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FORUM REVIEW ARTICLE

Impaired Redox Signaling and Antioxidant Gene Expression in Endothelial Cells in Diabetes: A Role for Mitochondria and the Nuclear Factor-E2-Related Factor 2-Kelch-Like ECH-Associated Protein 1 Defense Pathway

Xinghua Cheng, Richard C.M. Siow, and Giovanni E. Mann

Abstract

Type 2 diabetes is an age-related disease associated with vascular pathologies, including severe blindness, renal failure, atherosclerosis, and stroke. Reactive oxygen species (ROS), especially mitochondrial ROS, play a key role in regulating the cellular redox status, and an overproduction of ROS may in part underlie the pathogenesis of diabetes and other age-related diseases. Cells have evolved endogenous defense mechanisms against sustained oxidative stress such as the redox-sensitive transcription factor nuclear factor E2-related factor 2 (Nrf2), which regulates antioxidant response element (ARE/electrophile response element)-mediated expression of detoxifying and antioxidant enzymes and the cystine/glutamate transporter involved in glutathione biosynthesis. We hypothesize that diminished Nrf2/ARE activity contributes to increased oxidative stress and mitochondrial dysfunction in the vasculature leading to endothelial dysfunction, insulin resistance, and abnormal angiogenesis observed in diabetes. Sustained hyperglycemia further exacerbates redox dysregulation, thereby providing a positive feedback loop for severe diabetic complications. This review focuses on the role that Nrf2/ARE-linked gene expression plays in regulating endothelial redox homeostasis in health and type 2 diabetes, highlighting recent evidence that Nrf2 may provide a therapeutic target for countering oxidative stress associated with vascular disease and aging. *Antioxid. Redox Signal.* 14, 469–488.

Introduction

TYPE 2 DIABETES, characterized by hyperglycemia and insulin resistance, affects ~4% of the population worldwide. The incidence of type 2 diabetes is tightly correlated with age, gender, family history, and an unhealthy lifestyle (224). Diabetes affects both macrovascular and microvascular beds leading to vascular pathologies such as retinopathy, atherosclerosis, and stroke (84, 147). In the past decades, endothelial dysfunction and increased oxidative stress have been accepted as major pathological changes in diabetes. Although recent evidence implicates uncoupled endothelial nitric oxide synthase (eNOS), mitochondrial reactive oxygen species (ROS), and the interaction of NO and ROS in the development of diabetic vascular complications, the underlying molecular mechanisms remain to be elucidated. In fact, endothelial dysfunction and increased ROS levels may occur even before the onset of disease in the elderly. Notably, decreased levels of antioxidants such as glutathione (GSH) and the cysteine/cystine potential have been detected in the plasma of aged animals or humans (64, 95), suggesting that cellular redox regulation may provide important insights for elucidating the molecular mechanisms underlying altered vascular function in type 2 diabetes (Fig. 1).

ROS are short-lived molecules generated as by-products of metabolism and react rapidly with cellular components, causing damage to membrane lipids, proteins, and DNA (69). Endothelial and smooth muscle cells can generate $O_2^{\bullet -}$ and hydrogen peroxide (H_2O_2) from xanthine oxidase, peroxidases, lipoxygenase, cyclooxygenase (COX), eNOS, heme oxygenase-1 (HO-1), and NADPH oxidases (61). Under physiological conditions, vascular cells generate ROS principally *via* membrane-associated NADPH oxidase(s) (13, 29), with mitochondrial ROS providing further regulation of redox signaling (64, 137). Cellular defenses against ROS-mediated damage include enzymes such as superoxide dismutase, catalase (Cat), and GSH peroxidase, as well as, nonenzymic scavengers such as GSH, ascorbic acid, and carotenoids.

Cardiovascular Division, School of Medicine, King's College London, London, United Kingdom.

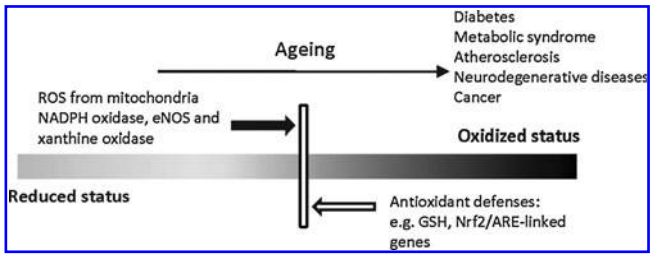

FIG. 1. Cellular redox status and age-related diseases. Cardiovascular and age-related diseases are characterized by altered cellular redox homeostasis resulting from an imbalance between oxidative stress and endogenous antioxidant defenses. The process of aging shifts the cellular redox balance to a more oxidized status, thereby increasing the incidence of type 2 diabetes and other cardiovascular diseases. ARE, antioxidant response element; eNOS, endothelial nitric oxide synthase; GSH, glutathione; Nrf2, nuclear factor E2-related factor 2; ROS, reactive oxygen species.

Dismutation of $O_2^{\bullet-}$ by a cytosolic copper-zinc form of superoxide dismutase (SOD), mitochondrial SOD (MnSOD), and extracellular copper-zinc form of SOD generates H_2O_2, which is converted to H_2O and O_2 in the presence of catalase (Cat) and GSH peroxidase (69). In addition, enzymes such as NAD(P)H:quinone oxidoreductase 1 (NQO1), GSH-*S*-transferase (GST), and HO-1 metabolize ROS and toxic compounds to readily exportable forms (74, 128, 136).

The redox-sensitive transcription factor nuclear factor E2-related factor 2 (Nrf2) serves as a regulator of cell survival, and the coordinated induction of defense enzymes is controlled through a *cis*-acting element designated antioxidant response element (ARE) or electrophile response element within the regulatory regions of target genes (86, 87). Nrf2 is normally targeted for proteasomal degradation *via* its cytosolic regulatory protein Kelch-like ECH-associated protein 1 (Keap1). ROS and electrophilic agents lead to spatial alterations in the Nrf2-Keap1 complex (88, 100, 132), resulting in nuclear accumulation of Nrf2 and upregulation of ARE-linked gene expression. Although Nrf2 null mice are viable (32), they produce smaller litter sizes and are more sensitive to hyperoxic lung injury and ischemia-reperfusion damage in stroke (40, 169). There is convincing evidence that expression of Nrf2 and cytoprotection against oxidative stress diminishes with aging (43, 114, 182), leading to redox imbalance and subsequently mitochondrial and vascular dysfunction (141, 154). This review focuses on the role that Nrf2/ARE-linked gene expression plays in regulating endothelial redox homeostasis in health and type 2 diabetes, highlighting recent evidence that Nrf2 may provide a therapeutic target for countering oxidative stress associated with vascular disease and aging.

Endothelial Dysfunction in Diabetes

Cardiovascular disease is associated with type 2 diabetes, since vascular cells are vulnerable to elevated glucose. This is because in response to hyperglycemia, glucose transporters are downregulated in other cell types to maintain intracellular glucose homeostasis, whereas in vascular cells this mechanism is weaker (23, 130). Common diabetic cardiovascular complications include microvascular disease (*e.g.*, retinopathy and nephropathy) and macrovascular disease (*e.g.*, stroke and atherosclerosis) (147). Among them, atherosclerosis accounts for almost 50% of diabetes-associated death (16). It is well recognized that endothelial dysfunction is involved in the pathogenesis of atherosclerosis in diabetic patients (see Fig. 2) (157). Reduced endothelial-dependent vasodilation in insulin resistant and obese individuals (101, 179) is largely a consequence of diminished NO generation and/or bioavailability (195). NO is a labile vasodilator synthesized in endothelial cells from the cationic amino acid L-arginine, requiring tetrahydrobiopterin, NADPH, flavin adenine dinucleotide, and flavin mononucleotide as additional cofactors for its activity (130, 135). Under conditions of oxidative stress and/or limited substrate supply, eNOS becomes uncoupled and generates superoxide ($O_2^{\bullet-}$) (46, 61, 167). In this context, endothelial ROS generation in diabetes, derived from mitochondria, NADPH oxidase, and uncoupled eNOS, can react with NO to form peroxynitrite (149), which itself can disrupt the NOS

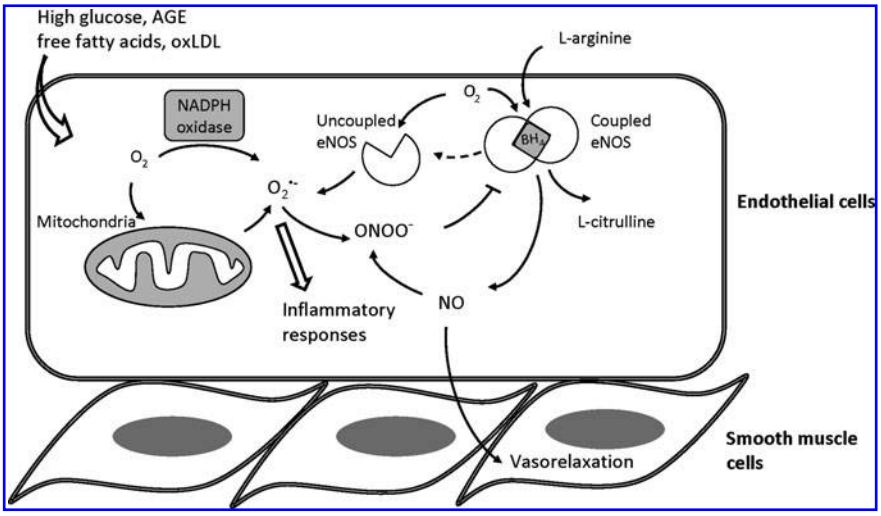

FIG. 2. Endothelial dysfunction and ROS production in diabetes. Diabetes is characterized by endothelial dysfunction. Under normal redox balance, NO produced *via* eNOS modulates vascular relaxation and Nrf2/ARE-linked antioxidant gene expression. Under more oxidized conditions (*e.g.*, diabetes and insulin resistance), increased intracellular ROS can react with NO to form peroxynitrite ($ONOO^-$), which in turn may uncouple eNOS to produce superoxide ($O_2^{\bullet-}$) instead of NO, leading to vascular dysfunction. AGE, advanced glycation end products; BH_4, tetrahydrobiopterin; oxLDL, oxidized low-density lipoprotein.

dimer by oxidizing tetrahydrobiopterin leading to further increases in ROS generation in the diabetic vasculature (112). Thus, in diabetes uncoupling of eNOS and/or reduced bioavailability or sensitivity to NO will impair vascular reactivity (2, 8, 52, 130, 197). Antioxidants such as SOD and Cat restore endothelial responses to acetylcholine in arteries from diabetic dogs (6), and 5-methyltetrahydrofolate, a circulating form of folic acid, improves endothelial function by preventing eNOS uncoupling due to peroxynitrite scavenging property (8). These findings highlight the importance of ROS in endothelial dysfunction and NO signaling. Apart from the NO pathway, increased ROS in diabetes also inhibit glyceraldehyde-3-phosphate dehydrogenase (54) and activate COX-2, leading to an inflammatory status (105) and cardiovascular complications associated with diabetes mellitus (22).

NADPH Oxidase Mediated ROS Generation

ROS-induced damage is mediated by superoxide ($O_2^{\bullet-}$), H_2O_2, and their secondary products hydroxyl and peroxyl radicals, peroxynitrite, and hydrochlorous acid (69). The double-edged sword of ROS in modulating NADPH oxidase in diabetes has recently reviewed (61). NADPH oxidases are a group of complexes consisting of several subunits (*e.g.*, gp91phox, Rac1, p22phox, and p67phox) that transfer electrons from NADPH to molecular oxygen generating $O_2^{\bullet-}$ (13, 29). The main subtypes of NADPH oxidases in endothelial cells are NOX2 and NOX4, which are predominantly localized in perinuclear area and on the membrane of the endoplasmic reticulum (196). NADPH oxidase-derived ROS may also function as physiological signaling molecules, as vascular endothelial growth factor and transforming growth factor-β-induced angiogenesis and endothelial cell proliferation are markedly attenuated in NOX2/4-deficient cells (80, 194). However, in humans and animal models, diabetes is characterized by increased expression of NADPH oxidase activity resulting in increased ROS production and impaired endothelial function (12, 61). Inhibition of NADPH oxidase reduces oxidative stress and improves vascular relaxation in arteries from diabetic humans and rats (67, 104). Thus, the dual role of NADPH oxidase in diabetes requires further investigation to establish whether this enzyme is the primary source of increased ROS in diabetes.

Mitochondrial ROS Generation and Redox Signaling

The role of the mitochondria as the main energy source in eukaryotic cells in health and disease has been well described (26, 126, 141, 153). Mitochondria are important sources of ROS, since ~1%–2% of mitochondrial oxygen consumption can be converted to $O_2^{\bullet-}$ due to electron leakage at complexes I and III of the respiratory chain (34, 140, 191). Mitochondrial ROS are critical for a variety of vascular cell functions, including shear-mediated vasorelaxation (70, 121), adaptive endogenous antioxidant responses (64, 159), and insulin signaling (131, 180). As summarized in Figure 3, mitochondrial ROS generation depends on the mitochondrial membrane potential and redox status. The respiratory chain contains (i) electron transport chain (complexes I–IV) that transfers electrons from reducing equivalents (NADH and flavin adenine dinucleotide H_2) to O_2 and pumps out protons to create a proton gradient across inner membrane and (ii) a proton gradient reducing system involving ATP synthase to generate ATP and uncoupling proteins (UCP), allowing protons to leak back to the matrix and maintain a appropriate proton gradient (141, 217). As $O_2^{\bullet-}$ accumulate in mitochondria, the mitochondrial permeability transition pore opens, initiating a burst of $O_2^{\bullet-}$ production in the mitochondrial matrix (201). Thus, the mitochondrial membrane potential is critical in the regulation of electron transport through respiratory

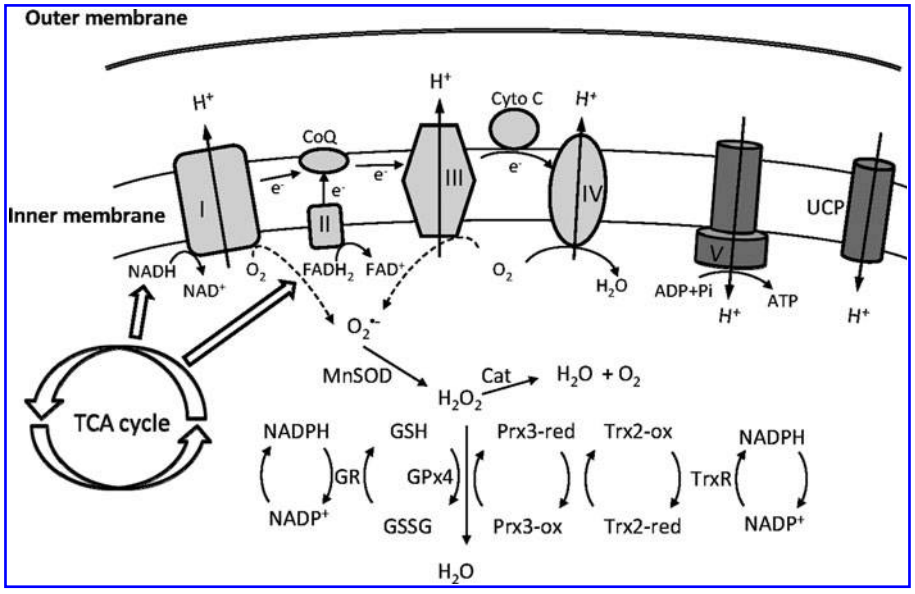

FIG. 3. Mitochondrial electron transport chain and redox regulation. Mitochondria are an important source of ROS in vascular cells. $O_2^{\bullet-}$ is produced along with reduction of O_2 during oxidative phosphorylation in the mitochondrial respiratory chain. $O_2^{\bullet-}$ generation depends on the mitochondrial respiratory rate and membrane potential. In depolarized mitochondria, the electron transport chain is slowed down, leading to an excess of $O_2^{\bullet-}$ production. Mitochondria also have their own redox modulators such as MnSOD, GSH, Prx3, Trx-2, and GPx4. ADP, adenosine diphosphate; ATP, adenosine triphosphate; FAD, flavin adenine dinucleotide; Cat, catalase; CoQ, coenzyme Q; Cyto C, cytochrome C oxidase; GPx4, glutathione peroxidase 4; GR, glutathione reductase; GSSG, disulfide glutathione; H_2O_2, hydrogen peroxide; MnSOD, mitochondrial superoxide dismutase; Prx3-ox, oxidized peroxiredoxin 3; Prx3-red, reduced peroxiredoxin 3; TCA cycle, tricarboxylic acid cycle; Trx-2-ox, oxidized thioredoxin 2; Trx-2-red, reduced thioredoxin 2; TrxR, thioredoxin reductase; UCP, uncoupling proteins.

chain and also largely determines mitochondrial ROS production. In dysfunctional mitochondria, protons accumulate outside the inner membrane and more electrons are transferred to molecular oxygen to produce $O_2^{\bullet -}$ (26, 48).

The oxidative status of mitochondria is also regulated by antioxidant defenses such as MnSOD, which dismutates $O_2^{\bullet -}$ to H_2O_2. The latter can diffuse freely across the mitochondrial inner and outer membrane and function as a signaling molecule by modulating mitochondrial thiol groups (*e.g.*, thioredoxin-2 [Trx-2], glutaredoxin-2 [Grx-2], and peroxiredoxin-3 [Prx-3]) and protein cysteine residues (cys) (191). An overproduction of H_2O_2 can be reduced by mitochondrial GSH as well as Cat to restore redox balance (58, 145). Emerging evidence establishes that the cellular redox status is critical for mitochondrial function and ROS-mediated signaling. In cardiomyocytes, depletion of intracellular GSH markedly increases mitochondrial ROS production and induces mitochondrial membrane depolarization (63). Moreover, mitochondrial ROS production is influenced by changes in the plasma redox potential (64). Go *et al.* have shown in murine aortic endothelial cells that a more oxidized plasma redox potential increases mitochondrial ROS production and oxidizes mitochondrial Trx-2, whereas this increase in ROS is abrogated by overexpression of Trx-2 in transgenic mice (64). Thus, interactions between the mitochondrial respiratory chain and antioxidant defenses constitute an important dynamic loop in physiological redox signaling and in type 2 diabetes (126, 141, 156).

Mitochondrial ROS and type 2 diabetes

Under hyperglycemic conditions, mitochondrial ROS appear to be the common mediator linking increased glucose metabolism *via* the polyol pathway, accumulation of advanced glycation end products (AGEs), protein kinase C (PKC) activation, and increased activity of the hexosamine pathway with vascular damage in diabetes (23). A key piece of evidence implicating a central role for mitochondrial ROS in diabetic vascular complications is the finding that upregulation of MnSOD and UCP-1, which reduces the mitochondrial membrane proton gradient, abolishes hyperglycemia-induced PKC activation, formation of AGEs, and NF kappa B (κB) activation in endothelial cells (141). Moreover, hyperglycemia in humans or animal models of diabetes is associated with increased mitochondrial ROS production in vascular cells (48, 141) leading to apoptosis and endothelial dysfunction. Interestingly, overproduction of ROS by mitochondria may be more prevalent in the diabetic vascular tissue than in other tissues, as there are negligible differences in mitochondrial ROS generation in skeletal muscle from lean healthy and type 2 diabetic subjects even though ATP production is lower in skeletal muscle from diabetic patients (1). Oxidative stress is also less evident in pancreatic β cells than in vascular cells in diabetic animals, possibly because islets are able to defend themselves against hyperglycemia *via* the upregulation of endogenous defenses such as GSH (110). Beneficial effects of reducing mitochondrial ROS in diabetes either by upregulating mitochondrial antioxidants or UCP-1 have been reported by several groups (66, 141).

However, mitochondrial ROS may also serve as important mediators in insulin sensitization and its downstream pathways. Mice deficient in UCP-2, which also decreases the mitochondrial membrane potential and ROS production and bypasses ATP synthesis, show a remarkable improvement in insulin secretion and reduced blood glucose level in obesity-induced diabetes (217). This is consistent with the report that overexpression of GSH peroxidase in transgenic mice, which reduces cellular H_2O_2, leads to insulin resistance (131). In addition, studies in a rat liver cell line show that mitochondrial fragmentation is required for high-glucose-induced ROS production, indicating that the mechanism underlying mitochondrial ROS production may require cross-talk with other signaling pathways (213). Thus, dysregulation of mitochondrial ROS production may thus underlie the development of diabetic vascular complications (22).

Nrf2 a Key Regulator of Cellular Defense and Survival

Normal cell function requires a stable oxidation–reduction environment, and eukaryotic cells have evolved endogenous antioxidant defenses to counteract oxidative stress. Among them, the Nrf2/ARE pathway has emerged as the most important transcription mechanism involved in upregulating antioxidant genes and maintaining cellular redox homeostasis (136). ARE is a *cis*-acting element located in the 5′-flanking region of genes encoding phase II detoxifying enzymes and antioxidant enzymes, including NQO1, GST, glutamate-cysteine ligase (GCL) subunits, HO-1, and the cystine-glutamate transporter (xCT) (87). Activation of these genes is involved in quinone reduction, detoxification of xenobiotics, GSH synthesis, and heme metabolism. Nrf2, along with Nrf1, Nrf3, Bach1, Bach2, and p45, is a member of the CNC (cap 'n' collar) family of transcription factors that possess a b-zip binding motif (136). As Nrf2 null mice survive until adulthood, this has enabled researchers to study the role of this transcription factor in redox signaling *in vivo* (32).

Although Nrf2 is not essential for survival, activation of Nrf2/ARE pathway is fundamental for the maintenance of the intracellular GSH and the induction of antioxidant defense enzymes in response to stress. Nrf2-deficient mice are more susceptible to liver damage due to diminished basal GSH levels and an impaired compensatory induction GSH synthesis (30). Moreover, decreased expression of GCL catalytic subunit (GCLC) and GCL modifier subunit (GCLM) and activity of GST in Nrf2 null mice renders these animals more vulnerable to oxidative stress (30, 31, 33, 136). Notably, in macrophages and vascular smooth muscle cells isolated from Nrf2 knockout mice, induction of HO-1 and cystine-glutamate transport activity is diminished in response to electrophilic stress (86, 87). HO-1 is an important antioxidant enzyme in the cardiovascular system and induction of L-cystine transport elevates intracellular levels of cysteine, the rate-limiting precursor for GSH synthesis (87, 174). In this context, overexpression of Nrf2 significantly increases expression of endogenous antioxidants, for example, HO-1 and NQO1, *via* activation of ARE in liver and vascular cells (3, 198). Recent evidence also highlights a protective role of Nrf2 against oxidative stress in aging (170, 182, 186), with diminished Nrf2 expression and activity contributing to the development of age-related diseases such as atherosclerosis, cancer, and stroke.

Regulation of Nrf2/ARE-linked gene transcription

Under basal conditions, Nrf2 is tethered by a cytosolic protein, Keap1, and targeted for ubiquitin-dependent pro-

teasomal degradation (88, 97). Keap1 is the main cytosolic inhibitor of Nrf2, as Keap1 knockout mice exhibit constitutive Nrf2 nuclear accumulation and a marked upregulation of antioxidant genes (97). Keap1 possesses a number of cys residues and modification of these residues by ROS and electrophilic agents leads to spatial alterations in the Nrf2-Keap1 complex (88, 132, 199), resulting in nuclear accumulation of Nrf2 and upregulation of ARE-mediated gene expression both *in vitro* and *in vivo* (50, 106). Thus, the Nrf2-Keap1 complex serves as a master regulator of the cellular redox status, and dietary compounds contained in cruciferous vegetables, such sulforaphane, can activate Nrf2 signaling by modulating cys on Keap1 (51).

Modification of the Nrf2-Keap1 complex and phosphorylation may be essential for Nrf2 activation, as mutation of Nrf2 phosphorylation sites or critical cys of Keap1 abolishes ROS-induced Nrf2 translocation (56, 143). In liver carcinoma cell lines, PKC has been shown to regulate Nrf2 activation, with phosphorylation of Ser40 on Nrf2 by PKC implicated as an essential step in unlocking Keap1 binding by *t*-butyl hydroquinone and phorbol 12-myristate 13-acetate (19, 81, 143). Phosphoinositide 3-kinase (PI3K)/Akt has also been reported to mediate activation of Nrf2 in response to sulforaphane (200), shear stress (76), hyperoxia (151), and insulin (111). Extracellular signal-regulated kinases 1/2 appear to be involved in regulation of Nrf2 by NO (25), oxidized lipids (9), and hyperoxia (151). Other mitogen-activated protein kinases such as c-Jun and p38 are also involved in the activation Nrf2 (9, 138). A recent study reported that Nrf2 nuclear import requires phosphorylation of Nrf2 by casein kinase 2 (10), while nuclear export of Nrf2 may be regulated by phosphorylation *via* p38α or Src kinase family (90). Glycogen synthase kinase 3β (GSK-3β), a rate-limiting enzyme for glycogen metabolism and a key regulator of insulin signaling, cell proliferation, and apoptosis (42), has been shown to be a repressor of Nrf2 (89, 163). GSK-3β can directly phosphorylate Nrf2 *in vitro*, and by phosphorylating Fyn accelerates nuclear export of Nrf2 (89) (see Fig. 4). Dominant negative GSK-3β and specific inhibitors of the enzyme (LiCl and TDZD-8) increase Nrf2 nuclear accumulation, whereas nuclear translocation and antioxidant defenses are diminished in GSK-3β overexpressing human embryonic kidney 293T cells (163). Notably, GSK-3β can be phosphorylated and deactivated by PI3K/Akt after insulin treatment (42), implicating insulin in cellular antioxidant defenses against stress. By contrast, insulin-resistant cells may not respond to stress normally due to inhibition of Nrf2/ARE signaling by active GSK-3β (62).

Apart from regulation of Nrf2/ARE signaling, other signaling pathways may also affect Nrf2 transcriptional activity positively or negatively. As shown in Figure 4, Bach1 is another member of the CNC family and inhibits Nrf2 activity by competitively binding to the ARE sequence and silencing downstream antioxidant responses (184). Nuclear export of Bach1 is also controlled by phosphorylation but at a different time point from Nrf2. Dynamic changes in the phosphorylation of these two competitors in nuclei will influence regulation of ARE-linked gene expression (184). Dephosphorylation of Keap1 may also contribute to Nrf2 activation in response to H_2O_2, as dephosphorylated Keap1 results in a much faster nuclear and cytosolic degradation of this Nrf2 inhibitor (91). Meanwhile, under oxidative stress both Nrf2 nuclear activity and expression appear to be altered. Treatment of cardiomyocytes with H_2O_2 (100 μM 0–4 h) leads to increased Nrf2 protein levels with negligible changes in mRNA levels (155). The increase in Nrf2 protein expression was inhibited by cycloheximide implicating post-translational regulation of the Nrf2/ARE pathway (155).

Nrf2 and cellular redox regulation

Depletion of intracellular GSH leads to activation of Nrf2 and protects cells from further stress-induced damage (87, 109). Supplementation with *N*-acetylcysteine reverses GSH depletion and restores ARE-linked gene transcription to basal levels (109, 118). Appropriate intracellular ROS levels play a

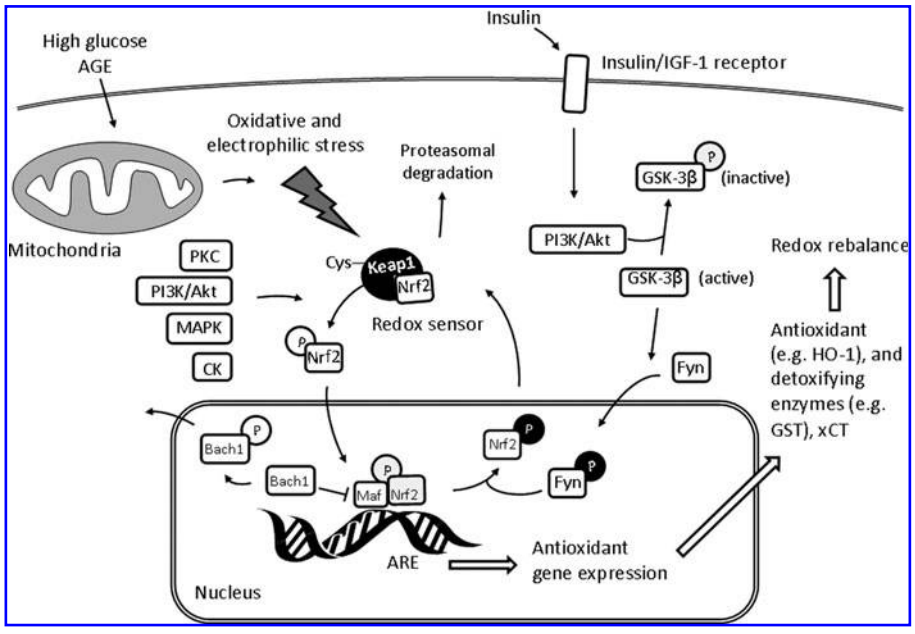

FIG. 4. Regulation of the Nrf2-Keap1 defense pathway. Regulation of Nrf2 involves nuclear import, nuclear binding to the ARE sequence, nuclear export, and ubiquitin-dependent proteasomal degradation. These processes are highly redox sensitive and involve phosphorylation. Insulin and increased ROS production induced by high glucose positively affect the regulation of Nrf2. Dysregulation of Nrf2 signaling renders cells more vulnerable to oxidative, nitrosative, and xenobiotic insults. CK, casein kinase 2; GSK-3β, glycogen synthase kinase 3β; GST, glutathione-*S*-transferase; HO-1, heme oxygenase-1; IGF-1, insulin-like growth factor; Keap1, Kelch-like ECH-associated protein 1; MAPK, mitogen-activated protein kinases; NQO1, NAD(P)H:quinone oxidoreductase 1; PI3K, phosphoinositide 3-kinases; PKC, protein kinase C; xCT, cystine-glutamate transporter.

key role in physiological redox signaling by activating and regulating endogenous defenses to protect cells from long-term oxidative, nitrosative, and electrophilic stress (61, 75, 113, 204). Notably, supplementation with exogenous antioxidants abolishes exercise-induced improvements in insulin sensitivity and antioxidant gene expression (93), highlighting the importance of ROS induced endogenous antioxidant enzymes in regulating and/or restoring physiological redox balance. Other redox-sensitive proteins can modify the binding activity of Nrf2 in nuclei. Overexpression of Trx-1 increases nuclear Nrf2 binding activity without changing Nrf2 levels in nuclei (103). Overexpression of Trx suppresses the progression of insulin resistance in both type 1 and type 2 diabetic animal models (206), whereas higher glucose reduces Trx activity in mouse cardiomyocytes *via* nitration (125). Although modulation of Nrf2 signaling in response to an altered redox status has not been fully elucidated, triggering endogenous antioxidant defenses is integral for the maintenance of cellular redox homeostasis. Activation of the Nrf2/ARE pathway is also modulated by oxygen tension, since in human microvascular cells Nrf2 activity is elevated by hyperoxia (65). Table 1 summarizes the antioxidant protection afforded by the activation of Nrf2-linked genes.

Nrf2 and vascular protection

Activation of Nrf2 and upregulation of the downstream enzymes provides vascular protection in oxidative stress (53, 116) and inhibits inflammatory responses *via* suppression of NF-κB pathway and expression of vascular cell adhesion molecule-1 (VCAM-1) (82). In smooth muscle cells, Nrf2 suppresses cell proliferation and growth, thereby preventing neointimal thickening (82). Studies *in vivo* have shown that athero-susceptible regions of the aorta exposed to disturbed flow have reduced levels of Nrf2 expression and upregulation of VCAM-1, with the latter attenuated by enhancing Nrf2 activity with sulforaphane (215). However, Nrf2-deficient $ApoE^{-/-}$ mice fed a high-fat diet have a significantly reduced size of atherosclerotic plaque, which the authors attributed to decreased uptake of oxidized low-density lipoprotein (oxLDL) *via* the scavenger receptor CD36 (185). It is worth noting that these Nrf2-deficient $ApoE^{-/-}$ mice have raised plasma levels of oxLDL, triglycercides, and glucose and decreased HO-1, NQO1, and GSH levels in vasculature (185). As summarized in Table 2, the protective role of Nrf2 in the vasculature is largely due to the upregulation HO-1, enzymes involved in GSH synthesis (*e.g.*, GCLM and GCLC), and induction of the xCT (87). HO-1 is the rate-limiting enzyme in heme degradation, and its role in vasculature has been reviewed extensively (123, 127, 174). In the presence of O_2 and NADPH, heme is metabolized to biliverdin, Fe^{2+}, and carbon monoxide by HO-1 (127). Biliverdin can be further converted to bilirubin and these end products have antioxidant and anti-inflammatory properties. The role of HO-1 in normal vascular function is highlighted in angiogenesis, where it mediates vascular endothelial growth factor-induced tissue repair but inhibits leukocyte infiltration and inflammatory angiogenesis (123). Under pathological conditions, the consensus is that HO-1 is involved in the protection against atherosclerosis, stroke, myocardial infarction, and diabetes.

In endothelial cells, Nrf2 is activated by laminar shear stress *via* ROS and PI3K/Akt signaling pathways (38, 47). Disturbed blood flow diminishes the Nrf2-mediated activation of ARE-linked genes, which predisposes the endothelium to a proatherogenic situation (78). Laminar flow also potentiates statin-induced antioxidant responses in endothelial cells, which enhances anti-inflammatory actions of the drug in atherosclerosis (5). Reduced activity of Nrf2 in en-

Table 1. General Functions of Nuclear Factor E2-Related Factor/Antioxidant Response Element-Linked Antioxidant Enzymes

Nrf2/ARE-linked genes	*Functions*	*References*
HO-1	Key enzyme involved in heme degradation, generates biliverdin/bilirubin providing cytoprotection and inhibition of NADPH subunit assembly; involved in angiogenesis and vascular protection	(3, 9, 41, 60)
NQO1	Catalyzes reduction of quinone to hydroquinones, involved in detoxifying xenobiotics and protection of membrane phospholipids from oxidative damage *via* reduction of coenzyme Q	(108, 161, 198)
GST	Detoxifying enzyme, catalyzes conjugation of GSH to peroxidized lipid and xenobiotics	(33, 74)
GCLC	Catalytic subunit of GCL, the rate-limiting enzyme in glutathione synthesis	(124)
GCLM	Modifier subunit of GCL	(124)
xCT	Cystine-glutamate transporter and maintains intracellular cysteine levels, which are rate-limiting for GSH synthesis	(86, 87)
AKR	Detoxifies toxic aldehyde, protects cells from carbonyl stress	(49, 142, 222)
GR	Key enzyme in the GSH/GSSG turnover and GSH recycling	(223)
TrxR	Reduction of disulfide form of Trx	(162, 189)
Trx	Reduced Trx reacts with oxidized Prx to maintain redox homeostasis	(162, 189)
Prx I	Scavenges peroxide and maintains redox homeostasis, may also involved in ROS signaling	(86, 87)

AKR, Aldo-keto reductase; ARE, antioxidant response element; GCL, glutamate-cysteine ligase; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit; GR, glutathione reductase; GSH, glutathione; GSSG, disulfide glutathione; GST, glutathione-*S*-transferase; HO-1, heme oxygenase-1; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, nuclear factor E2-related factor; Prx, peroxiredoxin; ROS, reactive oxygen species; TrxR, thioredoxin reductase; xCT, cystine-glutamate transporter.

Table 2. Cytoprotective Actions of Nuclear Factor E2-Related Factor in the Vasculature

	Cell type	*Nrf2 function*	*References*
Endothelial cells	HUVEC	↑HO-1, GST, GPx3, GCLM, GCLC, GR, and NQO1, defends against oxidative stress; protects mitochondria from HOCl-induced dysfunction and apoptosis. ↓VCAM-1, ↓NF-κB activity, ↓p38, ↓endothelial cells activation, ↓atherosclerosis; ↑NO but transiently, ↓eNOS expression	(5, 77, 82, 109, 215)
	HMEC	↓hyperglycemia-induced activation of PKC and hexosamine pathways; ↓accumulation of glycating agent methylglyoxal; ↓high-glucose-induced ROS generation	(205)
	HAEC	↑GSH, NQO1, HO-1 levels; protect cells from oxidative stress; ↓TNF-α-induced MCP-1, VCAM-1, monocytic cell adherence; ↓atherosclerosis; ↑half life of IL-8 mRNA	(37, 78, 219)
	HBEC	Hyperglycemia interrupts cytosolic and mitochondrial thiol-protein balance while insulin induces upregulation of GCLC *via* Nrf2 reversing adverse effects of hyperglycemia and rebalancing redox status	(145)
	BAEC	↑Trx and TrxR	(162)
Smooth muscle cells	HASMC	↑HO-1, NQO1, GCLM, GCLC, GSH, ↓proliferation, ↓oxidative stress (ROS and oxLDL), ↓inflammatory response (MCP-1, macrophages invasion, apoptosis); ↓p53 induced apoptosis; ↓TNF-α-induced inflammation; maintenance of mitochondrial membrane potential during challenge with H_2O_2	(9, 24, 41, 116, 218)
	RASMC	↑HO-1, aldose reductase ↓oxidative stress ↓ER stress ↓apoptosis and proliferation. ↓neointimal hyperplasia after balloon injury	(96, 102, 119, 120, 150)
	MAoSMC	↑A170, HO-1, and Prx I leading to ↓neointimal hyperplasia, SMC migration and proliferation after balloon injury	(86, 181)
$Nrf2^{-/-}$ mice		Reduced loss of tight junctional proteins and EC leading to decreased blood barrier permeability after brain injury	(221)

BAEC, bovine aortic endothelial cells; EC, endothelial cells; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; Gpx3, glutathione peroxidase; HAEC, human aortic endothelial cells; HASMC, human aortic smooth muscle cells; HBEC, human brain endothelial cells; HMEC, human microvascular endothelial cells; HPAEC, human pulmonary artery endothelial cells; HUVEC, human umbilical vein endothelial cells; H_2O_2, hydrogen peroxide; IL-8, interleukin 8; MAoSMC, murine aortic smooth muscle cells; MASMC, mouse airway smooth muscle cells; MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor kappa B; oxLDL, oxidized low-density lipoprotein; PKC, protein kinase C; RASMC, rat aortic smooth muscle cells; SMC, smooth muscle cells; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1.

dothelial cells under disturbed flow is possibly due to relatively lower Trx levels compared with cells exposed to laminar flow (207), and diminished DNA binding activity of Nrf2 in nuclei (71). Notably, in a clinical study from Japan, HO-1 expression was markedly upregulated in atherosclerotic lesions in the coronary artery of diabetic subjects, suggesting that enhanced activity of HO-1 may play a role in the initial defense against inflammation and oxidative stress in the vasculature (176). The Nrf2/ARE pathway in endothelial cells can also be activated by NO or nitrosative stress (128, 129, 173). Interestingly, upregulation of Nrf2-linked HO-1 expression is thought to maintain eNOS in a coupled state by increasing NO production and transiently reducing eNOS expression to reduce uncoupled eNOS (77).

Nrf2 and mitochondria

Mitochondria are vulnerable to oxidative stress and mitochondrial dysfunction is implicated in the aging process, insulin resistance, type 2 diabetes, and cardiovascular diseases (48, 126). As a key defense mechanism against oxidative stress, the Nrf2/ARE pathway also confers protection in mitochondria. Although basal ROS and GSH levels are similar in murine wild-type and $Nrf2^{-/-}$ embryonic fibroblasts, treatment with diquat, a mitochondrial ROS stimulus, markedly reduces GSH levels and the viability of $Nrf2^{-/-}$ compared with wild-type cells (148). Similar findings have been reported in astrocytes and diminished Nrf2 activity is implicated in the pathogenesis of neurodegenerative diseases (27, 169). In contrast, overexpression of Nrf2 or preactivation of Nrf2 with *tert*-butyl hydroquinone protects astrocytes from toxicity of the mitochondrial complex II blocker 3-nitropropionic acid, implicating Nrf2 in the defense against mitochondrial oxidative stress (27, 169). Nrf2-mediated protection may involve an upregulation of GSH levels (124), since depletion of GSH leads to a depolarization of the mitochondrial membrane potential and increased ROS production (160), which are both associated with diabetes and insulin resistance (22).

Oxidative and electrophilic stress induce *de novo* GSH synthesis *via* Nrf2-mediated upregulation of the xCT anionic amino acid transporter and HO-1. Under normal conditions, the mitochondrial GSH pool is maintained even when the cytosolic redox balance is disturbed (63, 203). However, in diabetes mitochondrial GSH levels are decreased (15, 164, 203), and mitochondria are unable to recover after cytosolic GSH depletion (63). Diminished mitochondrial antioxidant defenses in diabetic cardiomyocytes render these cells more sensitive to apoptosis after an oxidant insult (63). These findings may

explain why the elderly, with diminished Nrf2 activity (182) and endogenous antioxidant defenses, have a higher incidence of type 2 diabetes and cardiovascular problems.

Upregulation of HO-1 confers protection for mitochondria against oxidative stress. In rat livers, HO-1 is found in mitochondrial fractions and catalyzes heme degradation (44). Recent evidence suggests that carbon monoxide, a product of HO-1-catalyzed heme degradation, increases mitochondrial biogenesis (183). In summary, Nrf2 signaling appears to be important in maintaining mitochondrial homeostasis and cellular protection against mitochondrial-generated ROS.

Mitochondrial ROS May Be Important for Triggering Nrf2 Activation

Accumulating evidence shows that mitochondrial ROS activate downstream protective mechanisms, including the Nrf2/ARE pathway (83, 169), and thus interactions between mitochondria and Nrf2 merit further investigation. As summarized in Figure 5, recent evidence suggests that the Nrf2-Keap1 complex may be tethered on the mitochondrial outer membrane by a mitochondrial located protein PGAM5 and thereby directly sense ROS released from mitochondria (122). In fact mitochondrial ROS are critical in shear-induced HO-1 expression in endothelial cells, since ROS scavengers targeted to mitochondria significantly inhibit the induction of HO-1 (70). In human hepatoma cells, glucose deprivation upregulates HO-1 expression *via* ROS production, and scavenging mitochondrial ROS abrogates metabolic stress-induced HO-1 expression (35).

Electrophilic lipids such as HNE and 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ_2) are potent inducers of the Nrf2/ARE pathway (78, 86). ROS production is significantly increased in bovine aortic endothelial cells treated with these lipid peroxidation products, and mitochondria are the most likely source due to mitochondrial deposition of these compounds and the reported depolarization of the mitochondrial membrane potential (159). Mitochondria targeted thiol reactive compounds such as (4-iodobutyl)triphenyl-phosphonium prevent HO-1 induction by 15d-PGJ_2 and hemin without affecting mitochondrial ROS production. Notably, in this latter study inhibition of HO-1 induction was correlated with reduced nuclear Nrf2 expression (159). These observations suggest that Nrf2 activation *in vivo* may be regulated in a mitochondrial- and ROS-dependent manner and influenced by the cellular thiol status.

The extracellular redox potential, for example, plasma cysteine/cystine couple [E(h)CySS], is associated with diabetes and aging-induced cardiovascular disease. An increased extracellular plasma redox potential could oxidize mitochondrial thiol groups and increase mitochondrial ROS by modifying membrane proteins, for example, integrins and the cytoskeleton, without changing the cytosolic redox potential in endothelial cells (64). These authors proposed a dynamic model for ROS-mediated cell signaling, involving plasma/membrane oxidation, cytoskeletal mechanical transduction, and mitochondrial ROS generation. Interestingly, in fibroblasts oxidation of the plasma redox potential also leads to Nrf2 activation *via* mitochondrial ROS and overexpression of mitochondrial Trx-2 inhibits ROS production and Nrf2 activation induced by an oxidized extracellular redox potential (83). Thus impaired Nrf2 responses in diabetes or aging (43, 95) may be due to mitochondrial dysfunction leading to a delayed recovery of redox balance after oxidative stress.

Nrf2 as a Regulator of Oxidative Stress in Diabetes and Obesity

Diabetic complications are associated with increased ROS generation resulting from elevated blood glucose and free fatty acids (85, 141, 157). Nrf2 counteracts high-glucose-induced damage and downstream inflammatory responses in diabetes (205). In diabetic patients, Nrf2 and HO-1 levels are increased in the kidney, suggesting that activation of Nrf2 provides an adaptive response to counteract oxidative stress in the disease (94). Further, markers of oxidative stress such as oxLDL (9), 4-hydroxynonenal (HNE) (86, 115, 134), and transforming growth factor-β (41) are elevated in diabetic patients and have all been shown activate Nrf2/ARE-linked gene transcription.

Nonenzymatic reactions of glucose with N-terminal amino acid residues or ε-amino groups of proteins form Schiff base adducts that rearrange into Amadori-modified products and

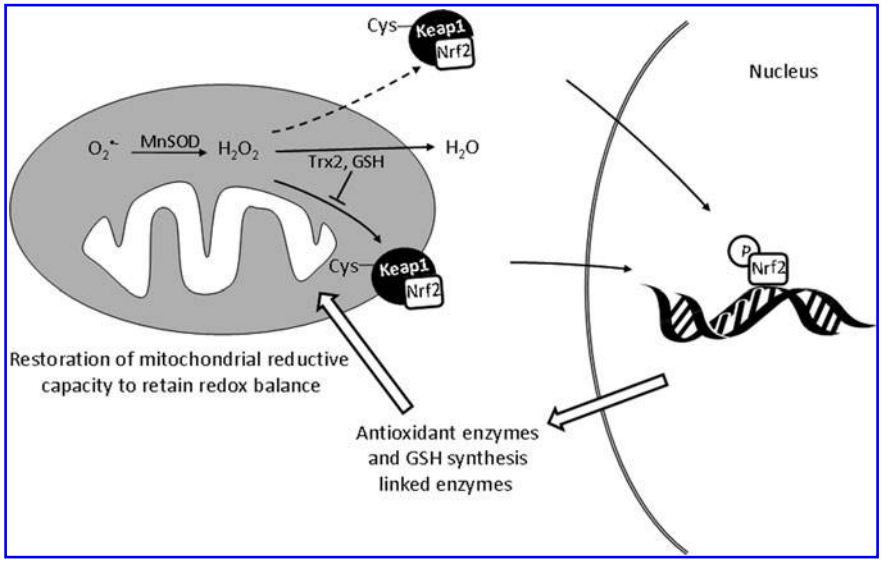

FIG. 5. Mitochondrial regulation of Nrf2. The interruption of mitochondrial redox homeostasis may activate Nrf2-Keap1 sensors anchored on mitochondria and the cytoskeleton. Nuclear accumulation of Nrf2 in turn leads to an upregulation of ARE-linked antioxidant enzymes to restore the redox balance in mitochondria and the cytosol.

advanced AGEs (208, 209). In diabetes, AGE levels are elevated in the vasculature and kidney due to their accelerated rate of formation under sustained hyperglycemia (21). AGEs play a detrimental role in the progression of diabetic vascular disease and nephropathy by modifying the extracellular matrix (99) and activating intracellular signaling pathways after an interaction with the receptor for AGEs expressed on the surface of cells (210). In endothelial cells, AGEs activate the NF-κB transcription pathway (17) and increase expression of pro-inflammatory markers such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) (166, 168). AGEs also increase ROS generation *via* NADPH oxidase (202) and mitochondria (45). Our recent studies in cultured human and bovine endothelial cells (75) have established that high glucose and AGEs increase ROS production and Nrf2-dependent HO-1 expression (Fig. 6). Treatment of bovine endothelial cells with AGEs led to nuclear accumulation of Nrf2, and inhibition of ROS production with the $O_2^{\bullet -}$ scavenger Tiron or flavoprotein inhibitor diphenyleneiodonium abrogated AGE-induced HO-1 expression (Fig. 6). Thus, AGE-induced upregulation of Nrf2-linked antioxidant enzyme activity may confer protection against sustained oxidative stress in diabetes (75).

Diminished Nrf2 activity may render endothelial cells more vulnerable to oxidative stress predisposing patients to diabetic vascular complications. Notably, in streptozotocin-induced diabetic Nrf2$^{-/-}$ mice diminished defenses against oxidative and/or nitrosative stress may account for a greater deterioration of renal function compared with wild-type mice (94, 212). In human endothelial cells, although high concentrations of glucose (30 m*M*, 6 h) do not significantly induce Nrf2 translocation, activation of this pathway by sulforaphane significantly reduces hyperglycemia-induced ROS generation and protein glycosylation (205).

Hydrogen sulfide (H_2S) is another vasodilator (187) and may be involved in diabetic vascular dysfunction *via* the Nrf2

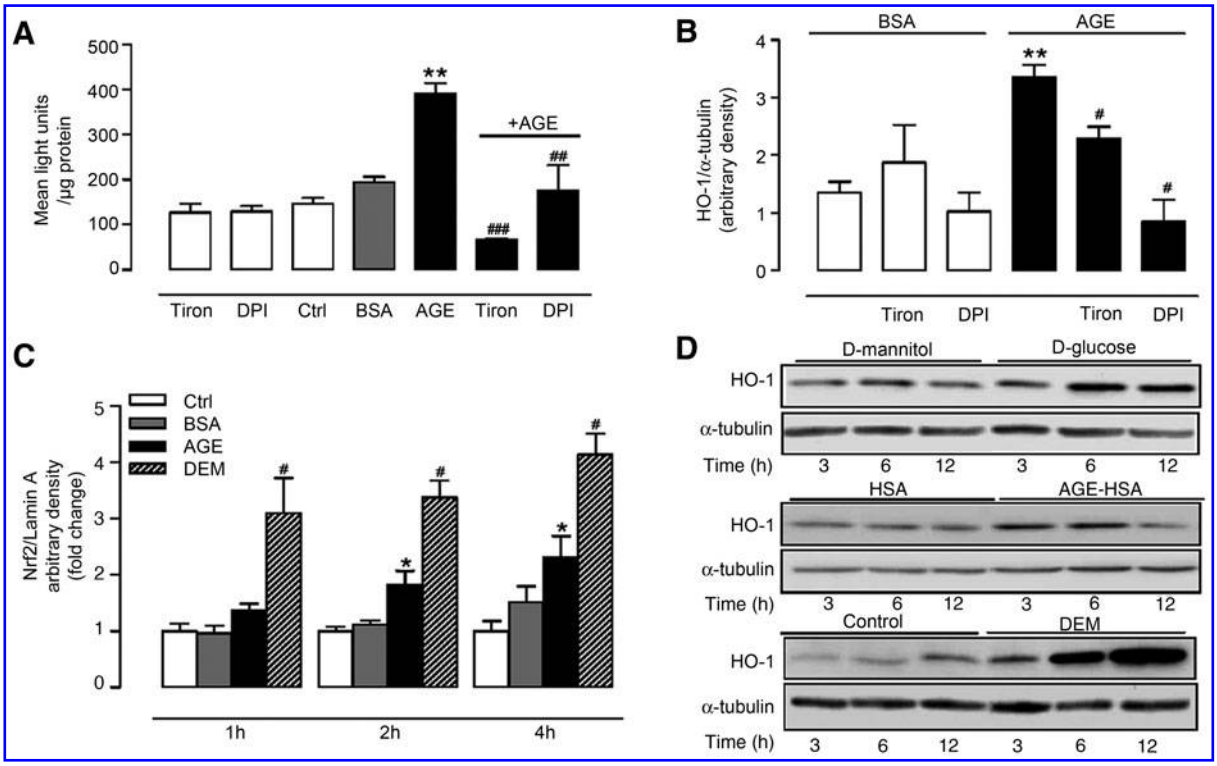

FIG. 6. Induction of HO-1 by elevated glucose, AGE, and electrophilic stress in endothelial cells. (**A**) BAEC were treated acutely with medium (Control, Ctrl) and Tiron (10 μ*M*), DPI (10 μ*M*), BSA (100 mg ml^{-1}), or AGE-BSA (100 mg ml^{-1}) in the absence or presence of the ROS scavengers. ROS generation was measured immediately over 0–40 min after incubation of cells in Krebs buffer containing the luminescent probe L-012 (100 μ*M*) in the continued absence or presence of BSA or AGE-BSA. (**B**) BAEC were treated for 24 h with native BSA (100 mg ml^{-1}) or AGE-BSA (100 mg ml^{-1}) in the absence or presence of Tiron (10 μ*M*) or DPI (10 μ*M*) and cell lysates immunoblotted for HO-1 expression relative α-tubulin. (**C**) AGE-BSA-induced nuclear accumulation of Nrf2 in BAEC. Cells were equilibrated for 24 h in DMEM and then treated for 1, 2, and 4 h with DMEM (Ctrl) or DMEM containing BSA (100 mg ml^{-1}) or AGE-BSA (100 mg ml^{-1}) or DEM (100 m*M*). Nrf2 protein levels relative to Lamin A/C (loading control) in three separate BAEC cultures were analyzed by densitometry. (**D**) Elevated D-glucose (25 m*M*), AGE-modified HSA, and the electrophilic agent DEM (100 μ*M*) increase HO-1 expression in human umbilical vein endothelial cells. Cells were challenged for 3–12 h with stress agents and cell lysates immunoblotted for HO-1 expression relative to α-tubulin. D-mannitol (5 m*M* glucose + 20 m*M* mannitol) and HSA (100 μg ml^{-1}) were used as controls for elevated D-glucose (25 m*M*) and AGE-HSA (100 μg ml^{-1}). Immunoblots are representative of three independent cultures. All other data are expressed as mean ± standard error of the mean, $n = 3$–4 different cultures, $^{*}p < 0.05$ *versus* BSA, $^{**}p < 0.01$ *versus* BSA, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *versus* AGE alone or for DEM $^{\#}p < 0.05$ *versus* control and $^{*}p < 0.05$ *versus* Ctrl. BAEC, bovine aortic endothelial cells; BSA, bovine serum albumin; DEM, diethylmaleate; DMEM, Dulbecco's modified Eagle's medium; DPI, diphenyleneiodonium; HSA, human serum albumin.

pathway. Endogenous H_2S synthesis is catalyzed by cystathionine β-synthase and cystathionine γ-lyase using L-cysteine as a substrate (187), whereas intracellular levels of cysteine are tightly regulated by the Nrf2-linked xCT anionic amino acid transporter (14, 165). Thus, a deficit of Nrf2-mediated cystine transport may affect *de novo* H_2S synthesis and contribute to vascular dysfunction. In fact, H_2S plasma levels are reduced in diabetic patients and streptozotocin diabetic rats (92). However, another study observed a nonsignificant decrease in plasma H_2S levels in streptozotocin-diabetic rats (214), with an increased formation of H_2S from exogenous L-cysteine in the liver and pancreas (but not kidney) of diabetic rats. Administration of H_2S (Na_2S 100 μg/kg, intravenous injection 24 h before reperfusion injury) in mice activates Nrf2 signaling and increases expression of HO-1 and Trx-1, rendering animals more resistance to oxidative stress and ischemia/reperfusion-induced injury (28). Although there are limited reports to date, it seems likely that an interaction between Nrf2 and H_2S may influence vascular reactivity and the pathogenesis of diabetes.

Insulin and Nrf2-linked gene expression

Insulin increases HO-1 expression in an Nrf2-dependent manner in mouse primary tubular epithelial cells and renal adenocarcinoma cells *via* activation of the PI3K/Akt pathway (72). This finding is consistent with reports in bovine retinal endothelial cells and pericytes that insulin receptor substrate 2 and Akt are responsible for insulin-induced HO-1 expression (62). Insulin may also protect endothelial cells against oxidative stress by reducing apoptotic and inflammatory responses *via* HO-1 induction (62). In human brain endothelial cells exposed to elevated glucose or *tert*-butylhydroperoxide, insulin-induced activation of PI3K and Nrf2 may afford protection against ROS by restoring redox homeostasis *via* upregulation of GCLC (111, 144). In streptozotocin diabetic Nrf2 null mice insulin resistance is exacerbated, as evidenced by higher blood glucose, free fatty acids and triglyceride levels, increased urine output, and prolonged hyperglycemia after a glucose challenge (4). Although experimental diabetes activates Nrf2-linked NQO1 expression in wild-type mice, this protection is abrogated in knockout mice (4). These findings implicate Nrf2 in preserving insulin sensitivity, and interruption of insulin-Nrf2 interactions may contribute to insulin resistance in diabetes.

Nrf2 and obesity

Obesity associated with metabolic syndrome is a potent risk factor for diabetes. In wild-type mice fed a high-fat diet, a model of obesity associated with diabetes and mitochondrial dysfunction (177), obesity is prevented by activation of Nrf2 with the triterpenoid analog oleanolic acid, and the beneficial effect of oleanolic acid is abrogated in $Nrf2^{-/-}$ mice (171, 188). The reduction of fat accumulation by Nrf2 may result from an inhibition of adipocyte differentiation as observed in 3T3-L1 cells (188). Interestingly, Nrf2-disrupted mice are generally more resistant to obesity and their weight gain is much slower compared with wild-type mice fed a high-fat diet (171). Although plasma-free fatty acid levels are increased in diabetic $Nrf2^{-/-}$ mice, synthesis of free fatty acids in the liver may be reduced as a compensatory mechanism (4). In summary, basal Nrf2 activity is essential for adipose tissue metabolism and differentiation, whereas further activation of Nrf2 inhibits fat accumulation in response to a high-fat diet or diabetes.

Nrf2 and Redox Regulation in Aging

Biological aging is associated with a marked increase in the incidence of type 2 diabetes, cardiovascular diseases, neurodegenerative diseases, and cancer (68). A study in the offspring of centenarians establishes that longevity is characterized by a delayed onset of diabetes, hypertension, and stroke, indicating that biological change during aging may contribute to the development of these diseases (190). Notably, biological aging is accompanied by an accumulation of oxidative stress and decline of antioxidant defenses (43, 95), implicating long-term oxidative stress as a major contributor to aging-related diseases and its effects on longevity (59). Decreased intracellular GSH levels predispose human umbilical vein endothelial cells to a presenescent status (193). Apart from increased ROS production during the aging process, dysregulation of endogenous antioxidant responses merit further investigation, since impaired Nrf2 activation may be associated with senescence and the onset of aging-related diseases.

Nrf2 homolog SKN-1 in Caenorhabditis elegans and longevity

SKN-1, a homolog of Nrf2 in *Caenorhabditis elegans*, is responsible for the activation of detoxifying and antioxidant enzymes, and SKN-1 mutants have a shorter lifespan and die as a consequence of impaired antioxidant defenses (7). Further studies link SKN-1 with an extended lifespan caused by caloric restriction, and mutations of this transcription factor completely abrogate the benefit of dietary restriction (18). Activation of SKN-1 by the antidiabetic drug metformin mimics the effects of caloric restriction on longevity (146). Moreover, recent findings reveal that the insulin/insulin-like growth factor-1 signaling pathway has a direct inhibitory impact on SKN-1, with abrogated insulin signaling leading to prolonged longevity associated with activation of SKN-1 (192). Moreover, as in mammalian cells, GSK-3β can prevent SKN-1 from constitutively inducing phase II gene expression in *C. elegans* (192).

Nrf2 and longevity in mammals

The antioxidant functions of Nrf2 are strictly conserved in higher mammals, but regulation of this pathway and its role in longevity are much more complicated. Accumulating evidence has established that the aging process correlates with a decline in Nrf2-mediated antioxidant responses in the liver, cardiovascular, nervous, and immune systems (43, 55, 169). Clinical evidence from liver transplantation donors reveals that Nrf2 expression is higher in livers from young donors (40.5 years average, range 28–53) than in those from old donors (216). Moreover, reduced nuclear Nrf2 activity and induction of detoxifying genes may in part be responsible for the decreased GSH levels in the liver of aged mice (170). Similar findings have been reported in smooth muscle cells isolated from old rats, where activation of Nrf2 in response to tumor necrosis factor-α and high glucose is diminished possibly due to dysregulation of the extracellular signal-regulated kinases pathway (117). Senescent fibroblasts exhibit lower mRNA and protein expression of Nrf2 and NQO1 (98). In this same study, Nrf2 knockdown using siRNA slowed cell proliferation and increased mRNA expression of the

senescent markers β-galactosidase activity and p16. Moreover, continuous activation of Nrf2 seems to prolong the lifespan of fibroblasts and their resistance to oxidative stress (98). To our knowledge, this is the first evidence in a mammalian cell system that Nrf2 activation can reverse the aging process and extend longevity.

Nrf2 knockout and wild-type mice have a similar lifespan when reared in a nonstressful environment (32). In mice, caloric restriction prolongs longevity, delays aging, increases liver NQO1 activity and GSH levels, and improves insulin sensitivity (152). In contrast to *C. elegans*, dietary restriction prolongs lifespan in both wild-type and $Nrf2^{-/-}$ mice (152), suggesting that other compensatory mechanisms may be involved in the aging process in mammals. The benefit of Nrf2 activation under less stressful conditions may be indirect involving, for example, signaling pathways leading to increased NO production (73). NO donors or overexpression of eNOS in endothelial cells delays senescence, as evidenced by reduced β-galactosidase-positive cells and an increase in telomerase activity (73). $Nrf2^{-/-}$ mice are more sensitive to oxidative/environmental stress and consequently have a reduced survival rate (152). Interestingly, knock down of the detoxifying enzyme GSH transferase mGSTA4-4 (a Nrf2 target gene) in mice extends lifespan, yet expression and activity of Nrf2 are upregulated indicating that adaptive responses in the Nrf2/ARE signaling influence longevity after metabolic perturbations (172). Genetically enhanced ARE genes may increase longevity in animal models, as the longer lifespan of snell dwarf mice is associated with increased basal Nrf2-linked gene expression (172). The regulation of Nrf2 and its response to oxidative stress may be more important in the elderly, as weakened endogenous antioxidant defenses may render these individuals more vulnerable to oxidative stress.

Nrf2 and stress-induced acceleration of senescence

Atherosclerosis is associated with endothelial cell senescence as characterized by deficits in DNA repair and telomere shortening, and vascular senescence is more prevalent in aged individuals (57, 133). Hyperglycemia, hyperinsulinemia, and other high-risk cardiovascular conditions are accompanied by increased ROS generation, which may accelerate the aging process and lead to stress-induced premature senescence (36, 133, 178). Impaired antioxidant defenses would exacerbate the aging process, and in this context metabolic syndrome is associated with an enhanced risk for atherosclerosis and senescence. Endothelial senescence is increased in mice fed a high-fat diet, and aged mice (12 months) are characterized by a decline in Nrf2 associated with an impaired metabolic status, diabetes, and accelerated atherosclerosis (43). A high-fat diet may also accelerate the aging process in young animals. In contrast, Nrf2 activators such as ebselen and rosiglitazone largely reverse premature senescence and rescue the vascular function in obesity-induced diabetic mouse models (20, 43).

Therapeutic Prospects

Nrf2/ARE and its downstream target genes constitute a key cellular defense against oxidative stress and xenobiotic insults. Under physiological conditions Nrf2/ARE signaling is critical for metabolic and redox homeostasis. Dysregulation of Nrf2-linked redox signaling may lead to and/or exacerbate vascular pathology in diabetes. Targeting the Nrf2 pathway may provide a strategy to counteract sustained oxidative stress in diabetes and other cardiovascular diseases such as stroke (61). HO-1, as a key Nrf2 regulated antioxidant enzyme, was recently highlighted in the therapy for diabetic vasculopathy and insulin resistance (79, 139), and has been shown to protect the neurovascular unit in a mouse model of stroke (220). In the context of translation of basic research to the clinic, we propose that targeting the Nrf2/ARE pathway may provide a useful therapy in the treatment of diabetes and other cardiovascular diseases, including stroke (220, 221).

Polymorphisms in the promoter of HO-1 are related with an increased incidence of cardiovascular complications in diabetic patients (39), suggesting that activation of this enzyme, probably *via* the Nrf2 pathway, is involved in the defense against oxidative stress. Notably, overexpression of HO-1 significantly enhances the survival of transplanted pancreatic islet cells and improves the outcome of surgery (158). Upregulation of HO-1 reduces oxidative stress and prevents endothelial cell sloughing in diabetic rats (107, 158, 175), restores mitochondrial transport carriers and cytochrome C oxidase activity in the diabetic kidney and vessels (107), and diminishes high glucose and tumor necrosis factor-α-induced ROS production and apoptosis in endothelial cells (11). Systemic upregulation of HO-1 using either adenoviral transfection or chemical induction ameliorates insulin resistance (139) restores glucose metabolism (4) and the immune response (79), all of which delay the progression of diabetes.

In summary, low physiological ROS levels activate the redox-sensitive Nrf2-Keap1 pathway to maintain redox homeostasis, whereas an overproduction of ROS, from either endogenous or exogenous sources, results in uncoupling of eNOS, mitochondrial dysfunction, and redox dysregulation (61). As recently reviewed (60), biliverdin/bilirubin generated from heme metabolism *via* HO-1 has been reported to inhibit NADPH oxidase activity, providing a mechanism by which ROS-induced activation of Nrf2-linked HO-1 activity provides vascular protection against NADPH and mitochondrial-mediated oxidative stress in diabetes.

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Address correspondence to:
Prof. Giovanni E. Mann
Cardiovascular Division
School of Medicine
King's College London
Franklin-Wilkins Building (Room 3.01)
150 Stamford St.
London SE1 9NH
United Kingdom

E-mail: giovanni.mann@kcl.ac.uk

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Abbreviations Used

15d-PGJ_2 = 15-deoxy-$\Delta^{12,14}$-prostaglandin J2
AGEs = advanced glycation end products
AKR = Aldo-keto reductase
ARE = antioxidant response element
ATP = adenosine triphosphate
BAEC = bovine aortic endothelial cells
BH_4 = tetrahydrobiopterin
BSA = bovine serum albumin
Cat = catalase
CK2 = casein kinase 2
CoQ = coenzyme Q
COX-2 = cyclooxygenase-2
Ctrl = control
cys = Cysteine residues
Cyto C = cytochrome C oxidase
DEM = diethylmaleate
DMEM = Dulbecco's modified Eagle's medium
DPI = diphenyleneiodonium
EC = endothelial cell
eNOS = endothelial nitric oxide synthase
ER = endoplasmic reticulum
FAD = flavin adenine dinucleotide
GCL = glutamate-cysteine ligase
GCLC = glutamate-cysteine ligase catalytic subunit
GCLM = glutamate-cysteine ligase modifier subunit
GPx = glutathione peroxidase
GR = glutathione reductase
GSH = glutathione
GSK-3β = glycogen synthase kinase 3β
GSSG = Disulfide glutathione
GST = glutathione-*S*-transferase
H_2O_2 = hydrogen peroxide
H_2S = hydrogen sulfide
HAEC = human aortic endothelial cells
HASMC = human aortic smooth muscle cells
HBEC = human brain endothelial cells
HMEC = human microvascular endothelial cells
HO-1 = heme oxygenase-1
HPAEC = human pulmonary artery endothelial cells
HSA = human serum albumin
HUVEC = human umbilical vein endothelial cells
IGF-1 = insulin-like growth factor
IL-8 = interleukin 8
Keap1 = Kelch-like ECH-associated protein 1
MAoSMC = murine aortic smooth muscle cells
MAPK = mitogen-activated protein kinases
MASMC = mouse airway smooth muscle cells
MCP = monocyte chemotatic protein-1
MnSOD = mitochondrial superoxide dismutase
NF-κB = nuclear factor kappa B
NO = nitric oxide
NOX = NADPH oxidase
NQO1 = NAD(P)H:quinone oxidoreductase 1
Nrf2 = nuclear factor E2-related factor
oxLDL = oxidized low-density lipoprotein
PI3K = phosphoinositide 3-kinases
PKC = protein kinase C
Prx = peroxiredoxin
Prx3-ox = oxidized peroxiredoxin 3
Prx3-red = reduced peroxiredoxin 3
RASMC = rat aortic smooth muscle cells
ROS = reactive oxygen species
SKN = SKiNhead (transcription factor)
SMC = smooth muscle cells
SOD = superoxide dismutase
TCA cycle = tricarboxylic acid cycle
TNF-α = tumor necrosis factor-α
Trx = thioredoxin
TrxR = thioredoxin reductase
Trx-2-ox = oxidized thioredoxin 2
Trx-2-red = reduced thioredoxin 2
UCP = uncoupling proteins
VCAM-1 = vascular cell adhesion molecule-1
xCT = cystine-glutamate transporter

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