The role of Nrf2 in oxidative stress-induced endothelial injuries

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Abstract

Endothelial dysfunction is an important risk factor for cardiovascular disease, and it represents the initial step in the pathogenesis of atherosclerosis. Failure to protect against oxidative stress-induced cellular damage accounts for endothelial dysfunction in the majority of pathophysiological conditions. Numerous antioxidant pathways are involved in cellular redox homeostasis, among which the nuclear factor-E2-related factor 2 (Nrf2)/Kelch-like ECH-associated protein 1 (Keap1)–antioxidant response element (ARE) signaling pathway is perhaps the most prominent. Nrf2, a transcription factor with a high sensitivity to oxidative stress, binds to AREs in the nucleus and promotes the transcription of a wide variety of antioxidant genes. Nrf2 is located in the cytoskeleton, adjacent to Keap1. Keap1 acts as an adapter for cullin 3/ring-box 1-mediated ubiquitination and degradation of Nrf2, which decreases the activity of Nrf2 under physiological conditions. Oxidative stress causes Nrf2 to dissociate from Keap1 and to subsequently translocate into the nucleus, which results in its binding to ARE and the transcription of downstream target genes. Experimental evidence has established that Nrf2-driven free radical detoxification pathways are important endogenous homeostatic mechanisms that are associated with vasoprotection in the setting of aging, atherosclerosis, hypertension, ischemia, and cardiovascular diseases. The aim of the present review is to briefly summarize the mechanisms that regulate the Nrf2/Keap1–ARE signaling pathway and the latest advances in understanding how Nrf2 protects against oxidative stress-induced endothelial injuries. Further studies regarding the precise mechanisms by which Nrf2-regulated endothelial protection occurs are necessary for determining whether Nrf2 can serve as a therapeutic target in the treatment of cardiovascular diseases.

Introduction

The vascular endothelium synthesizes and releases multiple biologically active molecules that modulate vascular structure, vasodilation, vasoconstriction, and thrombolysis and form a natural barrier that maintains internal homeostasis. Vascular dysfunction elicits functional changes that lead to diminished nitric oxide bioavailability and the onset of cardiovascular disease (Tousoulis *et al.* 2013). Endothelial dysfunction is associated with the pathogenesis of common diseases. Oxidative stress, hypoxia, and flow disturbances are important factors that are related to endothelial dysfunction (Coleman *et al.* 2013).

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Reactive oxygen species (ROS) are free radicals and reactive metabolites that contain oxygen molecules with unpaired electrons. ROS exist primarily as singlet oxygen molecules ($1O_2$), oxygen free radicals ($O_2 \cdot^-$, $\cdot OH$, $\cdot HO_2$), peroxidases (H_2O_2, $\cdot ROOH$), and nitric oxides (NO). Endogenous sources of ROS include endothelial nitric oxide synthase (eNOS), xanthine oxidase, NADPH oxidase (NOX), and the mitochondrial respiratory chain. Exogenous sources include γ rays, ultrasonic waves, drugs, and pollutants. More than 90% of ROS are produced by the mitochondrial respiratory chain.

ROS are essential signaling molecules in the regulation of vascular homeostasis (Bachschmid *et al.* 2013). However, excessive ROS are a major cause of oxidative stress, the primary stimulus of vascular dysfunction. An initial consequence of increased ROS production is decreased NO availability, which results in decreased endothelium-dependent relaxation (Rochette *et al.* 2013). Excessive ROS generate large numbers of potentially harmful inter-mediates that cause cellular dysfunction and cell death resulting from alterations in metabolic activity, membrane structure, proteins, and DNA, which ultimately lead to imbalances between prooxidants and antioxidants that further result in aging and in numerous diseases. However, cellular evolution has enabled the development of adaptive antioxidant systems that scavenge excessive ROS. There are two types of antioxidants: enzymatic and nonenzymatic (or chemical). Enzymatic antioxidants are proteins, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase, and catalase (CAT), whereas chemical antioxidants include vitamins C and D and glutathione (GSH). Prooxidants include enzymes, such as NOX, cyclooxygenase 2 (COX2), and inducible nitric oxide synthase (iNOS) (Rajendran *et al.* 2014). When antioxidant activity is disrupted, it is no longer possible to maintain appropriate redox balance.

Nuclear factor-E2-related factor 2 (Nrf2), a transcription factor with a high sensitivity to oxidative stress, binds to antioxidant response elements (AREs) in the nucleus and promotes the transcription of a wide variety of antioxidant genes. Nrf2 is located in the cytoskeleton, adjacent to Kelch-like ECH-associated protein 1 (Keap1). Keap1 acts as an adapter for cullin 3 (Cul3)/ring-box 1 (Rbx1)-mediated ubiquitination and degradation of Nrf2, which decreases the activity of Nrf2 under physiological conditions. Oxidative stress causes Nrf2 to dissociate from Keap1 and to subsequently translocate into the nucleus, which results in its binding to AREs and the transcription of downstream target genes, including genes that encode antioxidants, detoxifying enzymes, antiapoptotic

proteins, and proteasomes (Niture *et al.* 2014). The aim of the present review is to briefly summarize the mechanisms that regulate the Nrf2 signaling pathway and the latest advances in understanding how Nrf2 protects against oxidative stress-induced endothelial injuries.

The origin and function of Nrf2

Moi *et al.* (1994) found that two basic leucine zipper (bZIP) transcription factor family members, Nrf2 and Nrf1, were powerful activators of RNA polymerase II. Several other family members, such as BTB–CNC, allogeneic Bach1 and Bach2 (Oyake *et al.* 1996), Nrf3 (Kobayashi *et al.* 1999), and p45-NFE2 (Pratt *et al.* 2002) have since been identified. Nrf2 is a leucine zipper/CNC protein, a polypeptide with a molecular weight of 66 kDa, and it is widely expressed in organs with hyperoxia consumption, such as the muscle, heart, vasculature, liver, kidney, brain, lung, skin, and digestive tract. Under normal conditions, Nrf2 remains in the cytosol at a low concentration. Under stressful conditions, Nrf2 translocates into the nucleus and serves as a transcription factor to maintain cellular redox homeostasis.

Nrf2 plays an important role in cellular resistance to oxidative stress and exogenous toxic substances, and it is closely linked to inflammatory reactions, respiratory system diseases, cardiovascular diseases, and malignant tumors. Following its translocation to the nucleus, Nrf2 heterodimerizes with Maf, JunD, c-Jun, and activating transcription factor 4 (ATF4) in a manner that is similar to other bZIP family members. It subsequently combines with AREs to trigger the transcription of more than 200 endogenous protective genes, including i) antioxidant genes, ii) phase II detoxification enzyme genes, iii) molecular chaperones, and iv) anti-inflammatory co-stimulating genes. These proteins play vital roles in strengthening cellular antioxidant defenses, and they protect tissues from harmful damage by exerting antitumor, anti-inflammatory, and antiapoptotic effects. Large amounts of data have demonstrated that the Nrf2–ARE pathway is one of the most powerful known intracellular antioxidative stress pathways.

The Nrf2/Keap1–ARE pathway

The protein structural domain of Nrf2

Nrf2 is the most potent member of the CNC transcription factor family, whose members share a highly conserved

bZIP structure. Studies have confirmed that Nrf2 is a polypeptide that contains 589 amino acid residues and six domains, Neh1–Neh6 (Nioi *et al.* 2005, Li *et al.* 2006, Satoh *et al.* 2006, Zhang *et al.* 2007, 2014*a*, Chowdhry *et al.* 2013), which are highly conserved among different species. Neh1 contains a bZIP motif through which Nrf2 interacts with Mafs and forms heterodimers with DNA sequences. Neh2 mediates the formation of heterodimers of Nrf2 and Keap1, the latter of which is the natural inhibitor of Nrf2 in the cytoplasm. Neh2 contains two motifs that combine with Keap1, an ETGE motif with strong affinity and a DLG motif with weak affinity. Neh2 also has a hydrophilic domain that is rich in lysine residues and is essential for Keap1-dependent ubiquitin-mediated degradation of Nrf2. The Neh3 domain is located at the carboxyl terminus of Nrf2. The Neh4 and Neh5 domains trigger the transcription of downstream ARE-dependent genes. The Neh4 and Neh5 domains combine with another transcriptional coactivator, CBP, which is involved in the regulation of Nrf2 transcription activation. The Neh6 domain is involved in non-Keap1-dependent regulation and degradation of Nrf2 (Fig. 1A). Because of the remarkable effects of Nrf2 on cell growth and apoptosis, DNA repair, inflammatory responses, and redox conditions, there is widespread interest in defining the factors and mechanisms that regulate its biological functions under physiological and pathological conditions. The discovery that Keap1 is the key negative regulator of Nrf2 represents an important milestone and the culmination of more than a decade of study and investigation.

The protein structural domain of Keap1

Under physiological conditions, Nrf2 is bound to its inhibitory protein, Keap1, and anchored to the actin cytoskeleton, which limits its transcriptional activity in the nucleus (Kansanen *et al.* 2013). Keap1 is a polypeptide composed of 624 amino acid residues and five domains: NTR (N-terminus), BTB/POZ, IVR, DGR, and CTR (C-terminus). The DGR domain contains six repetitive double-stranded glycine (Gly) sequences, the binding sites of both Nrf2 and actin. Keap1 contains two protein interaction motifs, BTB and Kelch, which are separated by the IVR domain (Fig. 1A and B). The BTB/POZ domain contributes to the formation of Keap1 homodimers, which are associated with Cul3/Rbx1–E3 ubiquitin ligase. Ubiquitin ligase, which is also known as E3 ubiquitin ligase, connects ubiquitin molecules to the lysine residues of proteins. Typically, ubiquitin ligase forms many ubiquitin chains and is degraded by the 20S catalytic subunit of the proteasome. The BTB/POZ domain has a highly conserved Ser104 residue, and mutations at this locus lead to disruptions in Keap1 homodimer formation, which weakens Nrf2 dissociation. The Kelch domain regulates the interaction between the ETGE motif and the DLG motif of the Neh2 domain. The IVR domain is rich in cysteine residues and is sensitive to electrophiles and external oxidation stressors. When it is exposed to oxidative stress, the IVR domain induces conformational changes that lead to the dissociation of Nrf2 from Keap1 (Fig. 1C).
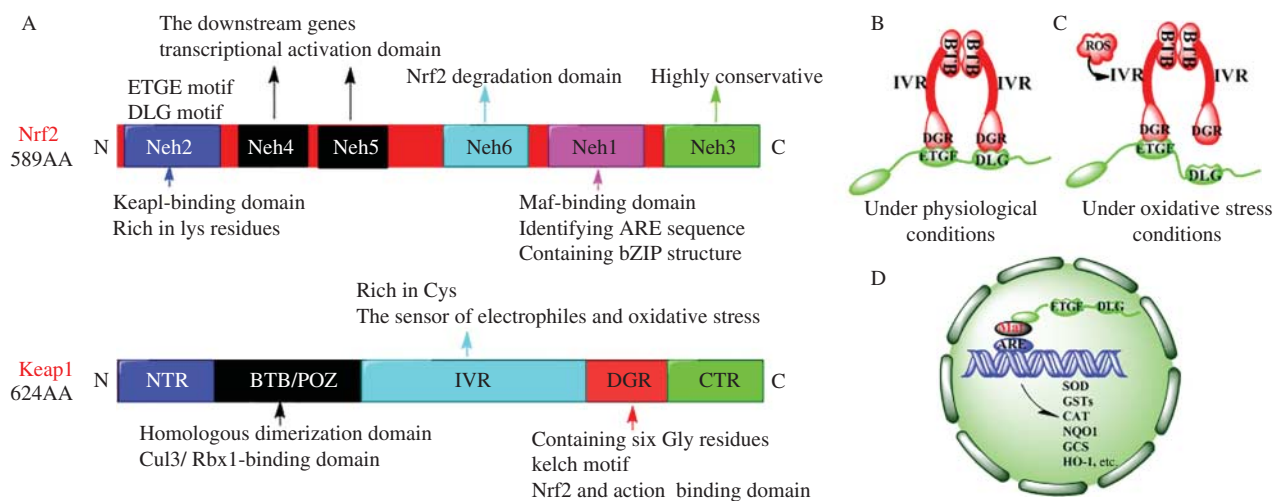
Figure 1

Nrf2 and Keap1 protein secondary structures. (A) The protein structural domains of Nrf2 and Keap1. (B) The spatial patterns of interaction between Nrf2 and Keap1 under physiological conditions. (C) The spatial patterns of interaction between Nrf2 and Keap1 under oxidative stress conditions. (D) The downstream target genes of the Nrf2–ARE pathway. A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-14-0662.

Keap1 is rich in cysteine residues, and there are 27 cysteines in human Keap1. Some of these cysteine residues are located near basic residues and are therefore prone to stimulation by electrophiles and oxidants. The modification of these cysteine residues by electrophiles is known as the cysteine code. The cysteine code hypothesis states that different Nrf2 activators act on different Keap1 cysteines. Cysteine modifications lead to conformational changes in Keap1, which disrupts the interactions between the Nrf2 DLG domains and the Keap1 Kelch domains, thereby inhibiting the polyubiquitination of Nrf2. The functional importance of Cys151, Cys273, and Cys288 has been established: Cys273 and Cys288 are required for the suppression of Nrf2, and Cys151 is required for its activation (Kansanen *et al.* 2013).

Antioxidant response elements

AREs with core sequences of TGA****GC are specific DNA-promoter sequences that are located at the 5′-terminal ends of the promoter sequences for SOD, CAT, GST, HO-1, and NQO1. The sequences are activated by a variety of electrophiles and oxidants, and they trigger the expression of phase II detoxification enzymes and antioxidant enzymes. Nrf2 is the most important activator of AREs. Under oxidative stress conditions, Nrf2 dissociates from Keap1, translocates into the nucleus, combines with the Maf protein to form a heterodimer, and recognizes the appropriate ARE sequence. ARE-mediated gene transcription is subsequently activated. This is the Nrf2/Keap1–ARE pathway. The Nrf2–ARE pathway inhibits Nrf2 degradation mediated by the ubiquitin proteasome, stabilizes cytoplasmic Nrf2 protein concentrations, promotes Nrf2 nuclear translocation, and increases Nrf2 transcriptional activity. The activation of Nrf2 forms a positive feedback loop. Nrf2 is a key transcription factor that regulates cells in response to invaders and oxidative damage. Degradation and inhibition of Nrf2 causes cells to become more sensitive, which then leaves them vulnerable to damage, even in low-stress environments. The Nrf2–ARE pathway is involved in a wide range of cellular protective functions, because it has antitumor, antioxidant, antiapoptotic, anti-inflammatory, and anti-atherosclerotic effects. Downstream genes regulated by the Nrf2–ARE pathway are presented in Table 1.

Two models to describe the interaction between Nrf2 and Keap1

Aside from endothelial injuries caused by oxidative stress, cellular antioxidant defenses depend primarily on Nrf2 dissociation from Keap1 and its subsequent translocation to the nucleus, where the activation of antioxidant genes occurs. Under physiological conditions, a homodimer composed of two Keap1 molecules combines with one Nrf2 molecule, which serves as an adaptive substrate for E3 ubiquitin ligase and promotes Nrf2 ubiquitination and 26S proteasome degradation (Fig. 2A). Nrf2 regulation depends primarily on the interaction between Nrf2 and Keap1. Under stressful conditions, the following two models describe the possible mechanisms that underlie this interaction: the Cul3 dissociation model and the 'hinge and latch' model (Fig. 2D and E). In the dissociation model, ROS and electrophiles result in the oxidation of Keap1 cysteine residues, which in turn causes a conformational change and the subsequent dissociation of Nrf2 from Keap1. This is the most commonly accepted explanation of how Nrf2 activation occurs (Bryan *et al.* 2013). The 'hinge and latch' model represents an alternative means by which this activation may occur. In this model, the DGR domains of the Keap1 homodimer combine with the DLG and ETGE domains of Nrf2 in the cell cytoplasm in preparation for ubiquitin-mediated degradation. The ETGE domain of Nrf2 remains connected to the DGR domain of the Keap1 DGR domain because of its higher affinity; therefore, Nrf2 is not recognized by ubiquitin ligase. Because the Keap1 binding site remains occupied by Nrf2, newly produced Nrf2 molecules are unable to bind with Keap1, so they instead enter the nucleus and combine with AREs. This ultimately results in the expression of downstream target genes (Kansanen *et al.* 2013).

Regulation of the Nrf2–ARE pathway

It is well known that the Michael addition reaction is involved in Nrf2 activation. Many chemical and phytochemical agents react with thiol groups and induce the phase II response through their reactivity with critical cysteine thiols of Keap1. Liu *et al.* (2008) reported that the anti-inflammatory and antioxidant potencies of a series of triterpenoids with Michael reaction centers were closely correlated with the potencies of these agents to induce the phase II response.

Chemopreventive flavonoids that promote the expression of NQO1 (Wang *et al.* 2015a) and HO-1 (Maydt *et al.* 2013) are triggered by the Nrf2–Keap1 signaling pathway and are initiated by the addition of chalcones to thiol groups of Keap1 via a Michael-type reaction. In addition to sulforaphane (SF), other electrophiles, including many Michael reaction acceptors, induce aldehyde

Table 1 Downstream genes regulated by the Nrf2–ARE pathway

Gene	Model	Up/down-regulation	References
Phase II detoxifying enzymes			
Glutathione *S*-transferase (GST)	Rat hepatocytes with ʟ-methionine starvation	Up	Lin *et al*. (2012)
NAD(P)H quinone oxidoreductase 1 (NQQ1)	Human intestinal epithelial LS180 cells	Up	Satsu *et al*. (2012)
UDP-glucuronosyl transferases (UDPGTs) UDP-glucuronic acid synthesis enzymes	Mouse liver (Nrf2-null, Keap1-knockdown mice)	Up	Wu *et al*. (2012)
Epoxide hydrolase 1 (Eh1)	Liver injury mediated by ROS	Up	Cornejo *et al*. (2013)
Aflatoxin aldehyde reductase (AAR)	Ischemia/reperfusion tridecemlineatus	Up	Morin *et al*. (2008)
Aflatoxin B1 aldehyde reductase	Hepatoma rat	Down	Kwak *et al*. (2001)
Heme oxygenase-1 (HO-1)	Caco-2 cells	Up	Zhai *et al*. (2013)
Antioxidant enzymes			
Gamma-glutamine cysteine synthase (γ-GCS)	Rats with chronic obstructive pulmonary disease	Up	Liu *et al*. (2012)
Superoxide dismutase (SOD)	HepG2 hepatoma cells	Up	Krajka-Kuzniak *et al*. (2015)
Catalase (CAT)	Hepatic injury mice induced by ᴅ-gal	Up	Yu *et al*. (2015)
Glutathione reductase (GR)	Copper-induced ctenopharyngodon idella	Up	Wang *et al*. (2015*b*)
Thioredoxin reductase (TR)	Green tea polyphenol (−)-epigallo-catechin-3-gallate triggered hepatotoxicity in mice	Up	Wang *et al*. (2015*c*)
Peroxiredoxin (Prx)	Preeclampsia (PE)	Down	Acar *et al*. (2014)
Glutathione peroxidase (GPx)	Cerebral ischemia/reperfusion rats	Down	Liu *et al*. (2015)
Molecular chaperones and proteases			
Heat shock protein 70 (HSP70)	Ethanol-induced human hepatic L02 cells	Up	Yao *et al*. (2015)
HSP90	6-OHDA-induced PC12 cells	Up	Alani *et al*. (2015)
HSP60	Tetrafluoroethylcysteine-induced cytotoxicity	Up	Ho *et al*. (2005)
HSP40	Methionine-deprived human cells	Up	Hensen *et al*. (2013)
Bip	Type 2 diabetic patients	Up	Mozzini *et al*. (2015)
20S proteasomes	Mammals, *Caenorhabditis elegans*, and *Drosophila melanogaster*	Up	Pickering *et al*. (2013)
DNA repair enzymes			
Poly (ADP-ribose) polymerase 1 (PARP1)	Fibroblasts	Up	Wu *et al*. (2014)
Flap endonuclease1 (Fen1)	Breast cancer	Down	Chen *et al*. (2014)
8-Oxoguanine glycosylase 1 (OGG1)	Estrogen-induced breast cancer	Down	Singh *et al*. (2013)
MRN complex	Whole-body exposure to low linear energy transfer (LET) ionizing radiations (IRs) damages	Up	Anuranjani & Bala (2014)
Anti-inflammatory response proteins and others			
Cyclooxygenase 2 (COX2)	DMBA mammary carcinogenesis	Down	Mandal & Bishayee (2015)
Nuclear factor-kappa B (NF-κB)	Oxidized LDL-induced monocyte adhesion	Down	Huang *et al*. (2015)
HO-1	Murine hippocampal and microglial cells	Up	Im *et al*. (2015)
iNOS	Lipopolysaccharide-induced mouse macrophage	Down	Ye *et al*. (2014)
Ferritin	AML cells	Up	Valenzuela *et al*. (2014)

dehydrogenase and parallel their activities in inducing NQO1, which is also Nrf2 dependent (Ushida & Talalay 2013). In contrast, tetrahydrocurcumin, which lacks a Michael reaction acceptor, was shown to have no effect on HO-1 expression, ARE activation, or vascular smooth muscle cell (VSMC) growth inhibition (Pae *et al*. 2007).

Phosphorylation plays a crucial role in the regulation of most transcription factors. Multiple protein kinases are involved in Nrf2 regulation as a result of their participation in Nrf2 phosphorylation. MAPK, protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), GSK3β, and casein kinase 2 (CK2) participate in
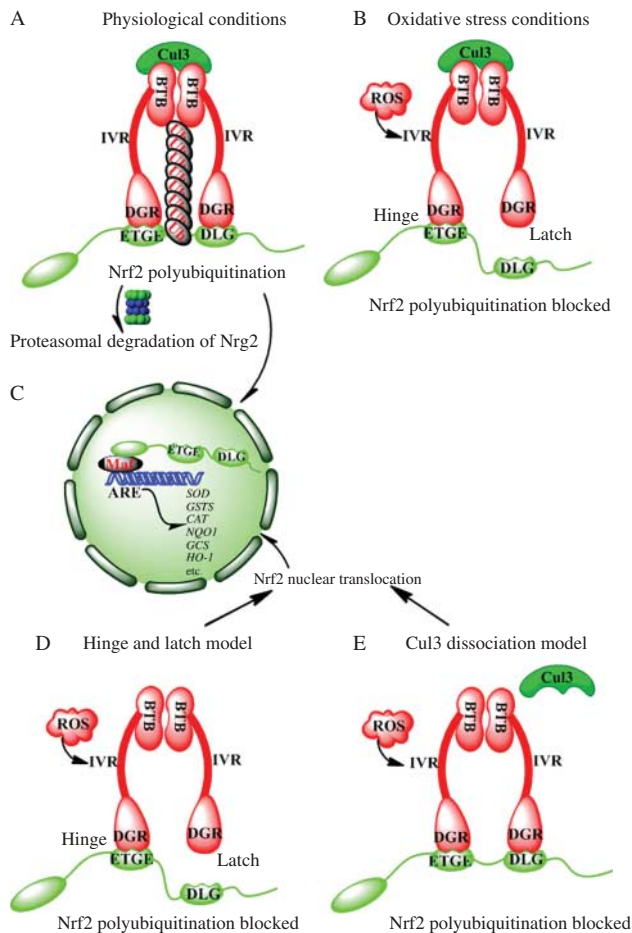
Figure 2

Two models to describe the interaction between Nrf2 and Keap1 under stress conditions. (A) The Nrf2–Keap1 complex under physiological conditions. (B) The Nrf2–Keap1 complex under oxidative stress conditions. (C) The downstream target genes of the Nrf2–Keap1 pathway, including *SOD*, *NQO1*, *HO-1*, *CAT*, *GCS*, *GSTS*, etc. (D) The 'hinge and latch' model under stress conditions. (E) The 'Clu3 dissociation model' under stress conditions. A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-14-0662.

the activation of the Nrf2–ARE pathway and regulate the expression of its downstream target genes (Fig. 3). Other protein partners, such as p21 and caveolin-1, as well as microRNA molecules such as microRNA-144, -28, and -200a, have been shown to affect Nrf2 activation and nuclear translocation by different means (Bryan *et al.* 2013).

Numerous reports indicate that PKC has the ability to activate Nrf2 both inside and outside of cells. PKC-mediated Nrf2 phosphorylation may be a key step in Nrf2 nuclear translocation. The PKCδ inhibitor, rottlerin, significantly inhibits ARE activation, as well as HO-1 expression, but the nonspecific PKCα inhibitor, GO6976,

does not affect these processes. Nonselective PKC agonists such as phorbal esters increase Nrf2 and HO-1 expression, which proves that PKCδ rather than PKCα activates Nrf2 (Zhang *et al.* 2013).

MAPKs are a group of Ser/Thr protein kinases that are activated by a variety of signals and are involved primarily in cell growth, differentiation, and adaption under conditions in which cells are exposed to a variety of external stimuli. The MAPK family consists of ERK, JNK, and p38. Rodriguez-Ramiro *et al.* reported that Nrf2 nuclear translocation involves the activation of two signaling proteins, ERK and p38. Utilizing ERK- and p38-specific inhibitors reduces Nrf2 nuclear translocation. These results suggest that ERK and p38 participate in the regulation of Nrf2 (Rodriguez-Ramiro *et al.* 2012).

The PI3K–Akt signaling pathway is involved in the regulation of cell migration, proliferation, and survival. PI3K is associated with Ser/Thr kinase activity and PI3K activity. The PI3K inhibitor, LY294002, has been shown to inhibit the transcription of ARE genes, whereas over-expression of PI3K activates ARE downstream target genes in a dose-dependent manner (Cong *et al.* 2013). Additional research has shown that the activation of PI3K may result in cytoskeletal reorganization (Koriyama *et al.* 2013) and may increase intracellular Ca^{2+} concentrations (Henke *et al.* 2012), which is an important step in Nrf2 nuclear translocation. PI3K- and PKC-specific inhibitors have been shown to inhibit Nrf2 and downstream HO-1 expression and Akt phosphorylation; this illustrates that CaS protects against oxidative stress as a result of HO-1 activation, which in turn is mediated by Nrf2 and is originally triggered by the PI3K–Akt pathway (Nguyen *et al.* 2013).

CK2 is also involved in the transcriptional regulation of Nrf2. CK2 interacts with two phosphorylated forms of Nrf2, specifically Nrf2-118 and Nrf2-98, the latter of which has transcription activity, which makes it easier for it to degrade (Pi *et al.* 2007). Additionally, glycogen synthesis kinase 3 (GSK3; Chowdhry *et al.* 2013, Mobasher *et al.* 2013) and RNA-dependent protein kinase endoplasmic reticulum enzyme also phosphorylate Nrf2 (Zhang *et al.* 2014*b*).

Tyrosine kinase Fyn is an Nrf2 suppressor. GSK3β modulates Fyn-mediated nuclear exportation of Nrf2 by phosphorylating Fyn threonine residues, which results in the accumulation of Fyn in the nucleus. Subsequently, Fyn induces Nrf2 Tyr568 residue phosphorylation and promotes Nrf2 removal from the nucleus to the cytoplasm, which results in its binding with Keap1 and in ubiquitin-mediated degradation of Nrf2 (Jain & Jaiswal 2007, Niture *et al.* 2014). In addition, nuclear

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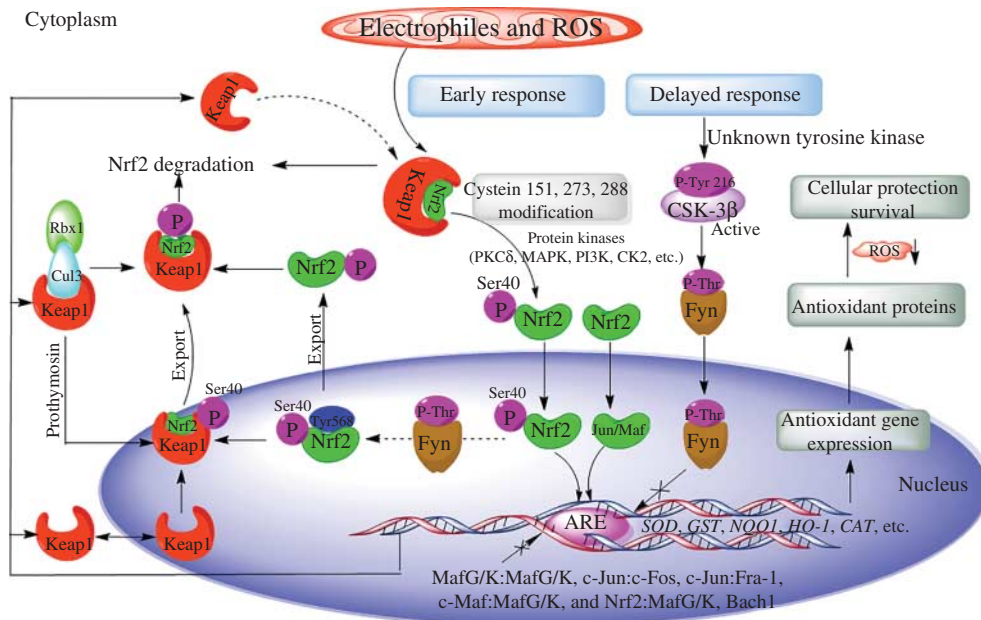
Figure 3

The mechanism of Nrf2 signaling in regulating the activation of ARE-mediated antioxidant gene expression, such as the expression of *SOD*, *GST*, *NQO1*, *CAT*, *HO-1*, etc. PKCδ and Jun/Maf are positive regulators of ARE-mediated gene expression. Cytosolic Keap1/Cul3/Rbx1 complex, nuclear MafG/K:MafG/K complex, c-Jun:c-Fos complex, c-Jun:Fra-1 complex, c-Maf:MafG/K complex, Nrf2:MafG/K complex, and Bach1 are negative regulators of ARE-mediated gene expression. Cys273 and Cys288 residues in Keap1 are required for the suppression of Nrf2, and Cys151 is required for the activation of Nrf2 by electrophiles and ROS. A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-14-0662.

MafG/K:MafG/K, c-Jun:c-Fos, c-Jun:Fra-1, and Nrf2:MafG/K (Jaiswal 2004) and Bach1 (Ho *et al.* 2013) are negative regulators of ARE-mediated gene expression.

Nrf2 in oxidative stress-induced endothelial dysfunction

The mechanism of oxidative stress-induced endothelial dysfunction

There is a large amount of evidence to suggest that endothelial dysfunction is the initial step in the pathogenesis of several cardiovascular diseases. Oxidative stress induced by hypertension, hypercholesterolemia, diabetes mellitus, aging, obesity, and smoking strongly correlates with endothelial dysfunction. The balance between NO, an endothelium-derived vasodilator, and ROS modulates endothelial function. Increased inactivation of NO, as well as decreased NO production by ROS, reduces NO bioavailability. This results in the generation of more ROS within vessels, which initiates a vicious cycle that impairs endothelial function. Decreases in NO production appear to be related to diminished activity of the PI3K–Akt pathway under pathologic conditions. The PI3K–Akt pathway causes intracellular calcium-independent eNOS phosphorylation and activation. Additionally, decreased levels of L-arginine, which acts as a substrate for the eNOS, contribute to reduced NO production. ROS activate membrane oxidases, which results in an increased level of asymmetric dimethylarginine, a derivative of arginine that competes for the active sites on eNOS and L-arginine transporters (Chien *et al.* 2014). In terms of normal ROS generation, numerous oxidase enzymes, such as NOX, xanthine oxidase, uncoupled eNOS, COX, glucose oxidase, and lipoxygenase, are engaged in this process. There are seven isoforms of NOX in mammals that are expressed in different cells, and NOX4 is the prominent isoform in endothelial cells (Konior *et al.* 2014). The mitochondrial electron transport chain is another important part of ROS generation. Small amounts of ROS are normally produced by mitochondrial respiration, and they play an important role in cellular processes, such as cell cycle and inflammatory responses. Several mechanisms have been linked to excess ROS generation in the vasculature. These include: i) NADH/NOX may activate via the up-regulation of $p22^{phox}$ mRNA expression, which is the predominant source of ROS in the vasculature (Zuo *et al.* 2014). ii) ROS may be produced by eNOS as a

result of tetrahydrobiopterin (BH4) deficiency, because BH4 is an essential cofactor for eNOS. Additionally, ROS degrades BH4 and exacerbates this deficiency (Mangge *et al.* 2014). iii) ROS superoxide may bind to NO to form a highly reactive intermediate, peroxynitrite ($ONOO^-$). $ONOO^-$ is involved in the activation of NADH/NOX, which in turn impairs eNOS function and influences the generation of other endothelial mediators (Forstermann & Li 2011). iv) Mitochondria are the predominant intracellular sites of ROS production as a natural byproduct of oxidative phosphorylation, which may be a result of the incomplete reduction of oxygen at sites of respiratory complexes I and III (Kuznetsov *et al.* 2011). In high glucose conditions, such as diabetes, the mitochondrial electron transfer in complex III is blocked when electron transport exceeds the threshold. These electrons then escape the electron transport chain to reduce molecular oxygen to superoxide (Newsholme *et al.* 2007). v) Antioxidant enzymes such as SOD, GPx, and CAT scavenge ROS in the vasculature. However, excess ROS, particularly free radicals, various oxidize molecules, lipid peroxidation, and protein oxidation, induce the overexpression of redox genes and pro-inflammatory genes, which results in intracellular calcium overload and DNA fragmentation as well as vascular smooth-muscle proliferation, inflammation, thrombosis, apoptosis, and vasculature remodeling. These damage VSMCs, endothelial cells, and myocardial cells (Darley-Usmar & Halliwell 1996).

The role of Nrf2 in protecting against oxidative stress-induced endothelial dysfunction

Physiological antioxidant defense mechanisms include multiple ROS scavenging enzymes, phase II detoxification enzymes, and other antioxidants: each of these bears AREs in its promoter regions. Nrf2 is a critical transcription factor that targets multiple ARE-regulated antioxidants. It is plausible that Nrf2 plays a crucial role in protecting the endothelium from ROS-related injuries. In the past decade, large amounts of evidence have indicated that Nrf2-driven free radical detoxification pathways are physiologically important endogenous homeostatic mechanisms of vasoprotection in the setting of aging, atherosclerosis, hypertension, diabetes, ischemia, and smoking-related cardiovascular diseases. In endothelial cells, Nrf2 is activated by laminar shear stress via an elevated ROS level and PI3K–Akt signaling pathway (Chen *et al.* 2003). HO-1, NQO1, GST, and Trx are some of the most important target genes in Nrf2/ARE-linked vasoprotective regulation. Chen *et al.* (2011) reported that

the expression of Nrf2 in human aortic endothelial cells resulted in marked increases in ARE-driven transcriptional activity. Increased protein levels of HO-1, GPx, and GSH intracellularly protected endothelial cells from H_2O_2-mediated cytotoxicity. An adenovirus-Nrf2 infection also suppressed tumor necrosis factor alpha, and interleukin 1 beta (IL1β) induced MCP1 and VCAM1 expression in both endothelial and mesangial cells, which suggests its potential as an anti-inflammatory agent (Chen *et al.* 2006). However, when blood flow becomes oscillatory under high flow rates, stenosis, or vessel branching, shear stress on the vascular wall is disturbed, which results in reduced NO production and promotes superoxide release (Hosoya *et al.* 2005). This diminishes the Nrf2-mediated activation of ARE-linked genes and predisposes the endothelium to a proathrogenic situation (Cheng *et al.* 2011). In respect to ROS origination, accumulated evidence shows that mitochondrial ROS may be critical for triggering Nrf2 activation, although the interaction between mitochondria and Nrf2 still remains unclear and needs further investigation. Recent evidence suggests that the Nrf2–Keap1 complex may be tethered on the mitochondrial outer membrane by a mitochondrial-located protein PGAM5 and may directly sense ROS that are released from mitochondria (Lo & Hannink 2008). In endothelial cells, specific mitochondrial ROS scavengers dramatically abrogate shear-induced HO-1 expression via the Nrf2–ARE pathway.

GSH is a well-known intracellular scavenger of free radicals with detoxification, and it can be biosynthesized in the body from the amino acids L-cysteine, L-glutamic acid, and glycine (Newsholme *et al.* 2011). GSH reduces disulfide bonds formed within cytoplasmic proteins to cysteines by serving as an electron donor. In the process, GSH is converted to its oxidized form glutathione disulfide. The ratio of reduced GSH to oxidized GSH within cells is often used as a measure of cellular toxicity (Newsholme *et al.* 2007). Protecting endothelial cells under oxidative stress by the GSH system is a key method for treating cerebrovascular disease, neurodegenerative diseases, and endothelial cell dysfunction. Nrf2 is able to promote cellular defenses against the cytotoxic ROS by regulating the transcription of antioxidant genes, including the catalytic subunit of glutamylcysteine ligase (GCLC), a rate-limiting enzyme that reduces GSH biosynthesis (Song *et al.* 2014). Similar impairments in Nrf2–Keap1–GCLC have been observed in endothelial cells that were exposed to high glucose and in retinas from donors with diabetic retinopathy (Zhong *et al.* 2013). Moreover, in diabetic retinopathy, histone methylation

at GClC–ARE4 plays an important role in regulating the Nrf2–GClC–GSH cascade (Mishra *et al.* 2014). In addition, GSH prevents the apoptosis of endothelial cells in response to oxidative stress. It has been reported to prevent free fatty acid-induced HBVEC apoptosis by activating the Akt pathway (Zhou *et al.* 2013).

Several *in vitro* experiments involving human umbilical vein endothelial cells (HUVECs), human coronary artery endothelial cells (HCAECs), and endothelial progenitor cells have successfully mimicked the cytotoxicity of ROS in cardiovascular diseases, such as atherosclerosis, through the administration of H_2O_2, hyperoxia (32% O_2) and oxidized LDL (oxLDL). Decreased nuclear localization and Nrf2 transcriptional activity were noted in these models, and they were accompanied by increased apoptosis and cell death. Additionally, the expression of HO-1, one of the most important ARE-driven antioxidant enzymes in endothelial cells, also combats H_2O_2-induced cytotoxicity in a dose-dependent manner.

Nrf2 and atherosclerosis

Atherosclerosis is a multifactorial process affected by all of the classic risk factors, such as smoking, diabetes, hypertension, and hyperlipidemia, and increased ROS production is the central pathogenic factor in this process. ROS can induce the oxidation of LDLs to oxLDL, and the accumulation of oxLDL in the arterial wall causes pro-inflammatory events, including the recruitment of macrophages and lymphocytes, which triggers subsequent atherosclerotic lesion formation (Van-Assche *et al.* 2011).

As an antioxidative transcription factor, Nrf2 is considered important in atherosclerosis resistance (Howden 2013). In cultured human ECs under different shear stress, it has been demonstrated that atheroprotective flow strongly activates Nrf2 in a PI3K/Akt-dependent manner, and Nrf2 is the determining factor for the alterations in redox homeostasis under hemodymatic forces (Hosoya *et al.* 2005, Dai *et al.* 2007). In mice models, increased Nrf2 expression has been shown to indirectly protect macrophages from oxLDL-mediated injury via phase II antioxidant enzyme activity, whereas an absence of Nrf2 increased foam cell formation and atherosclerosis progression (Zhu *et al.* 2008). It has been demonstrated that HO-1, the downstream target gene of Nrf2, plays a crucial role in Nrf2-mediated anti-atherosclerosis. HO-1 is capable of suppressing atherosclerotic lesion formation by reducing the oxLDL-induced transmigration of monocytes (Ishikawa *et al.* 2001), and it can also promote atherosclerotic plaque stability at advanced stages by

suppressing matrix metalloproteinase 9 (MMP9), which potentially helps with the avoidance of sudden, life-threatening coronary and cerebral events (Gough *et al.* 2006). Moreover, HO-1 is reported to protect against oxidative stress and inflammation in vascular tissue, which are the two predominant mechanisms in the pathogenic process of atherosclerosis (Van-Assche *et al.* 2011). It has been shown that HO-1 gene transfer inhibits atherosclerosis in apoliporotein E-deficient (ApoE$^{-/-}$) mice as well as graft arteriosclerosis and vascular remodeling in rat arteries (Juan *et al.* 2001, Bouchet *et al.* 2002). Apart from HO-1, other Nrf2 downstream targets, such as NQO1, GPx, and Prx I, appear to be involved in the reduction of oxidative damage in the endothelium or arteries during the atheroprotective process (Buijsse *et al.* 2012). Taken together, these data indicate that Nrf2 is an important component in protecting against the pathogenesis of atherosclerosis.

However, some contrary results reported by Sussan *et al.* confused the role of Nrf2 in atherosclerosis susceptibility. ApoE$^{-/-}$ mice with or without an Nrf2 deficiency were fed a high-fat diet for 20 weeks. Interestingly, ApoE$^{-/-}$/Nrf2$^{-/-}$ mice exhibited significantly smaller plaque areas than ApoE$^{-/-}$ controls did, and this was associated with a significant decrease in the uptake of modified LDL (AcLDL) and a decreased expression of the scavenger receptor CD36 by isolated macrophages from ApoE$^{-/-}$/Nrf2$^{-/-}$ mice (Sussan *et al.* 2008). Barajas *et al.* confirmed this finding by developing Nrf2 heterozygous and homozygous knockout mice in the ApoE$^{-/-}$ background. Homozygous knockout mice exhibited decreased levels of antioxidant genes and increased ROS generation, as was expected. However, they also exhibited a 53% reduction of aortic atherosclerosis as compared to their WT littermates, accompanied by decreased hepatic cholesterol and an increased expression of lipogenetic genes. This scenario was explained by their lower rate of cholesterol influx, which was mediated by an Nrf2 deficiency and resulted in the down-regulation of the scavenger receptor CD36 (Barajas *et al.* 2011). This suggests that inhibition of oxLDL macrophage uptake is more important than antioxidant capacity in atherosclerosis development. Other evidence supporting the proatherogenic role of Nrf2 exists in inflammation regulation. It has been found that cholesterol crystals activate Nrf2 and NLRP3 inflammasome, which results in vigorous IL1 response-mediated vascular inflammation. That study also showed that Nrf2-deficient ApoE$^{-/-}$ mice were highly protected against diet-induced atherogenesis (Freigang *et al.* 2011). Additionally, the cross-talk between Nrf2 and ATF4, an important

unfolded protein response factor associated with plaque formation, has been demonstrated in endothelial cells. Oxidized phospholipid- and oxLDL-induced up-regulation of ATF4 levels were Nrf2-dependent (Afonyushkin *et al.* 2010). Regarding the paradoxical role of Nrf2 in atherosclerosis, more detailed and precise investigations should be performed to provide new insights into the therapeutic application of Nrf2 in atherosclerosis.

Nrf2 and diabetic vascular disease

Hyperglycemia and hyperlipidemia promote oxidative stress in endothelial cells in the setting of diabetes mellitus, which contributes to the development of cardiovascular disease. Oscillating high glucose may be more detrimental to HCAECs than persistent high glucose, and this is most likely a result of the enhancements in oxidative stress and cellular apoptosis that are induced by frequent glucose fluctuations as a result of the inhibition of the Nrf2–HO-1 pathway (Liu *et al.* 2014). *In vivo* models of high-fat diet Nrf2$^{-/-}$ and Nrf2$^{+/+}$ mice revealed that a high-fat diet-induced increases in vascular ROS levels were greater in Nrf2$^{-/-}$ than in Nrf2$^{+/+}$ mice. A high-fat diet also elicited significant increases in the mRNA expression of GCLC and HO-1 in the aortas of Nrf2$^{+/+}$ mice but not Nrf2$^{-/-}$ mice, which suggests that adaptive activation of the Nrf2–ARE pathway confers endothelial protection in the setting of diabetes (Ungvari *et al.* 2011a). It is well known that mitochondrial GSH levels are deteriorated in diabetes, and mitochondria are unable to recover after cytosolic GSH depletion. Impaired mitochondrial antioxidant capability in the diabetic cardiovascular system has been shown to result in a more sensitive response to apoptosis after oxidative insult (Cheng *et al.* 2011). This is a reasonable explanation of why the elderly, who have diminished Nrf2 activity, have a higher incidence of type 2 diabetes and cardiovascular problems (Suh *et al.* 2004). Furthermore, it has been reported that in diabetic patients, Nrf2 and HO-1 levels are increased in the kidneys, and a clinical study from Japan showed that Nrf2-linked HO-1 expression was markedly up-regulated in atherosclerotic lesions in the coronary artery of diabetic subjects, which suggests that the enhanced activity of HO-1 is important for the initial defense against diabetic vasculature injury (Song *et al.* 2009). Advanced glycation end products (AGEs) play a detrimental role in the progression of diabetic vascular disease. In endothelial cells, AGEs activate the NF-κB pathway, increase pro-inflammation markers, and increase ROS generation (Bierhaus *et al.* 2001). It has been demonstrated that elevated AGEs in bovine endothelial cells lead to the nuclear accumulation of Nrf2 and consequently an increased expression of HO-1, which results in the inhibition of ROS production (He *et al.* 2011). Therefore, AGE-induced up-regulation of Nrf2-linked antioxidant enzyme activity may benefit protection against sustained oxidative stress in diabetes. Notably, in streptozotocin-induced diabetic Nrf2 knockout mice, exacerbated oxidative and/or nitrosative defense capability may cause greater deterioration in renal function, which suggests an important role for Nrf2 in the pathological processes of diabetes and diabetic complications (Jiang *et al.* 2010).

Nrf2 and age-related cardiovascular disease

Aging is the primary risk factor for cardiovascular disease, and impaired endothelial function is an early hallmark of arterial aging. Endothelial aging is a complicated process, and various factors contribute to it, including oxidative stress and inflammation. Mitochondria are vulnerable to oxidative stress, and mitochondria dysfunction is implicated in the aging process, insulin resistance, type 2 diabetes, and cardiovascular diseases. Previous studies have convincingly demonstrated that reduced Nfr2-mediated antioxidant responses and down-regulation of mitochondrial SOD2 account for the establishment of chronic oxidative stress in aging vessels (El Assar *et al.* 2013). Portions of this evidence were derived from non-human primate experiments. Ungvari *et al.* reported that the carotid arteries of aged rhesus macaques exhibited significant oxidative stress as compared to the vessels of younger monkeys. The activation of Nrf2 nuclear translocation and the subsequent expression of its downstream target genes (NQO1, GCLC, and HMOX1) did not occur in vessels of aged monkeys. Furthermore, when exposed to H$_2$O$_2$ and high-glucose environments, VSMCs derived from young monkeys exhibited significantly increased expression levels of Nrf2-regulated genes, whereas the activation of the Nrf2 pathway was blunted in the VSMCs of older monkeys (Ungvari *et al.* 2011b). Similar results were noted in aging rats, which exhibited decreased Nrf2 activity and Nrf2 target gene expression as well as increased NF-κB target gene expression in the vasculature (Ungvari *et al.* 2011c). Aging impairs Nrf2 function as part of a vicious cycle that exacerbates age-related oxidative stress-induced cellular damage.

Nrf2 and smoking-related vascular damage, angiogenesis, and hypertension

Smoking is a risk factor for cardiovascular disease. Increased oxidative stress has been demonstrated in

smokers. A study by Fratta Pasini *et al.* (2012) reported that HUVECs exposed to smokers' serum exhibited decreased NO and GSH concentrations as well as corresponding decreases in the levels of Nrf2, HO-1, and GCLC, which demonstrates that increases in oxidative stress in smokers may play a role in the repression of the Nrf2–ARE pathway (Fratta Pasini *et al.* 2012).

Angiogenesis plays an important role in myocardial repair following myocardial infarction, which is associated with significant morbidity and mortality. Recent investigations have demonstrated that Nrf2 also regulates the expression of genes involved in angiogenesis in rat cardiac microvascular endothelial cells (CMECs). Under hypoxic conditions, Nrf2 and HO-1 expression levels are temporarily up-regulated and the knockout of Nrf2 significantly suppresses vascular tube formation and the migration of rat CMECs in the setting of hypoxia; these findings may represent a new therapeutic strategy for the treatment of myocardial infarction (Kuang *et al.* 2013). The disruption of Nrf2 signaling in HCAEs has been shown to impair angiogenesis, insofar as these cells demonstrated reduced migration ability and became incapable of forming capillary-like structures. This mechanism may also be associated with microvascular rarefaction in the setting of aging (Valcarcel-Ares *et al.* 2012).

It is well established that oxidative stress is closely related to hypertension, as is evidenced by the increased levels of ROS in renin–angiotensin-induced hypertension. Nevertheless, the potential role of Nrf2 in blood pressure control has not been well defined (Howden 2013). Nrf2-induced expression of HO-1 has potential hypotensive effects. In spontaneously hypertensive rats, HO-1 expression is up-regulated, which suggests its role in hypertension (Chen *et al.* 2013). It has also been demonstrated that HO-1 is involved in the production of carbon monoxide (CO), a direct vasodilatory factor. A number of studies have shown reduced blood pressure in response to increases in HO–CO pathway activity in spontaneously hypertensive rats, which suggests that Nrf2 is a major regulator of HO-1 and may be important in blood pressure regulation (Ndisang *et al.* 2002). Regardless of the HO–CO pathway, whether the antioxidant capability of Nrf2 is part of its hypotensive effect is still unknown. Moreover, there are no significant differences in either basal blood pressure or angiotensin II-induced blood pressure elevation between $Nrf2^{-/-}$ and WT mice (D'Amario *et al.* 2011). The details of how Nrf2 influences blood pressure should first be established before Nrf2 can be considered as a target for clinical hypertension therapy.

Activators of Nrf2/Keap1–ARE pathways as clinical therapeutic tools

Intracellular redox exists in a state of dynamic equilibrium in which exogenous and endogenous antioxidants are not sufficient to offset the products of oxidation reactions that cause lipid peroxidation and protein and DNA damage. Nrf2 is one of the most important endogenous antioxidant proteins. A significant number of studies have utilized Nrf2 as a therapeutic target in oxidative stress-induced disease models undertaken at the cellular and animal levels. Because physiological Nrf2-dependent adaptive responses are relatively weak and cannot completely compensate for the increased cellular oxidative stress associated with several cardiovascular diseases, an opportunity exists for pharmacological intervention to boost the efficiency of Nrf2-driven homeostatic mechanisms. The pharmacological Nrf2 activators include: i) phenolic compounds, such as butylated hydroxyanisole, butylated hydroxytoluene, and tert-butyl hydroquinone; ii) 1,2-mercapto-3-sulfur ketone derivatives, such as oltipraz; iii) isopropyl sulfur cyanogen compounds, such as SF and its synthetic analogs; iv) natural compounds from plants, such as curcumin, resveratrol, tanshinone, plumbagin, puerarin, luteolin, oleanolic acid, mangiferin, and quercetin, etc.; v) hydrogen peroxide compounds, such as hydrogen peroxide, isopropyl benzene hydrogen peroxide, and 4-butyl hydroperoxide; and vi) compounds that are rich in arsenic, selenium, trace elements, and heavy metal ions, such as As_2O_3, ebselen, and Co^{2+} (Tkachev *et al.* 2011). To date, large amounts of evidence have established that a growing list of antioxidants exert their vasoprotective functions via Nrf2–ARE-related pathways. Resveratrol appears to have tremendous potential as a therapy for coronary artery disease, particularly in the setting of diabetes and aging; such therapy that facilitate increased transcriptional activity of Nrf2 and its target genes in a dose-dependent manner (Avila *et al.* 2013). Resveratrol encapsulated in novel fusogenic liposomes has been shown to activate Nrf2 and to attenuate oxidative stress in cerebromicrovascular endothelial cells in aging rats (Csiszar *et al.* 2014). Epoxyisoprostane E2 (EI) has been shown to induce oxidative stress in endothelial cells, which results in the increased expression of the oxidative stress response genes OKL38 and HO-1 via the Nrf2 signaling pathway in the setting of atherosclerosis (Yan *et al.* 2014).

Calorie restriction plays an important role in cardiovascular protection and may result in increased longevity. Increased Nrf2 activity and improved mitochondrial

function may both bolster its positive effects (Martin-Montalvo *et al.* 2011). Cimino *et al.* (2013) reported that anthocyanins protect human endothelial cells from mild hyperoxic damage via the modulation of the Nrf2 pathway. Tea flavonoids also protect endothelial cells against inflammation by inhibiting AhR and activating Nrf2-regulated genes (Han *et al.* 2012). Other vasoprotective components, such as docosahexaenoic acid (Ishikado *et al.* 2013*a*), 4-hydroxy hexenal (Ishikado *et al.* 2013*a*), and willow bark extract (Ishikado *et al.* 2013*b*), reduce oxidative stress through the activation of Nrf2 in vascular endothelial cells.

Although a large number of *in vitro* and *in vivo* studies have shed light on the translation of the Nrf2–ARE pathway into promising clinical application, the reported clinical trials in this area are still limited (Suzuki *et al.* 2013). The methyl ester derivative (CDDO-ME) is a potent inducer of Nrf2 at low nanomolar concentration; it robustly stimulates Nrf2-dependent cytoprotective processes. CDDO-ME has been previously studied in clinical trials under the generic name bardoxolone methy (Pergola *et al.* 2011) to assess its potential for the treatment of chronic diseases, type 2 diabetes, liver dysfunction, and certain cancers. Its positive clinical effects in a short-term (24 weeks) treatment in chronic kidney disease and type 2 diabetes were observed to persist for 52 weeks, which suggests that it has promising clinical application (Pergola *et al.* 2011). Unfortunately, a phase III trial was terminated in 2012 because of adverse events (Suzuki *et al.* 2013).

Protandim is an antioxidant supplement that consists of five ingredients: ashwagandha, bacopa extract, green tea extract, silymarin, and curcumin. It was demonstrated that the mixture of these components produced a strongly synergistic induction of HO-1 expression that greatly exceeded the sum of the individual parts. Evidence from an *in vitro* study showed that protandim-mediated HO-1 induction involved the nuclear translocation of Nrf2 and ARE activation (Velmurugan *et al.* 2009). A clinical study in healthy human subjects ranging in age from 20 to 78 years demonstrated the *in vivo* antioxidant effects of protandim, which included robustly declined thiobarbituric acid-reaction substances and increased erythrocyte SOD and CAT (Nelson *et al.* 2006).

Over the past decade, the small polyphenol resveratrol has received widespread attention as a promising anti-aging, anti-inflammation, and antioxidant reagent (Baur & Sinclair 2006). Notably, the resveratrol supplementation is capable of significantly increasing Nrf2 binding activity following meals and of up-regulating the expression of many downstream antioxidant genes (Ghanim *et al.* 2011). A number of publications have reported a relationship between resveratrol and cardiovascular disease, and the ability of the former to promote eNOS is considered to be a major mechanism (Wallerath *et al.* 2002). It has been reported that flow-mediated vasodilation was distinctly increased following trans-resveratrol treatment in obese individuals (Wong *et al.* 2011), and cerebral blood flow and hemoglobin status were increased in healthy young adults after they took high doses of trans-resveratrol (Kennedy *et al.* 2010). However, despite the increase in blood flow, resveratrol did not enhance cognitive functions (Smoliga *et al.* 2011).

Epilogue

Experimental research has shown that vascular endothelial oxidative stress plays an important role in disease prevention and treatment. Therefore, antioxidant responses have become more important. The Nrf2/Keap1–ARE signaling pathway is the most powerful endogenous antioxidative signaling pathway, and it effectively combats oxidative stress-induced injuries to endothelial cells. Nrf2 activity plays an important role in many diseases, because the molecule can be used to target oxidative stress and may also play a role in cancer prevention. Its downstream target genes have been studied extensively, but most of the experiments in question targeted only positive Nrf2 regulation. However, the relationship between Nrf2 and other antioxidant stress networks warrants further study. Timely activation or inactivation of Nrf2 appears to be the key to the regulation of endogenous cellular antioxidant defenses, because continuous Nrf2 activation may be fatal (Surh *et al.* 2008).

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