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STATE-OF-THE-ART REVIEW

Nrf2 and oxidative stress in liver ischemia/reperfusion injury

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In response to stress signal, nuclear factor-erythroid 2-related factor 2 (Nrf2) induces the expression of target genes involved in antioxidant defense and detoxification. Nrf2 activity is strictly regulated through a variety of mechanisms, including regulation of Keap1-Nrf2 stability, transcriptional regulation (NF-κB, ATF3, ATF4), and post-transcriptional regulation (miRNA), evidencing that transcriptional responses of Nrf2 are critical for the maintenance of homeostasis. Ischemia-reperfusion (IR) injury is a major cause of graft loss and dysfunction in clinical transplantation and organ resection. During the IR process, the generation of reactive oxygen species (ROS) leads to damage from oxidative stress, oxidation of biomolecules, and mitochondrial dysfunction. Oxidative stress can trigger apoptotic and necrotic cell death. Stress factors also result in the assembly of the inflammasome protein complex and the subsequent activation and secretion of proinflammatory cytokines. After Nrf2 activation, the downstream antioxidant upregulation can act as a primary cellular defense against the cytotoxic effects of oxidative stress and help to promote hepatic recovery during IR. The complex crosstalk between Nrf2 and cellular pathways in liver IR injury and the potential therapeutic target of the Nrf2 inducers will be discussed in the present review.

Abbreviations
4-HNE, 4-Hydroxynonenal; ADP, adenosine diphosphate; ALT, alanine transaminase; ARE, antioxidant response elements; ATF3, activating transcription factor 3; ATF4, activating transcription factor 4; BHA, butylated hydroxyanisole; CAT, catalase; CDDO-Im, imidazol derivative; CDDO-Me, bardoxolone-methyl; COX2, cyclooxygenase-2; Cul3, cullin 3; D3T, 3H-1,2-dithiole-3-thione; DMF, dimethyl fumarate; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; FGF10, fibroblast growth factor 10; Fpn1, ferroportin-1; G6PD, glucose-6-phosphate 1-dehydrogenase; GOH, geraniol; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione-s transferase; HO-1, heme oxygenase-1; HSP, heat shock proteins; IGL-1, institute george lopez solution; IL, interleukin; iNOS, inducible nitric oxide synthase; IR, ischemia-reperfusion; IRE1, inositol requiring enzyme 1; Keap1, kelch ECH associating protein 1; LC3, microtubule-associated proteins 1A/1B light chain 3B; LPS, lipopolysaccharide; MEI, microsomal enzyme inducers; miRNA, micro RNA; mtDNA, mitochondrial deoxyribonucleic acid; NASH, non-alcoholic steatohepatitis; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NLRP3, NLR family pyrin domain containing 3; NMP, normothermic machine perfusion; NQO, NADPH quinone reductase; NRF1, nuclear respiratory factor-1; Nrf2, nuclear factor-erythroid 2-related factor; OLT, orthotopic liver transplantation; PEG-35, polyethylene glycol 35; PERK, PKR-like endoplasmic reticulum kinase; PERK, PKR-like endoplasmic reticulum kinase; PGD, 6-phosphogluconate dehydrogenase; PINK1, PTEN-induced putative kinase 1; POMP, proteasome maturation protein; ROS, reactive oxygen species; SOD, superoxide dismutase; TLR4, toll-like receptor 4; TNF- α, tumor necrosis factor α; UCP, uncoupling proteins; UPR, unfolded protein response; YAP, yes-associated protein.

Introduction

Ischemia-reperfusion (IR) injury is an important cause of organ damage that occurs in different systems, as well as in human diseases and clinical conditions [1–3]. During liver surgery, the unavoidable interruption of blood flow causes ischemia followed by a subsequent tissue reperfusion. This results in a massive injury to the hepatocytes. Several studies have demonstrated the association between hepatic IR injury and reactive oxygen species (ROS) generation. During the initial phase of IR, ROS can also cause considerable harm to hepatocytes through lipid peroxidation, protein oxidation, mitochondrial dysfunction, and DNA damage. Subsequently, Kupffer cells and neutrophils are recruited in response to the death of hepatocytes, leading to liver inflammation. Under the IR process, the damage caused to the liver tissue causes the nonviability of the organ [4].

Since ROS regulation is suggested as a new therapeutic strategy for liver IR injury [5,6], a large number of antioxidant agents have been tested both clinically and experimentally. Vitamins, such as α-tocopherol [7,8] and resveratrol [9], have shown hepatoprotective effects against IR injury. Melatonin prevents oxidative stress and inflammatory cytokine release when added to storage solutions [10] and the glutathione precursor N-acetyl cysteine improves microcirculation in the treatment of liver IR [11].

Induction of several highly protective endogenous genes occurs early in ischemic injury. They are controlled at the transcriptional level by the nuclear factor-erythroid 2-related factor 2 (Nrf2) [12,13]. Nrf2 plays a protective role against many pathological conditions, such Alzheimer's disease, Parkinson's disease, epilepsy, inflammation, aging, and ischemia, all of them being closely related to oxidative stress [14–18]. Recently, it has been proposed that Nrf2 activation may induce clinical benefits against acute respiratory distress syndrome and pneumonia due to COVID-19 [19,20]. Therefore, advances in understanding the regulation of antioxidant systems by Nrf2 may provide exciting new potential therapeutic targets.

Upon detection of various stress factors, including ROS [21], the assembly of the inflammasome protein complex NLR pyrin domain containing protein 3 (NLRP3) results in activation and secretion of pro-inflammatory cytokines. The NLRP3 inflammasome has been involved in the pathogenesis of liver injury [22,23]. Recent evidence showed an inverse relationship between the Nrf2 pathways and the NLRP3 inflammasome at different levels [24–26].

In recent years, research on Nrf2 has described its role as the backbone of multifaceted cellular defense mechanisms. Oxidative stress activates the Nrf2 transcription factor, which in turn induces the expression of a series of cytoprotective gene products. This review addresses the role of Nrf2 in hepatic IR with the aim of therapeutically targeting Nrf2 for IR protection.

The Keap1-Nrf2 signal transduction pathway

Nrf2 is a basic leucine zipper protein, with a Cap "*n*" collar structure (CNC). Nrf2 is normally distributed in the cytoplasm of mammalian cells, where it is bound to its cytosolic repressor Kelch ECH associating protein 1 (Keap1) and E3 ubiquitin ligase. Under physiological conditions, Keap1 binds to Nrf2 in the cytoplasm and promotes ubiquitination by the E3 ubiquitin ligase. This process removes Nrf2 from the cytoplasm, where it is constantly degraded, preventing the translocation of Nrf2 to the nucleus, where it would have signaling effects (Fig. 1).

Under exposure to electrophiles, ROS and nitric oxide modification of the cysteine residues of Keap1 occurs [27] leading to conformational changes in Keap1 and thus inhibition of Nrf2 ubiquitination [28–30]. As a response, Nrf2 undergoes phosphorylation and translocates to the nucleus [31] where it attaches to Antioxidant Response Elements (AREs) [32].

Activation of Nrf2 pathways has been studied in different cellular compartments. Altered redox homeostasis can cause stress in the endoplasmic reticulum (ER) which, in turn, could induce ROS production in the ER. In order to limit oxidative damage, the ER-spanning transmembrane proteins PKR-like endoplasmic reticulum kinase (PERK) and inositol requiring enzyme 1 (IRE1) phosphorylate and activate Nrf2 [33]. The mechanisms of Nrf2 activation by ER stress will be discussed in detail below. Mitochondria also activate Nrf2, as recently discussed by Tsushima et al. [34], in response to multiple redox signals with the aim of protecting cells against oxidative damage derived from inflammation. Mitochondrial or ER stress stimulates activating transcription factor 4 (ATF4) and regulates redox homeostasis in the cells [35,36]. Nrf2 and ATF4 cooperatively upregulate cytoprotective genes [34,37].

In addition to this classical pathway, it has also been described that p62 is capable of enhancing the transcriptional activity of Nrf2 [38]. The p62 protein is a selective substrate for autophagy and regulates the formation of protein aggregates. Accumulation of inclusions positive for p62 and/or p62 has been reported in human liver diseases where its overproduction competes with the interaction between Nrf2 and Keap1, resulting in Nrf2 stabilization [38,39].

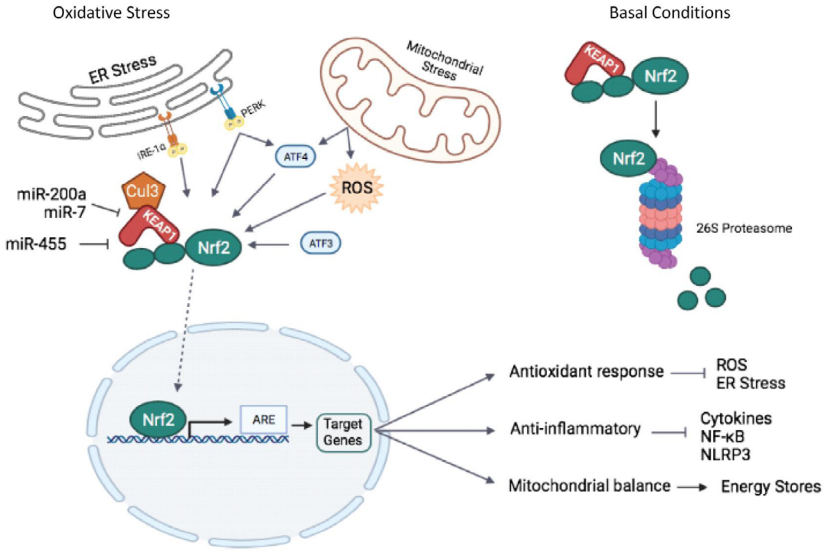

Fig. 1. The Keap1-Nrf2 signal transduction pathway. Under basal conditions, Nrf2 is polyubiquitinated by the ubiquitin ligase complex Keap1, becoming a substrate for the 26S proteasome. Under conditions of oxidative stress, Keap1 undergoes oxidation of its reactive cysteine residues, resulting in the inhibition of Nrf2 ubiquitination and thus in the cytosolic and nuclear accumulation of Nrf2. Nrf2 can also be regulated at the post-transcriptional levels. Activation of the Nrf2-ARE pathway triggers the transcription of multiple genes. This involves the activation and modulation of antioxidant and radical scavenging functions, anti-inflammatory and metabolic functions.

Activity of Nrf2 can also be modulated at the post-transcriptional level. miRNAs can target genes that indirectly modulate Nrf2 activation, mostly by targeting KEAP1 [40,41] and cullin 3 (Cul3) [42]. These studies suggest that miRNAs can constitute a novel strategy to provoke the activation of the antioxidant response through Nrf2 signaling [43].

Nrf2 as a transcription factor: cellular and molecular functions in the liver

The protective function of Nrf2 is due to the regulated expression of a battery of genes associated with oxidative stress, chronic inflammation, and cellular detoxification [13,44]. In fact, Nrf2 was originally identified for its cancer prevention function, through induction of Phase II enzymes. The search for Nrf2 targets throughout the genome has been validated by using knockout models. Furthermore, microarray technologies have been productively used to profile all genes under the control of Nrf2. This allowed the identification of many ARE regions that contain genes beyond the conventional antioxidant stress response. As shown in Fig. 2, Nrf2 affects multiple aspects of cellular functions through gene regulation or by transcription factors crosstalking.

During the last decade, several researchers reported that the activation of the Nrf2 transcription factor has a great impact on improving hepatic IR injury [45–50] (Table 1).

The hepatoprotective function of Nrf2 against liver IR injury was first described by Ke et al. [47]. They showed that the Keap1-Nrf2 complex alleviates oxidative injury in a mouse model of hepatic cold storage (20 h at 4 °C) followed by orthotopic liver transplantation (OLT). Ablation of Keap1 signaling reduced macrophage and neutrophil trafficking, pro-inflammatory cytokine programs, and hepatocellular necrosis and apoptosis, while simultaneously promoting anti-apoptotic functions in mouse orthotopic liver transplantation. The role of Nrf2 was also identified in Nrf2-deficient livers from mice after 60 min of ischemia followed by reperfusion [46]. Nrf2-deficient livers exhibited enhanced tissue damage; impaired glutathione-S transferase (GSTm1), and NADPH quinone reductase (NQO1) inductions; aggravated Tumor Necrosis Factor α (TNF-α) expression, when compared to wild-type mice. Recently, a crucial role for YAP (Yes-associated protein) effector in IR-mediated hepatocellular damage and liver fibrogenesis has been described [49]. Notably, YAP activation failed to protect Nrf2-deficient livers against IR-mediated damage, leading to extensive fibrosis.

The hepatic expression of Nrf2 could also be used as a criterion to rule out marginal liver allografts. In a recent work [50], discarded human donor livers were stratified into a high NRF2 and low NRF2 group by quantifying NRF2 expression and were then exposed to a normothermic perfusion machine (NMP). After 6 h NMP, high NRF2 livers had significantly reduced liver enzyme alterations and improved lactate clearance.

Nrf2-mediated response to oxidative stress and detoxification

It is well known that Nrf2, Keap1, and ARE genes are implicated in Phase II of the detoxification after the

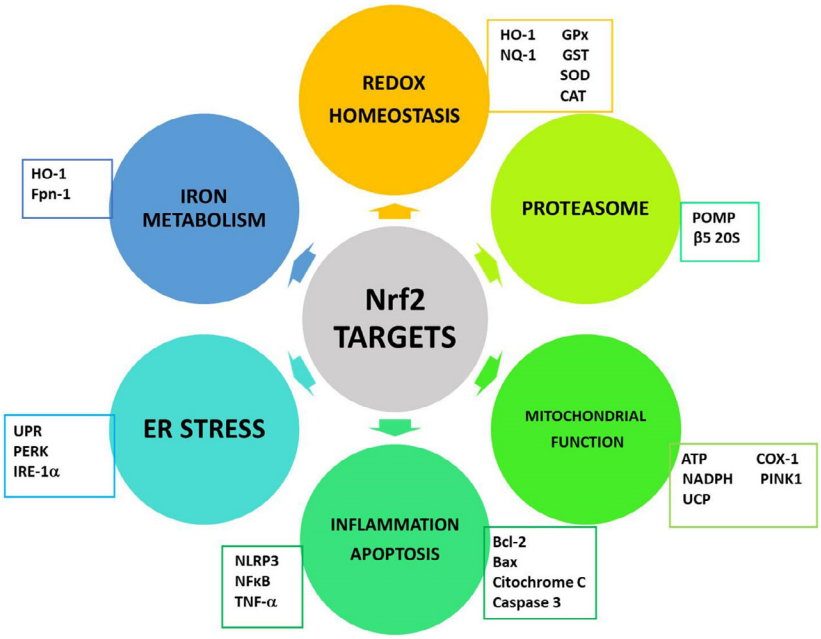

Fig. 2. Schematic representation of the cellular functions driven by Nrf2 target genes. Nrf2 targets are involved in a multiple of cellular processes, including: redox regulation, metabolism and mitochondrial function, inflammation and apoptosis, iron homeostasis, and proteasome assembly. As antioxidant response, Nrf2 promotes enzymatic activity of HO-1, GPx, GST, SOD, and CAT and attenuates inflammation targeting NLRP3 and NF-κB. Regarding energy storage, Nrf2 increases NADPH and ATP. Nrf2 also activates uncoupled proteins and COX-1, affecting mitochondrial function.

inflammatory process [46]. Most of the Nrf2 target genes are involved in providing cellular protection against damage by oxidizing molecules. ARE genes include antioxidant enzymes, like superoxide dismutase (SOD), glutathione-S transferase (GST), glutathione peroxidase (GPx), catalase (CAT), but also proteasomal proteins, as heat shock proteins (HSP), and anti-inflammatory proteins, heme oxygenase-1 (HO-1), and cyclooxygenase-2 (COX-2) [51,52]. One of the first antioxidant enzymes to be related to ARE genes was HO-1, an inducible enzyme that catalyzes the reaction of the heme group to biliverdin, which is reduced to the potent antioxidant bilirubin [53].

The experimental confirmation that phase II enzymes were under transcriptional control of AREs came from the research of Itoh et al. [54]. They used the phenolic antioxidant butylated hydroxyanisole, an inductor of phase II antioxidants, in the *nrf2* heterozygous and homozygous mutant mice. While the expression of phase II enzymes (e.g., GST, NQO) was markedly induced in wild-type and heterozygous mutant mice, the induction was eliminated in the liver and gut of homozygous *nrf2*$^{-/-}$ mutant mice [54]. After these experiments, they reported that Nrf2 regulates ARE.

Several subsequent studies have contributed to expanding the number of genes involved in detoxification after Nrf2 activation in the liver. In liver from *nrf2*$^{-/-}$ mutant mice, reduced transcription levels of UDP-glucuronyl-transferase 1A6, HO-1, and manganese superoxide dismutase (MnSOD) were obtained after induction of a cancer chemopreventive agent (D3T) confirming that these inducible detoxifying enzymes are regulated by Nrf2 [55].

Regarding the glutathione system, which is the main nonenzymatic cellular antioxidant, the *nrf2*$^{-/-}$ mouse model confirmed that Nrf2 is essential for constitutive and inducible expression of ARE, which mediates GST (Gsta1, Gsta2, Gstm1, Gstm2, Gstm3, and Gstm4) genes [56]. GST catalyzes the conjugation of reactive electrophilic species with glutathione (GSH) to reduce potential xenobiotics and to detoxify. Furthermore, higher levels of GSH were found in response to fisetin, a plant polyphenol from the flavonoid group, compared to the control group. This increase is granted by the transcription factor Nrf2 [57]. Thus, the increased sensitivity of Nrf2 knockout mice to xenobiotics can be partly attributed to the loss in constitutive expression of multiple GSH-dependent enzymes, which causes a reduction in intrinsic detoxification capacity in knockout animals. The attenuated induction of GSH-dependent enzymes found in *nrf2*$^{-/-}$ homozygous mice explains their lack of adaptation to the chronic exposure to chemical and oxidative stress.

Besides the liver, these Nrf2-mediated antioxidant mechanisms have also been described during IR in the brain [58–60], heart [61–63], and kidney [64–66].

Nrf2 regulates iron metabolism

Nrf2 play a critical role in mediating iron metabolism [67,68]. Tissue damage promotes hemolysis, and the resulting cytosolic heme can be catabolized into ferrous iron and biliverdin by HO-1, an Nrf2 target gene [53].

Table 1. Impact of Nrf2 transcription factor activation on liver IR injury.

Model	Nrf2 pathway	Mediators	Effects and outcome	References
Nrf2-deficient treated with 15d-PGJ2 subjected to hepatic IR (mice)	Amplification of Nrf2 activation	15d-PGJ2 protected livers from I/R injury via activation of Nrf2	Nrf2-deficient mice showed: Enhanced tissue damage, impaired GSTm1, NQO1, and GCLc inductions, disturbed redox state, and aggravated TNF-α	[46]
Brg1 overexpression in hepatic IR (mice)	Activation of Nrf2/HO-1 pathway	Increased antioxidants HO-1 and NQO1	Alleviate hepatic IRI Decrease 8-isoprostane levels	[48]
Liver IRI cold storage (20 h at 4 °C) followed by OLT in KEAP1 KO in the donor liver (mice)	Keap1-Nrf2 signaling pathway	Ablation of Keap1 signaling	Reduced macrophage/neutrophil trafficking, pro-inflammatory cytokine, and hepatocellular necrosis/apoptosis. Anti-apoptotic functions in OLTs	[47]
IR in Nrf2 deficient livers (human biopsies and mice)	Nrf2 required for hepatocellular protection	Intrinsic hepatic YAP expression	Reduction of Oxidative stress Reduction in apoptosis/necrosis Reduced inflammatory response Attenuates fibrosis	[49]
Mouse model of warm IRI	Nrf2 activation by FGF10 overexpression	Phosphatidylinositol-3-kinase/AKT pathway	Protective role of FGF10 in liver IRI and the role of Nrf2 in preventing oxidative stress	[45]
Human liver allografts with high Nrf2 expression exposed to 6-h of NMP	Nrf2 expression	Are not considered	Nrf2 improves liver function after NMP	[50]

Nrf2 also controls the heavy and light chains of the iron storage protein ferritin, as well as the protein responsible for the flux of iron out of the cell, ferroportin [67]. Harada et al. observed that Nrf2 activation by electrophilic compounds resulted in upregulation of ferroportin-1 (Fpn1) gene transcription in macrophages obtained from wild-type mice, but not from Nrf2 knockout mice [69]. Recently, it has been shown that the synthesis of hepcidin, the peptide hormone that maintains systemic iron homeostasis, is altered in Nrf2 knockout mice, increasing iron accumulation, and hepatic damage [70].

Moreover, Okada et al. proved the role of Nrf2 regulating iron metabolism in steatohepatitis. Using wild-type mice, Nrf2 gene-null mice and Keap1 gene-knockdown mice (sustained activation of Nrf2) they demonstrated that Nrf2 inhibits hepatic iron accumulation and counteracts against oxidative stress-induced liver injury in fatty livers [71]. It should be noted that dysregulation of iron metabolism can lead to cell damage and increased inflammation, as reactive ferrous iron can participate in Fenton reactions that result in the hydroxyl radical formation. Therefore, the role of Nrf2 in the regulation of iron metabolism could be considered as an antioxidant cytoprotective mechanism.

Proteasome regulation by Nrf2

The proteasome system is responsible for the removal of oxidized, misfolded and damaged proteins. The main proteasome activity is performed by 26S proteasome, which consist of a 20S core and two 19S regulatory subunits. Most of the genes encoding proteasome subunits and the proteasome maturation protein (POMP) contain ARE. Li et al. described that Nrf2 binds and activates the POMP promoter [72]. Furthermore, proteasome expression has been shown to be regulated by Nrf2 through ARE genes located on the proximal promoter region of several proteasome subunits in mouse cell lines [73]. Pickering et al. [74] found that Nrf2 translocated to the nucleus and bound to the ARE sequences of the proteasome β5 subunit gene, leading to increased expression of the 20S proteasome after the addition of H_2O_2.

Additionally, Nrf2 inducers also upregulate proteasome subunits and activity [30]. In senescent fibroblasts, treatment with an Nrf2 inducer results in increased transcription of proteasome subunits and increased levels of assembled proteasome. This results in elevated proteasome activities and functions, accompanied by reduced ROS levels and oxidized proteins, as well as lifespan extension [75].

Induction of proteasome subunits by the Nrf2 pathway could be relevant in liver IR, since the increase in proteasome activity has been observed [76,77], and is likely to be an important way, for a cell undergoing oxidative stress, to increase its capacity to remove damaged and oxidized proteins.

Nrf2 activation by ER stress

The production of ROS and the consequent oxidation of proteins is one of the main initiators of endoplasmic

reticulum (ER) stress. ER is an essential organelle for the synthesis, modification, and folding of proteins. Due to the system complexity, errors can occur and cause the accumulation of misfolded proteins, leading to ER stress. It arises in response to different physiological processes such as oxidative stress, Ca^{2+} imbalance, as well as associated diseases such as ischemia, atherosclerosis, and diabetes [78]. In response to ER stress, the GRP78 protein will lead to the unfolded protein response (UPR) by activating specific transmembrane receptors, associated with the initiation of the UPR signaling pathway, by binding to the lumen domains of the ER. Activation of the UPR system allows non-native polypeptides to fold or be removed; otherwise, as a last resort, apoptosis occurs [79].

Nrf2 can also be activated by UPR machinery [80]. Nrf2 has been proposed to be a direct PERK substrate and an indirect substrate for IRE1α kinase activity [33]. Following exposure to ER stress, the Nrf2 activation by PERK signaling promoted cell survival. The regulation includes phase II detoxifying enzymes induction, which maintains cellular redox homeostasis as well as the repression of genes that promote apoptosis. An alternative mechanism that drives Nrf2 activation after PERK activation has recently been described. This activation of PERK triggers ATF4-dependent control of Nrf2 mRNA abundance that enhances the cytoprotective function of Nrf2 [36]. Hence, the PERK pathway may involve different mechanisms to activate Nrf2 during ER stress.

Moreover, Nrf2 has been tested as an ER stress controller. In a recent study, the ability of the PERK/Nrf2/HO-1 axis to protect against IR damage in mice myocytes has been demonstrated [81]. Although HO-1 is only one of the Nrf2 response factors, it is essential in the response of ER. The same work showed that inhibition of HO-1 expression invalidated the roles of PERK overexpression in restricting signal transduction of the ER stress-mediated apoptotic cascade. A connection between UPR and Nrf2 was reported in the liver. Thus, Nrf2 has been observed to play a cytoprotective role in response to ER stress in animal models of nonalcoholic steatohepatitis (NASH) [71] and in hepatocellular carcinoma cells [82].

Regarding IR models, it has been described in rat brain that Nrf2 improves ER stress due to its antioxidant response and prevention of ROS formation. This protection is considered to be afforded by creating a positive feedback loop between p62/ZIP and Nrf2. p62/ZIP is an autophagy receptor and can transport misfolded proteins to a macroautophagy-lysosome system for degradation [83]. Although this mechanism is not entirely clear, it is associated with a dual role. On the one hand promoting the interaction between Keap1 and microtubule-associated proteins 1A/1B light chain 3B (LC3), thus activating autophagy and accelerating the elimination of protein aggregates. On the other hand, by the connection of a positive feedback loop between p62/ZIP and Nrf2, that can play a role in relieving ER stress in cerebral IR injury.

Nrf2 role in energy stores and mitochondrial balance

Nrf2 target genes affect multiple aspects of metabolism and mitochondrial function, such as glycolysis, pentose phosphate pathway, fatty acid metabolism [84], as well as genes for nucleotide synthesis [85], and NADPH synthesis [86].

Mitochondrion is an essential organelle for the energy and metabolic maintenance of cells. However, in stressful circumstances such as ischemia, mitochondria is known to be the main ROS generator, activating Nrf2 and leading to ARE genes transcription. Some of these genes prevents directly or through crosstalking, metabolic disruption, and consequently autophagy [84].

The first alterations during cellular ischemia occur in the mitochondria. The lack of oxygen as the final electron acceptor in the mitochondrial electron transport chain increases the reduced state of the organelle because of the increase in the $NADPH/NADP^{+}$ ratio and a decrease in the generation of ATP from ADP. During cellular anoxia, and independently of the decrease in the oxidative generation of ATP, there are intracellular mechanisms that lead to the inhibition of ATP synthase and the maintenance of the ionic gradient that preserve mitochondrial potential and integrity. This adaptive cellular response to anoxia is a consequence of the release of mitochondrial calcium in cultured hepatocytes [87–89]. When blood flow is restored, accumulated metabolites such as calcium and succinate will cause ROS overproduction and the consequent damage to liver tissue. Thus, avoiding the accumulation of ROS appears as a main therapeutic strategy, and in this sense it has been shown in recent years that Nrf2 can play an important role [64,90,91].

NADPH provides the reducing equivalents for biosynthetic reactions of oxido-reduction and protects against ROS, but also through the glutathione and thioredoxin systems. Nrf2 regulates 4 genes which are responsible for NADPH generation (G6PD, PGD, MEI, IDH) [86]. In a "gene dose-response" study, performed a different control test for Nrf2 in mice liver, testing the knockout or overexpression of Nrf2 and KEAP1. They showed that Nrf2 increased the

antioxidant response when overexpressed, as well as an increase in NADPH. Furthermore, they concluded that Nrf2 activation increases the availability of NADPH to counteract oxidative stress while decreasing its activity in metabolism.

Nrf2 not only increases the production of NADPH but also promotes the reduction through the activation of NADPH quinone reductase (NQO1 and NQO2), which are encoded in ARE genes [54]. NQO1 and NQO2 catalyze detoxification of quinones, which prevents the generation of reactive semiquinones, $O_{2,}^{-}$ and H_2O_2. Both constitutive and inducible expression of protective genes is regulated by the antioxidant response element (ARE) that is present in the upstream regions of these genes [92]. In a study of hepatic IR in mice, Li et al. demonstrated that overexpression of fibroblast growth factor 10 (FGF10) decreased oxidative stress and ROS generation, presumably due to upregulation of Nrf2. Among its results, it is worth highlighting the increase in the expression of the NQO1 enzyme [45]. In another study comparing the response of mice deficient in Nrf2 with respect to WT mice against IR damage, it was shown that those deficient in Nrf2 would provide greater tissue damage and less induction of the NQO1 enzyme, as well as greater oxidative damage [46]. The aforementioned studies point to the relevance of Nrf2 in the stress response to IR injury, as well as the importance of targeting Nrf2 as a therapeutic tool.

One of the strategies to prevent IR injury of the liver is to avoid ATP depletion [76,93]. In a model of rat liver cold ischemia, our group demonstrated that the solutions which best preserve graft viability prevented ATP depletion while increased Nrf2 levels [94]. Although Nrf2 has not been reported to have a direct effect on ATP synthesis, different studies support the importance of this factor for the preservation of ATP levels [95,96].

An additional antioxidant defense mechanism regulated by Nrf2 is through uncoupling proteins. Uncoupling proteins (UCP) are mitochondrial inner membrane proteins, whose function is to decrease the mitochondrial electrochemical potential leading to heat dissipation. In this way, the major function of UCP1 in rodents is to produce heat to maintain body temperature, while UCP-2 and UCP-3 can have different functions. Current findings suggest that UCP-2 and UCP-3 likely play a role in controlling the ROS production and regulating ATP synthesis [97]. UCP-2 improves the mitochondrial NAD^+/NADH ratio by suppressing ATP and ROS generation. The levels of NAD^+ directly control the activity of SIRT3 and in turn SIRT3 could regulate Nrf2 through PGC1α, although this pathway has not been directly demonstrated [98]. Upregulation of UCP-3 expression through Nrf-2 has been demonstrated [99]. 4-Hydroxynonenal (4-HNE) treatment in cardiomyocytes induced nuclear accumulation of Nrf2 and enhanced the expression of UCP-3. It is worth highlighting the importance of this finding, since the positive regulation of UCP-3 mediated by Nrf2 in response to 4-HNE, a final product of lipid peroxidation, could be important in the protection against oxidative stress in conditions of IR. The fact that Nrf2 stimulates the expression of UCP proteins while maintaining ATP levels could be explained given that some UCP homologues do not act as true uncouplers; instead, their activity goes beyond what was initially defined [100].

In the case of a high and irreversible mitochondrial damage or elevated ROS production, mitophagy occurs. In this process of clearance of dysfunctional mitochondria, Nrf2 will also play a key role. Usually, mitophagy is produced by a process of mitochondrial homeostasis and is mediated by the PTEN-induced putative kinase 1 (PINK1)/Parkin pathway targeting mitochondria lacking of membrane potential to autophagosomes. One of the activating pathways of mitophagy is through the autophagic adapter protein sequestome-1 (SQSTM/p62), which competes with Keap1 to bind Nrf2. p62 disrupts the Nrf2-Keap1 interaction and induces mitophagy independently of PINK/PARKIN recruitment.

Some studies described the regulation of mitophagy via Nrf2-PINK1. Murata et al. demonstrated in different cell lines that Nrf2 binds directly to the promoter regions of PINK1 [101] corresponding to ARE sequences, giving cells an advantage for cell survival. This model was also shown in diabetic kidney disease [102]. In an IR model of the mouse brain, Xu et al. tested the efficacy of esculetin as a neuroprotective therapy [103]. Their results showed increased Nrf2 expression related to a reduction in oxidative stress markers, and triggered mitophagy in the hippocampus. Until now, no studies have been reported linking the Nrf2-PINK1 pathway with hepatic ischemic damage.

Besides, another recent study demonstrates the role of Nrf2 on mitochondria being a regulator of nuclear respiratory factor-1 (NRF1) [62,104]. NRF1 was identified as a major transcriptional regulator that connects the regulation of nuclear-encoded genes and mitochondrial biogenesis. Nrf2 binds to NRF-1 promoter sites increasing NRF-1 expression and activity [62]. Accumulation of nuclear NRF-1 protein leads to gene activation for mitochondrial biogenesis and respiratory function [104].

Nrf2 is also a relevant factor in the regulation of PINK1 and NRF1, demonstrating its involvement in quality control and mitochondrial biogenesis. Although further studies will be needed to corroborate the role of Nrf2 as a regulatory mechanism of mitochondrial homeostasis in IR liver injury.

Nrf2 and inflammasome signaling

Nrf2 has been studied for its potential role in inflammation. Nrf2 knockout mice were hypersensitive to neuroinflammation induced by lipopolysaccharide (LPS) [105]. Moreover, Nrf2 deletion aggravated the damage caused by neuroinflammation and oxidative stress, increasing the pathogenesis of Alzheimer's Disease in mice [106]. In the liver IR, recent works evidenced an inverse relationship between the Nrf2 pathways and the NLRP3 inflammasome at different levels [24–26]. Inflammasomes are key components of the innate immune response [107]. Inflammasomes are multiprotein complexes that activate caspase-1, leading to the maturation of the pro-inflammatory cytokines interleukin 1B (IL-1B) and IL-18 and the induction of pyroptosis [107,108]. The most studied is the NLRP3 inflammasome [109]. Activation of NLRP3 has been shown to result in severe liver inflammation, fibrosis, and hepatocyte pyroptosis [110].

NF-κB signaling is a necessary input for adequate activation of the NLRP3 inflammasome [109,111]. In primary hepatocytes, LPS-induced expression of NLRP3 is directly related to activation of the NF-κB pathway, while inhibitors of NF-κB signaling are effective in inhibiting NLRP3 expression [109]. NF-κB is also a redox-regulated transcription factor, it is a protein complex mainly involved in cytokine production and cell survival [112]. In brain IR models, Nrf2 activation attenuates inflammation and apoptosis through inhibition of the Nrf2-mediated ROS/NF-κB pathway [113].

Activating transcription factor 3 (ATF3) is a stress-induced transcription factor that suppresses the expression of inflammatory genes in multiple cell types and diseases. Extracellular signals, such as ER stress, cytokines, chemokines, and LPS, are linked to ATF3 induction [114]. In a mice liver IR injury model, deletion of ATF3 aggravated liver damage, as evidenced by increased serum Alanine transaminase (ALT) levels, intrahepatic macrophage/neutrophil trafficking, hepatocellular apoptosis, and up-regulation of pro-inflammatory mediators [115]. Recent studies demonstrated ATF3-mediated Nrf2/HO-1 signaling in the regulation of Toll-Like Receptor 4 (TLR4) driven inflammatory responses in IR livers [116].

Nrf2 regulates apoptosis

Cellular apoptosis contributes to liver damage during hepatic IR. The fact that oxidative stress can trigger apoptotic cell death was first demonstrated in primary rat hepatocytes [117]. In a mouse model, the activation of a Tollip-ASK1-JNK/p38 pathway during hepatic IR injury lead to apoptosis [118]. Additionally it has been described that the MAPK signal transduction pathway plays a key role in the regulation of inflammatory and apoptotic processes in a rat model of prolonged cold hepatic IR [119].

Nrf2 also play a key role in preventing apoptosis. In mouse hepatoma and human hepatoblastoma cells exposed to the antioxidant tert-butylhydroquinone, Nrf2 was found to mediate the upregulation of the anti-apoptotic protein Bcl-2 that led to a decrease in Bax, cytochrome c release, activation of caspases, and increased cell survival [120]. A recent study demonstrates that fibroblast growth factor 10 (FGF10) inhibits hepatocellular apoptosis during liver IR [45]. The protein levels of total and nuclear Nrf2 were both upregulated in FGF10-overexpressing mouse liver tissues after IR, as well as NQO-1. Furthermore, FGF10 failed to protect the liver at 6 h post-IRI in Nrf2 knockout mice.

Targeting Nrf2: new opportunities for liver IR protection

The findings described so far provide a rationale for a new therapeutic strategy for the management of IR-induced liver injury. Different strategies that could effectively activate Nrf2/HO-1 pathway proved to lead a better outcome during hepatic IR. Table 2 shows several compounds that, by activating Nrf2 signaling pathways, protect the liver from IR injury [48,116,121–128].

In hepatic warm and cold IR models in mice, the activation of Nrf2 signaling using the organosulfur compound oltipraz, activated the anti-inflammatory functions of ATF3 [116]. Geraniol (a natural essential oil) was also used in a model of liver IR in rats. The hepatoprotective effect of the oral administration of geraniol, prior to liver IR, was mediated through the activation of Nrf2/HO-1 signaling, which resulted in the improvement of the antioxidant defense system with the reduction of oxidative stress [122]. Intraperitoneal pretreatment with the triterpenoid CDDO-imidazole activates Nrf2 and improves IR liver injury in mice by attenuating necrosis and apoptosis and reducing ROS levels [121]. These protective effects were attributed to the Nrf2/HO-1 signaling pathway.

Table 2. Compounds that activate Nrf2 signaling with potential protective effects on IR liver injury.

Compound	Model	Nrf2 signaling	Mediators Effects and Outcome		References
Oltipraz	Mice	Activation of Nrf2/HO-1 pathway	Activate ATF3 anti-inflammatory function	Regulate TLR4 innate immune response	[116]
CDDO-imidazolide	Mice	Activation of Nrf2/HO-1 pathway	Enhanced autophagy	Reduced mtDNA release Reduced ROS Reduced inflammatory response	[121]
Telluric acid	Rats	Activation of Nrf2	TLR4 and p13K/AKT pathway	Reduction of Oxidative stress Suppressed TNF-α, increased eNOS,	[134]
Geraniol	Rats	Activation of Nrf2/HO-1 pathway	Increased antioxidant capacity and GSH levels	Anti-inflammatory effects: Suppressed TNF-α, iNOS, COX-2. Antiapoptotic effects: reduced Bax, caspase 3-9	[122]
Bardoxolone-Methyl	Rats	NRF2 activator	Change in hepatocyte membrane transporters(HMT) regulation	Mitigate cholestasis associated with hepatic IRI	[123]
Sevoflurane	Rats and Hepatocytes	Activation of Nrf2/HO-1 pathway	Promoted Nrf2 nucleation Antioxidant and anti-inflammatory	Reduced LDH, MDA, TNF-α and IL-6, lowered apoptosis rate and inhibited ROS production	[126]
Fisetin	Mice and Hepatocytes	Activation of Nrf2/HO-1 pathway	Reduced oxidative stress and apoptosis	Reduced ROS, MDA, Bax, cleaved-caspase-3, cleaved-PARP, and enhanced Bcl-2.	[128]
Bracteanolide A	Rats	Upregulated expression of Nrf2, NQO1and HO-1.	Antioxidant and anti-inflammatory	Decreased the levels of ALT, AST,IL-1β and TNF-α	[124]

Accelerated autophagy and clearance of damaged mitochondria, reduced mtDNA release, and ROS overproduction, decreased inflammatory responses and subsequent secondary liver injury were the effects reported. Bardoxolone-Methyl, a synthetic triterpenoid derivative, mitigates tissue damages in mouse and rat models of hepatic IRI [46,123]. Bardoxolone-Methyl binds to Keap1, inhibits the Keap1-Nrf2 interaction, leading to the activation of NRF2 as a transcription factor. Sevoflurane, a volatile anesthetic, used as a pre- and post-conditioning agent, exhibits protective effects against liver IR injury [125] through a mechanism related to the Nrf2/HO-1 pathway regulation [126].

The potential of phytochemicals against IR liver injury has also been explored. The flavonoid fisetin alleviates IR-induced liver damage [127]. Inhibition of the Nrf2/HO-1 pathway reverses the effects of fisetin on cell viability, apoptosis, and oxidative stress [128]. Pretreatment of bracteanolide A, the major active compound of *Tradescantia albiflora*, attenuates oxidative stress, and reduced the levels of the inflammatory cytokines through activating Nrf2, and its downstream effectors, NQO1 and HO-1 [124].

Among the therapeutic measures that have been developed with the aim of protecting the liver from I/R injury, we have highlighted the usefulness of hypothermic preconditioning [4]. Treatment with mild hypothermia against cerebral IR promoted the nuclear localization of Nrf2, and increased the levels of NQO and HO-1 reducing oxidative stress and mitochondrial dysfunction [129]. In the hippocampus, hypothermia significantly elevated nuclear Nrf2 localization and HO-1 expression, reduced lipid peroxidation and increased SOD activity after cardiac arrest [130]. The effect of hypothermia treatment inducing a decrease in cytosolic Nrf2 was also confirmed in liver IR [131]. At the same time, the levels of TNF-α, IL-1β, IL-6, and IL-12 decreased and the levels of IL-10 increased [131]. In cell lines, the response to cellular hypothermia is associated with higher levels of Nrf2 that affect the antioxidant system. In contrast, NF-κB, a transcription factor involved in the control of immune and inflammatory responses, was not induced by hypothermia [132].

In view of the evidences that Nrf2 activation provides cytoprotection against liver IR, it is reasonable to ask whether Nrf2-inducing compounds could be used therapeutically for liver preservation. Some Nrf2-inducers are ongoing clinical trials in 2021 for the prevention or the treatment of different diseases (https://clinicaltrials.gov). Such is the case of synthetic triterpenoids, like bardoxolone methyl (CDDO-Me) in patients with chronic kidney disease (NCT04702997).

The imidazol derivative (CDDO-Im) has been proved to be protective during liver IR in mice [121]. An additional inducer of Nrf2 with ongoing clinical trials is dimethyl fumarate (DMF). DMF has several pharmacological benefits, including immunomodulation and fibrosis prevention, which are dependent on Nrf2 pathways [133]. DMF has been used since 1994 for the use in psoriasis and from 2013 has been approved by several countries as a first-line oral treatment for people with relapsing-remitting multiple sclerosis (RRMS).

The organosulfur compound oltipraz has been shown to be effective for the prevention of liver diseases in liver IR in mice [116] and in clinical trials of liver fibrosis and cirrhosis. Oltipraz is currently used in an ongoing trial for the reduction of liver fat in nonalcoholic fatty liver disease (NCT04142749). As such, it is highly likely that potential compounds that induce Nrf2 pathways could be candidates for avoiding IR injury in organ resection and liver transplantation.

Concluding comments and future perspectives

Research results of the last decade have established the importance of Nrf2 in regulation of redox balance, inflammation and cytotoxicity, and its involvement in many diseases, such as cancer, neurodegeneration, aging, and IR injury, all of them associated with oxidative stress. In addition to antioxidant and anti-inflammatory responses, Nrf2 is involved in diverse cellular processes, including metabolism. A review of the literature shows that the functions currently described go beyond those originally intended.

During liver IR, ROS cause lipid, protein and DNA oxidation, mitochondrial dysfunction, and subsequent liver inflammation, apoptosis, and necrotic cell death. Different strategies have been adopted to limit ROS damage in IR, including the administration of pharmacological agents, along with the use of preconditioning models. Advances in the study of the regulation of antioxidant and anti-inflammatory systems by Nrf2 could provide new and interesting potential therapeutic targets as in the case of organ transplantation, when there is an inherent damage due to the ischemia and reperfusion protocol. Therefore, therapeutic measures that activate Nrf2 signaling pathways have attracted the interest of new research.

Today's unhealthy lifestyles along with other factors such as aging are responsible for the accumulation of fat in the liver. This leads to varying degrees of hepatic steatosis. Steatotic livers show greater vulnerability against IR, increasing primary failure, and compromising the result of the graft after transplantation. Due to the lack of available organs, non-optimal livers will be required for transplantation regardless. Induction of Nrf2 against cold ischemic injury in fatty livers promises to be a good strategy to reduce the extent of subsequent oxidative damage against reperfusion (Fig. 3).

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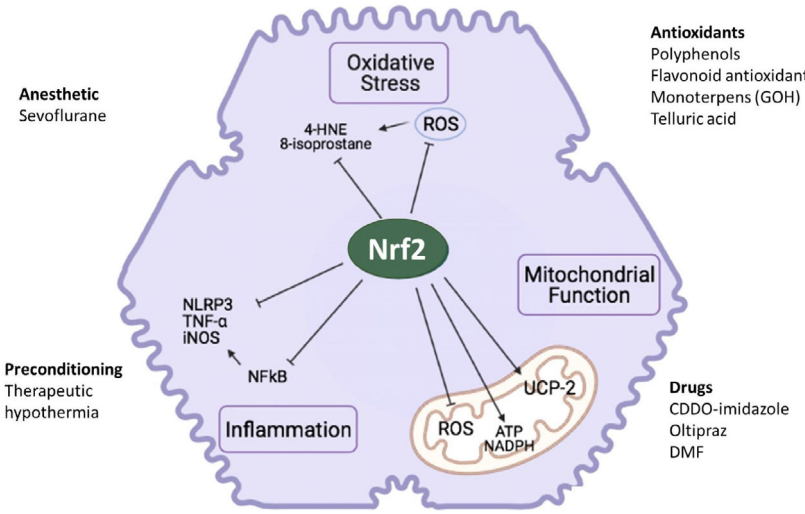

Fig. 3. Nrf2 activates different cellular mechanisms in response to IR damage. Nrf2-inducing compounds protection against IR injury has been shown at different cellular levels. First, against oxidative stress and ROS by activating phase II detoxification genes. Second, decreasing the production of ROS, through the UCP, and preventing the fall of ATP in the mitochondria. Finally, decreasing the inflammatory response through the regulation of NF-kB, cytokines, iNOS and NLRP3. Targeting Nrf2 through different treatments are good strategies to prevent liver damage.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

All the authors participate in the elaboration of the text. Conceptualization: TC; Writing-Original Draft Preparation: RGB and TC; Writing-Review and Editing: RGB; APR; S.S.N.; NA; JRC and TC, RGB; and TC designed figures.

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