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Nrf2 Weaves an Elaborate Network of Neuroprotection Against Stroke

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Abstract Nuclear factor erythroid 2-related factor 2 (Nrf2) is a neuroprotective transcription factor that has recently attracted increased attention. Stroke, a common and serious neurological disease, is currently a leading cause of death in the USA so far. It is therefore of vital importance to explore how Nrf2 behaves in stroke. In this review, we first introduce the structural features of Nrf2 and Kelch-like ECH-associated protein 1 (Keap1) and briefly depict the activation, inactivation, and regulation processes of the Nrf2 pathway. Next, we discuss the physiopathological mechanisms, upstream modulators, and downstream targets of the Nrf2 pathway. Following this background, we expand our discussion to the roles of Nrf2 in ischemic and hemorrhagic stroke and provide several potential future directions. The information presented here may be useful in the design of future experimental research and increase the likelihood of using Nrf2 as a therapeutic target for stroke in the future.

Keywords Nuclear factor erythroid 2-related factor 2 · Oxidative stress · Ischemia · Hemorrhage

Introduction

As the primary cause of adult disability in developed countries, stroke ranks only behind cancer and cardiac diseases [1]. Stroke represents the loss of brain function following a disturbance caused by ischemia or hemorrhage in the blood supply to the brain, which further causes permanent neurological damage or death [2]. Consequently, the affected area of the brain cannot function normally, which may result in an inability to move one or more limbs on one side of the body, a failure to understand or formulate speech, or a vision impairment on one side of the visual field [3]. Previous studies have demonstrated that isothiocyanate [4], an ester of pyruvic acid [5], and a metabolite of butylated hydroxyanisole [6] exhibit characteristic neuroprotective properties and can prevent brain injury. The underlying mechanisms of these interventions appear to involve a transcription factor referred to as nuclear factor erythroid 2-related factor 2, also known as Nrf2.

Nrf2 is a basic leucine zipper (bZIP) transcription factor with a cap'n'collar (CNC) structure [7]. It is ubiquitously expressed and undertakes a wide spectrum of functions in various organs and tissues, including the kidney [8], muscle [9], lung [10], heart [11], liver [12], and brain [13]. Most importantly, Nrf2 is involved in regulating the expression of antioxidant proteins, which protect against oxidative damage triggered by injury [14]. Currently, several upstream drugs or molecules that stimulate the Nrf2 pathway are under investigation for the treatment of stroke, including sulforaphane

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(SFN) [4], ethyl pyruvate (EP) [5], and tert-butylhydroquinone (tBHQ) [6]. Furthermore, Nrf2 regulates many downstream protective proteins, including hemeoxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase-1 (NQO1), glutathione-S transferase (GST), and other phase II antioxidant enzymes that conjugate drug metabolites or endobiotics [15]. These results indicate that Nrf2 offers neuroprotection via sophisticated signaling crosstalk and that Nrf2 is situated in the center of the entire pathway.

This review summarizes the recent research progress regarding the protective effects of Nrf2 in stroke. First, we briefly introduce the structural features of Nrf2 and the mechanisms regulating the Nrf2 pathway. We then discuss the basic physiopathological processes of Nrf2 in the brain. Next, we introduce several upstream mediators of Nrf2 and some of its downstream targets. We also highlight the particular roles of Nrf2 in ischemic stroke and hemorrhagic stroke. Finally, we discuss novel potential directions for Nrf2 studies. Because ischemic stroke is substantially more common than hemorrhagic stroke in terms of overall cases, we provide a greater focus on the former throughout this review. Taken together, the information compiled here may serve as a comprehensive reference for the currently identified actions of Nrf2 in the central nervous system (CNS). Furthermore, this information will hopefully facilitate the design of future experimental research in stroke and tap the potential of Nrf2 as a therapeutic target.

Molecular Mechanisms of the Nrf2 Pathway

Structural Characteristics of Nrf2

Nrf2 possesses six erythroid-derived CNC homology protein (ECH) domains, designated Neh1 to Neh6 [16]. The Neh1 domain is a CNC-bZIP domain that allows Nrf2 to heterodimerize with its transcriptional partners, small musculo-aponeurotic fibrosarcoma (Maf) proteins, and bind DNA as a heterodimer [17]. The Neh2 domain, which lies at the N-terminal region of Nrf2, controls the binding of Nrf2 to its cytosolic repressor, Kelch-like ECH-associated protein 1 (Keap1) [18]. The Neh3 domain, located at the C-terminus, binds the chromo-ATPase/helicase DNA binding protein family member CHD6, which functions as a transcriptional co-activator to promote the transcription of antioxidant response element (ARE)-dependent genes. Neh3 may thus act as a transactivation domain that is potentially involved in the interaction with components of the transcriptional apparatus to affect its transcriptional activity [19]. The Neh4 and Neh5 domains both individually and cooperatively bind to another transcriptional co-activator, cAMP-response element binding protein (CREB) binding protein (CBP), during acquisition of the potent transactivation activity of Nrf2. It should be noted that Neh5 interacts with CBP more strongly than does Neh4, which suggests that Neh5 plays a central role and Neh4 plays a supplementary role in CBP binding [20]. The Neh6 domain controls the Keap1-independent negative regulation of Nrf2. Notably, Neh2 is both necessary and sufficient for the degradation of Nrf2 in homeostatic cells; however, the turnover rate of the protein does not change in cells exposed to oxidative stress after the removal of Neh2. Thus, the degradation of the protein in stressed cells is predominantly mediated by the redox-insensitive Neh6 degron [21] (Fig. 1a).

Structural Characteristics of Keap1

Keap1, an indispensable molecule in Nrf2 pathway regulation, is a cysteine-rich protein that consists of three main domains: a broad-complex, tramtrack, and bric-à-brac (BTB) domain that contains cysteine 151 (C151), an intervening region (IVR) with four cysteine residues (C257, C273, C288, and C297), and a double glycine repeat (DGR) domain (also referred to as a Kelch domain). Both mutation analysis [22] and in vivo experiments [23] demonstrated that C273 and C288 of the IVR are essential for the repression of Nrf2 by Keap1 under basal conditions. Furthermore, the modification of these residues may decrease the rate of ubiquitination and degradation of Nrf2 rather than dissociating Keap1 from Nrf2 or Cullin 3 (Cul3). However, a mutant Keap1 protein with a single cysteine-to-serine substitution at C151 within the BTB domain is significantly resistant to inhibition by either quinone-induced oxidative stress or SFN, neither of which disrupts the association between Keap1 and Nrf2, suggesting that C151 in the BTB domain is crucial for the binding of Keap1 to Cul3 and the stabilization of Nrf2 [24]. Another study indicated that the induction of Nrf2 by arsenic is independent of C151 despite its necessity for Nrf2 activation by tBHQ or SFN, suggesting that Keap1 may sense various inducers in different ways [25]. The sensors in Keap1 can be divided into three categories. Each category is shown to be specific for certain types of inducers, with C151 required for nitric oxide (NO), SFN, and tBHQ reactivity; C288 responding to alkenals; and H225, C226, and C613 comprising a sensor for zinc [26]. As a consequence, these cysteine residues are hypothesized to serve as components of a molecular switch that enables Keap1 to regulate the steady-state levels of Nrf2 in response to perturbations in the intracellular redox environment [22] (Fig. 1b).

Regulation of the Nrf2 Pathway

Under quiescent conditions, two molecules of Keap1 form a homodimer and each dimer binds to Nrf2 via its Kelch domain [27]. The N-terminal segment of Keap1 binds to an E3 ubiquitin protein ligase, ring-box1 (Rbx1), via Cul3. Nrf2 is subsequently directed to the ubiquitination and constitutive

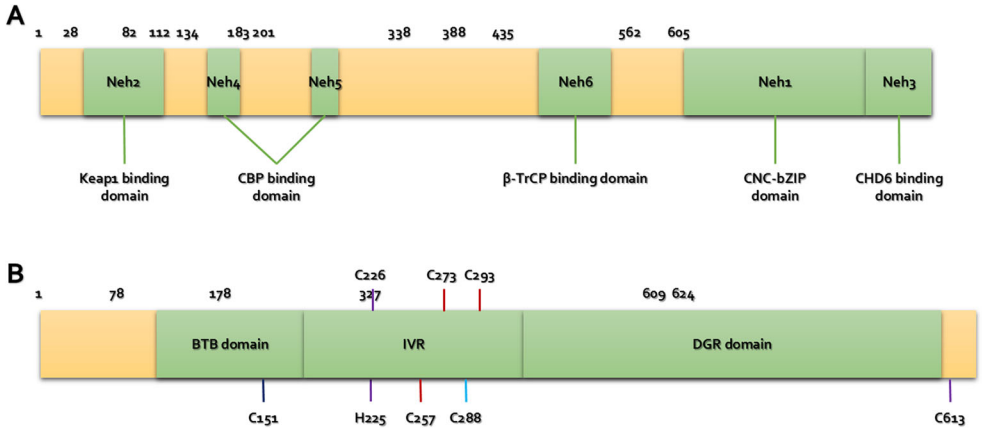

Fig. 1 Structures of Nrf2 and Keap1. **a** The six domains of Nrf2 that lie from the N-terminus to the C-terminus are Neh2, Neh4, Neh5, Neh6, Neh1, and Neh3, successively. Neh2 is in charge of binding Nrf2 to Keap1. Neh4 and Neh5 function as the CBP binding domain that renders transactivation of Nrf2. Neh6 binds Nrf2 to β-TrCP and regulates the Keap1-independent ubiquitination of Nrf2. Neh1 is a CNC-bZIP domain that heterodimerizes Nrf2 with small Maf proteins. Neh3 binds CHD6 and acts as a transactivation domain. **b** Keap1 consists of three major functional domains: the BTB, IVR, and DGR domains. The amino acid residues in Keap1 are marked in various colors to indicate the types of sensors that sense certain types of inducers. C151 in *dark blue* responds to NO, SFN, and tBHQ; C288 in *light blue* responds to alkenals; H225, C226, and C613 in *purple* function as a sensor for zinc. *Keap1* Kelch-like ECH-associated protein 1, *CBP* cAMP-response element binding protein (CREB) binding protein, *β-TrCP* β-transducin repeat-containing protein, *CNC* cap'n'collar, *bZIP* basic leucine zipper, *Maf* musculo-aponeurotic fibrosarcoma, *CHD6* chromo-ATPase/helicase DNA binding protein 6, *BTB* broad-complex, tramtrack, and bric-à-brac, *IVR* intervening region, *DGR* double glycine repeat. *NO* nitric oxide, *SFN* sulforaphane, *tBHQ* tert-butylhydroquinone, *Nrf2* nuclear factor erythroid 2-related factor 2 (color figure online)

degradation by the 26S proteasome [28]. Exposure to oxidants or electrophiles can initiate Nrf2-ARE pathway activation via the dissociation of the Nrf2-Keap1 complex [29]. Nrf2 is then translocated into the nucleus after its release from Keap1 in the cytoplasm. This nucleocytoplasmic shuttling of Nrf2 is mediated by the modification of nuclear shuttling signals or sequences identified in Nrf2 [30] and the phosphorylation by several protein kinases such as protein kinase C (PKC) [31]. After its entrance to the nucleus, Nrf2 heterodimerizes with a small Maf protein through its Neh1 domain and subsequently binds to ARE [16], which eventually promotes the expression of thousands of protective genes [32].

However, AREs are also present in the genes for Rbx1, Cul3, and Keap1. The activation of Nrf2 can upregulate the expression of the Rbx1-Cul3-Keap1 complex, which in turn mediates the ubiquitination and degradation of Nrf2 [33]. This negative feedback loop avoids excessive activation of the Nrf2-ARE pathway. Furthermore, inactivation of the Nrf2 pathway occurs via several other endogenous mechanisms. For example, glycogen synthase kinase-3β (GSK-3β) can mediate the ubiquitination of Nrf2 without the participation of Keap1. GSK-3β phosphorylates the Neh6 domain and results in the degradation of Nrf2 via the upregulation of β-transducin repeat-containing protein (β-TrCP), which is a scaffolding protein that binds Nrf2 to the Cul1-Rbx1 complex for ubiquitination [34]. Additionally, GSK-3β can phosphorylate Fyn, a member of the Src family, which then transports Nrf2 out of the nucleus through an interaction with exportin-1 (XPO-1) [35]. In addition, prothymosinα (ProTα), a Keap1 binding protein, mediates the intranuclear degradation of Nrf2 by importing the Rbx1-Cul3-Keap1 complex into the nucleus [36].

Taken together, Nrf2 is stimulated in a Keap1-dependent manner in stressed cells, whereas several compensatory mechanisms are simultaneously launched to limit overexpression of the Nrf2 pathway. These changes facilitate the return to normal conditions the maintenance of cellular homeostasis. The regulation of Nrf2 is an intricately coordinated process regulated at the levels of subcellular distribution, interaction with other proteins, phosphorylation, ubiquitination, transcription, and epigenetics. The more detailed modulatory mechanisms have been summarized at length in our previous work [13] (Fig. 2).

Physiopathological Roles of Nrf2 in the Cerebrum

Oxidative Stress

Oxidative stress is a pathological process during which the production of reactive oxygen species (ROS) exceeds the endogenous antioxidant defenses when exposed to various types of harmful stimuli. This disequilibrium causes an accumulation of oxidative damage, including the posttranslational modifications of lipids, proteins, and nucleic acids [37]. The cerebrum is particularly susceptible to oxidative damage because of its high lipid content and oxygen consumption [38]. Previous studies have demonstrated that the increased production of ROS during reperfusion is a major cause underlying

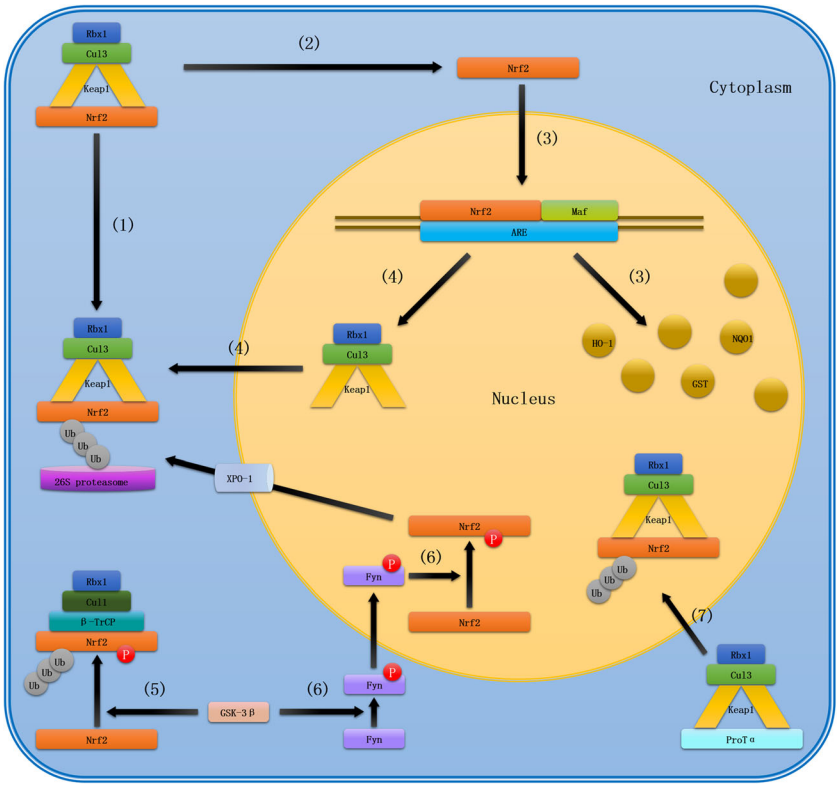

Fig. 2 The regulatory network of the Nrf2 pathway. Nrf2 is sequestered by the Rbx1-Cul3-Keap1 complex and ubiquitinated by the 26S proteasome in the cytoplasm under basal conditions (*1*). Nrf2 is dissociated from the Rbx1-Cul3-Keap1 complex under stressed conditions (*2*). After Nrf2 is released from Keap1, Nrf2 shuttles from the cytoplasm to the nucleus and heterodimerizes with a small Maf protein to bind ARE, which facilitates the transcription of a multitude of cytoprotective proteins, such as HO-1, NQO1, and GST (*3*). Rbx1, Cul3, and Keap1 are transcription products of ARE (*4*). They form the Rbx1-Cul3-Keap1 complex, which mediates the ubiquitination and degradation of Nrf2 via negative feedback. GSK-3β phosphorylates Nrf2 and mediates its degradation via the upregulation of β-TrCP, which binds Nrf2 to the Cul1-Rbx1 complex for ubiquitination (*5*). Moreover, GSK-3β phosphorylates Fyn, which transports Nrf2 out of the nucleus through an interaction with XPO-1 (*6*). ProTα mediates the intranuclear degradation of Nrf2 by importing the Rbx1-Cul3-Keap1 complex into the nucleus (*7*). *Rbx1* ring-box 1, *Cul3* Cullin 3, *Keap1* Kelch-like ECH associated protein 1, *Maf* musculo-aponeurotic fibrosarcoma, *ARE* antioxidant response elements, *HO-1* heme oxygenase-1, *NQO1* NAD(P)H: quinone oxidoreductase-1, *GST* glutathione-S transferase, *Ub* ubiquitination, *β-TrCP* β-transducin repeat-containing protein, *XPO-1* exportin-1, *GSK-3β* glycogen synthase kinase-3β, *ProTα* prothymosinα, *Nrf2* nuclear factor erythroid 2-related factor 2

the pathophysiology of cerebral ischemia-reperfusion (IR) injury and hemorrhage [39–41].

However, there is evidence that ischemic preconditioning (IPC) may induce brain ischemic tolerance, which is triggered by an initial oxidative stress [42]. The mechanisms appear to be related to the opening of mitochondrial ATP-sensitive potassium channels ($mitoK^{+}_{ATP}$), which occurs early in the preconditioning response and is required for IPC protection [43]. Hence, a delicate balance exists in the formation of ROS, i.e., high levels of ROS generated during IR are cytotoxic, whereas low levels of ROS generated by IPC are neuroprotective [44].

Most importantly, oxidative stress is regarded as a fundamental pathophysiological mechanism by which to understand the other pathophysiological processes, including mitochondrial dysfunction and endoplasmic reticulum (ER) stress, within brains subjected to IR injury. Nrf2 acts as a critical regulator of a multitude of genes, such as HO-1, NQO1, and GST, which are all involved in cytoprotection against various oxidative insults in the brain [14]. Ramos reported that the levels of GST and NQO1 are significantly reduced in Nrf2-deficient mice, and the induction of phase II genes is blunted by Nrf2 disruption [45]. At the cellular level, existing studies suggest that Nrf2 activation may benefit neurons, astrocytes, oligodendrocytes, and microglia regarding their susceptibility to oxidative damage, which indicates a high relevance of this therapeutic approach to the entire neurovascular unit [46].

Oxidative stress has also been proven to be responsible for contributing to the aggravation of secondary complications after subarachnoid hemorrhage (SAH) [47]. Evidence has demonstrated that genetic elimination of Nrf2 leads to an increased lipid peroxidation product malondialdehyde (MDA) and a decreased glutathione (GSH)/oxidized

glutathione (GSSG) ratio after SAH, suggesting that Nrf2 is also a favorable factor in the exacerbation of oxidative stress during SAH [48].

Therefore, Nrf2 plays an essential role in the fight against oxidative stress in both cerebral ischemia and hemorrhage.

Mitochondrial Dysfunction

Growing evidence has demonstrated that Nrf2 also participates in protecting against mitochondrial dysfunction, which has received considerable attention as a major contributor to both ischemia [49] and hemorrhage [50]. The breakdown of proper functioning of brain mitochondria may produce a severe energy insufficiency, increased production of ROS in neurons and ultimately cell death [51]. Mitochondria account for the majority of the ROS generated inside cells, primarily by complexes I and III of the mitochondrial electron transport chain [52].

Nrf2 activation has been demonstrated to strongly inhibit the effect of the mitochondrial uncoupler carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) on cytosolic Ca^{2+}, which leads to an improvement in mitochondrial function, inhibited production of ROS, and increased levels of the antioxidant enzymes [53]. In addition, the induction of Nrf2 may increase the levels of antiapoptotic protein B cell lymphoma 2 (Bcl-2) and inhibit the translocation of Bcl-2 associated X (Bax) to the mitochondria, thereby attenuating the release of cytochrome c from the mitochondria and the activation of downstream caspases [54]. Furthermore, recent findings suggest that the estrogen receptor-dependent activation of Nrf2 can inhibit apoptosis in primary cortical neurons subjected to cerebral ischemia via suppressing mitochondrial membrane potential disruption, caspase-3 activation, and deoxyribonucleic acid (DNA) fragmentation [55]. Interestingly, a retrospective interpretation of microdialysis data from SAH patients has indicated that mitochondrial dysfunction appears to be more frequent than ischemia in patients with SAH [50]. Taken together, Nrf2 is a potent mediator that addresses mitochondrial dysfunction through multiple mechanisms in cerebral ischemia and hemorrhage (Fig. 3).

ER Stress

When the levels of ROS overwhelm the antioxidant capacity of the organism, ROS may slow the folding of proteins, which leads to the accumulation of misfolded and/or unfolded proteins in the ER lumen, a condition referred to as ER stress [56]. Nrf2 is a direct substrate of protein kinase RNA-like ER kinase (PERK), a kinase that acts as a transducer of ER stress

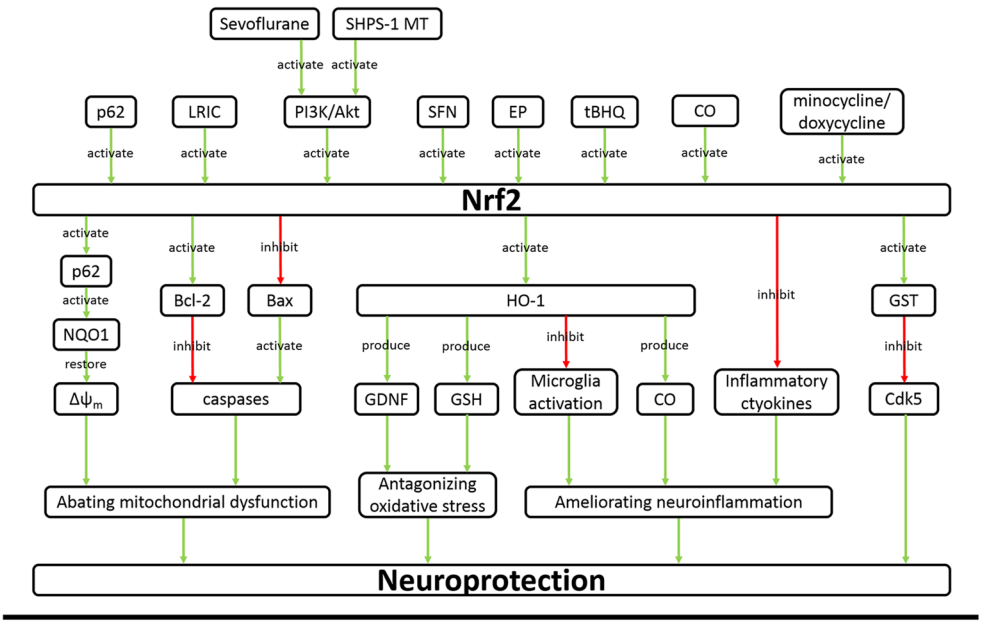

Fig. 3 Mechanisms of neuroprotection mediated by the Nrf2 signaling network. The potential upstream activators of Nrf2, a variety of downstream target genes and inflammatory responses, and their related effects with respect to cerebral protection. The regulation of gene expression by Nrf2 activity enables the activation and inhibition of signaling pathways involved in cerebral protection. *SHPS-1* src homology 2 domain-containing protein tyrosine phosphatase substrate-1, *LRIC* limb remote ischemic conditioning, *PI3K* phosphatidylinositol-4,5-bisphosphate3-kinase, *Akt* protein kinase B, *SFN* sulforaphane, *EP* ethyl pyruvate, *tBHQ* tert-butylhydroquinone, *CO* carbon monoxide, *NQO1* NAD(P)H: quinone oxidoreductase-1, $\Delta\psi_m$ mitochondrial membrane potential, *Bcl-2* antiapoptotic protein B cell lymphoma 2, *Bax* Bcl-2 associated X, *HO-1* hemeoxygenase-1, *GDNF* glial cell line-derived neurotrophic factor, *GSH* glutathione, *GST* glutathione-S transferase, *Cdk5* cyclin-dependent kinase-5, *Nrf2* nuclear factor erythroid 2-related factor 2

[57]. Thus, ER stress may trigger the Nrf2-dependent transcriptional regulation of phase II detoxifying enzymes [58].

Chronic activation of ER stress is considered a main pathogeny that causes neuronal disorders in ischemic stroke [59]. p62, also referred to as sequestosome 1, is a common component of protein aggregates, which are found in protein aggregation diseases that affect the brain [60]. Under oxidative stress, p62 interacts with Keap1 through its Keap1 interacting region, which activates Nrf2 and mediates the autophagic degradation of Keap1 [61]. Furthermore, Nrf2 can enhance p62 expression by binding the ARE sequence in its promoter region, forming a positive feedback loop between p62 and Nrf2 [62]. Wang and colleagues have also speculated that p62 may be involved in the regulation of autophagy through the Nrf2-Keap1 signaling pathway, where it may play beneficial roles in the removal of ROS, prevention of oxidative damage, and alleviation of ER stress during cerebral IR injury [63].

Experiments in the rat model of SAH have indicated that enhancing ER stress might improve neurological deficits, attenuate the expression of caspase-3, and reduce cell apoptosis [64]. Autophagy has been identified as a downstream mediator of the ER stress-induced protective effect. The suppression of autophagic activity with 3-methyladenine, an autophagy inhibitor, can inhibit this protection [64]. However, it remains to be elucidated whether a link exists between autophagy-associated ER stress and Nrf2-activated pathways in SAH.

These findings suggest a probable link between ER stress and the Nrf2-Keap1 pathway in neuroprotection. Targeting components of the ER signaling responses are potentially valuable methods to explore clinical treatment strategies or new drugs for cerebral ischemia and hemorrhage in the future.

Neuroinflammation

Neuroinflammation is glial cell activation triggered by limited neuronal insult without a breakdown of the blood-brain barrier (BBB), a specialized structure composed of astrocytes and endothelial cells, or concomitant leukocyte/blood monocyte infiltration [65]. The enhanced expression of Nrf2/HO-1 by artesunate is capable of abating microglia activation and pro-inflammatory cytokine expression [66]. The role of Nrf2 in neuroinflammation has been studied more frequently in neurodegenerative disorders, such as multiple sclerosis, than in stroke [67].

Compelling evidence demonstrates that ischemic stroke initiates a series of cellular responses that include both the activation of resident glial cells and the recruitment of inflammatory cells from systemic circulation. These responses have been confirmed to be detrimental to stroke-associated secondary brain damage and contribute to infarct evolution [68]. Research has indicated that the attenuation of cellular inflammatory mechanisms by increasing Nrf2/HO-1 expression to target macrophages/microglia might play a role in neuroprotection against cerebral ischemia [69]. However, it is unknown whether the downregulation of neuroinflammation is directly mediated by the activation of Nrf2 or is only one among multiple parallel outcomes of the interventions.

For the most part, the Nrf2-related mechanisms by which neuroinflammation ultimately contribute to the neuropathology of ischemic and hemorrhagic strokes have not been sufficiently elucidated. Hence, it remains uncertain whether targeting Nrf2 in the neuroinflammatory response represents an effective avenue for therapeutic interventions for stroke. Therefore, substantially more consequent studies should be instigated.

Upstream Activators of Nrf2

SFN

SFN is a well-studied isothiocyanate derived from the glucosinolate glucoraphanin, which is abundant in broccoli and broccoli sprouts. The initially identified and most studied mechanism for SFN-mediated chemoprevention occurs through the induction of phase II enzymes via Nrf2 signaling [4]. SFN is able to cross the BBB and accumulate in cerebral tissues with a peak level and disappearance after 15 min and 2 h, respectively, which suggests that SFN can rapidly reach the brain and exert its protective effects [70]. Multiple studies have demonstrated that SFN acts as a crucial activator upstream of Nrf2 during both cerebral ischemia and hemorrhage.

It has been reported that the upregulation of Nrf2 by SFN treatment prior to transient middle cerebral artery occlusion (MCAO) is associated with increased HO-1 expression in perivascular astrocytes in the peri-infarct regions and in cerebral endothelium in the infarct core, which prevents BBB breakdown and neurological dysfunction during ischemic stroke [71]. Remarkably, SFN has been proven to confer its neuroprotective effects on immature neurons. Recent studies show that SFN activates the Nrf2-ARE pathway to promote antioxidant defense and protects primary mouse hippocampal neurons from death caused by stress paradigms relevant to ischemic injury in the immature brain [72]. Studies regarding the kinetics of the SFN-induced Nrf2 response indicate that brief stimulation of the Nrf2 pathway by SFN produces a long-lasting increase of endogenous antioxidants in astrocytes. Part of this response can be amplified by repeated transient stimulation, which may explain how intermittent intake of SFN can result in long-term protection from radical damage [73]. However, single or repeated administration of SFN has no effect on the infarct volume, and it does not reduce the number of activated glial cells or proliferating cells after photothrombosis-induced permanent cerebral ischemia; these findings indicate SFN treatment does not interfere with key cellular mechanisms that underlie tissue damage in this type of stroke [74].

Treatment with SFN in prechiasmatic cistern SAH models also produced a series of beneficial effects related to amplification of the Nrf2-ARE pathway in various aspects, including the increase of enzymatic activity of downstream factors at both the pretranscriptional and posttranscriptional levels and the reduction of brain edema, BBB permeability, and apoptotic cell death [75]. Additionally, by promoting Nrf2-ARE pathway activation, SFN was able to inhibit the oxyhemoglobin-induced inflammatory responses in vascular smooth muscle cells (VSMCs) and further ameliorate cerebral vasospasm, which is considered an important factor that could induce poor outcomes associated with SAH [76].

It is noteworthy that SFN-induced autophagy via extracellular signal-regulated kinase (ERK) activation is independent of Nrf2 activity in neuronal cells [77]. Overall, as a potent inducer of Keap1-Nrf2 signaling and ARE-driven gene expression, SFN may exhibit a broad range of functions and be practical for the prevention or management of cerebral ischemia and hemorrhage.

EP

EP, a simple ester of pyruvic acid, is regarded as a novel Nrf2 activator and has been shown to exert robust neuroprotection against cerebral ischemia at various levels [5]. Previous studies have demonstrated that EP-mediated Nrf2 activation can lead to subsequent HO-1 induction, which then increases glial cell line-derived neurotrophic factor (GDNF) and GSH expression and ultimately enhances the viability of H_2O_2-treated primary astrocyte cultures and protects neurons exposed to oxidative insults [78]. Kim et al. demonstrated that EP suppresses microglia activation and inflammatory cytokine induction in primary microglia cultures. The anti-inflammatory effects are based on its antioxidant effects induced via a series of complex signaling pathways [79]. In addition, studies using ischemic models have demonstrated that EP significantly reduces infarct volumes and alleviates neurological deficits by scavenging ROS and suppressing microglial activation [80].

The latent mechanisms appear to be associated with EP-induced ERK and protein kinase B (Akt) activation, leading to Nrf2 upregulation in primary astrocytes [78]. However, a recent report claimed that EP inhibits p38 mitogen-activated protein kinase (MAPK), ERK, and Akt and suppresses matrix metalloproteinase (MMP)-9 expression in microglia [81]. This discrepancy might be attributed to the differences in the cell types and protocols. Moreover, novel anti-inflammatory and antioxidative mechanisms indicate that EP induces nuclear translocation of Nrf2, which binds to ARE along with p300, a transcriptional co-activator for both Nrf2 and p65, and hampers inducible nitric oxide synthase (iNOS) expression by making p300 unavailable to p65 [5].

However, it remains unknown whether the neuroprotective effects of EP are directly related to activation of the Nrf2 pathway. Current studies on EP have been largely limited to cellular levels, whereas experiments in animals, especially hemorrhagic models, are still far from sufficient to demonstrate the efficacy of EP in neuroprotection. Thus, the therapeutic potency of EP as a pharmacological primer for the Nrf2 pathway to prevent oxidative damage and inflammation in the brain deserves intensive research in the future, with a particular focus on studies of the complete pharmacokinetic and molecular mechanisms of EP activity in vivo.

tBHQ

tBHQ is a metabolite of the widely used food antioxidant butylated hydroxyanisole [82]. As an oral Nrf2 activator, tBHQ has been demonstrated to have neuroprotective effects in various models of CNS injury, such as ischemic stroke [6], brain trauma [83], and SAH [84]. It has been documented that tBHQ protects animals and cell lines against acute toxicity and oxidative insult, presumably through the induction of several cytoprotective and detoxifying enzymes such as epoxide hydrolase [85], GST [86], and glucuronosyltransferase [87]. This induction is dependent on the translocation of Nrf2 from the cytoplasm to the nucleus through an interaction with Keap1 [88].

A significant finding indicates that prophylactic tBHQ treatment improves functional recovery after transient MCAO in rats, suggesting that previous Nrf2 activation may reduce neuronal death during delayed apoptosis and inflammation long after stroke onset. Conversely, the loss of Nrf2 function in vivo exacerbates ischemic damage and abrogates the protective effects of tBHQ [6]. Experiments using rat SAH models indicate that the upregulated cortical levels of agents related to the Nrf2 signaling pathways are further activated with tBHQ treatment at both the mRNA and protein expression levels. The administration of tBHQ decreases early brain damage, including brain edema and BBB permeability, and ameliorates cortical apoptosis and necrosis attributable to SAH [84].

Together, these results strongly suggest tBHQ may have extensive clinical applications for the treatment of stroke.

Downstream Effectors of Nrf2

HO-1

HO is an enzymatic system responsible for heme degradation and formation of the α-isomer of biliverdin, which is converted to bilirubin-IX [89]. Increasing evidence indicates that the expression of HO-1, the constitutive isoform of HO, is induced in the brain after transient global ischemia [90], transient and permanent MCAO [91], and cortical photothrombosis [92].

However, whether HO-1 plays a beneficial or detrimental role in cerebral ischemia remains unclear. As a target gene of Nrf2, HO-1 is protective against brain injury. Many pharmacological treatments that confer beneficial effects against ischemic damage are associated with increased HO-1 expression [93]. In addition, an HO-1 knockout in mice exacerbates infarcts [94], whereas HO-1 overexpression reduces infarcts [95]. Wang et al. demonstrated that carbon monoxide (CO), a gaseous second messenger produced when HO enzymes catabolize heme, can be therapeutic in cerebral IR injury and permanent ischemic stroke [96]. However, certain protective therapies are associated with the repression of HO-1 expression. Pretreatment with WY14643, the selective peroxisome proliferator-activated receptor-α (PPAR-α) agonist, suppresses HO-1expression and protects the brain against excessive oxidative stress and inflammation [97]. The underlying regulation of HO-1 gene expression is sophisticated because it is positively regulated by Nrf2 [98] and negatively regulated by BTB and CNC homology 1 (BACH1) [99]. BACH1 is a transcription factor that binds ARE-like sequences and functions as a transcriptional repressor in a subset of ARE-regulated genes, thereby antagonizing the activator function of Nrf2 under quiescent conditions [100]. Recent studies have demonstrated that HO-1 induction after ischemia is relevant to the downregulation of BACH1. When HO enzymatic activity is inhibited by the HO competitive inhibitor zinc protoporphyrin IX (ZnPP), the induction of HO-1 is associated with a poor outcome after cerebral ischemia [101]. Therefore, the mechanism and related factors that determine the role of HO-1 in cerebral ischemia require further investigation.

The mechanism of HO-1 activity after intracerebral hemorrhage (ICH) also remains controversial. HO-1 has been reported to exacerbate striatal injury after experimental ICH in mice. The injury volume is smaller in HO-$1^{-/-}$ mice compared with wild-type mice early after ICH, and protection in HO-$1^{-/-}$ mice is associated with decreased inflammation and free radical levels [102]. In contrast, increasing evidence demonstrates that HO-1 may contribute to the protection of vascular cells and astrocytes from heme-mediated oxidative injury [103]. The contradiction may lie in the use of various models or experimental protocols. In total, HO-1 overexpression is associated with heme-mediated oxidative stress in ICH models, indicating that it may be a sensitive marker of secondary brain injury after ICH [104].

The Nrf2/HO-1 axis is a representative downstream pathway in Nrf2-mediated neuroprotection. Nevertheless, further clarification of the specific mechanisms of this pathway may help develop new strategies for stroke treatment.

NQO1

NQO1, an antioxidant flavoprotein regulated by the Nrf2-ARE pathway, is an inducible enzyme that catalyzes the two-electron reduction of quinones to hydroquinones [105]. In the healthy brain, NQO1 is predominately expressed in astrocytes and a subset of oligodendrocytes [106]. Although NQO1 is primarily located in the cytoplasm, small amounts localize to the mitochondria, endoplasmic reticulum, and nucleus [107]. NQO1 is highly inducible by many stimuli, including electrophilic metabolites and oxidative stress, and its induction is considered to be transcriptionally regulated by Nrf2 [105]. Intriguingly, $p62^{-/-}$ tissues exhibit attenuated expression of NQO1, whereas the expression of other Nrf2 target genes are not altered; these findings suggest that p62 may support the basal activation of Nrf2 through the modulation of Keap1 stability and confer a higher steady-state expression of NQO1 [108]. Furthermore, ectopic expression of NQO1 completely restored the mitochondrial membrane potential ($\Delta\psi_m$) and oxidant concentration in the p62- or Nrf2-knockdown cells [108].

Evidence that NQO1 acts as a vital downstream target of the Nrf2 pathway in neuroprotection against cerebral ischemia and hemorrhage is gradually accumulating. Experiments using ischemic models have indicated that a rapid increase in the intracellular expression of NQO1 induced by oxygen and glucose deprivation (OGD) is enhanced by posttreatment with curcumin, an extensively studied exogenous activator of Nrf2, which parallels an attenuation of cell injury [109]. Another study has indicated that exposure to sevoflurane remarkably upregulates the expression of phospho-Akt and NQO1, which is accompanied by an increase in the nuclear translocation of Nrf2 and the DNA-binding of Nrf2 to the ARE sequence. However, the addition of LY294002, a signaling inhibitor for phosphatidylinositol-4,5-bisphosphate3-kinase (PI3K), reduces NQO1 expression in MCAO rats. These results reveal an important role of the PI3K/Akt pathway involving Nrf2 in the neuroprotective effects of NQO1 against cerebral IR injury [110]. Nevertheless, the activity of the Nrf2/NQO1 pathway during cerebral hemorrhage has not been studied as extensively as cerebral ischemia. Limited evidence has shown that the administration of SFN can lead to a significant increase in the simulated SAH-induced expression of Nrf2 and NQO1 [76].

These findings demonstrate that NQO1 plays an essential role in counteracting oxidative stress and preserving mitochondria function in neuroprotection. However, the therapeutic potential of the Nrf2/NQO1 pathway in cerebral hemorrhage requires further investigation.

GSTs

GSTs, which are among the most inducible Nrf2-dependent genes, are best known for their ability to catalyze conjugation of the reduced form of GSH to xenobiotic substrates for detoxification [111]. The significance of the GSTs has been demonstrated by their wide distribution, with specific isoforms

abundant in the cytoplasm, endoplasmic reticulum, and mitochondria [112]. The homeostatic levels of various GST isoforms are reduced in mice null for Nrf2, which suggests that Nrf2 mediates the basal expression of GST by endogenous thiol-active endobiotics [113].

Growing evidence has suggested that GSTs participate in neuroprotection. GST Pi 1 (GSTP1), the most abundant member of the GST family, has been identified as a negative regulator of cyclin-dependent kinase-5 (Cdk5), which is implicated in many neurological disorders. Research has demonstrated that Cdk5 hyperactivation during ischemia promotes neuronal death, whereas pharmacological inhibition or conditional knockout of Cdk5 prevents neuronal death and dramatically reduces infarctions following MCAO [114]. Furthermore, Cdk5 silencing restores neurovascular unit integrity after cerebral ischemia [115]. Thus, an increase in GSTP1 level by Nrf2-ARE pathway activation is of potential therapeutic relevance and may represent an alternative approach to modulate Cdk5 signaling and eliminate oxidative stress.

To date, the Nrf2/GST axis has been studied most widely in the liver, whereas research on its roles in cerebral ischemia and hemorrhage is quite limited [116]. As a result, more attention should focused on the elucidation of Nrf2 mechanisms in the regulation of GSTs of various classes, as well as their therapeutic potential for stroke.

Research Progress Regarding Nrf2 in Stroke

Ischemic Stroke

Ischemic stroke is typically triggered by a decrease in blood supply to part of the brain, which causes brain tissue dysfunction in the corresponding area [3]. A growing number of studies have been focused on the dynamic variations in Nrf2 across time and space following cerebral ischemia. Compared with sham-operated rats, Nrf2 is upregulated at the gene and protein levels in ischemic brains, which begins at 3 h and peaks at 24 h after MCAO [117]. Notably, Tanaka et al. identified the spatial differences in Nrf2 and Keap1 expression between the peri-infarct regions and the regions destined to infarct. In the peri-infarct regions, a steady level of Keap1 exhibited reduced expression at 2 h of reperfusion, whereas Nrf2 exhibited a significant elevation at 2 h with a peak at 8 h of reperfusion after transient MCAO. However, in the regions destined to infarct, a similar trend of expression changes compared with the peri-infarct regions is observed in Keap1 and Nrf2 with substantially less pronounced reactions [118].

Furthermore, there are two major zones of injury within the ischemic cerebrovascular region, the core ischemic zone and the penumbra. The latter region represents a rim of ischemic but still viable cerebral cells that are functionally stable and can be protected from cell death, depending not only on the residual flow level in the ischemic phase but also the flow disturbance duration [119]. Dang et al. reported a significant increase in Nrf2 expression in the ischemic penumbra compared with no Nrf2 detected in the core ischemic zone; these findings indicate that the penumbra represents ischemia-related tissue where Nrf2 activation is believed to be beneficial and might subsequently contribute to cell protection and survival [120].

In the brain, src homology 2 domain-containing protein tyrosine phosphatase substrate-1 (SHPS-1) has been identified as a neural adhesion molecule that participates in neuronal survival via Akt activation [121]. Previous studies have demonstrated that oxidative stress is mitigated and neural injury is inhibited in SHPS-1 mutant mice subjected to MCAO. SHPS-1 deficiency increases Akt phosphorylation, which subsequently stimulates Nrf2 activity and eventually leads to the decline of oxidative stress [122]. These findings indicate that the activity of SHPS-1 may act more upstream in regulation of the Nrf2 pathway.

Consequently, the expression of Nrf2 varies with temporal and spatial features and might be relevant to upper mediators. These findings guide us to a better and deeper understanding of how Nrf2 participates in the pathological processes of ischemic stroke, thereby providing novel insight for the treatment of acute ischemic stroke.

Hemorrhagic Stroke

Hemorrhagic stroke is caused by the bleeding of blood vessels in the brain, either directly into the brain parenchyma or into the subarachnoid space surrounding brain tissue; these conditions are known as ICH and SAH, respectively [123].

Research using ICH models has confirmed that both pretreatment and posttreatment with Nrf2 activators are uniquely effective in upregulating the expression of many Nrf2-regulated antioxidative proteins, causing a reduction in oxidative burden to brain tissue and ultimately improving neurological functional recovery [124]. However, $Nrf2^{-/-}$ mice exhibit a larger injury volume, more severe neurological deficits, increases in leukocyte infiltration, the production of reactive oxygen species, DNA damage, and cytochrome c release during the early phase of the post-ICH period [125]. Remarkably, in contrast to ischemic stroke, a considerably longer therapeutic window may exist for the prevention of secondary injury caused by toxic hemolytic products after hemorrhagic stroke. Preconditioning via Nrf2 activation prior to the initial hemolytic events could prime the ICH-affected brain to better handle the noxious hemolytic products via the upregulation of antioxidant enzymes in all affected brain cells to increase their resistance to oxidative stress, as well as the upregulation of detoxification proteins to neutralize the toxic hemolysis products

Table 1 Effects of regulating Nrf2 on in vivo models of cerebral ischemia

Methods of regulating Nrf2	Models	Effects	References
Intraperitoneal administration of SFN	MCAO in rats	Protective	Alfieri et al. 2013 [71]
Intraperitoneal administration of SFN	Rose Bengal photothrombotic stroke in mice	No effects	Porritt et al. 2012 [74]
Intraperitoneal administration of EP	MCAO in rats	Protective	Yu et al. 2005 [80]
Intracerebroventricular infusion of tBHQ	MCAO in rats	Protective	Shih et al. 2005 [6]
Intraperitoneal administration of tBHQ	MCAO in rats	Protective	Shih et al. 2005 [6]
Dietary administration of tBHQ	Penumbral stroke in mice	Protective	Shih et al. 2005 [6]
Nrf2 KO	MCAO in Nrf2$^{-/-}$ mice	Suppressed phase II enzyme activities and exacerbated cortical infarction	Shih et al. 2005 [6]
Intragastrical administration of (S)-ZJM-289	MCAO in rats	Protective	Zhang et al. 2013 [54]
Intraperitoneal administration of Notoginsenoside R1	MCAO in rats	Protective	Meng et al. 2014 [55]
Intraperitoneal administration of DHA	MCAO in rats	Protective	Chang et al., 2013 [69]
CO exposure	MCAO in mice	Protective	Wang et al., 2011 [96]
Intraperitoneal administration of curcumin	MCAO in rats	Protective	Wu et al. 2013 [109]
Sevoflurane exposure	MCAO in rats	Protective	Li et al. 2014 [110]
Intracerebral transplantation of minocycline-preconditioned NSCs	MCAO in rats	Protective	Sakata et al. 2012 [129]

around the hematoma [46]. Recently, Zhao et al. demonstrated that Nrf2 plays a pivotal role in the regulation of phagocytic functions of microglia in an experimental model of ICH and appears to be essential to hematoma clearance [126].

Noticeably, recent studies using SAH models have demonstrated that the Nrf2-ARE pathway is activated in the cortex during an early stage of SAH in rats, suggesting that Nrf2-ARE signaling could participate in the pathogenesis of early brain injury (EBI) induced by SAH [75]. As the most common cause of disability and death in patients who suffer from SAH, EBI is considered a major treatment target in the management of patients who survive SAH [127]. This finding provides novel ideas for pursuing therapeutic agents for SAH-induced EBI. However, Nrf2 deficiency exacerbates brain injury with increased MDA, BBB disruption, neural apoptosis, and higher expression of tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β; these findings indicate that Nrf2 plays an important role in the attenuation of SAH-induced secondary complications via the regulation of excessive oxidative stress and the inflammatory response [48].

Therefore, Nrf2 stimulation or preconditioning can protect against hemorrhagic stroke through multiple means, including reducing oxidative stress and antagonizing neuroinflammation, providing a novel therapeutic target for hemorrhagic stroke. Details regarding the upstream regulators and

Table 2 Effects of regulating Nrf2 on in vivo models of cerebral hemorrhage

Methods of regulating Nrf2	Models	Effects	References
Intraperitoneal administration of SFN	SAH in rats	Protective	Chen et al. 2011 [75]
Intraperitoneal administration of SFN	ICH in rats	Protective	Zhao et al. 2007 [124]
Intraperitoneal administration of SFN	ICH in mice	Protective	Zhao et al. 2014 [126]
Oral administration of tBHQ	SAH in rats	Protective	Wang et al. 2014 [84]
Nrf2 KO	SAH in Nrf2$^{-/-}$ mice	Exacerbated BBB disruption and neural apoptosis	Li et al. 2014 [48]
Nrf2 KO	ICH in Nrf2$^{-/-}$ mice	Exacerbated neurologic deficit and increased leukocyte infiltration	Wang et al. 2007 [125]
Nrf2 KO	ICH in Nrf2$^{-/-}$ mice	Impaired hematoma resolution	Zhao et al. 2014 [126]

SFN sulforaphane, *tBHQ* tert-butylhydroquinone, *SAH* subarachnoid hemorrhage, *ICH* intracerebral hemorrhage, *BBB* blood-brain barrier, *Nrf2* nuclear factor erythroid 2-related factor 2

downstream enzymes in both ischemic stroke and hemorrhagic stroke have been discussed in previous sections.

Other Potential Directions

Of the many recent studies on Nrf2, some promising findings indicate potential directions for future studies and may be useful for the treatment of stroke.

The transplantation of neural stem cells (NSCs) during the acute stage of stroke often reduces lesion size and inhibits apoptosis in the penumbra area by providing neuroprotective paracrine factors that enhance host cell survival and function [128]. However, a substantial loss of transplanted NSCs is a major limitation of cell transplantation therapy for stroke. A recent study demonstrated that preconditioning with minocycline, a semisynthetic tetracycline, reprogrammed NSCs to tolerate oxidative stress and express higher levels of paracrine factors through Nrf2 upregulation after ischemic reperfusion injury. The underlying mechanism involves Nrf2 overexpression at both the mRNA and protein levels and the induction of NQO1 and HO-1. Moreover, Nrf2-siRNA suppresses the antioxidant capacity and cytoprotection provided by minocycline, which suggests that Nrf2 and Nrf2-regulated antioxidant genes play a critical role in these minocycline effects [129]. Interestingly, Nrf2 recognizes a functional ARE in the promoter of Notch1, which regulates processes such as proliferation and cell fate decisions [130]. This finding indicates that minocycline may enhance NSC proliferation through cross-talk between the Nrf2 and Notch1 signaling pathways [129]. Further in vitro evidence has demonstrated that preconditioning NSCs with doxycycline, a tetracycline-derived antibiotic, produces similar trends in Nrf2 variations under both normal and OGD/reoxygenation conditions [131]. Thus, the beneficial effects of minocycline/doxycycline preconditioning make NSC transplantation highly appealing for future clinical applications in ischemic stroke.

In recent years, many groups have confirmed the effectiveness of limb remote ischemic conditioning (LRIC) as a physiological strategy to harness endogenous protective capabilities against IR injury in the CNS [132, 133]. Zhang et al. reported that LRIC significantly increases the expression of Nrf2 and HO-1 after retinal IR and proposed that the Nrf2/HO-1 pathway is directly involved in the retinal protection induced by LRIC [134]. Because LRIC is a noninvasive neuroprotective strategy, it is of great significance to identify the potential of this pathway and its favorable effects following ischemia and reperfusion. However, the current literature offers insufficient evidence of the beneficial effects of Nrf2 conferred by LRIC in ischemic and hemorrhagic stroke. Therefore, additional research is required to differentiate the changes in Nrf2 and HO-1 in LRIC when considering clinical applicability and patients.

Table 3 Effects of regulating Nrf2 on in vitro models

Methods of regulating Nrf2	Cell lines	Effects	References
SFN	OGD in immature murine hippocampal neurons	Protective	Soane et al. 2010 [72]
SFN	Rat cortical astrocytes	Protective	Bergstrom et al. 2011 [6]
SFN	OxyHb-induced rat VSMCs	Protective	Zhao et al. 2013 [76]
SFN	Murine microglial cells	Protective	Zhao et al. 2014 [126]
Nrf2 siRNA + SFN	Murine cortical neurons	No effects on SFN-induced autophagy	Jo et al. 2014 [77]
EP	BV2 microglial cells	Protective	Kim et al. 2013 [5]
EP	Rat cortical astrocytes	Protective	Shin et al. 2012 [78]
EP	Murine BV2 microglial cells	Protective	Kim et al. 2008 [79]
tBHQ	IMR-32 neuroblastoma cells	Protective	Li et al. 2005 [88]
tBHQ	Murine microglial cells	Protective	Zhao et al. 2014 [126]
tBHQ	Rat cortical astrocytes	Protective	Shih et al. 2005 [6]
Nrf2 KO + tBHQ	$Nrf2^{-/-}$ murine cortical astrocytes	No effects	Shih et al. 2005 [6]
Anhydroexfoliamycin	Murine cortical neurons	Protective	Leiros et al. 2014 [53]
Notoginsenoside R1	OGD/reoxygenation in rat cortical neurons	Protective	Meng et al. 2014 [55]
Artesunate	BV2 microglial cells	Protective	Lee et al. 2012 [66]
Curcumin	OGD in rat cortical neurons	Protective	Wu et al. 2013 [109]
Doxycycline	OGD in rat NSCs	Protective	Malik et al. 2013 [131]

SFN sulforaphane, *EP* ethyl pyruvate, *tBHQ* tert-butylhydroquinone, *OGD* oxygen and glucose deprivation, *OxyHb* oxyhemoglobin, *VSMCs* vascular smooth muscle cells, *NSCs* neural stem cells, *Nrf2* nuclear factor erythroid 2-related factor 2

Conclusions

In summary, we originally postulated an elaborate network of several pathways and probable mechanisms of Nrf2 in neuroprotection against stroke. Exposure to oxidative stress triggers Keap1-dependent activation and nuclear translocation of Nrf2, which are followed by the transcription of a series of target genes, including HO-1, NQO1, and GST. Thereafter, these cytoprotective proteins mitigate oxidative stress, ameliorate mitochondrial dysfunction, alleviate ER stress, and antagonize neuroinflammation in the CNS. Nrf2 is simultaneously modulated by various proteins at multiple levels to achieve homeostasis within cells. Eventually, Nrf2 confers neuroprotection against cerebral ischemia and hemorrhage. The effects of Nrf2 in the in vitro and in vivo models previously discussed have been fully summarized in the tables attached to this review (Tables 1, 2, and 3).

Currently, accumulating evidence suggests that Nrf2 stimulation by exogenous activators, such as SFN, EP, and tBHQ, may represent a promising method for stroke therapy. However, many problems regarding the role of Nrf2 in cerebral pathways remain unresolved. For example, the pathways initiated by the upstream mediators and downstream effects of various targets might overlap with each other, indicating unknown mechanisms of the Nrf2 network. Moreover, future studies must focus on the changes of Nrf2 in neurons, astrocytes, and microglia to accurately elucidate the individual roles and interactions between neural and nonneural cells in brains subjected to stroke. In addition, intensified efforts should be extended to physiological and pathological animal models of various species to further address how to promote recovery and improve neurological performance over the short, medium, and long term. These problems must be resolved before Nrf2 can be considered a valuable therapeutic target for stroke.

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