months [59]. Therefore, the discrepancy in ethanol delivery may also account for these contradictory reports.

Nrf-2 deficiency aggravates ethanol-induced liver damage

Lamle et al. found that *Nrf-2*$^{-/-}$ mice displayed dramatically increased mortality, significantly reduced ability in detoxifying acetaldehyde, marked steatosis, upregulation of sterol regulatory element binding protein 1c (SREBP-1c), depletion of total and mitochondrial GSH, and aggravated inflammatory response, when exposed to ethanol at a dose which was tolerated by wild-type mice [62]. Wu et al. compared acute ethanol-induced liver toxicity in *Nrf-2* null mice, wild-type mice, *Keap-1*-knockdown (*Keap-1* KD) mice, and *Keap-1*-hepatocyte knockout (*Keap-1*-H-KO) mice [69]. They found that acute ethanol-induced increase of serum alanine transaminase (ALT) and lactate dehydrogenase (LDH) activities, triglyceride (TG) and thiobarbituric acid-reactive substances (TBARS) contents in *Nrf-2*-null and wild-type mice, but not in Nrf-2-enhanced mice (*Keap-1*-KD and *Keap-1*-H-KO mice). Besides, acute ethanol-induced decrease of mitochondrial GSH level and increase of ROS in hepatocytes disappeared in Nrf-2-enhanced mice. Furthermore, the basal mRNA and protein levels of SREBP-1c, the major nuclear transcription factor regulating the transcription of a battery of genes involved in fatty acid synthesis, were decreased with graded Nrf-2 activation. These results suggest that Nrf-2 activation can prevent acute ethanol-induced oxidative stress and accumulation of free fatty acids in liver by increasing genes involved in antioxidant defense and decreasing genes involved in lipogenesis [69].

Nrf-2 activators significantly attenuate ethanol-induced liver injury

Over the past few years, many bioactive natural compounds including sulforaphane, quercetin, curcumin, and diallyl disulfide have been shown to exhibit hepatoprotective effects against ALD, which may be related with the induction of Nrf-2 (Table 1).

Sulforaphane Sulforaphane is a well-known Nrf-2 activator affecting the cysteine residues in Keap-1 and affecting the phosphorylation of Nrf-2 [52]. The study by Zhou et al. demonstrated that sulforaphane could prevent binge drinking (3 g/kg body weight, twice daily, for 5 days)-induced liver steatosis by upregulating Nrf-2-mediated antioxidant defense and increasing autophagy activity in mice [64]. Another study showed that sulforaphane increased the Nrf-2 nucleus translocation, the protein and mRNA levels of HO-1, NQO-1, and GST-P in Hepa1c1c7 cells, and attenuated chronic ethanol (5 g/kg boy weight, twice daily, for 27 days)-induced increase of serum ALT and aspartate transaminase (AST) activities and improved the hepatic pathological changes (steatosis, necrosis, lymphocyte infiltration, loss of cellular boundaries) in male Sprague–Dawley rats [70].

Quercetin Quercetin is one of the most abundant dietary flavonoids, which has been demonstrated to protect against ethanol-induced oxidative damage in a variety of studies [21, 71–76]. Mechanism studies revealed that quercetin increased nucleus translocation of Nrf-2 and the activation of HO-1, which could be blocked by SB203580 (p38MAPK inhibitor) and PD98059 (ERK inhibitor), suggesting that p38MAPK and ERK-mediated Nrf-2/HO-1 activation might account for the protective effects of quercetin against ALD [75]. A recent study revealed that quercetin could prevent ethanol-induced hepatotoxicity by inducing p62-mediated non-canonical activation of Nrf-2 pathway, as p62 siRNA

Table 1 Effects of various phytochemical compounds/extracts on ethanol-induced liver injury via regulating Nrf-2 signaling pathways

Compounds	ALD models	Improved parameters	Evidences of Nrf-2 in the protection against ALD	References
Sulforaphane	Sprague–Dawley rats exposed to ethanol (5 g/kg bw) twice daily for 16 days	Serum ALT, AST, LDH; histological changes	Increased Nrf-2 nucleus translocation; increased mRNA and protein levels of HO-2, NQO-1, and GST-P	[70]
Sulforaphane	CYP2E1-expressing HepG2; Male SV129 humanized CYP2E1 knockin mice exposed to 3 g/kg bw ethanol twice daily for 5 days	Liver TC and TG levels; liver steatosis	Increased protein levels of Nrf-2 and HO-1; increased Nrf-2 activity	[64]
Curcumin	Sprague–Dawley rats exposed to ethanol (56%, 10 ml/kg bw) twice daily for 4 weeks; LO2 cells exposed to 100 mM ethanol	Necroptosis; serum ALT	Nrf-2 knockdown by shRNA lentivirus abrogated the hepatoprotective effect of curcumin	[66]
Curcumin	Sprague–Dawley rats exposed to ethanol (56%, 10 ml/kg bw) once daily for 9 weeks; LO2 cells exposed to 100 mM ethanol	Serum ALT, AST, ALP, LDH, TG, TC, LDL-C, HDL-C; Liver TG, TC; ALT, AST, and LDH in culture medium; Cellular lipid level; Steatosis	Increased protein levels of Nrf-2; Nrf-2 siRNA attenuated the protective effects, while Nrf-2 overexpression enhanced the protection.	[67]
Curcumin	Male Balb/C mice exposed to ethanol (56%, 10 ml/kg bw) orally for 6 weeks	Serum ALT, AST; Liver TG, TC, LDL-C, HDL-C; Steatosis	Increased Nrf-2 nucleus translocation; increased mRNA levels of NQO1, HO-1, and GCLc	[80]
Quercetin	LO2 cells exposed to ethanol	Cell viability	Induced nucleus translocation of Nrf-2 and p62; increased expression of GCL and HO-1; p62 siRNA blocked the protective effects	[76]
Quercetin	Human hepatocytes exposed to ethanol	GSH and MDA, LDH and AST activity in culture medium, and EC50 of ethanol	Nrf-2/HO-1 inhibitor abrogated the protective effects of quercetin	[75]
Diallyl disulfide	Male Kunming mice exposed to 3 doses of ethanol (5 g/kg bw); LO2 cells exposed to 25–200 μM ethanol	LDH, AST in culture medium; apoptosis	Increased the Nrf-2 nucleus translocation; increased protein and mRNA levels of HO-1; HO-1 inhibitor abrogated the protective effects of DADS	[25]
Dihydromyricetin	C57BL/6 mice fed with Lieber–DeCarli liquid diet for 6 weeks	Hepatic enzyme release, lipid peroxidation; TG deposition; inflammatory cytokines; hepatic pathological changes	Increased the protein levels of Nrf-2 and p62	[97]
Wuzhi tablet	Male C57BL/6 mice exposed to chronic-plus-binge model; or exposed to 6 g/kg bw ethanol for 3 times	Serum ALT and AST; Liver steatosis	Increase the protein levels of Nrf-2, GCLc, GCLm and HO-1	[99]
Triticum aestivum sprout-derived polysaccharide	Male C57BL/6 mice were exposed to ethanol for 10 days	Serum ALT and AST; Liver TG and TG; steatosis; apoptosis	Upregulated the expression of Nrf-2 and HO-1	[96]
Ligustrazine	Male ICR mice exposed to ethanol (56%, v/v, 10 ml/kg bw) once daily for 4 weeks; LO2 cell exposed to ethanol (100 mM)	Serum ALT, AST, ALP, LDH; liver inflammation and steatosis	Nrf-2 knockdown abrogated the hepatoprotective effects	[91]

Table 1 (continued)

Compounds	ALD models	Improved parameters	Evidences of Nrf-2 in the protection against ALD	References
Polydatin	Male Wistar rats exposed to ethanol (7 ml/kg bw) orally every 12 h at 5 different time points	Serum ALT, AST, ALP, LDH; liver inflammation, steatosis, necrosis, apoptosis	Increased protein levels of Nrf-2 and Nrf-2-targeted HO-1	[65]
Baicalin	Chronic-plus-binge model (Gao-Bin model)	Serum ALT and AST; liver TG; liver steatosis, inflammation, apoptosis, necrosis	Enhanced nuclear translocation of Nrf-2 and increased mRNA levels of Nrf-2 target genes including HO-1 and NQO-1	[92]
Hoveniae semen cum fructus extracts	Male C57BL/6 mice exposed to ethanol (5 g/kg bw) for 14 days	Serum ALT, AST, albumin, ALP, TG, γ-GT (γ-glutamyl transferase); liver TG, TNF-α; liver steatosis	Suppressed ethanol-induced decline of Nrf2 mRNA level and the decrease of hepatic GSH level and SOD and CAT activity	[93]
Glycycoumarin	Chronic plus binge drinking-induced chronic ALD and acute ALD model in C57BL/6 mice	Serum ALT and AST; liver TG level	Increased the protein levels of Nrf-2, HO-1, and GCLc; Nrf-2 activation lead to upregulation of p62.	[102]
Ethanolic extract of Sida cordifolia	Male Sprague–Dawley rats were gavaged with ethanol (4 g/kg bw) for 90 days	Serum ALT, AST, GGT; liver ALT, AST	Increased the nucleus translocation of Nrf-2 and the mRNA level of γ-GCS.	[94]
Tetramethylpyrazine	Ethanol-exposed LO2 cells	Cell viability, ALT and AST in culture medium; cellular TG, TC; apoptosis,	Increased Nrf-2 expression and nucleus translocation; overexpression of Nrf-2 enhanced the protective effects, while Nrf-2 siRNA eliminated the protective effects.	[68]
Citrus aurantium extract	Male C57BL/6 mice exposed to 5 g/kg bw ethanol for 3 doses	Serum ALT, AST, TG; liver steatosis, necrosis, apoptosis	Increased the protein levels of Nrf-2, NQO-1 and γ-GCSc	[63]
Chlorella ethanol extract	Sprague–Dawley rats were gavaged with ethanol 5 g/kg bw twice daily for 16 days	Serum ALT, AST, γ-GT, LDH	Increased Nrf-2 nucleus translocation and increased mRNA and protein levels of HO-2, NQO-1, and GST-P.	[70]
Oleanolic acid	Sprague–Dawley rats were gavaged with 4 g/kg bw ethanol for 30 days	Serum ALT, AST; liver ATP, TG, MDA	Increased the nucleus translocation of Nrf-2, and the protein expression of HO-1, SOD, and GR	[70]
Antroquinonol	Ethanol-exposed HepG2 cells	ALT, AST, ROS, MDA, NO	Increased the mRNA and protein levels of HO-1, Nrf-2 nucleus translocation, and ARE binding activity	[100]
Lucidone	Ethanol-exposed HepG2 cells	ALT, AST, NO, TNF-α, MDA, ROS	Increased the mRNA and protein levels of HO-1, Nrf-2 nucleus translocation and ARE binding activity	[101]

ALD alcoholic liver disease; *ALP* alkaline phosphatase; *ALT* alanine aminotransferase; *ARE* antioxidant response element; *AST* aspartate aminotransferase; *CAT* catalase; *GCL* glutamate–cysteine ligase; *GR* glutathione reductase; *GSH* glutathione; *GST* glutathione S-transferase; *γ-GT* γ-glutamyl transferase; *HDL-C* high-density lipoprotein cholesterol; *HO-1* heme oxygenase 1; *LDH* lactic dehydrogenase; *LDL-C* low-density lipoprotein cholesterol; *MDA* malondialdehyde; *NO* nitrogen oxide; *NQO-1* NAD(P)H quinone oxidoreductase 1; *Nrf-2* nuclear factor erythroid-derived 2-like 2; *ROS* reactive oxygen species; *SOD* superoxide dismutase; *TG* triglyceride; *TC* total cholesterol; *TNF-α* tumor necrosis factor α

abrogated quercetin-associated hepatoprotection against ALD [76].

Curcumin Curcumin, extracted from dry rhizome of *Curcuma longa*, attenuated chronic ethanol-induced liver injury by attenuating oxidative stress and suppressing the expression of nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) [77–79]. A series of studies have been conducted to investigate the roles of Nrf-2 activation and the hepatoprotective effects of curcumin against ALD [66, 67, 80]. Lu et al. found that curcumin could suppress ethanol-induced disturbance of SREBP-1c and peroxisome proliferator-activated receptor α (PPAR-α), and simultaneously induce the expression of Nrf-2 and farnesoid X receptor (FXR) in liver; the gain- and loss-of-function analyses in LO2 hepatocytes revealed Nrf-2 and FXR mediated the effect of curcumin on cellular lipid deposition, and curcumin modulated the expression of FXR by Nrf-2 [67]. Their following study showed that curcumin dose dependently ameliorated ethanol-caused hepatocyte necroptosis, which was blocked by Nrf-2 knockdown using shRNA lentivirus [66].

Organosulfur compounds from garlic Garlic is one of the most widely used herbal medicines in the world and is honored as "nature's protection against physiological threats" [81, 82]. Many organosulfur compounds in garlic including diallyl sulfide, dially disulfide and diallyl trisulfide have all been demonstrated to induce Nrf-2 activation and could protect against ALD [24, 83–90]. In one of our studies, we found that diallyl disulfide could suppress ethanol-induced elevation of LDH and AST activities, decrease of GSH level, and increase of MDA level, and apoptosis in LO2 cell, which could be blocked by Nrf-2/HO-1 inhibitor, ZnPPIX. The in vivo study showed that diallyl disulfide dose dependently increased the protein levels of HO-1 in mice liver [25].

Other phytochemical compounds/extracts A large number of other phytochemical compounds/extracts including oleanolic acid, polymethoxy flavonoid-containing citrus aurantium extract (CAE), tetramethylpyrazine (TMP), ethanolic extract of sida cordifolia, hoveniae semen cum fructus extract, baicalin, polydatin, ligustrazine, triticum aestivum sprout-derived polysaccharide (TASP), dihydromyricetin, baccharis trimera, and schisandra sphenanthera extract have been demonstrated to attenuate binge or chronic ethanol-induced liver/hepatocytes injury in various ALD models, which might be associated with the activation of Nrf-2 antioxidant system [63, 65, 68, 70, 91–102]. However, it should be noted that whether the hepatoprotective effects of these compounds/extracts are mainly attributed to Nrf-2 activation remains to be elucidated. As the authors only detected the activation of Nrf-2 antioxidant system (such as the increased nucleus translocation of Nrf-2 and the increased mRNA and protein levels of Nrf-2 targeted genes including HO-1, GCL, NQO-1) in many studies, the involvement of other mechanisms cannot be completely excluded.

Nrf-2 activation on the gut–liver axis and the adipose–liver axis in ALD

In addition to the direct impairment on hepatocytes, the deleterious effects of ethanol on adipose tissues and the hepatic resident macrophages (the Kupffer cells, KCs) have also been demonstrated to play crucial roles in ethanol-induced liver injury. Ethanol could stimulate lipolysis in adipose tissues, and the adipose TG then transports and deposits in liver forming steatosis [103, 104]. Besides, ethanol could impair the secretion of adiponectin, a 30-kD protein hormone, which has been demonstrated to provide protection against ALD via adiponectin–sirtuin-1(SIRT1)–AMP-activated kinase (AMPK) pathway [105, 106]. In addition, ethanol exposure could lead to intestinal hyperpermeability of intestinal mucosa and alter the gut microbiota favoring the production of pro-inflammatory endotoxin/lipopolysaccharide (LPS) [107, 108]. LPS translocates to liver and activates the toll-like receptor 4 (TLR-4) signaling pathway in KCs. The M1-type-polarized KCs can produce a large amount of ROS and pro-inflammatory cytokines including tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) [109, 110]. Animal studies showed that suppressing LPS-producing bacteria by probiotics, intestinal sterilization by antibiotics, and knockout of LPS receptor could suppress ethanol-induced liver injury, which supports that the gut–liver axis plays critical roles in the pathogenesis of ALD [111–114], and thus, pharmacological intervention targeting M2 type polarization of KCs has been considered as an attractive strategy for the limitation of ethanol-induced inflammation and hepatocyte injury [115, 116]. Previous studies suggest that oxidative stress could impair adiponectin secretion and promote lipolysis in adipose tissues, and is crucial for LPS-mediated KCs M1 type polarization [117–120]. Therefore, it appears plausible that the activation of Nrf-2 in adipose tissues may be beneficial for ALD protection by rescuing the adiponectin secretion and blocking lipolysis, while the Nrf-2 activation in KCs may suppress the activation of KCs and the production of pro-inflammatory cytokines. The effects of Nrf-2 on the deleterious effects of ethanol on adipose tissues and hepatic KCs need to be further investigated in future studies (Fig. 2).

The crosstalk between Nrf-2/Keap-1 pathway and autophagy in ALD

Complementing the Nrf-2/Keap-1 pathway, autophagy (macrophage) is another important defense mechanism against cellular oxidative stress [121]. Autophagy is a highly conserved intracellular catabolic pathway which is responsible for the degradation of oxidatively modified proteins, accumulated lipids and damaged organelles [122, 123]. Interestingly, accumulating evidence indicate a

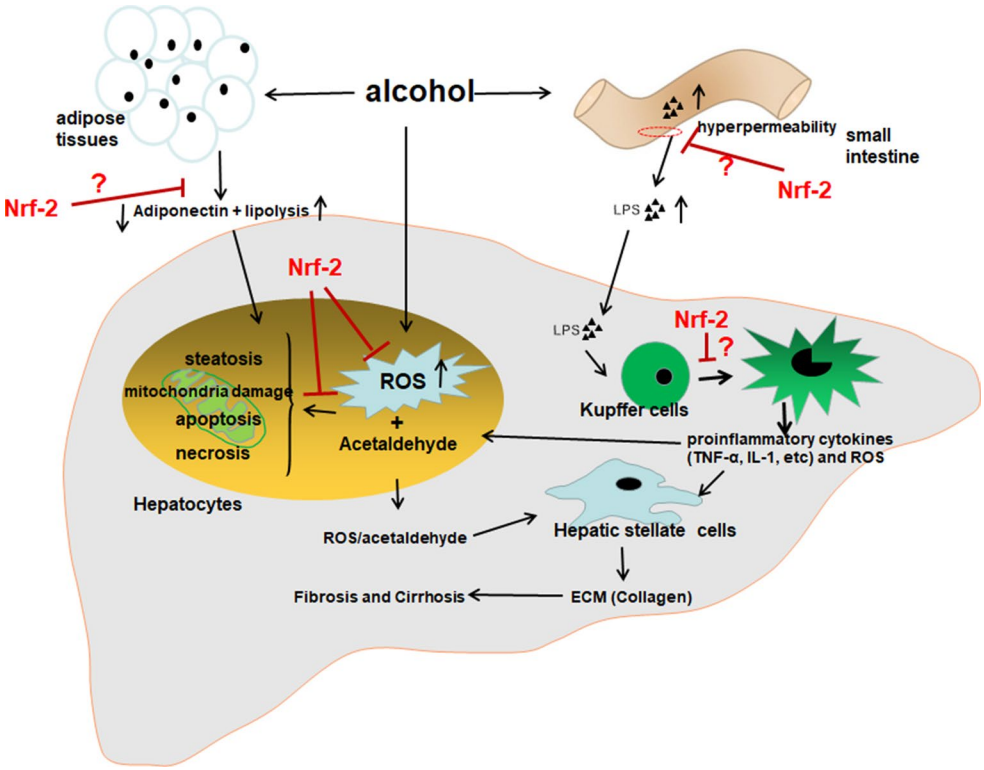

Fig. 2 Potential molecular targets for the hepatoprotection of Nrf-2 activators against alcoholic liver disease (ALD). (1) Nrf-2 activation in hepatocytes could eliminate ethanol-induced reactive oxygen species (ROS) and mitigate the subsequent deleterious effects of ROS in hepatocytes; (2) Nrf-2 activation in adipose tissues may block ethanol-induced lipolysis and the decreased secretion of adiponectin; (3) Nrf-2 activation in intestine may suppress ethanol-induced intestinal damage, and thus reduce the translocation of gut-sourced lipopolysaccharide (LPS) to liver; (4) garlic may suppress LPS-induced Kupffer cells (KCs) activation and reduce the production of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α)

crosstalk between autophagy and Nrf-2/Keap-1 pathway. As described before, p62 could competitively bind with Keap-1, leading to the non-canonical activation of Nrf-2 [43, 44]. Therefore, autophagy blockage, either via genetic ablation of key autophagy initiation proteins (Beclin-1, ATG5, or ATG7) or exposure to some environmental toxicants such as arsenate, results in the activation of Nrf-2 [47]. On the other hand, Nrf-2/Keap-1 pathway may also regulate the activity of autophagy [124]. p62 and nuclear dot protein 2 (NDP52) have been demonstrated to be targets of Nrf-2 [125, 126]. Furthermore, Keap-1 binding to p62 may be involved in p62-mediated autophagy of unbiquitinated proteins, as genetic ablation of Keap-1 led to accumulation of ubiquitin aggregates and defective activation of autophagy [127]. However, Nrf-2 activity seems to negatively regulate the autophagy activity, although some opposite results also exist [124, 128–130]. The negative regulation of Nrf-2 on autophagy activity may be not unexpected, as both Nrf-2 and autophagy play similar roles in mitigating oxidative stress. If the cellular antioxidant system is at a higher level due to the activation of Nrf-2, then it would be reasonable that autophagy, another antioxidant pathway, may maintain at a relatively lower level (Fig. 3).

Similar to the alteration of Nrf-2 activity in ALD, the activities of autophagy in ALD models remain inconsistent [131]. However, pharmacological activation of autophagy could attenuate ethanol-induced liver injury, suggesting that autophagy plays protective roles against ALD [131, 132]. Specially, PTEN-induced putative kinase 1 (PINK-1) and Parkin-associated mitophagy (responsible for degradation of damaged mitochondria) have been demonstrated to play critical roles in the protection against ALD by removing damaged mitochondria, maintaining a healthy mitochondria population for the efficient β-oxidation in the hepatocytes [133–136]. Much more works are needed to clarify the interactions between Nrf-2/Keap-1 pathway and PINK–Parkin-induced mitophagy pathway.

Could Nrf-2 activators be used for the prevention and therapeutic treatment of human ALD?

The intricacy of the human anatomy, along with the existence of many other variables in association with alcohol abuse in humans, makes it extremely difficult to replicate all facets of human drinking in laboratory models [137]. It is well known that the rodents (rats and mice) are more resistant to the effects of alcohol as compared with humans [138]. The currently available ALD animals models using ethanol-containing liquid diet or by intragastric feeding ethanol could only induce the early stages of ALD (e.g., steatosis, steatohepatitis, mild fibrosis), while the late stage of ALD (e.g., severe fibrosis, cirrhosis and hepatic carcinoma) could not be induced without the addition of secondary or multiple insults [137, 139–142]. Therefore, although a significant number of studies have illustrated the protective role of Nrf-2 against ALD in vitro and in animal models, whether

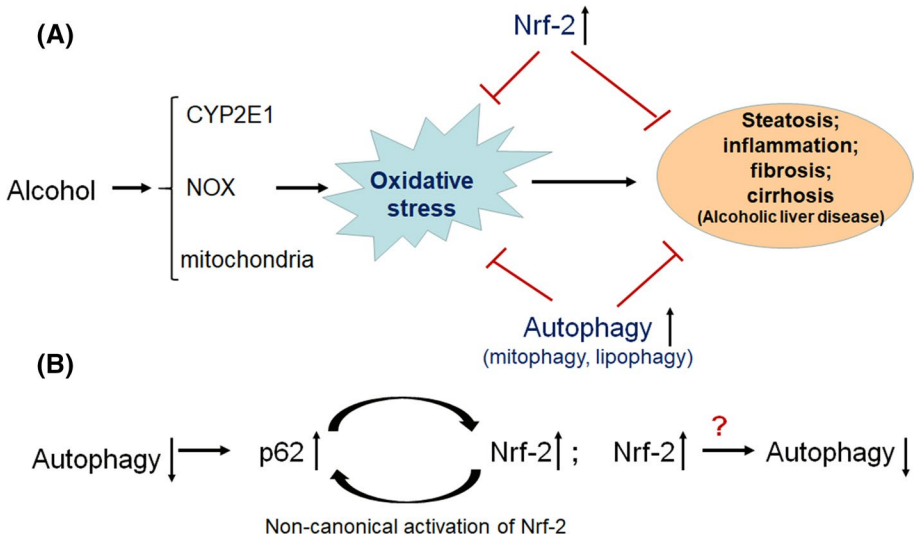

Fig. 3 The crosstalk between Nrf-2/Keap-1 pathway and autophagy in ALD. (1) Accumulating evidence demonstrates that both Nrf-2 activation and autophagy activation can significantly alleviate ethanol-induced oxidative stress and the subsequent liver damage; (2) autophagy suppression resulted in the non-canonical activation of Nrf-2 via accumulated p62, while Nrf-2 may negatively regulate the autophagy activity

these experimental data can be directly translated to human beings should be questioned due to the lack of clinical trials.

However, there has been accumulating evidence indicating the possible causative involvement of oxidative stress in the pathophysiology of human ALD [20]. For example, ethanol consumption increased the oxidative stress biomarkers including F2-isoprostanes and 4-HNE in the serum and urine of ALD patients [143, 144]. Besides, alkylation of proteins by hydroxyethyl radicals was detected in patients with AC [145]. Unexpectedly, a randomized, double-blind, placebo-controlled clinical trial found that S-adenosylmethionine (SAM), a well-characterized antioxidant, was not effective than placebo in the treatment of ALD [146], although an earlier study showed that SAM could improve survival or delay liver transplantation in patients with AC, especially in those with less advanced liver disease [147]. Other antioxidants including vitamin E and NAC also failed to show their efficacy in improving alcoholic hepatitis [148, 149]. The poor response of these conventional antioxidants in human ALD may be related to the reduced efficiency in enhancing the antioxidant activity in ALD patients. For example, there has been evidence for the less efficiency of reasonable levels of supplementation with vitamin E in enhancing the antioxidative status of healthy persons [150, 151]. In addition, clinical trials usually enrolled ALD patient with severe hepatitis, fibrosis, and/or cirrhosis. The severely impaired hepatocytes in patients with advanced ALD may not make full use of these exogenous antioxidants and thus poorly respond to the supplementation of antioxidants [146].

Interestingly, several studies have suggested that Nrf-2 activators could induce antioxidant enzymes in humans and ameliorate several chronic diseases which were associated with oxidative stress and inflammation. For example, protandim, a composition consisting of extracts of five widely studied medicinal plants, has been demonstrated to induce endogenous antioxidant enzymes (including SOD and catalase) and lowered oxidative blood markers in runners [152, 153]. Bardoxolone methyl, a novel synthetic Nrf2 activator, has been demonstrated to improve the kidney function in patients with advanced chronic kidney disease and type 2 diabetes in a phase II double-blind, randomized, placebo-controlled clinical trial, although a follow-up phase III trial was terminated due to undisclosed safety concerns [154, 155]. Another Nrf-2 activator, compound BG-12 (dimethyl fumarate), reduced brain magnetic resonance imaging activity and lesions associated with multiple sclerosis as compared with patients who received placebo in a phase IIb clinical trial [156].

Collectively, induction of endogenous Nrf-2-regulated antioxidant system may represent a promising approach for the prevention and treatment of human ALD. Considering the less efficiency of conventional antioxidants such as vitamin E and other potential therapeutic drugs such as TNF-α inhibitors (e.g., pentoxifylline, infliximab, and etanercept), well-designed clinical trials are warranted to investigate the roles of Nrf-2 activators in ALD patients [109, 153, 157–159].

Nrf-2 and other liver diseases

Non-alcoholic fatty liver disease (NAFLD) shares similar mechanisms with ALD, and the general consensus is that the gut microbiota, oxidative stress and mitochondrial damage may play key roles in the pathogenesis of both ALD and NAFLD [160, 161]. Knockout of Nrf-2 in mice profoundly exacerbated NAFLD, while activation of Nrf-2 by knockdown of Keap-1 or by pharmacological agents protected NAFLD [162–168]. However, there are some studies providing conflicting results. For example, genetic activation of Nrf-2 in mice by knockdown of Keap-1 aggravated

NAFLD induced by long-term high-fat diet feeding [169]. A more recent study showed that oxidative stress-induced Nrf-2 might be responsible for the upregulation of hepatic very low density lipoprotein (VLDL), which plays an important role in the development of hepatic steatosis [170]. Why Nrf-2 plays different roles in different NAFLD models remains to be elucidated.

The roles of Nrf-2 in the protection of chemical-induced liver injury have also been proposed, as oxidative stress serves as a common and important mechanism for the liver injury induced by various chemicals. It has been demonstrated that Nrf-2 activation offered significant protection against the liver injury caused by carbon tetrachloride (CCl_4), acetaminophen, microcystin, cadmium, and diquat [171–174]. Furthermore, some recent studies have provided clues that Nrf-2 may be also involved in the deleterious effects of HBV and hepatitis C virus (HCV) on liver. In hepatocytes, HBV could stimulate the expression of glucose-6-phosphate dehydrogenase (G6PD) by promoting HBV x protein (HBx) expression in an Nrf-2-dependent manner, which might play important roles in the development of HBV-associated hepatocarcinoma [175]. HCV could interfere with the crosstalk between Nrf-2/Keap-1 pathway, elevated ROS levels and autophagy, which was required for the release of infectious viral particles [176].

Conclusions and future research perspectives

Nrf-2-regulated antioxidant system has been demonstrated to play core roles in mitigating ethanol-induced oxidative stress. Nrf-2 activation by genetic manipulation or pharmacological compounds could effectively attenuate both binge and chronic ethanol-induced hepatocytes/liver damage in vitro and in animal studies, which suggest that Nrf-2 is a promising targeting molecule for the prevention and treatment of human ALD. However, there are some issues which should be addressed in future studies.

First, it should be noted that the currently available data are obtained from animal studies of ethanol-induced early stage of ALD or from in vitro studies. These results may be interpreted as Nrf-2 activator could prevent ethanol-induced early liver disease. Therefore, it remains to study whether Nrf-2 activator can improve advanced stage of ALD such as the fibrosis and cirrhosis. It may be necessary to use primate models to investigate the roles of the Nrf-2 activator in the pathogenesis of alcoholic fibrosis and cirrhosis, as excessive ethanol consumption alone could result in liver fibrosis and cirrhosis in baboon [137, 141]. Additionally, hybrid rats/mice models of fibrosis/cirrhosis induced by ethanol and other hits such as high-fat diet or hepatotoxicants (e.g., CCl4) can be also considered, as both CCl4 and ethanol may induce hepatocyte damage through some common mechanisms [142, 177–179].

Second, the roles of Nrf-2 in the hepatoprotection of many phytochemical compounds/extracts against ALD need to be further confirmed. Many studies only reported the increase of Nrf-2 nucleus translocation and increased expression of Nrf-2 target antioxidant genes. Whether Nrf-2 activation played the major roles for the protection of these compounds/extract against ALD remained to be clarified, as other mechanisms such as reducing ROS production, maintaining the intestinal barrier integrity, and improving the adipose–liver axis may be also involved. For example, DADS has been suggested to suppress CYP2E1 activity in human hepatocytes and also induce the activation of Nrf-2 [84, 180]. Besides, the isolation and preparation of these hepatoprotective compounds/extracts should be standardized and the bioavailability of these compounds/extracts must be evaluated, as uncharacterized crude extracts may lead to the difficulty in reproducing the results [181]. This is particularly important as there is an increasing public and scientific interest in this natural-derived substance [182]. In addition, it will be interesting to investigate the roles of combination of these Nrf-2 activators and KCs polarizing modulators in ALD models, as M1-type-polarized KC induced by gut-sourced LPS has been demonstrated to be another key contributor to ALD. Furthermore, well-designed clinical trials are urgently needed to evaluate the efficiency of these Nrf-2 activators on ALD patients.

Third, it has been demonstrated that the non-canonical activation of Nrf-2 may be associated with some negative outcomes such as tumor progression and chemotherapy resistance, namely the "dark side" of Nrf-2 [49, 183, 184]. For example, autophagy deficiency led to the formation of protein aggregates, liver fibrosis, inflammation and tumorigenesis, which could be blocked by knockout of p62 or Nrf-2 [49, 185]. As such, long-term use of these Nrf-2 non-canonical activators should be carefully considered.

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