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Nrf2 Activation, an Innovative Therapeutic Alternative in Cerebral Ischemia

Carlos Silva-Islas, Ricardo A. Santana,
Ana L. Colín-González and Perla D. Maldonado
*Patología Vasculiar Cerebral, Instituto Nacional de Neurología y Neurocirugía Manuel
Velasco Suárez
México*

1. Introduction

Cerebrovascular disease is the second cause of death and the most frequent cause of non-traumatic disability in adults worldwide, according to the World Health Organization (WHO, 2005). Noteworthy, acute ischemic stroke accounts for about 85% of all cases (Diez-Tejedor et al., 2001). The most common cause of stroke is a sudden occlusion of a blood vessel, resulting in activation of a series of biochemical events eventually leading to neuronal death (Dirgnal et al., 1999). Although return of blood flow (reperfusion) in ischemic brain tissue is essential for restoring normal function, paradoxically it can result in a secondary damage, where oxidative stress mediators play a critical role (Wong & Crack, 2008).

Antioxidant therapies have been used to determine whether oxidative stress may constitute a valuable therapeutic target in cerebral ischemia. Indeed, free radical scavengers (direct antioxidants) and agents that decrease free radicals production reduce damage in experimental models of cerebral ischemia. Despite experimental evidence supports the concept that free radicals production represents a valuable therapeutic target in stroke, negative results have been obtained in a number of clinical trials when some direct antioxidant agents have been evaluated (Aguilera et al., 2007). At present, this discrepancy is unclear; however, administration of treatment outside the temporal window of efficacy and difficulties in the establishment of the onset of ischemia and reperfusion in humans (Hsu et al., 2000) are factors that likely contributing to these differences. Clearly, development of preclinical testing must consider these factors in order to improve successful transition to clinical studies.

NF-E2-Related Factor-2 (Nrf2) is a transcription factor that play a crucial role in the cellular protection against oxidative stress. Nrf2 is referred to as the "master regulator" of the antioxidant response due to the fact that it modulates the expression of several genes including phase 2 and antioxidant enzymes playing an important role in detoxification of reactive oxygen species (ROS) and electrophilic species, including heme oxygenase-1, NAD(P)H:quinone oxidoreductase, glutathione-S-transferase, gamma-glutamyl cysteine ligase, glutathione reductase, etc. Recent studies demonstrate that dysfunction of Nrf2-driven pathways impairs cellular redox state thus oxidative stress.

Since ischemia and reperfusion insults generate an oxidative stress state, and considering that up to date there is no effective treatment to reverse morphological and behavioral alterations induced by stroke, it is conceivable that administration of antioxidants may limit oxidative damage and ameliorate progression of the disease. In this context, Nrf2 inducers are promising indirect antioxidant agents that are effective to attenuate oxidative stress and tissue/cell damage in different *in vivo* and *in vitro* experimental paradigms; therefore, here we review some compounds capable of inducing cellular antioxidant responses in order to understand their usefulness in prevention and treatment of cerebral ischemia-induced damage through activation of the Nrf2/ARE pathway.

2. Mechanism related to cerebral ischemic damage

Brain tissue requires high and constant supply of oxygen and glucose provided for the vascular system to maintain its viability and normal functions. Vascular obstruction – either transitory or permanent – of cerebral blood flow (ischemia) is accompanied by an immediate drop in neurological activity ultimately leading to cell death. The brain is not affected homogeneously and so, cerebral ischemia generates differentially damaged areas. Complete loss of blood flow produces an *infarct zone* where necrotic cell death is observed. The infarct area is surrounded by a *penumbra zone*, which is located between the *infarct zone* and the non-damaged area, or normally irrigated tissue. Cells belonging to the *penumbra zone* are still irrigated by collateral arteries, which maintain them viable for a variable period of time, although not functional (Figure 1). This is the area that shall be rescued, and the potential target for intervention with neuroprotective treatments (Dirgnal et al., 1999).

The return of blood flow (reperfusion) is associated with a decrease in the infarct size and clinical outcome. Although reperfusion is determinant for cell function recovery, after prolonged periods of ischemia, it also exerts negative side-effects. If blood flow is not restored within hours, the penumbra region will become part of the infarct zone. In some patients, reperfusion may exacerbate brain injury (e.g., some patients show edema or intracranial hemorrhage) (Kuroda & Siesjo, 1997). In animal models, reperfusion can induce larger infarct areas that can be associated with permanent vessel occlusion (Aronowski et al., 1997).

The reduction and return of blood flow triggers a cascade of events further leading to neuronal death (Dirgnal et al., 1999; Durukan & Tatlisumak, 2007). Such sequence includes:

1. *Energy failure.* This is the first event of the ischemic cascade. Cells need oxygen and glucose to undergo oxidative phosphorylation for energy production, consequently during ischemia ATP production is decreased (Figure 2).
2. *Depolarization of membrane.* The impairment of ATP production disrupts Na⁺/K⁺-ATPase and Ca²⁺/H⁺-ATPase pumps and reverses the Na⁺/Ca²⁺-transporter. Upon these conditions, cells are unable to maintain membrane potential and Ca²⁺ voltage-dependent channels are activated, leading to depolarization of cellular membrane (Figure 3).
3. *Excitotoxicity and increase in intracellular Ca²⁺ levels.* After depolarization, excitotoxic amino acids – mostly glutamate – are released to the synaptic cleft. Glutamate activates N-methyl-D-aspartic acid (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), and metabotropic glutamate receptors, thereby increasing intracellular

Ca²⁺ levels. In turn, voltage gated Ca²⁺ channels together with reverse operation of the Na⁺/Ca²⁺ exchanger also increase intracellular Ca²⁺ levels (Figure 3). Once in the cytoplasmic domain, Ca²⁺ activates a variety of Ca²⁺ dependent enzymes, including protein kinase C, phospholipase A2, phospholipase C, cyclooxygenase-2, Ca²⁺-dependent nitric oxide synthase, proteases and endonucleases, hence triggering protein phosphorylation, proteolysis, and mitochondrial damage.

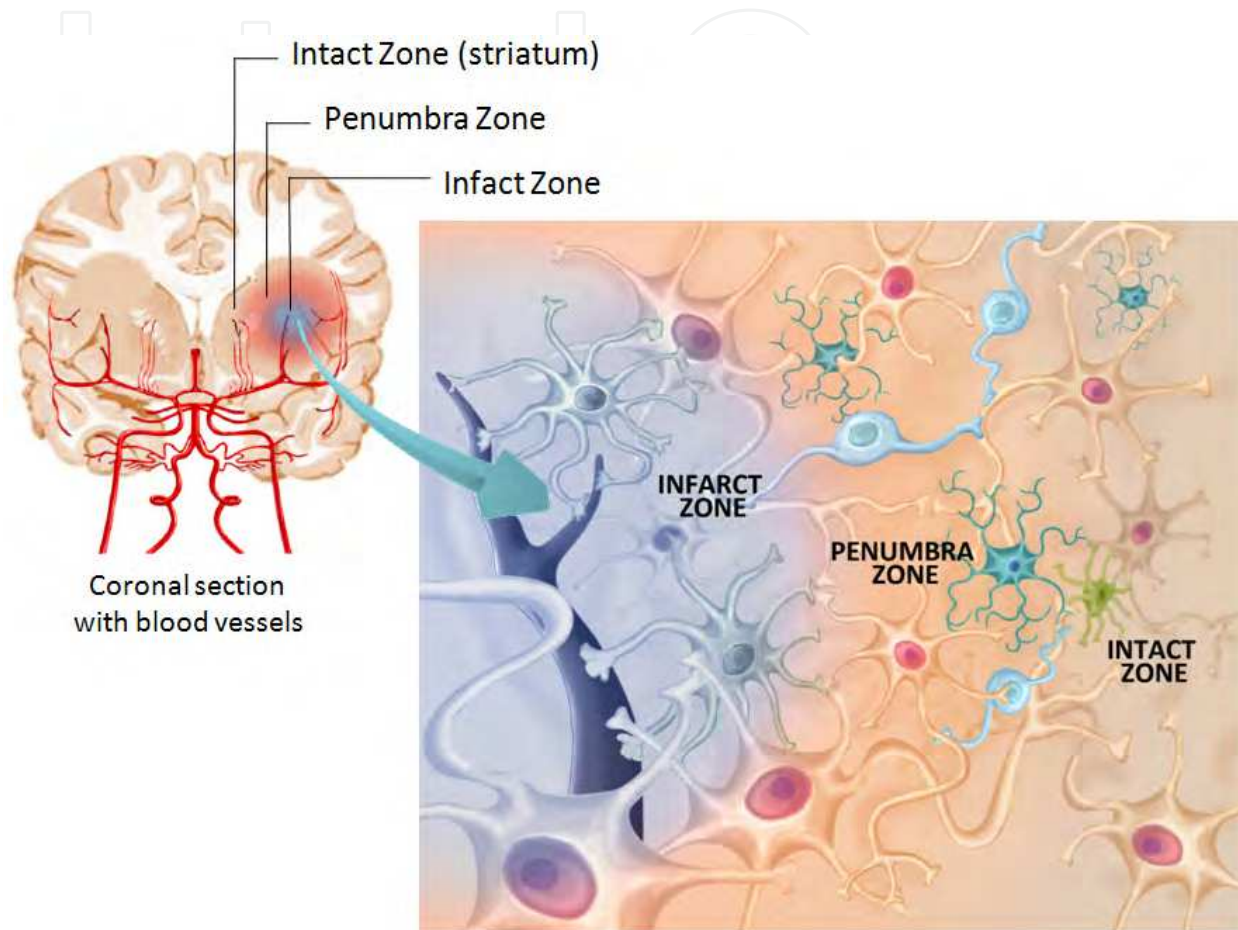


Fig. 1. Vascular obstruction of cerebral blood flow (ischemia) is accompanied by an immediate drop in neurological activity ultimately leading to cell death (*infarct zone*). Infarct core is surrounded by an area supplied with oxygen and glucose by collateral blood vessels (*penumbra zone*). Cells from the penumbra area are not functional; however, they remain viable for a variable period of time.

4. *Generation of free radicals and oxidative stress.* Reactive oxygen (ROS) and nitrogen (RNS) species generation is increased during ischemia, but particularly during reperfusion, and they eventually lead to oxidative stress. ROS and RNS cause lipid peroxidation, membrane injury, disruption of cellular processes, and DNA damage. Moreover, oxidative stress contributes to the disruption of the blood-brain barrier, hence allowing the infiltration of neutrophils and other cells (see below) (Chan, 2001).
5. *Inflammation and apoptosis.* Cerebral injury is a potent triggering of inflammatory cytokines and proteases secretion by microglia, leukocytes and resident cells of the neurovascular unit. Once the neurovascular barriers are breached, multiple neuroinflammatory cascades are activated, further leading to secondary brain injury

(Danton & Dietrich, 2003). Post-ischemic inflammation contributes to brain injury and has been linked to apoptosis. Cell death in cerebral ischemia is mainly dependent of the localization of the cells. For instance, in the core region, cell death is caused mainly by necrosis, while apoptosis predominates in the penumbra area.

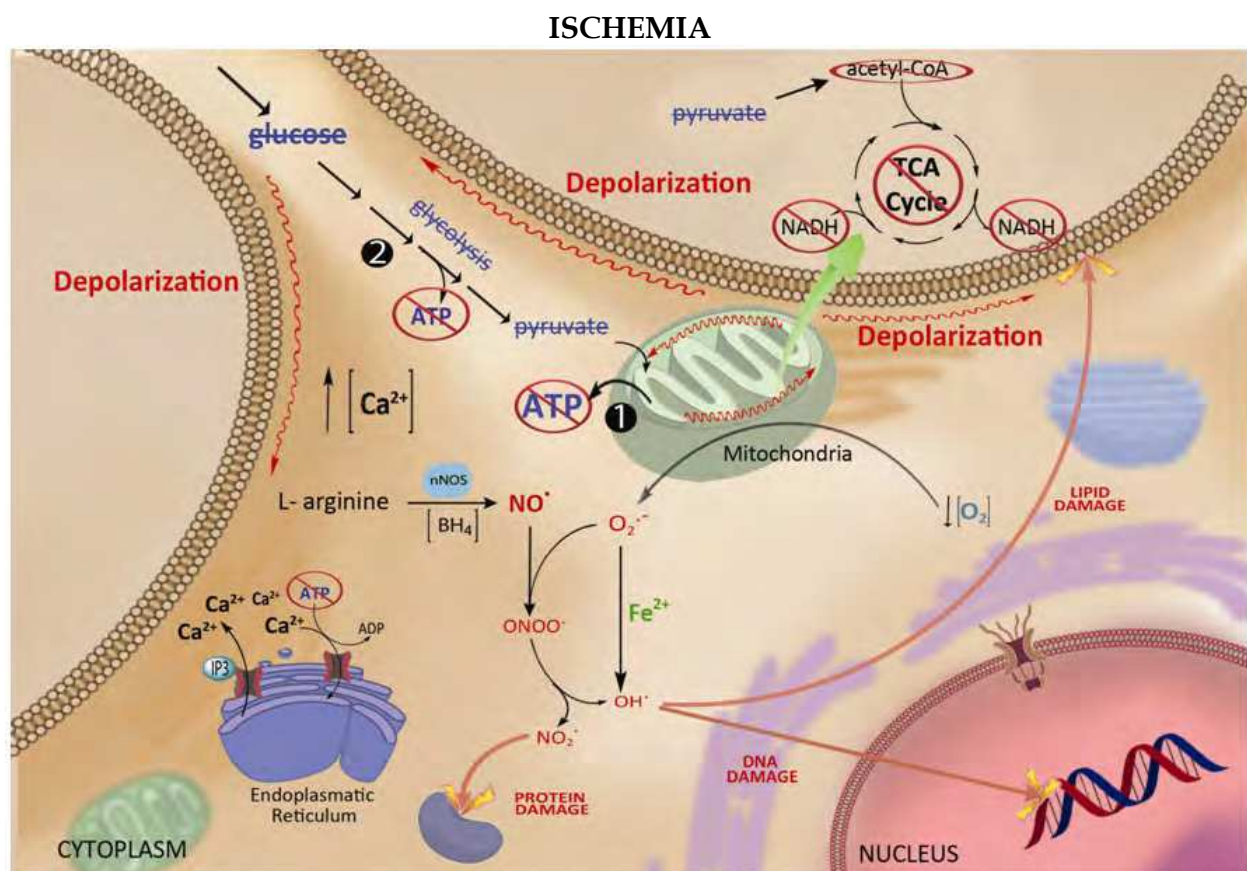


Fig. 2. The reduction of blood flow decreases oxygen and glucose levels; consequently, ATP production (*Energy failure*) (❶), glycolysis(❷) and ATP-dependent processes are blocked. Upon these conditions, oxidative damage is generated by residual oxygen in mitochondria. Pathways that are inhibited during ischemia are crossed out in the image. TCA cycle, tricarboxylic acid cycle; nNOS, neuronal nitric oxide synthase.

3. Oxidative stress is one of the most important events in ischemia/reperfusion-induced cerebral damage

In cells, the predominant ROS and RNS produced are superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\bullet OH$), nitric oxide ($\bullet NO$), peroxynitrite anion ($ONOO^-$), and nitrogen dioxide ($\bullet NO_2$). In normal conditions, natural defense against ROS and RNS is provided by antioxidant molecules such as glutathione (GSH), ascorbic acid, α -tocopherol, and a number of antioxidant enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). SOD converts $O_2^{\bullet-}$ to H_2O_2 , whereas GPx and CAT convert H_2O_2 to H_2O . However, an imbalance in the formation and clearance of ROS and RNS can lead to oxidative stress and subsequent changes affecting the cell dynamics (Aguilera et al., 2007; Margail et al., 2005).

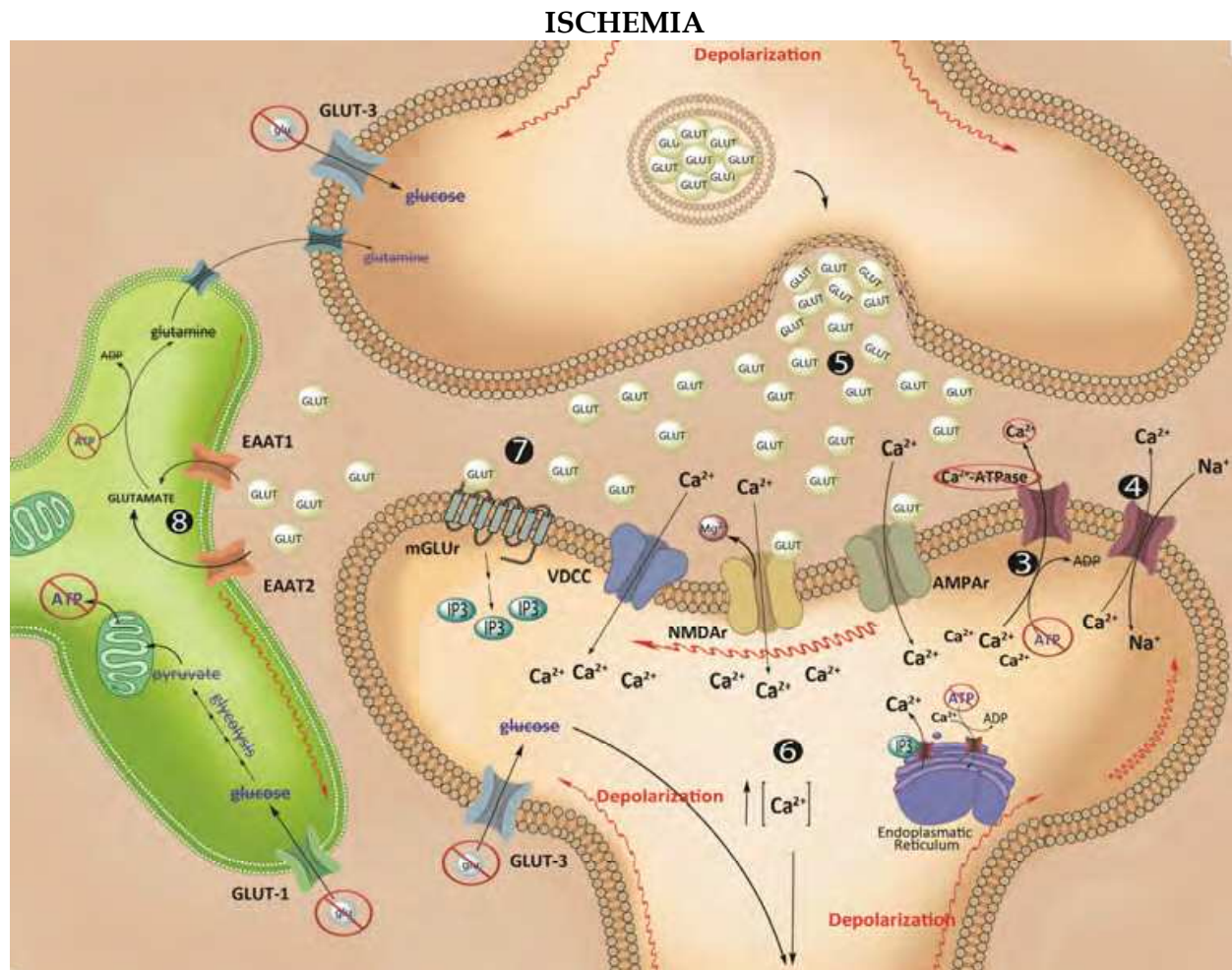


Fig. 3. Reduction of blood flow decreases ATP production, disrupts ATP-dependent pumps (③) and reverses the $\text{Na}^+/\text{Ca}^{2+}$ transporter (④). Upon these conditions, cells are unable to maintain membrane potential (*Depolarization of membrane*). After depolarization, glutamate (GLUT) is released and activates N-methyl-D-aspartic acid (NMDAr) and α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPAr) receptors (⑤, *Excitotoxicity*), hence directly increasing intracellular Ca^{2+} levels (⑥). On one hand, GLUT activates metabotropic glutamate receptors (mGLUr) (⑦), which releases inositol 1,4,5-triphosphate (IP₃), a molecule that binds to its receptor at the endoplasmic reticulum to release more Ca^{2+} (⑥, *Increase of intracellular Ca^{2+} level*). On the other hand, voltage gated Ca^{2+} channels (VDCC) and the reverse operation of the $\text{Na}^+/\text{Ca}^{2+}$ -exchanger increase intracellular Ca^{2+} levels. Energy disruption also affects astrocytes, causing a deficient activity of glutamate transporters (EAAT1 and EAAT2) (⑧).

ROS and RNS produce cellular damage through lipid peroxidation, nucleic acid alteration and inactivation of enzymes (Figure 4); they also modify cellular signaling and gene regulation, contributing to breakdown of the blood-brain barrier and edema generation (Moro et al., 2005). Oxidative stress can ultimately induce neuronal damage, leading to neuronal death by apoptosis or necrosis (Loh et al., 2006).

The brain is particularly sensitive to oxidative stress since 20% of the total oxygen consumed by the body is used by this organ, which constitutes only 2% of the total body weight. This

feature makes the brain the major generator of ROS and RNS when compared with other organs (Dringen, 2000). Moreover, in brain there are numerous conditions favoring ROS and RNS production, including: 1) a high unsaturated lipid content, 2) chemical reactions involving dopamine oxidation (Heiss, 2002; Hou & MacManus, 2002), 3) high concentrations of iron in various regions, and 4) lower antioxidant systems than other organs such as kidney or liver (Dringen, 2000).

As previously described, physiopathological mechanisms leading to neuronal injury in cerebral stroke are complex and multifactorial. However, several studies suggest that oxidative stress, secondary to ROS and RNS production, actively participates during post-ischemic brain damage (Peters et al., 1998; Rodrigo et al., 2005). During ischemia, free radical production in the infarct zone decreases or remains without change, while it increases during reperfusion. However, free radical production in the penumbral zone increases during both events (Liu et al., 2003). Despite the low oxygen tension produced during ischemia, exist an increase in ROS formation after 1.6 h of ischemia, the highest ROS production ($489 \pm 330\%$ of control) occurs after 20 min of reperfusion, and remains increased at least for 3 h (Peters et al., 1998). Christensen et al. (1994) reported that ROS production is maximal during the first hour of reperfusion.

Main sources of ROS, RNS, and free radicals during reperfusion are summarized as follows (Aguilera et al., 2007; Margail et al., 2005):

1. Mitochondrial respiratory chain generates O_2^{\bullet} .
2. Xanthine oxidase produces O_2^{\bullet} when it catalyzes oxidation of hypoxanthine to uric acid.
3. Cyclooxygenase 2 (COX-2) produces O_2^{\bullet} during oxidative metabolism of arachidonic acid, a delayed process in ischemia reperfusion.
4. NADPH oxidase (NOX) produces O_2^{\bullet} during NADPH oxidation.
5. Nitric oxide synthases (NOS) produce $\bullet NO$ in normal conditions. $\bullet NO$ produced can react with O_2^{\bullet} and generate the strong oxidant $ONOO^-$. Tetrahydrobiopterin (BH4) is an important regulator of NOS function because it is required to maintain enzymatic coupling. Loss or oxidation of BH4 to 7,8-dihydrobiopterin (BH2) is associated with NOS uncoupling, resulting in the production of O_2^{\bullet} rather than $\bullet NO$ (Crabtree & Channon, 2011) (Figure 4).

4. Direct and indirect antioxidants

Living systems have developed multiple lines of defense against oxidative stress. Cellular protection against oxidative stress is a process more complex than cellular protection against electrophiles. In this process two types of molecules participate (Dinkova-Kostova et al., 2007):

1. *Direct antioxidants*. Compounds of low molecular weight (ascorbate, glutathione, tocopherols, lipid acid, ubiquinones, carotenes) that can undergo redox reactions and scavenge reactive oxidation products (peroxides), as well as ROS and RNS ($\bullet OH$, $ONOO^-$). Direct antioxidants are consumed or modified in the process of their antioxidant action (ROS scavenger). Thus, it is necessary to replenish or regenerate them.
2. *Indirect antioxidants*. These agents may or may not have redox activity, and exert many of their effects through upregulation of phase 2 and antioxidant enzymes. In turn, these enzymes act catalytically, exhibit long half-lives, and display a wide variety of antioxidant activities, in addition to their capacities to detoxify electrophiles.

REPERFUSION

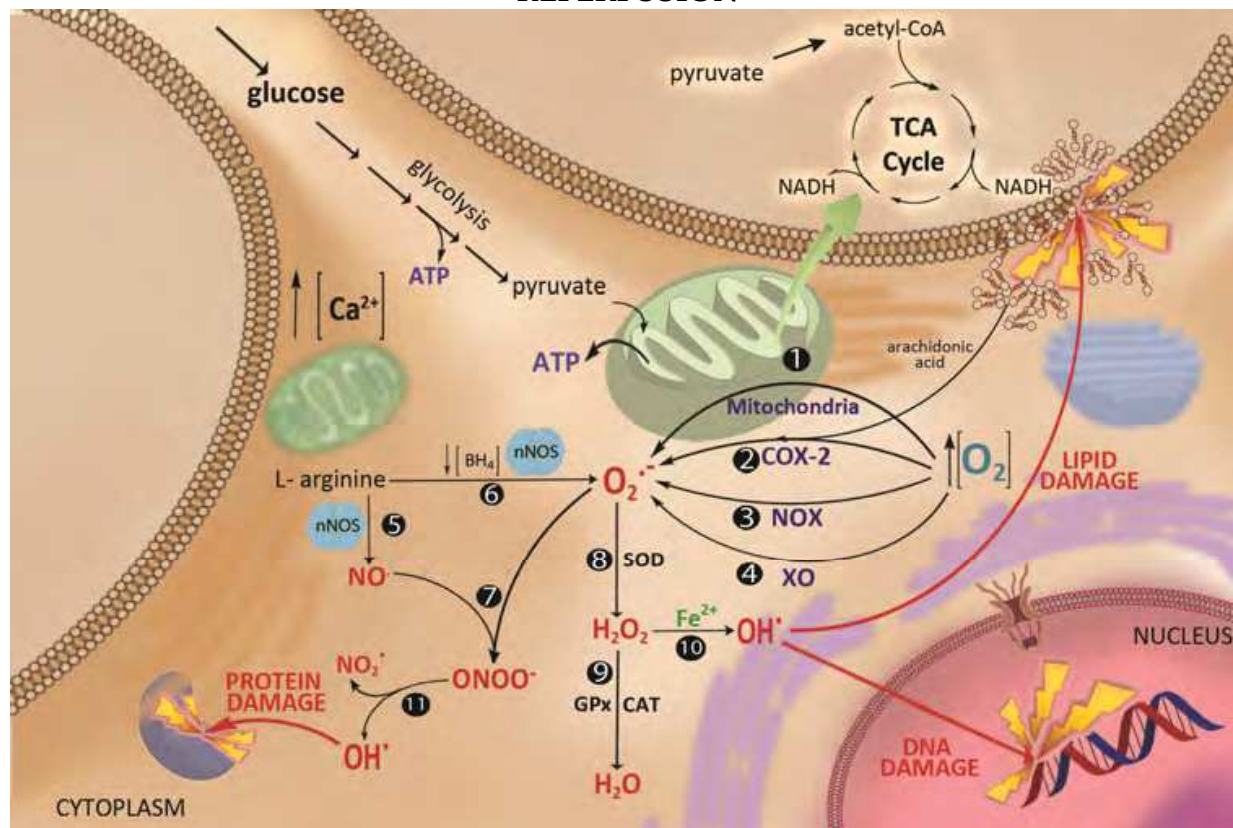


Fig. 4. Main sources of superoxide anion ($O_2^{\bullet-}$) during reperfusion are summarized as follows: mitochondrial respiratory chain ①; cyclooxygenase-2 (COX-2) ②; NADPH oxidase (NOX) ③; xanthine oxidase (XO) ④; and nitric oxide synthase (NOS), responsible for nitric oxide ($\bullet NO$) formation ⑤, or $O_2^{\bullet-}$ if tetrahydrobiopterin (BH₄) is deficient ⑥. $O_2^{\bullet-}$ can react with $\bullet NO$ to generate peroxynitrite anion (ONOO⁻) ⑦, or be degraded by superoxide dismutase (SOD) to hydrogen peroxide (H₂O₂) ⑧. Then, H₂O₂ can be catabolized by glutathione peroxidase (GPx) or catalase (CAT) to H₂O ⑨, or react with Fe²⁺ to form hydroxyl radicals ($\bullet OH$) via the Fenton reaction ⑩. ONOO⁻ can be degraded to nitrogen dioxide radical (NO₂ \bullet) and $\bullet OH$ (11), responsible for damaging lipids, proteins and DNA.

However, the distinction between direct and indirect antioxidants is complicated by a close reciprocal relation between these two types of agents, as is showed in the following examples (Dinkova-Kostova et al., 2007):

1. Whilst glutathione is the main protective direct antioxidant present in high concentrations (mM) in tissues, its rate of synthesis is controlled by γ -glutamyl cysteine ligase (GCL), a typical phase 2 enzyme that is upregulated by phase 2 inducers which are, by definition, indirect antioxidants. The complexity of this reciprocal relation is further enhanced by the mandatory participation of glutathione in activities of several antioxidant enzymes (glutathione peroxidase, glutathione-S-transferases, glutathione reductase).
2. At least one phase 2 enzyme, heme oxygenase-1 (HO-1) generates carbon monoxide and biliverdin/bilirubin, which are small direct antioxidant molecules.
3. Some direct antioxidants are inducers of the phase 2 response; e.g., the vicinal dithiol lipoic acid and reduced Michale reaction acceptors such as hydroquinones.

4. Phase 2 enzymes NADPH:quinone oxidoreductase-1 (NQO1) and glutathione reductase are responsible for regeneration of reduced and active forms of oxidized tocopherols, and ubiquinone and glutathione, respectively.

5. Indirect antioxidants induce a cytoprotective phase 2 response

Aerobic cells have developed an elaborated mechanism for their protection against oxidative stress, known as "phase 2 response" (Dinkova-Kostova & Talalay, 2008; Kensler et al., 2007; Kobayashi & Yamamoto, 2006; Motohashi & Yamamoto, 2004). Phase 2 response involves a group of genes that are regulated by a common molecular signaling pathway depending of the transcription factor Nrf2, and can be coordinately induced by a variety of synthetic and natural agents (Dinkova-Kostova et al., 2005a; Talalay, 2000). Extensive studies on chemistry of inducers have disclosed that all are chemically reactive without having common structural features (Dinkova-Kostova et al., 2004), and all react with sulfhydryl groups (Dinkova-Kostova et al., 2001) of highly reactive cysteine residues of Keap1, the cellular sensor that is integrally involved in the mechanism of induction (Itoh et al., 2003; Wakabayashi et al., 2004). The known inducers belong to at least nine chemical classes (Dinkova-Kostova et al., 2004): (i) diphenols, phenylenediamines and quinones; (ii) Michael reaction acceptors; (iii) isothiocyanates/dithiocarbamates; (iv) 1,2-dithiole-3-thiones/oxathiole oxides; (v) hydroperoxides; (vi) trivalent arsenicals; (vii) heavy metals; (viii) vicinal dimercaptans; and (ix) carotenoids.

It is now widely recognized that the up-regulation of the phase 2 response is a powerful, highly efficient and promising strategy for protection against several diseases including ischemic stroke (Alfieri et al., 2011; Talalay, 2000). Experimental evidence shows the powerful protective effects of phase 2 response: (i) its up-regulation protects cells, animals, and humans against a wide variety of damaging agents including ROS, RNS, carcinogens, electrophiles, and radiation (Kensler et al., 2007; Kobayashi & Yamamoto, 2006; Motohashi & Yamamoto, 2004; Talalay et al., 2007); (ii) when the phase 2 response is disrupted, cells are much more susceptible to oxidative damage; and (iii) numerous anticarcinogens have been identified and isolated from natural sources by bioassays that monitor induction of Nrf2-dependent enzymes such as NAD(P)H:quinone oxidoreductase (NQO1) (Kang & Pezzuto, 2004; Zhang et al., 1992).

5.1 Phase 2 proteins and enzymes

In the past, enzymatic protection against oxidants focused largely on classical enzymes such as SOD, CAT, and various types of peroxidases (Halliwell & Gutteridge, 1999), now this is changing. Phase 2 proteins were originally perceived as only promoters of xenobiotic conjugation with endogenous ligands (e.g., glutathione, glucuronic acid) to generate more water-soluble and easily excretable products. This restricted view of the nature and functions of phase 2 proteins and enzymes has gradually been expanded. Nowadays, several genes are considered part of the phase 2 response. Enzymes encoded by these genes have chemically versatile antioxidant properties, share common regulatory mechanisms, and are highly inducible by a variety of agents including dietary components (Ramos-Gomez et al., 2001; Talalay, 2000).

Phase 2 proteins catalyze diverse reactions that collectively result in broad protection against the continuous damaging effects of ROS, RNS and electrophiles. They are expressed

at low basal levels, but can be markedly elevated by various small molecules (indirect antioxidants).

Using an oligonucleotide microarray analysis, Lee et al. (2003a) reported that *tert*-butylhydroquinone (t-BHQ), a well known Nrf2 inducer, stimulated a group of genes responsible for conferring protection against oxidative stress or inflammation in primary cortical astrocytes. The major functional categories are detoxification enzymes, antioxidant proteins, NADPH-producing proteins, growth factors, defense/immune/inflammation-related proteins, and signaling proteins (Table 1). It has been proposed that proteins within these functional categories are vital to cell's defense system, suggesting that an orchestrated change in the modulation of Nrf2/ARE pathway would stimulate a synergistic protective effect.

Proteins and enzymes directly related with an antioxidant protective effect can be divided into 3 major groups (Lee et al., 2003a):

Group 1. Genes involved in glutathione (GSH) homeostasis. GSTs catalyze the nucleophilic addition of GSH to an electrophilic group of a broad spectrum of xenobiotic compounds. GPx and PRx metabolize H_2O_2 to H_2O and oxidized GSH (GSSG), and GR regenerates GSH. Ideally, in association with an increased utilization of GSH, there would also be an increased production of GSH. The rate-limiting step in the GSH biosynthesis is mediated by GCLM/GCLC. The coordinate regulation of these genes can evoke a synergistic effect in the maintenance of GSH levels, as well as in detoxification of reactive intermediates (Figure 5).

Group 2. Genes involved in H_2O_2 detoxification and iron homeostasis. SOD and HO-1 are very important for cellular defense against oxidative stress. SOD detoxifies O_2^{\bullet} resulting H_2O_2 , and HO-1 generates a potent radical scavenger, bilirubin. However, SOD and HO-1 can induce more oxidative stress because they increase the cellular concentrations of H_2O_2 and free iron, respectively; which together can generate $\bullet OH$ through the Fenton reaction. For complete detoxification of superoxide, H_2O_2 should be further metabolized to H_2O by GPx, CAT, or PRx. CAT directly detoxifies H_2O_2 , whereas PRx uses GSH (Figure 6) and/or thioredoxin (Trx) as an electron donor for peroxidation of H_2O_2 , resulting in generation of GSSG or oxidized thioredoxin, respectively (Figure 6). GSSG and oxidized thioredoxin are converted to their reduced forms by GR and TXNRD1, respectively. In addition, proper management of free iron is also important for minimizing oxidative stress, and this can be best achieved by ferritin. Ferritin converts Fe^{2+} to Fe^{3+} (ferroxidase activity) and sequesters it, thereby avoiding the participation of Fe^{2+} in the Fenton reaction (Orino et al., 2001). Thus, up-regulation of HO-1 together with ferritin constitutes a physiological strategy to increase the antioxidant potential while $\bullet OH$ formation is minimized.

Group 3. Genes involved in NADPH homeostasis. NQO1, GR, and TXNRD1 are important in detoxifying quinones and maintaining the cellular redox balance. One common feature of these proteins is the fact that they use NADPH as an electron donor. So, for efficient detoxification and maintenance of cellular redox status, it would be beneficial to up-regulate these proteins together with the appropriate reducing potential (NADPH) to support enzymatic reactions. G6PD/malic enzyme can directly generate NADPH, and transketolase/transaldolase can increase NADPH production by regenerating substrates for G6PD (Figure 7). These Nrf2-dependent genes would also contribute to cell's detoxification potential and cellular redox balance.

GENE	GENE	GENE
Detoxification	Antioxidant/reducing potential	Transcription
✓ NAD(P)H:quinone oxidoreductase-1 (NQO1) ^a	✓ γ -glutamate cysteine ligase modifier subunit (GCLM) ^a	✓ CCAAT/enhancer-binding protein- β
✓ Glutathione-S-transferase (GST) A4 ^a	✓ γ -glutamate cysteine ligase catalytic subunit (GCLC) ^a	✓ Zinc finger protein of cerebellum-2
✓ GST Pi2 ^a	✓ Hemo oxygenase-1 (HO-1) (decycling) ^a	✓ TG-interacting factor
✓ GST Mu1 ^a	✓ Thioredoxin reductase-1 (TXNRD-1)	✓ MafG
✓ GST Mu3 ^a	✓ Thioredoxin (Trx) ^a	✓ Activating transcription factor-4
✓ GST Omega1 ^a	✓ Ferritin light chain-1 ^a	Growth
✓ GST microsomal-1 ^a	✓ Ferritin H subunit ^a	✓ Proliferin
✓ UDP glycosyltransferase 1A6 ^a	✓ Type I peroxiredoxin (PRx)	✓ Proliferin-2
✓ Epoxide hydrolase-1 ^a	✓ 1-Cys PRx protein-2	✓ Nerve growth factor- β
✓ Aldehyde dehydrogenase-2	✓ Transferrin receptor	✓ Platelet-derived growth factor- α
✓ Aldehyde dehydrogenase-9	✓ Cu, Zn superoxide dismutase (CuZnSOD) ^a	Defense/immune/inflammation
✓ Aldehyde oxidase-1	✓ Catalase-1 (CAT)	✓ Macrophage C-type lectin
✓ Cytochrome P450 1B1	✓ Glutathione peroxidase-4 (GPx)	✓ EST, similar to dithiolethione-inducible-1
Signaling	✓ Glutathione reductase-1 (GR)	✓ PAF acetylhydrolase
✓ Protein kinase, cAMP-dependent regulatory, type I β	✓ Glucose-6-phosphate dehydrogenase (G-6PD), X-linked	✓ P lysozyme structural
✓ AW125016 4 1.9 0.07 NR	✓ G-6PDH-2	✓ Lysozyme M
✓ Mitogen-activated protein kinase-10	✓ Transaldolase-1	✓ Prostaglandin-endoperoxide synthase-2
	✓ Transketolase	✓ Matrix metalloproteinase-12
	✓ Solute carrier family-1/4	
	✓ Glycine transporter-	
	✓ Malic enzyme, supernatant ^a	

^aKnown to contain or to potentially have an ARE sequence.

Modified of Lee et al., 2003a.

Table 1. Nrf2-dependent genes induced by *tert*-butylhydroquinone in primary cortical astrocytes

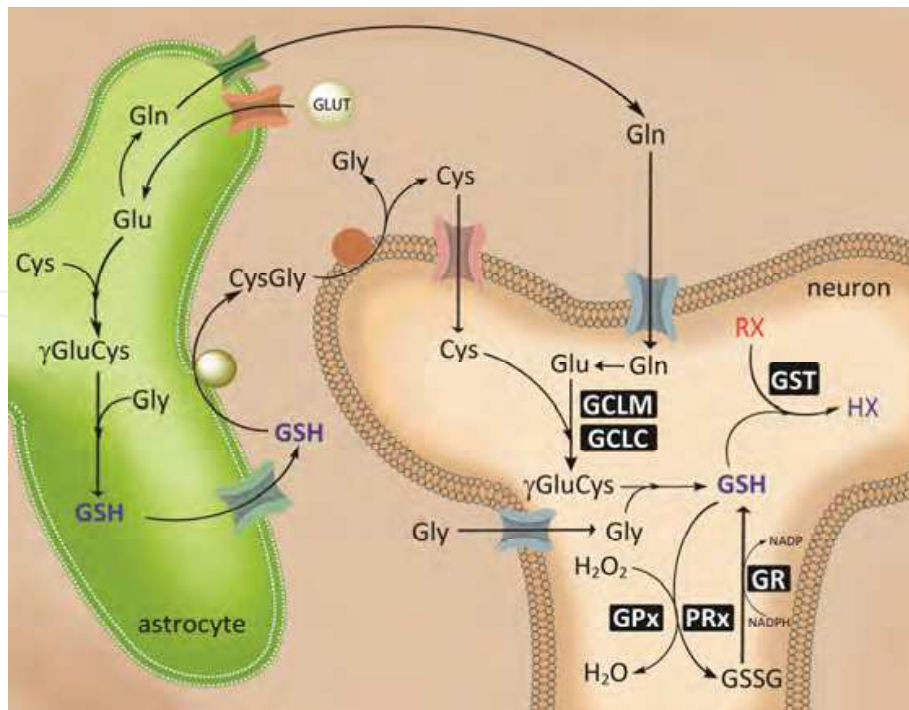


Fig. 5. Genes involved in glutathione (GSH) homeostasis are indicated in black boxes. GST, glutathione-S-transferase; GCLM, γ -glutamate cysteine ligase modifier subunit; GCLC, γ -glutamate cysteine ligase catalytic subunit; GPx, glutathione peroxidase; PRx, peroxiredoxin; GR, glutathione reductase.

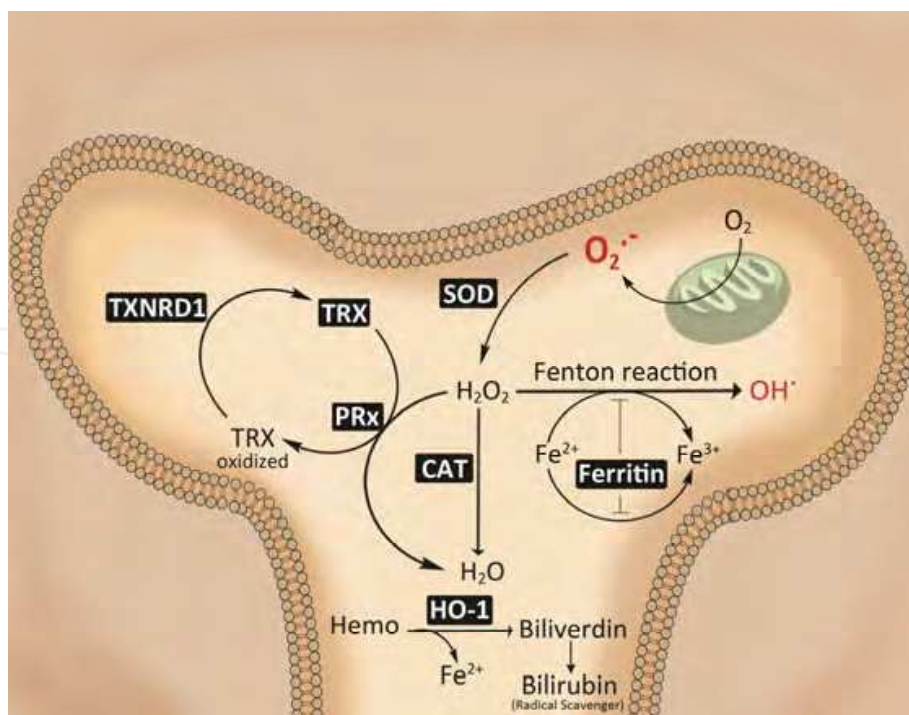


Fig. 6. Genes involved in H_2O_2 detoxification and iron homeostasis are indicated in black boxes. SOD, superoxide dismutase; CAT, catalase; PRx, peroxiredoxin; Trx, thioredoxin; HO-1, hemo oxygenase-1; TXNRD1, thioredoxin reductase-1.

Together, these coordinately regulated gene clusters presented in Figures 5, 6 and 7 strongly support the hypothesis that Nrf2-dependent gene expression is crucial for an efficient detoxification of reactive metabolites and ROS, as well as for the cellular capacity to counteract stressing events such as inflammation.

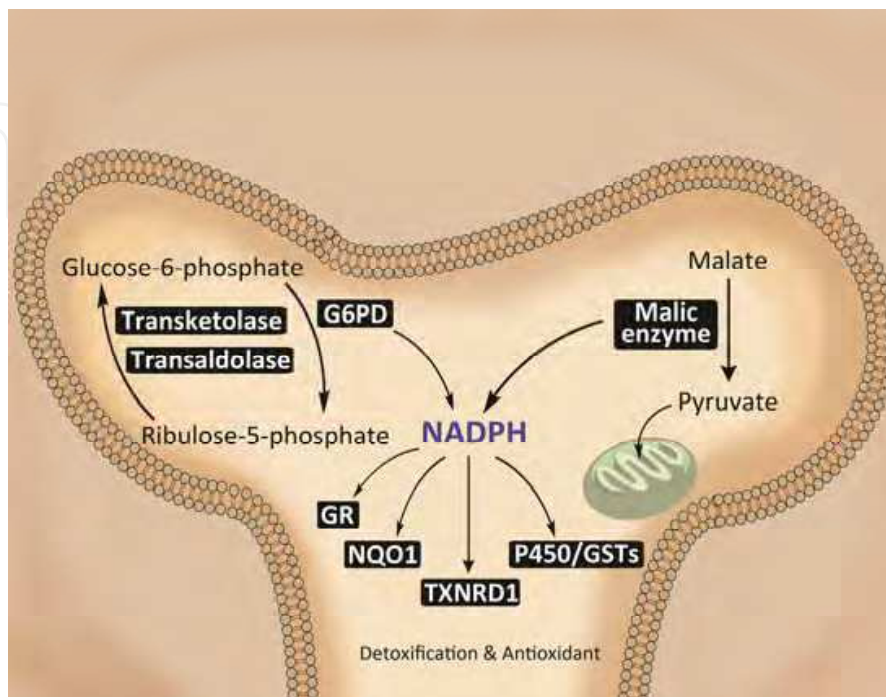


Fig. 7. Genes involved in NADPH homeostasis are indicated in black boxes. P450, cytochrome P450; GST, glutathione-S-transferase; TXNRD1, thioredoxin reductase-1; NQO1, NAD(P)H:quinone oxidoreductase-1; GR, glutathione reductase; G6PD, glucose-6-phosphate dehydrogenase.

6. Nrf2 characteristics

The transcription factor Nrf2 (Nuclear factor-E2-related factor 2) is the guardian of redox homeostasis because it regulates basal and inducible expression of array wide of antioxidant and cytoprotective genes, providing a level of protection required for normal cellular activities and against various oxidative stress-related pathologies, including ischemic stroke (Cho & Kleeberger, 2009; Nguyen et al., 2004; Van Muiswinkel & Kuiperij, 2005). Nrf2 is highly expressed in detoxification organs - such as liver and kidney - and organs exposed to the external environment - such as skin, lung and digestive tract - (Motohashi et al., 2002), whereas in the brain its levels are low (Moi et al., 1994).

Nrf2 is a member of the cap 'n' collar (CNC) family basic region-leucine zipper transcription factor (Katsuoka et al., 2005; Sykiotis & Bohmann, 2010). Nrf2 protein has six highly conserved regions, called Nrf2-ECH homology (Neh) domains. Neh1 is located in the half C-terminal of the molecule and constitutes the basic DNA binding domain and the leucine zipper for dimerization. Neh2 domain is located in the proximal N-terminus of Nrf2 and represents the region through which Nrf2 associates with the cytoplasmic protein Keap1 (kelch-like ECH-associated protein 1) (Itoh et al., 1999). Neh6 is a redox-insensitive degron, which is essential for maximal turnover of Nrf2 in stressed cells, as well as for its

degradation (McMahon et al., 2004). Neh3 domain is required for transcriptional activation of the protein (Nioi et al., 2005). Neh4 and Neh5 domains are required for its binding to ARE (Figure 8, upper panel).

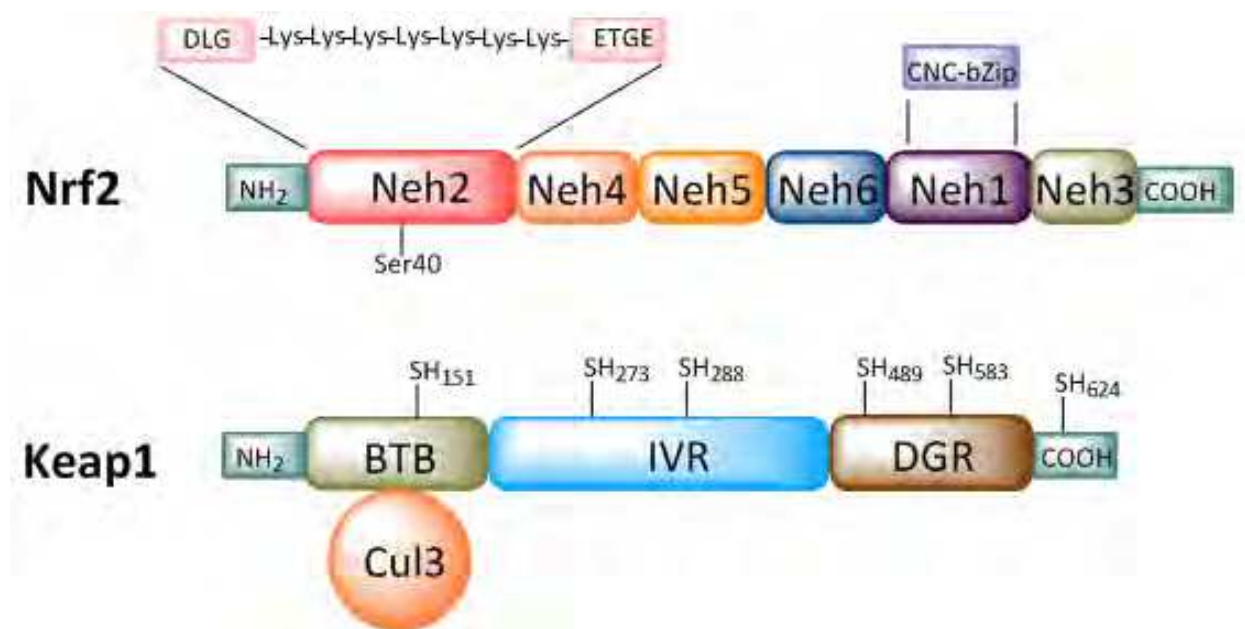


Fig. 8. Nrf2 and Keap1 domains. *Upper panel*: in Nrf2, Neh1 is the basic DNA binding domain and the leucine zipper for dimerization. Neh2 is the Keap1 (kelch-like ECH-associated protein 1) binding domain. Neh3 is required for transcriptional activation of the protein. Neh4 and Neh5 domains are required for the binding to ARE. Neh6 is essential for both Nrf2 turnover in stressed cells and for its degradation. *Lower panel*: in Keap1, BTB domain functions as a substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. IVR domain is a domain of intervention which is distinguished for its high number of cysteine residues. DGR domain is associated with actin filaments, giving stability to Keap1.

Under oxidant conditions, Nrf2 binds with high affinity to the *cis*-acting enhancer sequence called Antioxidant Response Element (ARE, 5'-GTGACnnnGC-3'), located in the 5'-flanking regions of a broad range of antioxidant and cytoprotective genes that act against oxidative/electrophilic damage (Nguyen et al., 2004; Rushmore et al., 1991). The binding of Nrf2 to ARE requires its heterodimerization with small Maf proteins (Katsuoka et al., 2005), which stimulates transcription of downstream genes, with participation of transcriptional co-activators - mainly CREB-binding protein (CBP) -, through the Neh4 and Neh5 domains (Figure 8, upper panel) in the transcription factor. These co-activators act synergistically to attain maximum its activity (Kato et al., 2001).

7. Regulation of Nrf2: Keap1 (ARE elements)

Nrf2 activity is primarily regulated by suppressor protein Keap1 (Figure 8, lower panel), a member of the BTB (Broad complex/Tramtrack/Bric-a-brac)-Kelch protein family (Cullinan et al., 2004), that under normal conditions (unstressed) forms a complex with Nrf2 within the cytosol. This complex is associated with actin filaments through its double glycine repeat

exhibit different affinity for Keap1; the affinity of ETGE is greater than DLG (Tong et al., 2006b). The term “hinge” indicates that the interaction of high affinity is not affected by inducers; in contrast, inducers abolish the low-affinity interaction mediated by the “latch”, thereby disrupting the presentation of Nrf2 to the ubiquitination machinery of Keap1 (Li & Kong, 2009) (Figure 10, right panel). Other models that describe the interaction between Nrf2 and Keap1 have provided conflicting information when contrasted with the “hinge and latch” model (Lo & Hannink, 2006; 2008).

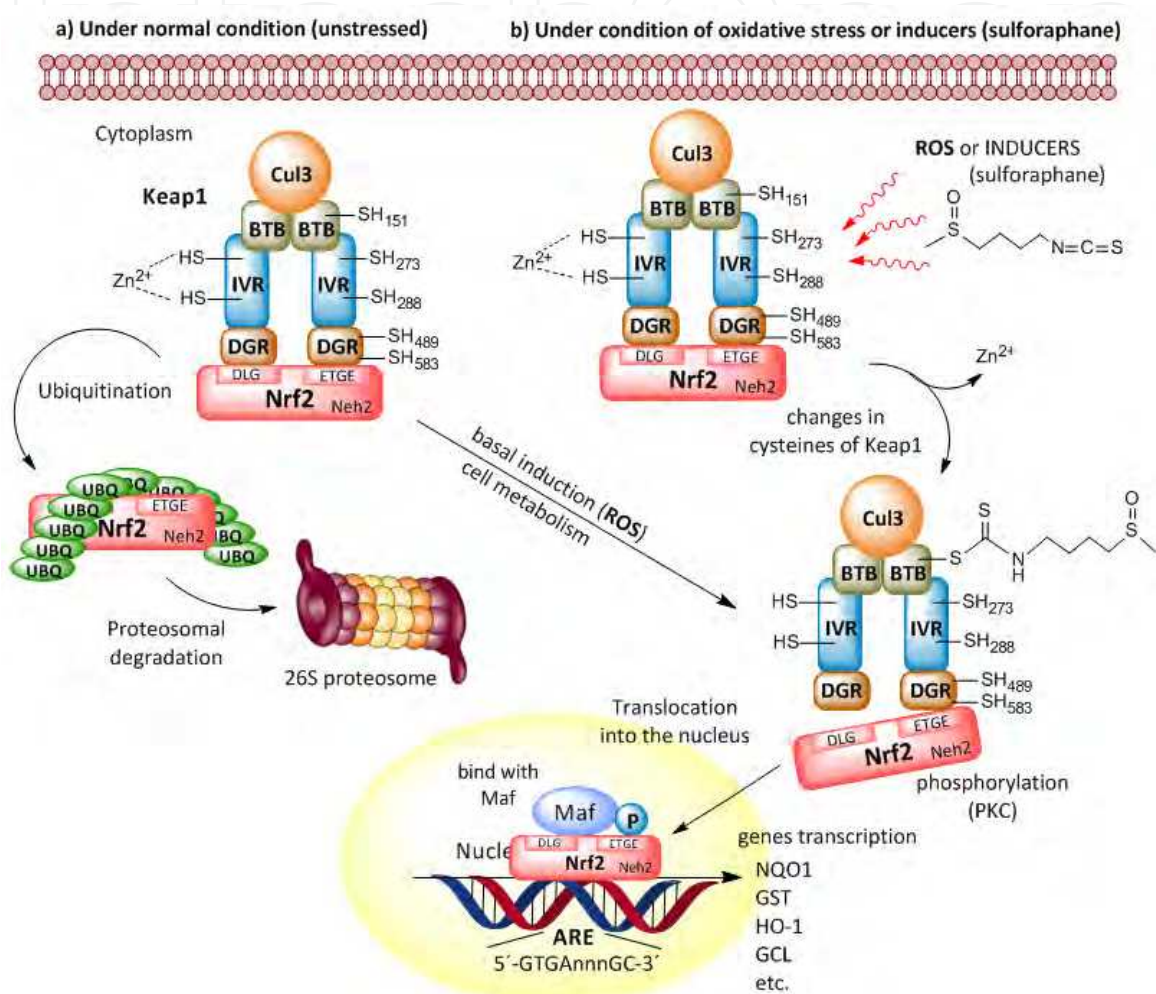


Fig. 10. Effect of sulforaphane on Nrf2/Keap1 complex. *Left panel:* Upon unstressed conditions, this complex is dissociated and Nrf2 can either suffer proteasomal degradation or respond to stimuli typical of basal cell metabolism. In the later, Nrf2 is phosphorylated and translocated to the nucleus forming heterodimers with Maf and acting on ARE. *Right panel:* Under stress oxidative conditions, or in the presence of inducers, several cysteine residues suffer changes inducing its Nrf2 dissociation and further translocation of this factor to nucleus, where it will induce phase 2 genes transcription.

Sulforaphane induces a phase 2 response as a result of gene expression modulation through Nrf2/ARE pathway. ARE-driven targets include NAD(P)H:quinone oxidoreductase (NQO1), heme oxygenase-1 (HO-1) and γ -glutamylcysteine ligase (γ GCL). The induction of these enzymes has been observed both in *in vivo* and *in vitro* experiments after sulforaphane treatment.

8. Nrf2 in cerebral ischemia

Nrf2 has been detected in neuronal and glial cells (Chen et al., 2011; Li et al., 2011; Shah et al., 2010; Yang et al., 2009). Previous studies using gel-shift assay found that ischemic brains selectively upregulate ARE-mediated gene expression, whereas binding activities of other stress response elements were unchanged, including metal response element, interleukin-6, and STAT (signal transducer and activator of transcription) response elements (Campagne et al., 2000).

Middle cerebral artery occlusion (permanent or transient) is a classical and well-characterized model inducing cerebral ischemia in rats that involves a cytotoxic response occurring within few minutes from the onset of cerebral ischemia, and encompasses oxidative stress, pro-inflammatory responses and cell death (Ikeda et al., 2003; Longa et al., 1989; Simonyi et al., 2005). Yang et al. (2009) used permanent focal ischemia to detect the expression of Nrf2. They found that Nrf2 protein and mRNA were upregulated when compared with normal control, showing a peak at 24 h and localizing with nuclei and cytoplasm of neurons and astrocytes. Alternatively, Nrf2 was presented in the injured regions of cortices with cerebral ischemic/reperfusion, and markedly increased in both cytoplasm and nuclei (Li et al., 2011). Meanwhile, Keap1 immunoreactivity was significantly reduced. Besides, an altered expression of thioredoxin, glutathione, and heme oxygenase was detected (Tanaka et al., 2011).

Oligemia is another model that was used to determine Nrf2 localization. It consists in a reduction in the mean arterial pressure to 30-40 mm Hg, resulting in a 50% reduction in cerebral blood flow after reperfusion. This blood flow reduction presents an increase in oxidative stress through lipid peroxidation (Heim et al., 1995; L  er et al., 1993) and an augmented $\cdot\text{OH}$ production during the reperfusion phase (Heim et al., 2000). In this model, Nrf2 was specifically upregulated 1 h after the surgery. Nrf2-positive neurons were found in the Purkinje cells of the cerebellar cortex and in the pyramidal neurons of the cingulate cortex (Liverman et al., 2004).

Additionally, Nrf2 knockout (Nrf2^{-/-}) mice have been used to understand the role of Nrf2 during ischemia-mediated oxidative brain insult.

In vitro studies showed that neurons and astrocytes from Nrf2 knockout (Nrf2^{-/-}) mice were more sensitive to oxidative stress, Ca²⁺ influx and mitochondrial toxicity than neurons and astrocytes from wild type animals; however, when the cells were transfected with a functional Nrf2 construct, they became less prone to oxidative stress (Kraft et al., 2004; Lee et al., 2003a; Lee and Johnson, 2004). Consistent with these results, dominant negative-Nrf2 stable cells and Nrf2-sensitized neuroblastoma cells silenced with siRNA were more amenable to apoptosis induced by nitric oxide (Dhakshinamoorthy & Porter, 2004). Also, increasing Nrf2 activity in mixed neuronal/glial cultures was highly neuroprotective in *in vitro* models that simulated components of stroke damage, such as oxidative glutamate toxicity, H₂O₂ exposure, metabolic inhibition by rotenone, and Ca²⁺ overload (Duffy et al., 1998; Kraft et al., 2004; Lee et al., 2003b; Murphy et al., 1991; Shih et al., 2003).

In vivo, using permanent middle cerebral artery occlusion by cauterization, Shih et al. (2005) did not observe significant difference in infarct size between Nrf2^{-/-} and Nrf2^{+/+} mice 24 h after stroke. However, 7 days after permanent focal ischemia, they observed a two-fold increase in infarct volume with Nrf2^{-/-} mice, while the infarct size of Nrf2^{+/+} mice did not increase in size between 24 h and 7 days. On the other hand, Nrf2 knockout (Nrf2^{-/-}) mice subjected to 90 min middle cerebral artery occlusion followed by 24 h reperfusion, showed

an infarct volume and neurological deficit significantly larger than in wild type mice (Shah et al., 2007).

Taking together, these data suggest that Nrf2 is upregulated in permanent ischemia and ischemic/reperfusion, an augment that is related with a decreased expression of Keap1 and an altered expression of antioxidant proteins. Thus, this upregulation may be due to an alteration in the redox state, a mechanism through which cells active an antioxidant response to protect themselves from future oxidant damage. Moreover, it has been demonstrated that Nrf2 activation induces the expression of the Nrf2 gene itself (Lee et al., 2005), indicating that the administration of Nrf2 inducers may be an important neuroprotective antioxidant mechanism that can limit stroke damage.

9. Effect of Nrf2 inducers in cerebral ischemia

A wide range of dietary phytochemicals or supplements with medicinal properties have been reported to activate adaptive stress responses related with the induction of cytoprotective genes through Nrf2 induction (Surh et al., 2008). The mechanism of action of such phytochemicals can therefore be considered as a form of hormesis where a stressor triggers an adaptive response which increases resistance to more severe stress and disease (Calabrese et al., 2007). Unfortunately, few of these compounds have been tested in brain ischemic models; some of them are sulforaphane, curcumin and ter-butylhydroquinone, among others.

Sulforaphane

Sulforaphane is a natural dietary isothiocyanate present in cruciferous vegetables of the genus *Brassica* such as broccoli, brussel sprouts, cauliflower, cabbage, etc. Several studies have shown the neuroprotective properties of sulforaphane against ischemia/reperfusion damage. It has been found that sulforaphane (5 mg/kg) reduced the cerebral infarct volume in a carotid/middle cerebral artery occlusion common model in rodents when it was administered 15 min after injury (Zhao et al., 2006). Other groups reported that an injection of sulforaphane (5 mg/kg) 30 min before the onset of ischemia reduced the infarct size in a neonatal hypoxia-ischemia model (Ping et al., 2010). In both studies, the protective effects of sulforaphane were associated with its well-known capacity to induce the expression of HO-1 mRNA and protein through Nrf2/ARE pathway.

Other *in vivo* studies support the ability of sulforaphane as inducer of phase II enzymes in brain increasing HO-1, NQO1 and GST mRNA levels (Chen et al., 2011). It has also shown in *in vitro* studies that pretreatment and post-treatment with sulforaphane reduced hippocampal death of astrocytes and neurons induced by transient exposure to O₂ and glucose deprivation. This protective effect was associated with nuclear accumulation of Nrf2 accompanied by an increase in NQO1, HO-1 and GCL mRNA levels, and a decrease in DNA oxidation (Danilov et al., 2009; Soane et al., 2010). Altogether, these studies indicate that sulforaphane could be considered as a useful tool for pre- and post-treatment of brain injury due its well-know capacity as inducer of Nrf2.

Curcumin

Curcumin is a diferuloylmethane derived from the rhizomes of turmeric (*Curcuma longa* Linn, Zingiberaceae) widely used in Indian curry with a favorable safe profile. Its chemopreventive effects have been related with its antioxidant and anti-inflammatory

properties (Surh & Chun, 2007; Thangapazham et al., 2006). However, its mechanism of action is still poorly understood.

Curcumin has a protective effect against neurodegeneration in cerebral ischemia through the preservation of the blood-brain barrier integrity, and a decrease of the ischemia-induced lipid peroxidation, mitochondrial dysfunction and anti-apoptotic effects (Sun et al., 2008).

Yang et al., (2009) observed that the systematic administration of curcumin (100 mg/kg) 15 min after middle cerebral artery permanent occlusion increased Nrf2 nuclear translocation and Nrf2 and HO-1 gene and protein levels at 24 h onset of reperfusion. Curcumin reduced neurologic deficit, brain edema and infarct volume at 24 h after stroke. These results show that curcumin maybe an effective therapeutic drug for the treatment of brain injury toward a potential mechanism of upregulation Nrf2/ARE pathway at gene and protein levels.

However, the bioavailability of curcumin is very limited due to poor absorption, rapid metabolism and quick systemic elimination. Moreover, it has a poor blood-brain barrier penetration following acute administration. To improve its bioavailability, pharmacokinetics and interaction with multiple viable targets, new curcumin derivatives are being synthesized (Lapchak, 2001).

tert-Butylhydroquinone (t-BHQ)

tert-butylhydroquinone (t-BHQ), a metabolite of the widely used food antioxidant butylated hydroxyanisole, has already been approved for human use (Food and Agriculture Organization of the United Nations/World Health Organization, 1999; National Toxicology Program, 1997). t-BHQ possesses an oxidizable 1,4 diphenolic structure that confers its potent ability to dissociate Keap1/Nrf2 complex (Van Ommen et al., 1992). T-BHQ can protect neuronal cells against the oxidative insult initiated by dopamine, H₂O₂, *tert*-butyl hydroperoxide, NMDA and glutamate (Duffy et al., 1998; Kraft et al., 2004; Li et al., 2002; Murphy et al., 1991; Shah et al., 2007).

Shih et al., (2005) determined the neuroprotective effect of tBHQ in ischemic injury in two different ischemia/reperfusion models - middle cerebral artery occlusion and endothelin-1 vasoconstriction - in rats and mice, using different routes of administration: intracerebroventricular, intraperitoneal, and dietary. Intracerebroventricular administration of t-BHQ (1 μL/h) during 3 days before rats were subjected to 1.5 h of ischemia and 24 h reperfusion showed a significant reduction of infarction in the cortex and a significant reduction in the neuronal scores. Intraperitoneal administration of t-BHQ (16.7 mg/Kg; 3 times/8h) 24 h before middle cerebral artery occlusion improved functional recovery up to 1 month after MCAO, showing a long-term benefit in ischemic damage and sensorimotor deficit. Nrf2^{+/+} and Nrf2^{+/-} mice fed with 1% t-BHQ during one week before permanent focal ischemia did not show changes in infarct area after 7 days, while Nrf2^{-/-} mice were less tolerant to the diet, losing 20% body weight and showing a continuous growth of infarct area, thus suggesting that loss of Nrf2 function promotes peri-infarct zone. Finally, Nrf2^{+/+} and Nrf2^{-/-} mice were fed with t-BHQ after endothelin-1 administration into cortical parenchyma. Nrf2^{+/+} mice showed a decrease in endothelin-1-induced infarction while Nrf2^{-/-} mice showed an exacerbated injury (Shih et al., 2003; 2005).

Collectively, these data suggest that t-BHQ may have a therapeutic potential for ischemic injury by increasing brain antioxidant capacity through the up-regulation of Nrf2 expression.

10. Presumable protective effect of garlic compounds in cerebral ischemia

Numerous studies have shown that garlic and its compounds exhibit a diverse biological activity, including anti-tumorigenic, anti-atherosclerosis, detoxification, anti-inflammatory, and antioxidant (Aguilera et al., 2010; Ali et al., 2000; Fisher et al., 2007; Fukushima et al., 1997; Mathew & Biju, 2008). The effect of different garlic preparations (aged garlic extract, aqueous garlic extract, garlic oil) and isolated compounds (S-allylcysteine) in cerebral ischemia, has been associated to its ability to scavenge ROS, acting as direct antioxidants (Kim et al., 2006a).

Gupta et al. (2003) found that garlic oil administration 90 min before the ischemia/reperfusion diminished the infarct area and associated this effect to its antioxidant properties. Saleem et al. (2006) showed that aqueous garlic extract treatment increased neurobehavioral score, decreased malondialdehyde levels, increased GSH content, and prevented the depletion in GPx, GR, GST and Na⁺/K⁺-ATPase activities. Moreover, CAT and SOD activities were increased by aqueous garlic extract. Aguilera et al. (2010) reported that the major protective effect exerted by aged garlic extract was observed when it was administered at the onset of reperfusion. In this work, aged garlic extract prevented the ischemia/reperfusion-induced increase in nitrotyrosine levels and the decrease in GPx, SOD and CAT activities both in cortex and striatum.

Numagami et al. (1996) demonstrated that aged garlic extract compounds that present a thioallyl group (particularly S-allylcysteine) exhibited a strong antioxidant capacity in a model of cerebral ischemia in rats. Indeed, S-allylcysteine reduced the infarct volume and brain edema, while prevented ONOO⁻ formation and lipid peroxidation (Numagami & Ohnishi, 2001). More recently, S-allylcysteine (300 mg/kg, i.p.) produced a protective effect on cerebral ischemic injury in rats due to the inhibition of extracellular signal-regulated kinase activity (Kim et al., 2006a). The fact that S-allylcysteine can cross the blood-brain barrier turned it soon of potential interest to be tested in neurotoxic models. In fact, the prophylactic impact and rescue properties of S-allylcysteine in ischemia/reperfusion injury are being recently discussed and reinforced (Sener et al., 2007). In addition, S-allylcysteine is a stable compound (Lawson, 1998) and is easily absorbed by gastrointestinal tract after oral administration (Kodera et al., 2002). One of its advantages in regard to other garlic compounds, such as allicin and diallyl sulfide, is its limited toxicity established by its higher lethal oral dose (Amagase et al., 2001). Pharmacokinetic studies demonstrate fast absorption and distribution phases followed by a slow elimination phase for oral administration, as well as fast distribution and slow elimination phases for i.v. administration (Nagae et al., 1994; Yan & Zeng, 2005). Pharmacokinetics of S-allylcysteine in humans by oral garlic administration revealed a half-life of 10 h and clearance time of 30 h (Kodera et al., 2002), suggesting a high bioavailability. After its oral administration, S-allylcysteine is absorbed by gastrointestinal tract, and its higher concentrations are detected in plasma and kidney up to 8 h post-intake (Nagae et al., 1994; Yan & Zeng, 2005).

On the other hand, garlic oil-derived organosulfur compounds such as diallyl trisulfide, diallyl disulfide, and diallyl sulfide provide significant protection against carcinogenesis, and this protection is likely related with their antioxidant properties (Maldonado et al., 2009). Moreover, the lipophilic characteristics of these compounds allow crossing the blood-brain barrier as follows: diallyl sulfide crosses the blood-brain barrier easier than diallyl disulfide > diallyl trisulfide > S-allylcysteine (Kim et al., 2006b).

Recently, it has been reported that some garlic compounds (diallyl trisulfide, diallyl disulfide, diallyl sulfide and S-allyl-L-cysteine) are able to activate Nrf2 factor in liver, kidney, intestine and lung. (Chen et al., 2004; Fisher et al., 2007; Fukao et al., 2004; Gong et al., 2004; Guyonnet et al., 1999; Kalayarasan et al., 2008; 2009; Wu et al., 2002). However, there is no information on Nrf2 induction by these garlic compounds in the brain.

Altogether, these data indicate that S-allyl-L-cysteine, diallyl trisulfide, diallyl disulfide, and diallyl sulfide may be alternative treatments for cerebral ischemia through Nrf2 upregulation.

11. Conclusion

Nowadays is widely recognized that up-regulation of phase 2 response is a powerful, highly efficient and promising antioxidant strategy for protection against several diseases, including ischemic stroke. A wide range of dietary phytochemicals with medicinal properties have been reported to activate adaptive stress responses related with the induction of cytoprotective genes through Nrf2/ARE pathway. Unfortunately, few of these compounds (sulforaphane, curcumin, ter-butylhydroquinone) have been tested in cerebral ischemia experimental models. Moreover, these compounds have characteristics that limit their use as therapeutic agents in ischemic stroke. For example, sulforaphane is expensive, while curcumin poorly crosses the blood-brain barrier. Due to this, new agents should be evaluated. In this context, some garlic compounds (diallyl sulfide, diallyl disulfide, diallyl trisulfide and S-allylcysteine) could be promising agents for treatment of ischemic stroke because their physicochemical properties are promising, their absorption is high and most of them can easily cross the blood-brain barrier. Moreover, they have the ability to active Nrf2 factor and induce a phase 2 response in several models of hepatic and renal damage.

12. Acknowledgements

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13. Abbreviation list

ARE	Antioxidant Response Element
BH2	Dihydrobiopterin
BH4	Tetrahydrobiopterin
CAT	Catalase
G6PD	Glucose-6phosphate dehydrogenase
GCLC	Glutamate cysteine ligase catalitic subunit
GCLM	Glutamate cysteine ligase modifier subunit
GPx	Glutathione Peroxidase
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
HO-1	Heme oxygenase-1
NQO1	NADPH:quinone oxidoreductase-1
Keap1	Kelch-like ECH-associated protein 1
Nrf2	Nuclear Factor-E2-related Factor 2
RNS	Reactive Nitrogen Species

ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
tBHQ	tert-butylhydroquinone
TXNRD1	Thioredoxine Reductase-1

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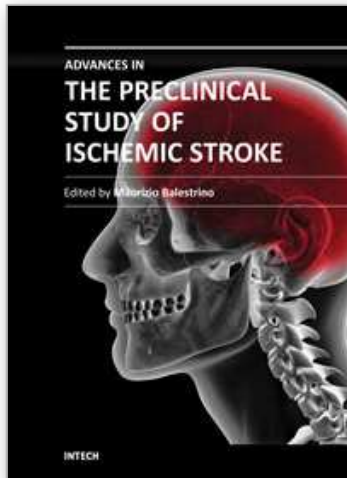
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This book reports innovations in the preclinical study of stroke, including - novel tools and findings in animal models of stroke, - novel biochemical mechanisms through which ischemic damage may be both generated and limited, - novel pathways to neuroprotection. Although hypothermia has been so far the sole "neuroprotection" treatment that has survived the translation from preclinical to clinical studies, progress in both preclinical studies and in the design of clinical trials will hopefully provide more and better treatments for ischemic stroke. This book aims at providing the preclinical scientist with innovative knowledge and tools to investigate novel mechanisms of, and treatments for, ischemic brain damage.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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