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Nuclear Factor (Erythroid-Derived 2)-Like-2 Factor (Nrf2), a Key Regulator of the Antioxidant Response to Protect Against Atherosclerosis and Nonalcoholic Steatohepatitis

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Abstract Tissue oxidative stress is a common hallmark of atherosclerosis and non-alcoholic steatohepatitis (NASH), 2 conditions linked epidemiologically and pathophysiologically. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is the master regulator of inducible antioxidant responses, that can attenuate cellular injury from oxidative stress induced by obesity and other redox insults. Nrf2 expression and activation is reduced in mouse and human vessels that harbor accelerated atherosclerosis and in livers with histologic criteria of NASH. Systemic antioxidants have thus been attractive therapeutic targets, but clinical trials have been largely unsuccessful in improving cardiovascular health. Macrophage-selective Nrf2 activation may, however, provide an approach to reduce vascular and hepatocyte injury without the complications of systemic antioxidants, since macrophages play key roles in the development and progression of both atherosclerosis and NASH. In this article, we review the common mechanisms of oxidative stress and inflammation in atherosclerosis and NASH, and discuss the role of Nrf2 in vascular and hepatocyte protection.

Keywords Nrf2 · Oxidative stress · Atherosclerosis · Nonalcoholic steatohepatitis · NASH

Introduction

In developed countries, the epidemic of obesity in aging populations is setting the stage for a massive health care crisis. In the US alone, obesity-related health-care costs are expected to rise to $276 billion by 2030 [1]. Atherosclerosis and non-alcoholic steatohepatitis (NASH) are among the most common complications of obesity and are often found in the same patients. Both are characterized by increased inflammation and lipid and cholesterol deposition in their affected tissues [2]. Individuals with these conditions often share common risk factors, including metabolic syndrome components of central obesity (≥40” males, ≥36” females), elevated triglycerides (≥150 mg/dL), low high-density lipoprotein (HDL) cholesterol (<40 mg/dL males, <50 mg/dL females), hypertension (≥130/80 mm Hg) and glucose intolerance (fasting plasma glucose ≥110 mg/dL) as defined by the US National Cholesterol Education Program Adult Treatment Panel [3]. Age, another important risk factor for atherosclerosis is also associated with increased risk for NASH [4, 5].

Oxidative Stress is Enhanced in Metabolic Syndrome

Oxidative stress arises from both excess production and insufficient neutralization of toxic reactive oxygen species (ROS). In metabolic syndrome patients, increases in free fatty acids, mitochondrial fatty acid beta-oxidation, and inflammation as well as reduced high density lipoprotein (HDL) cholesterol, hypertension, hyperglycemia, and hyperglycemia-induced increases in advanced glycosylated end-products (AGEs), all amplify tissue oxidative stress. Mitochondrial damage associated with both aging and obesity leads to inefficient mitochondrial respiration resulting in increased mitochondrial ROS production [6]. High free fatty acid levels in obese and insulin resistant individuals stimulate increased mitochondrial beta-oxidation, however, due to the lack of corresponding energy demand, this results in the accumulation of incomplete fatty acid breakdown products that can increase mitochondrial ROS and H_2O_2 production [7, 8]. Fatty acids, particularly saturated fatty acids, can

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activate a number of proinflammatory signaling pathways such as the Toll-like receptor pathway [9], which activate inhibitors of κB Kinase and nuclear factor κB that induce proinflammatory gene expression. These changes promote enhanced recruitment and activation of inflammatory cells [10]. As part of their characteristic activation response, inflammatory cells increase local production of ROS. Hypercholesterolemia has been shown to increase superoxide and peroxynitrite levels in the heart, leading to cardiac dysfunction over time [11]. Oxidative stress has been implicated as a potential pathophysiologic mechanism underlying hypertension since oxidative stress markers such as plasma and urine 8-isoprostane were positively correlated, whereas plasma antioxidant capacity, plasma vitamin C levels, and erythrocyte GSH/GSSG ratio were negatively correlated with blood pressure in hypertensive individuals [12]. Hyperglycemia markedly increases glucose concentrations in cells that passively take up glucose, such as endothelial cells, to stimulate protein glycation reactions resulting in AGE formation [13]. AGE modifications can alter the function of affected cellular proteins and their interactions with extracellular matrix components. Receptors for AGE (RAGE) on macrophages recognize AGE-modified plasma proteins to activate inflammatory pathways that further increase oxidative stress [14, 15]. AGE mechanisms are particularly operative in the macro- and microvascular complications of diabetes [16]. Other mechanisms by which hyperglycemia increases oxidative stress include activation of aldose reductase and the polyol pathways by glucose, which decrease NADPH/NADP+ ratios [17]. Hyperglycemia may also activate protein kinase C which activates NADPH oxidase to reduce NADPH/NADP+ ratios [18]. Reduced NADPH levels suppress the regeneration of reduced glutathione from oxidized glutathione, an important cellular antioxidant [19].

Multiple Sources of Oxidative Stress Contribute to Vascular Damage

Endothelial cells normally secrete both vasodilators and vasoconstrictors to regulate vascular tone. Vascular oxidative stress, however, can induce endothelial dysfunction, characterized by numerous abnormalities, including decreased vasodilatory capacity. We have demonstrated that vascular oxidative stress, manifested as coronary circulatory abnormalities of nitric oxide-mediated, endothelium-dependent vasomotion, occurs in young insulin resistant people and progresses with age and glucose intolerance [20]. These changes occurred in the absence of the metabolic syndrome, but appeared associated with obesity-induced inflammation. Endothelial cells normally secrete nitric oxide (NO) in response to shear stress to promote vasodilation through NO-mediated actions on vascular smooth muscle cell (VSMC) relaxation. However, increases in exposure to inflammation and many of the above described risk factors can induce oxidative stress to reduce the amount of bioactive NO by chemical inactivation to form toxic peroxynitrile. Peroxynitrile in turn can lead to dysfunction of NO synthase such that it generates superoxides, which form ROS [21]. Impaired NO signaling and toxic peroxynitriles lead to endothelial dysfunction [22]. In addition, obesity is associated with upregulation of many components of the renin-angiotensin system [23]. The vasoconstrictor angiotensin II stimulates vascular NADH/NADPH oxidase to produce superoxide, which decreases NO bioavailability and increases vasoconstriction. Angiotensin II also increases vascular expression of vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), and pro-inflammatory cytokines, including MCP-1, to promote monocyte invasion and VSMC migration that result in vascular remodeling, and thus promote atherosclerotic plaque development [24, 25].

Atherosclerotic plaques result from macrophage accumulation in the sub-endothelial regions of arteries. Hypercholesterolemia results in the accumulation of low density lipoprotein (LDL) cholesterol in the sub-endothelial microenvironment, which becomes converted to oxidized LDL (oxLDL) due to vascular oxidative stress [26]. Vascular macrophages ingest oxLDL causing them to differentiate into foam cells that release inflammatory cytokines that recruit more macrophages and other pro-inflammatory cells to the sub-endothelial site. Enlarged oxLDL-laden foam cells ultimately undergo apoptosis, which is markedly enhanced by oxidative stress, and which leads to the development of advanced atherosclerotic plaques. In advanced lesions, oxidative stress-induced macrophage apoptosis interferes with efferocytosis, ie, the recognition and clearance of dead cells by appropriate phagocytic cells [27•]. The inability to clear foam cell debris results in formation of necrotic lipid cores (NLC), which are a critical feature defining the atherosclerotic plaque. NLCs contain oxidized lipid species, which are highly thrombogenic in the event of plaque rupture [28]. Therefore, macrophage-selective attenuation of oxidative stress and apoptosis may be a plausible strategy to suppress atherosclerosis.

Despite strong evidence linking oxidative stress to atherosclerosis events *in vitro*, the role of oxidative stress *in vivo* in atherosclerosis is unclear. Several clinical trials have tested oral antioxidants such as Vitamins E and C, beta-carotene, and *N*-acetylcysteine (NAC) as therapeutic approaches to mitigate oxidative stress associated with age and obesity, but these trials have shown little or no effect to reduce the severity of vascular damage [29–32]. While the underlying reasons for the failure of these treatments have not been completely resolved, it has been proposed that Vitamin E may require a co-oxidant to prevent oxidation of low-density lipoproteins (LDL) and that 1–2 years of antioxidant treatment may be inadequate to reverse the

effect of 40–50 years of oxidative stress [29]. ROS also act as important and necessary signaling molecules to regulate key health preserving biological processes [33], so it is not completely surprising that non-targeted ROS suppression has been shown to produce detrimental effects. For example, high-dose vitamin E treatment was associated with increased mortality in several cardiovascular disease populations and was found to antagonize the HDL-lowering effects of statins [34], while the ROS scavenging effects of vitamin C supplementation was found to partially negate the effects of exercise to improve insulin sensitivity in healthy normal-weight young men [35]. These data suggest that non-targeted attenuation of systemic oxidative stress may not be desirable in all patient populations. In contrast, some studies have shown beneficial cardiovascular effects of vitamin E supplementation in select patient populations. For example, vitamin E treatment was found to decrease the incidence of non-fatal myocardial infarction in male smokers, patients with prevalent atherosclerosis, hemodialysis patients and women over 65 year of age [34]. In the Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) Study, vitamin E and slow-release vitamin C supplementation was also found to reduce carotid artery intima media thickness in hypercholesterolemic persons [36].

Oxidative Stress is a Key Mediator of Liver Injury

Non-alcoholic fatty liver disease (NAFLD), frequently found in obesity, is defined by the development of hepatic steatosis, a condition in which triacylglycerols (TAGs) accumulate within hepatocytes as microscopic lipid droplets. Liver normally demonstrates a limited degree of lipid storage, but in NAFLD the capacity of the liver to safely store TAGs is surpassed, and excess lipid leads to 'lipotoxicity', resulting in hepatocyte dysfunction and death. Fatty acid oxidation in excess of demand increases ROS production in the overburdened hepatic mitochondria, resulting in chronic hepatic oxidative stress that is common in the liver of obese subjects [37]. Oxidative stress also diminishes insulin sensitivity, preventing hepatic TAG export from the liver, and exacerbating hepatic lipotoxicity [38]. NAFLD livers exhibit increased oxidative damage to lipids, proteins, and DNA [39], and a concomitant reduction in the expression and activity of antioxidant enzymes such as catalase and glutathione S-transferase [40]. Lipid accumulation and oxidative stress induce the activation of Kupffer cells, specialized liver-resident macrophages, as well as infiltration of other pro-inflammatory cells. Chronic inflammation and oxidative stress then activate stellate cells to promote liver fibrosis which if extensive and longstanding can progress to cirrhosis and end-stage liver disease.

Approximately, 10 %–20 % of Americans have NAFLD, and 2 %–5 % of Americans have NASH, and these numbers are prominently increased by obesity, such that ~30 % of obese subjects have NASH [41]. If left untreated, NASH can advance to liver cirrhosis or hepatocarcinoma, and is currently the second leading cause of liver transplantation in the US. Despite its increasing prevalence and increasing role in end-stage liver failure, there is no approved treatment for NASH. Following lipid accumulation in the liver (first "hit"), oxidative stress has been directly implicated as the stimulus (second "hit") of progression from NAFLD to NASH [42]. Consequently, reducing oxidative stress is expected to decrease progression to NASH. In agreement with this hypothesis and in contrast to the mixed findings in atherosclerosis studies, vitamin E treatment reduced hepatic steatosis, lobular inflammation, serum liver injury markers, and NASH scores in patients with biopsy-proven disease, although it did not reverse fibrosis [43•].

Nrf2 is a Major Regulator of the Antioxidant Response

Free radical detoxification, which is essential to reduce ROS-induced cell injury, is mediated by multiple well-coordinated antioxidant enzymes that are responsible for maintaining cellular redox balance. ROS exposure stimulates cells to increase the expression of antioxidant and cytoprotective enzymes. Nuclear factor erythroid 2-related factor 2 (Nrf2), a redox-regulated transcription factor, is the master regulator of the cellular response to excess ROS. Similar to regulatory factors controlling other rapid responses to changing cellular environmental conditions (ie, NF-κB and HIF1α); Nrf2 is primarily regulated at the post-translational level [44]. Nrf2 is normally constitutively expressed but retained in the cytoplasm by interaction with an inhibitor protein, Keap1, which promotes rapid Nrf2 ubiquitinylation and degradation under normal redox conditions. ROS exposure modifies a critical cysteine residue in Keap1 [45] to stimulate both Nrf2 release and Keap1 ubiquitinylation and degradation. Released Nrf2 protein translocates to nucleus, where it dimerizes with other bZIP proteins, such as small Maf proteins [46], to form transactivation complexes on antioxidant response elements (AREs) and induce the transcription of hundreds of antioxidant and cytoprotective enzymes [47] (Fig. 1).

Selective siRNA-mediated Keap1 knockdown is sufficient to promote Nrf2-dependent gene expression, increase glutathione levels, and suppress tumor necrosis factor α (TNFα)-induced intracellular H_2O_2 production [48], indicating that Keap1 suppresses Nrf2 activity. A recent study showed that autophagic degradation of Keap1 is induced by Sestrins (*Sesn*1 and 2), products of 2 p53 target genes. Ablation of *Sesn2* in mice blocked Keap1 degradation, thereby preventing Nrf2 activation and increasing the susceptibility of the liver to oxidative damage [49]. However,

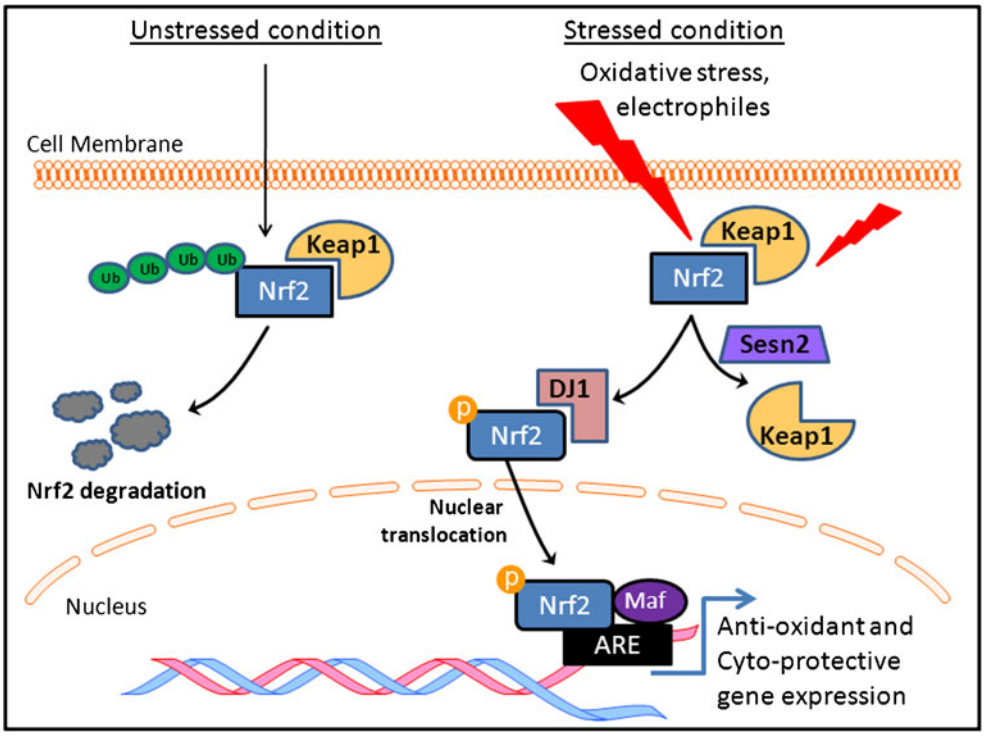

Fig. 1 Nrf2 regulation and activation under stressed and unstressed conditions. Under unstressed conditions, Keap1 facilitates cytoplasmic localization and ubiquitin-mediated degradation of Nrf2. Under conditions of oxidative stress, Sens2 induces degradation of Keap1; releasing Nrf2. Nrf2 is phosphorylated and activated under the protection of DJ1, followed by nuclear translocation. In the nucleus, active Nrf2 binds to antioxidant response elements (ARE) and in coordination with several coactivators (Maf) induces expression of antioxidant genes

Nrf2 activity is also regulated by interaction with the redox-sensitive protein PARK7/DJ-1 (Fig. 1), which can inhibit the Nrf2-Keap1 interaction to attenuate Nrf2 proteolysis [50]. PARK7/DJ-1 mutations are associated with Parkinson's disease [51], in which oxidative stress plays an important role in neurodegeneration.

Nrf2-Deficient Mice Have Increased Tissue Injury

Nrf2 plays an important role in detoxification reactions, especially in the liver, where it directly regulates the expression of several key drug metabolizing enzymes and xenobiotic transporters, including phase 1 enzymes such as aldo-keto reductases, and phase 2 enzymes, such as glutathione transferases and UDP-glucuronosyltransferases [52]. Nrf2-deficiency increases oxidative burden and susceptibility to redox-related injury and carcinogenesis due to reduced expression of a battery of Nrf2-regulated detoxification, redox and xenobiotic genes [53, 54]. Nrf2-deficient mice manifest greater sensitivity to the hepatotoxicity of acetaminophen than wild-type mice [55], whereas mice with a hepatocyte-specific deletion of Keap1 are resistant to high doses of acetaminophen, consistent with increased nuclear Nrf2 protein levels and greater hepatic expression of the Nrf2 target genes, Nqo1, and Gst [56]. Nrf2-deficient mice are more sensitive to chemical carcinogens [57], tobacco smoke [58], bacterial endotoxins [59], butylated hydroxytoluene [60], and other toxins. Similarly, DJ-1 deficiency is associated with a deficit in the scavenging of mitochondrial H_2O_2 and increased susceptibly to tissue injury, especially in neurons [61]. In contrast, Nrf2 activation in an endotoxemic mouse model by Keap1 knockdown suppressed ICAM-1, VCAM-1 and TNFα expression, resulting in significant protection against liver and lung injury [48].

Nrf2 knockout studies to examine obesity-associated atherosclerosis and NASH have been challenging mainly because *Nrf2*$^{-/-}$ mice are partially protected from high fat diet (HFD)-induced obesity [62], making them less insulin resistant than wild-type mice. These mice also have a greater expression of fibroblast growth factor 21 (FGF21) [63], which is known to increase insulin sensitivity in mouse models of obesity [64]. In contrast to global *Nrf2*$^{-/-}$ mice, myeloid Nrf2-deficiency is not sufficient to protect mice from HFD-induced adiposity and insulin resistance [65]. These data agree with our results where we found no difference in body fat, body weight, or measurements of insulin resistance between HFD-fed wild-type and *Nrf2*$^{-/-}$ bone marrow transplant mice [66••], suggesting that Nrf2 acts primarily through non-myeloid cells to affect HFD-induced mouse phenotypes. Treatment of differentiating adipocytes with the potent Nrf2 activator 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im) prevents lipid accumulation in a Nrf2-dependent manner, apparently via stimulating aryl hydrocarbon receptor signaling that has been implicated in the repression of adipocyte differentiation [67]. Consistent with this *in vitro* observation, CDDO-Im treatment prevented HFD-induced obesity and increased energy expenditure, in wild-type mice but not in Nrf2-deficient mice [68].

Nrf2 Activity is Decreased in Aging

One of the hallmarks of aging is the reduced capacity to protect the body against a variety of oxidative and pathological stressors. Reduced Nrf2 expression and activation is at least in part responsible for the impaired cellular mechanisms to combat stress in old age [69]. For example, aged macaques have greater levels of 8-iso-PGFa and 4-HNE in their carotid arteries than young macaques, indicative of increased oxidative stress [70], which was accompanied by reduced carotid expression of Nrf2 and its target genes *Nqo1*, *Gclc*, and *Hmox1*. Further, cultured VSMC of young macaques but not aged macaques were able to elicit an antioxidant response to H_2O_2 and high glucose [70]. In rodents, old Fischer 344 X Brown Norway rats have greater free oxygen radical production than young rats in response to hyperglycemia, corresponding to decreased Nrf2 expression, activation, and expression of its downstream genes in the old rats, while aortas of young rats retained an adaptive increase in Nrf2 activation to hyperglycemia [71].

We reported that aging and metabolic syndrome are associated with an inability to mount an antioxidant response in an age- and diet-induced mouse model of both human-like advanced atherosclerosis and NASH. This metabolic syndrome mouse model is a middle-aged $Ldlr^{-/-}$ mouse given a high fat, high cholesterol western diet (HFD). These mice get 4 of the 5 criteria of metabolic syndrome as described for humans, including obesity, impaired glucose tolerance, reduced HDL cholesterol, and high triglycerides [72]. While young $Ldlr^{-/-}$ mice fed HFD revealed increased expression of *Sod2*, *Cat*, *Gpx1*, *4* in vessels in response to HFD-induced oxidative stress, middle-aged $Ldlr^{-/-}$ mice fed HFD demonstrated a global drop in antioxidant gene expression associated with decreased *Nrf2* expression [72]. This age-related impairment in Nrf2-mediated antioxidant responses is not restricted to the vasculature, since we also found that livers of HFD-fed middle-aged $Ldlr^{-/-}$ mice have reduced antioxidant capacity compared with young mice. Furthermore, while young mice develop fatty livers, middle-aged mice develop more severe hepatic steatosis, along with inflammation and fibrosis and represent a unique mouse model of metabolic syndrome-associated NASH [73]. Thus, Nrf2 dysfunction in aging exacerbates the age-related oxidative stress and increases susceptibility of vascular and hepatic cellular damage. In the brain of aging mice, DJ-1 protein is progressively altered to inactive forms, which leads to accelerated loss of Nrf2 [74]. It is possible that excess oxidative stress related to aging combined with the presence of multiple risk factors leads to altered DJ-1 and impaired Nrf2 activity.

Nrf2 Has Yin/Yang Effects on Atherosclerosis

Nrf2 activity plays several critical roles in the development and progression of atherosclerotic plaques. Nrf2 knockdown impairs the proliferation, adhesion, and migration of cultured human coronary arterial endothelial cells, indicating that Nrf2 is essential for normal angiogenic endothelial processes [75]. On the other hand, Nrf2 activation suppresses VCAM-1 expression, which plays an important role in leukocyte-endothelial interactions [76]. The Nrf2 activator magnesium lithospermate B has also been shown to inhibit glucose-induced proliferation and migration of aortic VSMCs and to prevent neointimal hyperplasia after catheter-induced arterial injury in diabetic rats [77]. Furthermore, siRNA knockdown of *Nrf2* or the Nrf2-regulated antioxidant gene *Nqo1* reversed the protective effects of magnesium lithospermate B in cultured aortic VSMCs, strongly suggesting a direct role for Nrf2-regulated *Nqo1* expression in its vascular protective effects [77]. Hyperglycemia-mediated oxidative stress also induces Nrf2 and its target genes in coronary arterial endothelial cells, and HFD-induced vascular ROS and endothelial dysfunction is more severe in $Nrf2^{-/-}$ mice, indicating that activation of Nrf2 pathways confers endothelial protection under diabetic conditions [71]. $Ldlr^{-/-}$ mice fed a high-fat, high-cholesterol Western diet are an established model of atherosclerosis, although young $Ldlr^{-/-}$ mice used for these studies develop only fatty streaks in their aortae when fed Western diet [72]. Middle-aged $Ldlr^{-/-}$ mice, however, develop more complex atherosclerotic lesions containing NLCs with fibrous caps and, unlike young $Ldlr^{-/-}$ mice that display robust diet-induced antioxidant responses, fail to mount Nrf2-mediated antioxidant responses in both their vasculature and liver [72].

Macrophages are central actors in the development and progression of atherosclerosis, and are thus attractive candidates for targeted Nrf2 activation to both prevent atherosclerosis development and regress established lesions. We recently reported that atherosclerosis-prone young $Ldlr^{-/-}$ mice receiving bone marrow transplants (BMT) from $Nrf2^{-/-}$ mice developed larger and more complex atherosclerotic lesions than mice receiving BMT from wild-type mice, which developed only fatty streaks [66••]. The vascular "aging" of the mice with $Nrf2^{-/-}$ transplant was associated with increased vascular pro-inflammatory gene expression, and $Nrf2^{-/-}$ macrophages displayed increased MCP-1-induced migration, greater lipopolysaccharide-stimulated expression of chemoattractant and proinflammatory genes, and markedly increased susceptibility to H_2O_2-induced apoptosis [66••].

In contrast to the HFD-fed, $Ldlr^{-/-}$ mice model which develops obesity, Nrf2-deficiency in $ApoE^{-/-}$ mice, another popular model of atherosclerosis but not associated with

obesity had attenuated atherosclerotic plaque formation and progression. This result was attributed to decreased macrophage expression of the scavenger receptor CD36 in $Nrf2^{-/-}ApoE^{-/-}$ mice, resulting in decreased uptake of modified LDL by macrophages and reduced foam cell formation [65]. Correspondingly, peritoneal macrophages of $Nrf2^{-/-}$ mice challenged with electronegative LDL (LDL(-)) had greater ROS production and apoptosis, but lacked LDL(-)-induced *Cd36* expression seen in peritoneal macrophages of wild-type mice, suggesting that Nrf2 regulates *Cd36* expression and protects macrophages against ROS-induced apoptosis [78]. However, we did not observe a drop in vascular CD36 expression in middle-age HFD-fed $Ldlr^{-/-}$ [79]. Nrf2-deficiency may also have other effects to attenuate atherosclerosis. For example, $ApoE^{-/-}Nrf2^{-/-}$ mice had reduced plasma cholesterol levels, corresponding with decreased aortic lesion area, suggesting that changes in cholesterol metabolism could at least partially explain the anti-atherosclerotic effect of Nrf2-deficiency [80]. Further, in $ApoE^{-/-}$ mice, Nrf2 is also an essential positive regulator of cholesterol crystal-induced inflammasome activity in macrophages, and Nrf2 was reported to drive IL-1 production, IL-1-dependent vascular injury, and atherosclerosis [81]. These studies suggest Nrf2 has opposing effects on 2 hallmark features of atherosclerosis, cholesterol uptake, and inflammation/oxidative stress, and in $ApoE^{-/-}$ mice the effects of Nrf2-deficiency on macrophage cholesterol uptake appear to dominate, resulting in an anti-atherosclerotic phenotype. In contrast, overexpression of catalase, an Nrf2 target gene, attenuates atherosclerosis in $ApoE^{-/-}$ mice, corresponding with decreases in plasma oxidative stress, indicating that reduced oxidative stress also has beneficial effects on atherosclerosis in these mice [82]. Thus, Nrf2 expression may have positive or negative effects on vascular inflammation, depending upon the genetic and environmental context.

Nrf2 in Liver: Implications for NASH

NASH has been referred to as "atherosclerosis of the liver" primarily due to recognition of the risk factors shared by these 2 diseases and their similar pathological presentations of lipid accumulation, inflammation, and oxidative stress [2]. Indeed, elevated plasma concentrations of the liver enzymes aspartate and alanine aminotransferase (AST and ALT) in metabolic syndrome patients are considered a risk factor for both coronary artery disease and NASH [83]. NASH is characterized pathologically by steatosis, inflammation, fibrosis, and hepatocyte ballooning degeneration [84]. The mechanism for progression of NAFLD into NASH is thought to occur through a '2 hit' injury response, with hepatic lipid accumulation (insulin resistance) representing the first insult and oxidative stress the second injury [42]. Accumulation of triglycerides is followed by impairment in mitochondrial function, resulting in overproduction of ROS [85]. Chronic ROS generation, reduced superoxide dismutase and catalase activity, and depletion of mitochondrial glutathione leads to increased lipid peroxidation within hepatocytes [86], which can hinder nucleotide and protein synthesis to promote hepatocyte apoptosis [87]. Chronic oxidative stress and lipid accumulation induce inflammation and activation of Kupffer cells and stellate cells resulting in more inflammation and fibrosis, respectively.

Nrf2 knockout mouse studies provide strong evidence that Nrf2 activity regulates NASH progression. Nrf2-deficient mice have more rapid steatohepatitis progression than wild-type mice when fed high-fat high-cholesterol diet [88]. Methionine choline deficient diet, frequently used to induce hepatic inflammation and NASH in mice, also causes a more rapid NASH onset in Nrf2-deficient than wild-type mice [89]. NASH in $Nrf2^{-/-}$ mice is associated with greater expression of fatty acid metabolism genes (*Lxr, Srebf1, Acc1, Scd1, Fasn*), pro-inflammatory cytokine genes (*Tnfα* and *IL-1β*) and fibrosis-associated genes (*Tgfβ1* and *α-Sma*) [88]. Nrf2 regulates several stages of NASH progression, since Nrf2-deficiency increases hepatic steatosis [68], the hepatic expression and action of inflammatory cytokines and hepatic oxidative stress [88], while Nrf2 activation attenuates hepatic stellate cell activation and reduces fibrosis [90, 91]. Nrf2 activation by Keap1 knockdown protects mice from liver injury induced by the superoxide-generating herbicide diquat, while diquat-treated $Nrf2^{-/-}$ mice reveal increased lipid peroxidation and death [92]. Similarly, therapeutic approaches that activate Nrf2 to reduce ROS levels have been shown to prevent hepatic ischemia-reperfusion injury [93]. In humans, Nrf2 and Nrf2-regulated redox genes, such as glutamate-cysteine ligase and glutathione-S-transferase, are reduced in end-stage liver disease, suggesting that Nrf2 pathway impairment may critically impact hepatic detoxification processes [94]. Thus, Nrf2 plays a central role in maintaining liver health in response to various stressors.

NASH research has been hindered by the lack of an animal model that adequately mimics human NASH, which is closely linked to insulin resistance. Nearly 5 out of 6 NASH patients have the metabolic syndrome [95, 96]. However, most mouse models of metabolic syndrome do not develop NASH, while most mouse NASH models do not develop fatty liver or insulin resistance. We have recently identified that middle-aged male $Ldlr^{-/-}$ mice fed Western diet develop metabolic syndrome associated with multiple characteristics of human NASH [73]. This model has a dramatic Nrf2 down-regulation, resulting in reduced expression of hepatic *Sod2*, catalase and other antioxidant enzymes and an increase in oxidative stress. Liver injury

associated with inflammation and oxidative stress is often diagnosed by elevated plasma AST and ALT [97], which are also dramatically increased in this model. Mitochondrial dysfunction, associated with increased oxidative stress [85], is also markedly increased in this model. Treatment with rosiglitazone, an insulin-sensitizing peroxisome proliferator-activated receptor γ (PPARγ) agonist, strongly attenuated hepatic oxidative stress, inflammation, fibrosis, and markers of liver injury, increased *Nrf2* expression, and normalized hepatic antioxidant gene expression, and improved mitochondrial function, enhancing fatty acid oxidation [73]. Moreover, beneficial results of PPARγ ligand treatment on NASH have also been observed in human trials [43•], suggesting that results from this mouse model may translate to human disease. NASH improvements in these mouse and human studies may result from improving the hepatic redox balance to remove the '2nd hit' for NASH progression, suggesting that Nrf2 could be considered a potential therapeutic target for NASH.

Kupffer cells are specialized hepatic macrophages that drive inflammation in response to stimuli such as a high fat diet. Nrf2 appears to play an important role in attenuating this response, since $Nrf2^{-/-}$ BMT $Ldlr^{-/-}$ mice fed Western diet develop greater liver inflammation and fibrosis than wild-type BMT $Ldlr^{-/-}$ control mice [66••]. One hypothesis is that increased cytokine secretion by $Nrf2^{-/-}$ Kupffer cells and other invading $Nrf2^{-/-}$ myeloid cells may increase inflammatory cell infiltration and the activation of stellate cells to increase fibrosis. Thus, myeloid Nrf2 activation may represent an attractive therapeutic target for the treatment of NASH if a myeloid-targeted activation approach can be used to circumvent the potential complications of systemic Nrf2 activation.

Conclusion: Nrf2 as a Potential Therapeutic Target

Nrf2 may be a promising target for treatment of NASH. However, its anti-atherosclerotic effects are model-dependent. In $ApoE^{-/-}$ mice, a model driven primarily by hypercholesterolemia, Nrf2 promotes liver cholesterol synthesis and enhances cholesterol activation of the NLRP3 inflammasome [81]. In aging, obese $Ldlr^{-/-}$ mice, oxidative stress in the setting of metabolic syndrome drives plaque formation [72], so that loss of Nrf2 antioxidant effects in macrophages enhances atherosclerosis extent and complexity [66••]. Further studies are necessary to dissect the complex function of Nrf2 in regulating pro- and anti-inflammatory responses. Systemic ROS attenuation has not shown positive results in multiple clinical trials of antioxidants. Thus, a possible approach for future antioxidant studies may be to attenuate ROS in a cell-specific manner, using either agents that demonstrate cell-selective uptake or targeted delivery systems such as nanoparticles, in order to attenuate oxidative stress in critical cells such as macrophages and Kupffer cells that contribute to atherosclerosis and NASH.

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Christopher J. Lyon declares that he has no conflict of interest.

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- • Of importance
- •• Of major importance

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