

## Reactive Oxygen Species Generation and Atherosclerosis

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Atherosclerosis is the key component of most cardiovascular diseases, including stroke and myocardial infarction.<sup>1–3</sup> An inflamed endothelium recruits inflammatory cells, such as monocytes, via the expression of various mediators and chemokines.<sup>4–6</sup> This, in addition to the accumulation of cholesterol and smooth muscle cells (SMCs) in the intima, leads to the transformation of monocytes into foam cells, which consume dead cells and lipids.<sup>7–9</sup> The atherogenic process involves multiple cell types, that is, endothelial cells (ECs),<sup>10</sup> SMCs,<sup>11</sup> immune cells,<sup>12</sup> and stem/progenitor cells,<sup>13</sup> in which levels of both intracellular and extracellular reactive oxygen and nitrogen species play a fundamental role in vascular cell homeostasis and eventually affects the development of atherosclerosis.<sup>14</sup> Fine-tuning of cellular redox status is a prerequisite for the well-being of vascular system. Although too much oxidative stress can be detrimental, some basal levels are crucial for proper cell signaling. Recently, a number of publications in *ATVB* and other journals have demonstrated substantial progress in research into oxidative stress vascular disease, especially atherosclerosis.<sup>14–17</sup> In the present article, we highlight these updated publications, providing insights into the mechanisms of reactive oxygen species (ROS) generation in pathophysiological conditions of the vessel wall, and the contribution of redox imbalance to lesion formation via influencing vascular cell (dys)functions.

Oxidative stress is defined as a cellular condition where the damaging effect of oxidant is greater than the beneficial effect of antioxidants. Major oxidants are based on O<sub>2</sub> molecules, which are taken in during respiration, with higher reactivity than molecular O<sub>2</sub>, and are known as ROS.<sup>14</sup> ROS are, thus, broadly defined as oxygen-containing chemical species with higher reactive properties. Major ROS include superoxide (O<sub>2</sub><sup>•-</sup>) and hydroxyl (HO<sup>•</sup>) free radicals, as well as nonradical molecules, such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Primary sources of oxidative stress in vessel wall are mitochondria,<sup>18</sup> uncoupled nitric oxide synthase,<sup>19</sup> lipoxygenase,<sup>20</sup> myeloperoxidase,<sup>21</sup> xanthine oxidase (XO),<sup>22</sup> and importantly

NAD(P)H oxidases<sup>23</sup> (Figure 1). However, the influence of ROS-producing enzymes, especially NADPH oxidases, in the development of atherosclerosis is ambiguous.<sup>23</sup> Nox4-derived mitochondrial ROS were detrimental in older mice with atherosclerosis,<sup>24</sup> and dominant negative mutant form of Nox4 decreased atherosclerosis formation.<sup>25</sup> On the other hand, *Nox4* knockout aggravated atherosclerosis,<sup>26</sup> especially in diabetic mice.<sup>27</sup>

High levels of oxidative stress can be counteracted by complex antioxidant cell systems that are crucial for the maintenance of redox balance. The key player that plays a primary role in the regulation of antioxidant gene response is nuclear factor erythroid 2-related factor 2 (Nrf2),<sup>28</sup> encoded by the *Nfe2l2* gene. However, depending on the mouse model used and cell type analyzed or even sex of animals, Nrf2 showed both pro- and antiatherogenic properties.<sup>29</sup> Wire injury induces higher neointima formation in *Nfe2l2*<sup>-/-</sup> mice than in control animals.<sup>30,31</sup> Global knockout of *Nfe2l2* in *Apoe*<sup>-/-</sup> mice decreased the formation of atherosclerotic lesions.<sup>32–35</sup> Transplantation of *Nfe2l2*<sup>-/-</sup> bone marrow to *Apoe*<sup>-/-35</sup> or *Ldlr*<sup>-/-36</sup> recipients attenuated atherosclerosis, what underlines proatherogenic activity of Nrf2 in myeloid cells. However, increased formation of plaque in *Ldlr*<sup>-/-</sup> mice transplanted with *Nfe2l2*<sup>-/-</sup> bone marrow was also reported.<sup>37</sup> On the other hand, activation of Nrf2 in SMCs<sup>38,39</sup> or ECs<sup>40</sup> was protective against atherosclerosis. Furthermore, knockouts of potent antioxidant enzymes, for example, *Gpx1*,<sup>41</sup> *Prdx2*,<sup>42</sup> *Hmox1*,<sup>43</sup> can also aggravate plaque formation. Importantly, basal levels of ROS were crucial for the activation of endoplasmic reticulum (ER) stress response,<sup>44</sup> maintenance of SMC contractile phenotype,<sup>45</sup> or differentiation of SMC from stem cells.<sup>46,47</sup> Thus, the regulation of oxidative stress is complex, and investigation of its role in the pathogenesis of atherosclerosis remains an important subject of many studies. Therefore, the aim of this article is to summarize the latest advances in the research on the role of oxidative stress in the modulation of cells that can affect the development of atherosclerosis.

### Endothelial Cells

The healthy endothelium is key for the functional maintenance of vascular system.<sup>48,49</sup> Sustained ROS levels can contribute to the endothelial dysfunction, and further to its senescence and activation of an inflammatory response, and in turn lead to the development of atherosclerosis. NADPH oxidases, especially Nox4, play an ambiguous role in the development of atherosclerosis. Craig et al<sup>50</sup> reported recently that *Apoe*<sup>-/-</sup> mice with endothelial-specific *Nox4* overexpression (*Apoe*<sup>-/-</sup> Nox4<sup>EC</sup>) showed significantly smaller lesions than control *Apoe*<sup>-/-</sup> animals. *Apoe*<sup>-/-</sup> Nox4<sup>EC</sup> aortas contained, however, similar numbers of macrophages and did not differ from *Apoe*<sup>-/-</sup> mice in expressions of macrophage or inflammatory markers, that is, E- and P-selectin, VCAM1 (vascular

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## Nonstandard Abbreviations and Acronyms

<b>EC</b>	endothelial cell
<b>ER</b>	endoplasmic reticulum
<b>ROS</b>	reactive oxygen species
<b>SMC</b>	smooth muscle cell

cell adhesion molecule 1), and ICAM1 (intercellular adhesion molecule 1). On the contrary, *Apoe*<sup>-/-</sup> *Nox4*<sup>EC</sup> aortas contained higher T<sub>reg</sub> numbers, while numbers of effector T cells and plasma CXCL9 (chemokine [C-X-C motif] ligand 9) concentration were lower than in *Apoe*<sup>-/-</sup> controls. Both mouse lung ECs and human aortic ECs overexpressing *Nox4* showed attenuated CXCL9 induction after interferon- $\gamma$ .<sup>50</sup>

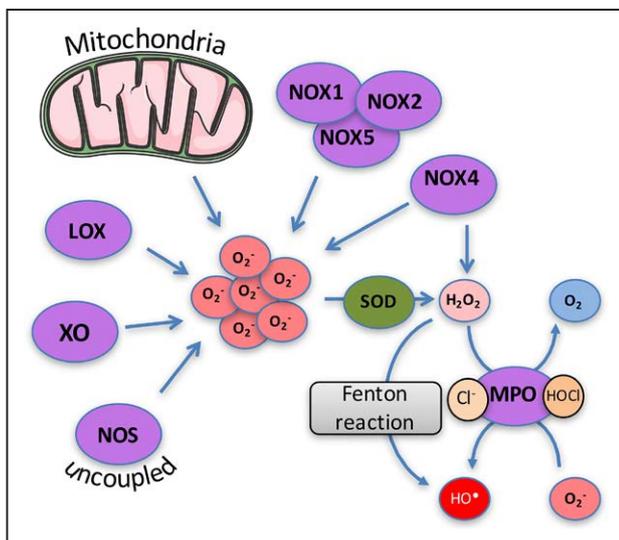
In another study, downregulation of *Nox4* in human aortic ECs, cultured under high glucose conditions, resulted in the increase of profibrotic CTGF (connective tissue growth factor), while endothelial H<sub>2</sub>O<sub>2</sub> decreased.<sup>51</sup> On the other hand, *Nox4* overexpression caused a significant reduction in p-SMAD3 (phosphorylated mothers against decapentaplegic homolog 3). In diabetic *Apoe*<sup>-/-</sup> mice, aortic expression of both *Nox1* and *Nox4* increases after 10 weeks of diabetes mellitus. Further development of atherosclerosis in diabetic *Apoe*<sup>-/-</sup> mice led to the decline in *Nox4* expression, while levels of *Nox1* remained high. Importantly, *Nox4* expression in carotid plaques is lower in patients with cardiovascular events or diabetes mellitus. *Nox4* deletion in diabetic *Apoe*<sup>-/-</sup> mice increased, while deletion of *Nox1* in diabetic *Apoe*<sup>-/-</sup> attenuated plaque formation in comparison to control diabetic *Apoe*<sup>-/-</sup> mice. *Nox4*-deficient *Apoe*<sup>-/-</sup> diabetic mice showed lower levels of aortic H<sub>2</sub>O<sub>2</sub> while superoxide levels were increased. *Nox1* deletion resulted in a decrease in both superoxide and tyrosine nitration. Furthermore, diabetic *Nox4*<sup>-/-</sup> *Apoe*<sup>-/-</sup> showed elevated serum and aortic MCP-1 (monocyte chemoattractant protein 1) concentrations, as well as macrophage accumulation in the vessel wall. *Nox4* knockdown also led to the increase of

fibrillar collagens I and III in plaques, which was associated with elevated transforming growth factor- $\beta$  expression and p-SMAD3 levels.<sup>51</sup>

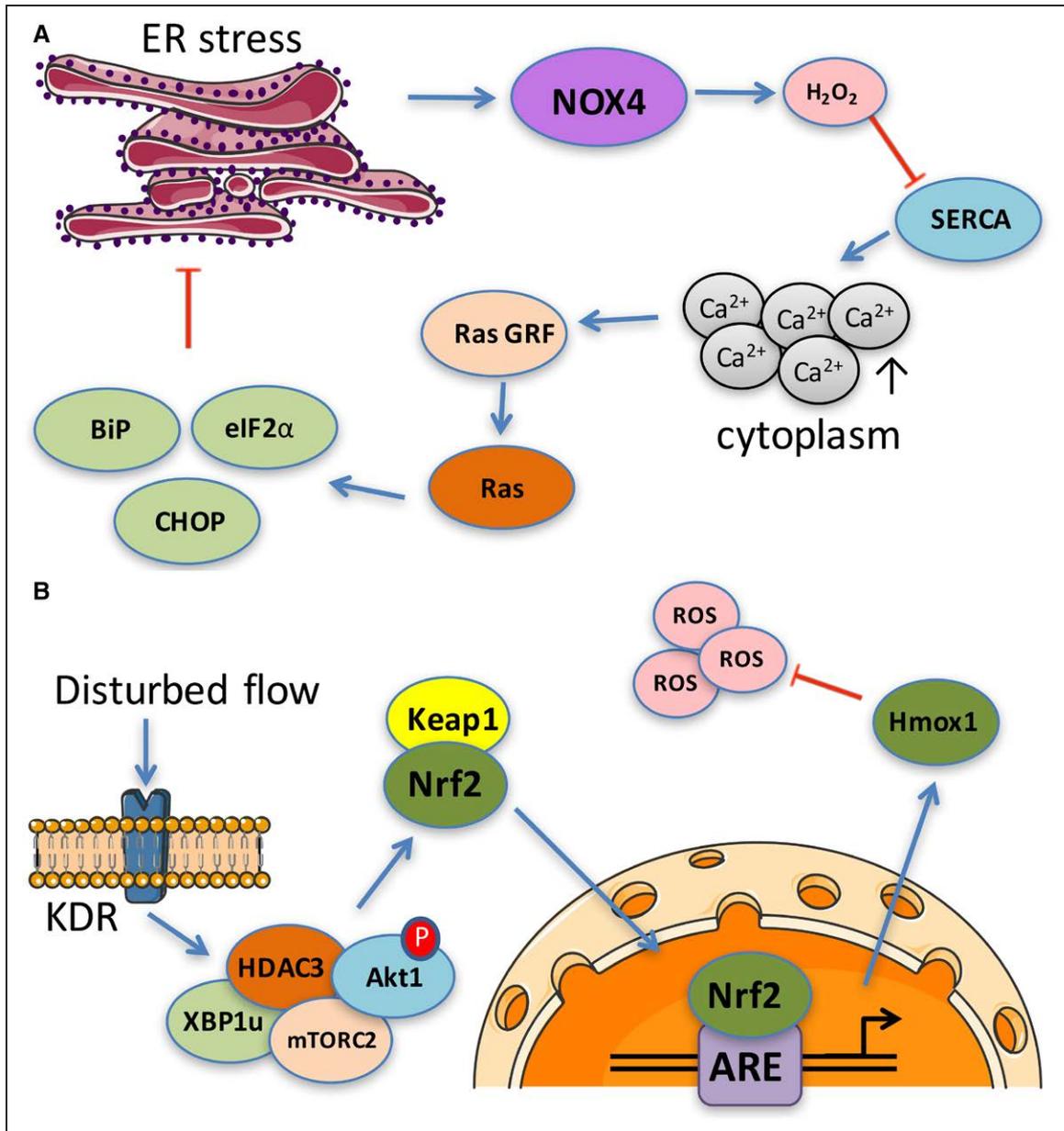
*Nox4* and H<sub>2</sub>O<sub>2</sub> regulate the response to ER stress, which is crucial for the induction of unfolded protein response.<sup>44</sup> Importantly, both ER stress and response to it are activated during the development of atherosclerosis.<sup>52</sup> Wu et al<sup>44</sup> showed that tunicamycin-induced ER stress elevated H<sub>2</sub>O<sub>2</sub> in ER in the *Nox4*-dependent way. Increased H<sub>2</sub>O<sub>2</sub> concentrations led in turn to the oxidation of sarco/endoplasmic reticulum Ca<sup>2+</sup>-ATPase and an increase in cytosolic calcium concentration. High levels of Ca<sup>2+</sup> caused next the activation of RasGRF (Ras-specific guanine nucleotide releasing factor), which then induced via Ras UPR (unfolded protein response), that is, BiP (binding immunoglobulin protein), CHOP (CCAAT enhancer-binding protein homologous protein), and phosphorylation of eIF2 $\alpha$  (eukaryotic initiation factor 2 $\alpha$ )<sup>44</sup> (Figure 2A). Another important player in the ER stress response is XBP1 (X-box-binding protein 1).<sup>53</sup> However, while spliced XBP1 (XPB1s), which positively regulates UPR, contributes to EC apoptosis and atherosclerosis formation, the unspliced XBP1 (XPB1u) can induce the antioxidant response.<sup>54</sup> Interestingly, disturbed flow elevated expression of antioxidant genes in ECs in XBP1u- and HDAC3 (histone deacetylase 3)-dependent manner. Increased expression of XBP1u and HDAC3 in HUVECs has decreased with KDR (kinase insert domain receptor) or PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase) inhibition. What is more, spliced XBP1 decreased HDAC3 levels, while overexpression of XBP1u protected it. Furthermore, high expression of XBP1u protected ECs from H<sub>2</sub>O<sub>2</sub>-induced oxidative stress. The latter effect was mediated by the increased stability of Nrf2 transcription factor and upregulation of its target—*Hmox1*<sup>54</sup> (Figure 2B).

Laminar shear stress increases expression of transcription factor EB (TFEB) in cultured human ECs.<sup>55</sup> TFEB is a basic helix-loop-helix transcription factor, which regulates lysosomal biogenesis.<sup>56</sup> Laminar shear stress induced TFEB translocation to the nucleus and decreased mTOR (mechanistic target of rapamycin) activity in ECs. Interestingly, atherosclerosis-resistant regions of mouse aorta had higher TFEB levels than atherosclerosis-prone ones. TFEB overexpression reduced levels of H<sub>2</sub>O<sub>2</sub> and superoxide, as well as expression of *SELE*, *MCPI*, *VCAM1*, *IL1B*, *IL6*, and *IL8*, in ECs and human coronary artery ECs. ECs overexpressing TFEB also had a higher expression of *HMOX1*, *SOD2*, and *TXN1*. Importantly, TFEB can bind to its putative binding sites in the *HMOX1* promoter and *SOD2* intron 2. However, knockdown only of *HMOX1* in TFEB-overexpressing EC potentially attenuated the anti-inflammatory effect of TFEB. Furthermore, although TFEB is known to regulate autophagy,<sup>56</sup> *ATG5* silencing or pharmacological inhibition of autophagy did not block TFEB anti-inflammatory activity. *mTie2-TFEB* mice overexpressing TFEB in ECs showed less leukocyte rolling after lipopolysaccharide treatment than wild-type (WT) animals. Finally, *Apoe*<sup>-/-</sup> *mTie2-TFEB* mice on high cholesterol diet had less atherosclerotic lesions than *Apoe*<sup>-/-</sup> control animals.<sup>55</sup>

Nrf2 in steady state conditions is sequestered by Keap1 and directed to the proteasomal degradation.<sup>57</sup> Therefore, the regulation of Nrf2 activity is of potential clinical interest.



**Figure 1.** Sources of ROS in the vascular wall. LOX indicates lipoxygenase; MPO, myeloperoxidase; NOS, nitric oxide synthase; NOX, NAD(P)H oxidase; SOD, superoxide dismutase; and XO, xanthine oxidase.



**Figure 2.** Endoplasmic reticulum (ER) stress leads to the activation of Nox4 on ER surface but not cell membrane and increases  $H_2O_2$  levels. Increased oxidative stress inactivates sarco/endoplasmic reticulum  $Ca^{2+}$ -ATPase (SERCA), what causes the increase of cytoplasmic  $Ca^{2+}$ , activation of RasGRF, and Ras. Ras induces then unfolded protein response, namely BiP (binding immunoglobulin protein), CHOP (CCAAT enhancer-binding protein homologous protein), and phosphorylation of  $eIF2\alpha$  (A). Disturbed flow causes ligand-independent activation of KDR, which induces formation of mTORC2-Akt1-XBP1u-HDAC3 complex and increases Akt1 phosphorylation, what contributes to the enhances stability of nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2, the regulator of antioxidant response, induces HMOX1 expression, which protects endothelial cells from oxidative stress (B).

Recent work by Xie et al<sup>58</sup> showed that hydrogen sulfide could enhance Nrf2 activity in diabetic atherosclerotic mice and ECs treated with high glucose and oxidized low-density lipoproteins (LDLs). Streptozotocin-treated *Ldlr*<sup>-/-</sup> mice on a high-fat diet had less atherosclerotic lesions when treated with GYY4137— $H_2S$  donor.  $H_2S$  treatment reduced ROS levels and VCAM1 and ICAM1 expressions and enhanced Nrf2 nuclear translocation in both diabetic atherosclerotic mice and ECs treated with high glucose and oxidized LDLs.  $H_2S$  did not show the protective effect in *Ldlr*<sup>-/-</sup> *Nfe2l2*<sup>-/-</sup> mice or in ECs with silenced *Nfe2l2*. Interestingly,  $H_2S$  enhanced Nrf2 activity by S-sulphydrylation of Keap1 at Cys151.<sup>58</sup>

Interestingly, lysophosphatidylcholines (LPC)—proinflammatory lipids—can contribute to the ROS formation in atherosclerosis.<sup>59</sup> The concentration of LPC, as well as expression of *Pla2g7* and *Pla2g4c*, were higher in *Apoe*<sup>-/-</sup> aortas than in control ones. LPC quickly induced both mitochondrial ROS and to lesser levels cytoplasmic ROS. Inhibition of NADPH oxidases further increased ROS levels. LPC increased cytoplasmic and mitochondrial  $Ca^{2+}$ , what was crucial for the induction of mitochondrial ROS production. LPC elevated mitochondrial proton leak but not ATP production and did not affect levels of mitochondrial superoxide dismutase or uncoupling protein 3. Furthermore, LPC increased

expression of proatherogenic genes, such as *ICAM1*, *IL6*, and *MMP2*, what was attenuated by mitoTEMPO—mitochondrial ROS scavenger. Finally, mitoTEMPO inhibited LPC-induced monocyte adhesion to ECs in vitro, and leukocyte rolling and numbers of total and inflammatory monocytes in *Apoe*<sup>-/-</sup> high-fat diet mice.<sup>59</sup>

Mitochondrial oxidative stress can be decreased by thioredoxin 2 (Txn2) that acts together with thioredoxin reductase 2 (Txnrd2) and peroxiredoxin 3 (Prdx3).<sup>60</sup> Importantly, the endothelial-specific knockout of *Txnrd2* impaired angiogenesis and arteriogenesis after femoral artery ligation and vascular function.<sup>61</sup> Lack of *Txnrd2* in ECs rendered them more proinflammatory and prothrombotic. Embryonic endothelial progenitor cells deficient in *Txnrd2* showed higher intracellular ROS levels and more positive mitochondrial potential. Such cells had also decreased angiogenic properties in vitro, what could be reversed by *Txnrd2* re-expression.<sup>61</sup> Therefore, the imbalance of redox-mediated signaling and the consequence results in endothelial dysfunction that is a key event for the development of atherosclerosis.

### Smooth Muscle Cells

Fine-tuning of SMC proliferation and differentiation status is crucial for the development of atherosclerosis<sup>62</sup> because they can contribute to the formation of neointima but, on the other hand, can stabilize the plaque and prevent its rupture.<sup>11,63</sup> Oxidative stress in atherosclerosis results primarily from the activity of NAD(P)H oxidases.<sup>64</sup> However, their role in SMCs, especially in the context of atherosclerosis, remains ambiguous. Mice lacking *Nox1* developed less neointima in femoral arteries after the wire injury than control animals.<sup>65</sup> On the other hand, SMC-specific overexpression of *Nox1* did not enhance neoplasia formation. *Nox1*-deficient SMCs were further characterized by lower proliferation rate and migration than WT SMCs. Overexpression of *Nox1* slightly enhanced both of the latter characteristics. Lower migration rate in *Nox1*-deficient cells was associated with changes in the regulation of actin cytoskeleton, that is, cofilin phosphorylation and mDia1 expression increased while PAK1 levels were lower.<sup>65</sup> Furthermore, expression of *Nox1* was higher in neointimal SMCs than in normal medial cells.<sup>66</sup> Elevated levels of Nox1 were associated with ERK1/2 (extracellular signal-regulated kinases 1/2) activation and enhanced MMP-9 (matrix metalloproteinase 9).<sup>66</sup>

Importantly, Nox1 forms canonical or hybrid systems with p47<sup>phox</sup> in place of NoxO1 in the hybrid system. Production of superoxide by hybrid Nox1 is regulated by ezrin–radixin–moesin-binding phosphoprotein 50 (EBP50).<sup>67</sup> Expression of EBP50 is elevated in SMC after the angioplasty.<sup>68</sup> EBP50 increased SMC proliferation with inhibiting antimitogenic parathyroid hormone type 1 receptor<sup>69</sup> and regulation of p21<sup>cip1</sup> and Skp2.<sup>70</sup> Al Ghoul et al<sup>67</sup> recently showed that *EBP50*<sup>-/-</sup> SMCs produce less superoxide than WT cells when treated with H<sub>2</sub>O<sub>2</sub> or angiotensin II. In line with that, aortic rings from both *EBP50*<sup>-/-</sup> or *Nox1*<sup>-/-</sup> mice show no increase in superoxide production after stimulation with angiotensin II. What is more, femoral arteries from *EBP50*<sup>-/-</sup> mice had lower lipid peroxidation after lipopolysaccharide treatment than control

animals. EBP50 binds to the p47<sup>phox</sup> subunit of the hybrid Nox1 system and promotes Nox1 activity.<sup>67</sup>

Expression of NADPH oxidases changes dynamically during the development of atherosclerosis.<sup>71</sup> Nox1 levels were increased in aortas *Apoe*<sup>-/-</sup>*Ldlr*<sup>-/-</sup> prior to the lesion formation and then normalized in later stages. Nox4 expression was elevated on later stages of atherosclerosis. SMCs isolated from mice with advanced atherosclerosis had lower Nox1 and higher Nox4 and p22<sup>phox</sup> levels and WT SMCs produced more superoxide or H<sub>2</sub>O<sub>2</sub>, but also expressed more catalase, SOD1 (superoxide dismutase 1), and SOD2. Furthermore, elevated Nox4 expression in SMCs increased their apoptosis in basal conditions and after stimulation with H<sub>2</sub>O<sub>2</sub>.<sup>71</sup> Vendrov et al<sup>24</sup> showed that while knockout of *Ncf1* gene, encoding for p47<sup>phox</sup>, an essential regulatory subunit of both Nox1 and Nox2, is protective in young *Apoe*<sup>-/-</sup> mice, the effect is lost in aged animals.<sup>24</sup> Areas of aortic atherosclerotic lesions, staining with DHE, levels of DNA oxidative damage, or macrophage infiltration were similar in aged *Apoe*<sup>-/-</sup> and *Apoe*<sup>-/-</sup>*Ncf1*<sup>-/-</sup> animals. What is more, SMCs from both types of aged mice showed similar upregulation of superoxide and H<sub>2</sub>O<sub>2</sub> when treated with thrombin. Importantly, neither xanthine oxidation inhibition, nor lipoxygenase inhibition but Nox1/4 inhibitor attenuated ROS induction after stimulation with thrombin. The mitochondrial source of elevated ROS in aged SMCs was confirmed with the analysis of H<sub>2</sub>O<sub>2</sub> levels in isolated mitochondria. Both SMC in aortas of aged animals or mitochondria in SMC from aged mice showed high staining against Nox4. When Nox4 was inhibited, levels of mitochondrial ROS in aged SMCs decreased. Interestingly, elevated levels of Nox4 were also detected in samples from older human donors. Last but not least, treatment with mitoTEMPO reduced thrombin-induced VCAM1 upregulation in SMCs in vitro, but also aortic wall stress and lesion area, and prevented oxidative damage in *Apoe*<sup>-/-</sup> mice.<sup>24</sup>

SMC-specific expression of a human-dominant negative form of NOX4 (NOX4 P437H) resulted in decreased neointima formation after carotid artery denudation in FVB/N mice<sup>25</sup> or lower atherosclerosis in *Apoe*<sup>-/-</sup> mice.<sup>72</sup> Protective effect of dominant negative NOX4 in *Apoe*<sup>-/-</sup> mice was also associated with lower numbers of infiltrating macrophages. Overexpression of NOX4 P437H resulted in inhibition of soluble epoxide hydroxylase-2 expression and decreased proinflammatory signaling and levels of VCAM1, MCP1, and ICAM1, what was mirrored by lower macrophage adhesion.<sup>72</sup> Importantly, soluble epoxide hydroxylase-2 downregulation reduced the expression of thrombospondin-1,<sup>72</sup> which was crucial for the observed effects of NOX4 P437H on proliferation and migration in *Apoe*<sup>+/+</sup> mice.<sup>25</sup> On the other hand, Nox4 in *Apoe*<sup>-/-</sup> mice had a protective effect both in mice with spontaneous atherosclerosis development or after partial carotid artery ligation.<sup>26</sup> *Nox4* deletion led to the decrease in H<sub>2</sub>O<sub>2</sub> in mouse aortas, associated with the increase in superoxide levels. Microarray analysis of gene expression in WT or *Nox4*-deficient animals showed increased proinflammatory signaling in aortas from the *Nox4*-deficient mice.<sup>26</sup>

Nox4 was protective also in diabetic *Apoe*<sup>-/-</sup> mice with streptozotocin-induced diabetes mellitus. Such mice had lower

aortic expression of smooth muscle contractile markers, while levels of PDGF (platelet-derived growth factor), vimentin, and osteopontin increased.<sup>27</sup> *Nox4* deletion further increased aortic expression of PDGF, collagen I, and Ki-67. SMCs isolated from *Nox4*-deficient animals had lower expression of smooth muscle contractile markers, produced less H<sub>2</sub>O<sub>2</sub> but more superoxide and upregulated *Nox1*. Silencing of the latter gene resulted in the attenuation of PDGF-BB and osteopontin production and Ki-67 expression levels. Abnormal expression of PDGF-BB in *Nox4*<sup>-/-</sup> SMCs could also be reversed by PDGF inhibition or treatment with H<sub>2</sub>O<sub>2</sub>.<sup>27</sup> What is more, *Nox4* together with Nrf2 play significant roles in the CD38 signaling pathway, which is crucial for the maintenance of SMC contractile phenotype.<sup>45</sup> Coronary arterial myocytes isolated from *Cd38*<sup>-/-</sup> mice had lower calponin and SM22 $\alpha$  expression, accompanied by an increase in vimentin and PCNA (proliferating cell nuclear antigen). 7-Ketocholesterol, known atherogenic stimulus, further exacerbated the effect of *Cd38* knockout. Lack of CD38 caused a decrease in Nrf2 expression and activity. *Nfe2l2* silencing in coronary arterial myocytes enhanced reduction of contractile markers caused by 7-ketocholesterol. CD38 activity is crucial for the induction of *Nox4* and superoxide production, which is then necessary for the Nrf2 translocation to the nucleus and maintenance of arterial myocytes contractile phenotype.<sup>45</sup>

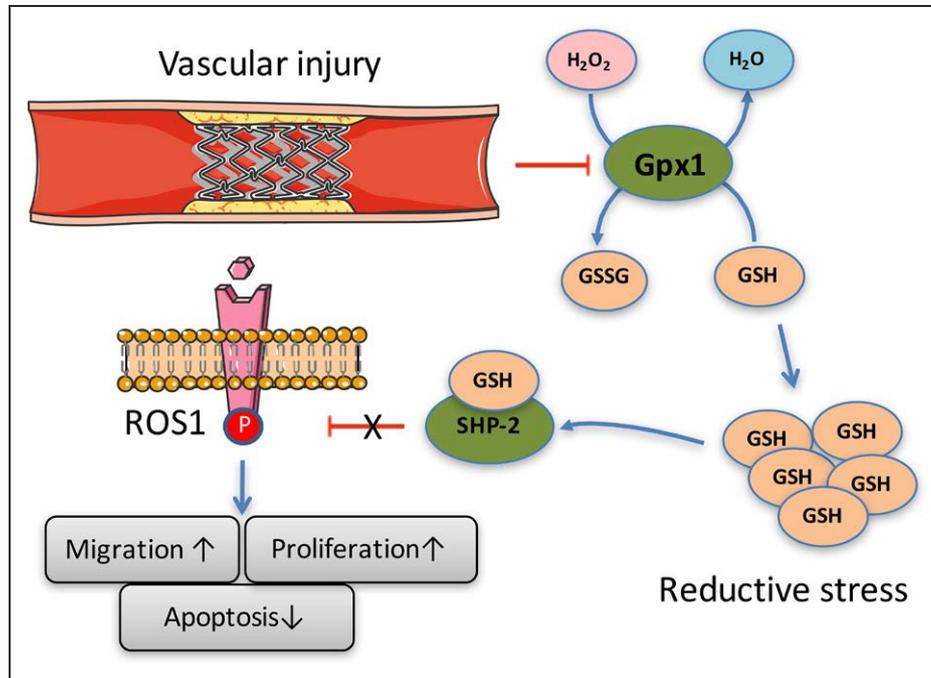
Interestingly, *Nfe2l2* overexpression in human aortic SMCs resulted in an increase of *GCLC*, *GCLM*, and *HMOX1* expression and decreased SMC proliferation.<sup>38</sup> The latter effect could be partially reversed with tin protoporphyrin IX, which is an HMOX1 (heme oxygenase 1) inhibitor. Furthermore, Ad *Nfe2l2* reduced oxidative stress, macrophage infiltration, and MCP-1 levels in balloon-injured aortas in rabbits. Finally, *Nfe2l2* gene transfer in vivo decreased both SMC proliferation and apoptosis, and therefore, it had no effect on neointimal neoplasia.<sup>38</sup> What is more, PDGF induces Nrf2 nuclear translocation and increases expression of its target genes: *Nqo1*, *Hmox1*, and *Txnrd1*.<sup>31</sup> *Nfe2l2* silencing enhances PDGF-induced migration of SMCs. Furthermore, it leads to prolonged activation of Rac1, which can increase NADPH oxidase activity and ROS production. *Nfe2l2* knockout also caused enhanced PDGF signaling, namely ERK1/2 phosphorylation, which can be inhibited with antioxidants, such as N-acetylcysteine. Femoral injury in *Nfe2l2*<sup>-/-</sup> mice resulted in decreased lumen area and higher neointima formation, with no significant changes in media and with greater ERK1/2 activation in neointima.<sup>31</sup> Sulforaphane, which is Nrf2 activity inductor, reduces neointima formation in injured femoral arteries of mice fed a Western diet.<sup>39</sup> In vitro, sulforaphane reduced leptin-induced SMC proliferation, cyclin D1 expression, and phosphorylation of both p70S6kinase and ribosomal S6 protein.<sup>39</sup> Ashino and et al<sup>30</sup> showed that injured femoral arteries contained regions of TUNEL<sup>+</sup> (terminal deoxynucleotidyl transferase dUTP nick-end labeling) apoptotic cells that overlapped with regions rich in Nrf2<sup>high</sup> cells. Silencing of Keap1 in rat SMC resulted in higher Nrf2 levels and activity, resulted in higher caspase-3/7 activity, and increased apoptosis. *Nfe2l2* silencing rescued the proapoptotic phenotype of Keap1-deficient cells. Finally, wire injury of femoral arteries in *Nfe2l2*<sup>-/-</sup> mice

led to the enhanced formation of neointima, which contained less apoptotic cells. Therefore, authors proposed that Nrf2 protect from neointima also because of the enhancement of SMC apoptosis.<sup>30</sup>

Interestingly, although *Atg7*<sup>-/-</sup> SMCs had higher Nrf2 activity, expression of its target genes, and resistance to oxidative stress, they showed accelerated senescence.<sup>73</sup> The latter effect was related to the defective autophagy and accumulation of Sqstm1/p62, which is a Nrf2 target, but also contributes to its activation.<sup>74</sup> In vivo, deletion of *Atg7* in SMCs caused enhanced senescence and promoted neointima formation.<sup>73</sup> On the other hand, carotid artery ligation in *Sqstm1*<sup>-/-</sup> mice caused more neointima formation.<sup>75</sup> SMCs isolated from *Sqstm1*<sup>-/-</sup> aortas proliferated and migrated faster.<sup>75</sup> Overexpression of HMOX1, enzyme degrading toxic heme to biliverdin, carbon monoxide and ferrous iron, and target of Nrf2 activity in SMCs decreased their migration in response to PDGF-BB.<sup>76</sup> Inhibitory effect of heme oxygenase-1 depended on its enzymatic activity and was mimicked by carbon monoxide. Increased activity of heme oxygenase-1 led to the upregulation of both *VEGFA* (vascular endothelial growth factor A) and *VEGFR1* (vascular endothelial growth factor receptor 1). The latter one formed a complex with PDGFR $\beta$ , which attenuated PDGFR $\beta$  signaling.<sup>76</sup> Activation of another Nrf2 target—NAD(P)H: quinone oxidoreductase-1—by  $\beta$ -lapochohne reduced neointima formation after balloon injury in rat carotid arteries and inhibited PDGF-induced SMC proliferation. Increased NQO1 (4-nitroquinoline 1-oxide) activity caused AMPK activation mediated by LKB1 but not CaMKK $\beta$  (Ca<sup>2+</sup>/calmodulin-dependent protein kinase kinase beta).<sup>77</sup> Taken together, Nrf2 is a crucial player in maintaining the hemostasis of the vessel wall via influencing inflammatory response, SMC proliferation, and neointimal formation.

Increased SMC apoptosis may lead to the plaque instability and rupture.<sup>78</sup> Wnt/ $\beta$ -catenin/WISP-1 (WNT1-inducible-signaling pathway protein 1) represents example of the pathway that can protect SMCs from apoptosis<sup>79</sup> and oxidative stress but, on the other hand, enhance SMC migration and contribute to intimal thickening.<sup>80</sup> Mill et al<sup>79</sup> showed recently that macrophage-derived Wnt5a could protect SMC from H<sub>2</sub>O<sub>2</sub>-induced apoptosis. Exogenous Wnt5a increased amounts of active and nuclear  $\beta$ -catenin in SMC and induced TCF (transcription factor) signaling. However, TCF pathway was attenuated when cells were costimulated with H<sub>2</sub>O<sub>2</sub>, but this effect was dependent on the active  $\beta$ -catenin. Protective activity of Wnt5a against H<sub>2</sub>O<sub>2</sub>-induced activity was lost after *LRP5/6* silencing, which suggests that it is regulated by Wnt/Frizzled/*LRP5/6* pathway. Importantly, Wnt5a induced cytoprotective WISP-1 in CREB-dependent manner. Interestingly, SMC in stable plaques in human coronary arteries showed higher staining for WISP-1 than those in unstable plaques.<sup>79</sup> Wnt2 also induced WISP-1 expression in SMC in  $\beta$ -catenin-dependent manner.<sup>80</sup> Levels of Wnt2 and WISP-1 were elevated in ligated carotid arteries. Furthermore, WISP-1 overexpression promoted intimal thickening, which was reduced in *Wnt2*<sup>+/-</sup> animals.<sup>80</sup>

Finally, although high glutathione levels and, that is, high ratio of reduced to oxidized glutathione are considered protective, too much of it can also lead to the enhanced



**Figure 3.** Reductive stress contributes to ROS1 activation. Vascular injury leads to the downregulation of Gpx1, what is accompanied with elevated GSH synthesis. The increase of cellular GSH leads to S-glutathionylation of SHP-2 phosphatase, what in turn inhibits its activity and leads to prolonged activation of ROS1 receptor tyrosine kinase. ROS1 activity contributes to enhanced migration and proliferation of SMCs.

vascular remodeling. Network analysis of gene expression in human carotid neointima showed that the most significantly downregulated network was the one with *GPX1* in its hub.<sup>81</sup> Furthermore, *ApoE*<sup>-/-</sup> mice with deleted *Gpx1* were characterized with increased atherosclerotic plaque, which contained more SMCs but not macrophages. However, increased macrophage infiltration in *Gpx1*<sup>-/-</sup>*ApoE*<sup>-/-</sup> was previously reported by Torzewski et al.<sup>41</sup> Furthermore, balloon angioplasty and stenting in *Gpx1*<sup>+/+</sup>*ApoE*<sup>-/-</sup> mice decreased *Gpx1* expression. Global knockout of *Gpx1* in *ApoE*<sup>-/-</sup> mice caused an increase in superoxide levels in media but not adventitia or endothelium. Effect of lack of *Gpx1* on SMCs was mediated by the high activity of ROS1 receptor tyrosine kinase. Inhibition of tyrosine kinase activity or ROS1 silencing could decrease enhanced proliferation and migration of *Gpx1*<sup>-/-</sup>*ApoE*<sup>-/-</sup> SMCs. *Gpx1*<sup>-/-</sup> SMCs contained more glutathione, which resulted from its higher synthesis. Elevated levels of glutathione caused reductive stress that contributed to S-glutathionylation and inactivation of SHP-2 (tyrosine-protein phosphatase nonreceptor type 11) phosphatase, what then inhibited ROS1 inactivation<sup>81</sup> (Figure 3). Interestingly, Izawa et al.<sup>82</sup> reported that buthionine sulfoximine, which is glutathione inhibitor, elevated vessel wall superoxide levels, but on the other hand decreased angiotensin II-induced vessel remodeling.

### Monocytes/Macrophages

Monocytes and macrophages play a significant role in the initiation and development of atherosclerosis.<sup>4,83,84</sup> At the beginning of atherosclerosis, monocytes, which are attracted by the chemokines secreted by resident vascular cells, migrate into the subendothelial area where they differentiate into

macrophages on growth factors stimulation.<sup>83</sup> In the atherosclerotic lesions, macrophages ingest oxidized LDL through scavenger receptors and become lipid-laden foam cells.<sup>85</sup> Under prolonged ER stress and extracellular stimuli, foam cells eventually undergo apoptosis and lead to the development of atherosclerosis. Although it has been well established that oxidative stress is involved in regulating monocyte migration, differentiation, and macrophage functions in atherosclerosis,<sup>86-88</sup> its origins and regulation in this process remain poorly understood.

Nox are primary sources of oxidative stress in macrophages. Although there is still no evidence showing that endogenous Nox in macrophage has a direct impact on the progress of atherosclerosis, many studies have revealed a significant role of Nox-derived ROS in regulation of monocyte differentiation and macrophage functions.<sup>89,90</sup> A recent study shows that tumor necrosis factor-like weak inducer of apoptosis (TWEAK), a proinflammatory cytokine, together with fibroblast growth factor-inducible 14 (Fn14) are colocalized with Nox2 in human advanced atherosclerotic plaques.<sup>91</sup> In vitro experiments showed that TWEAK/Fn14 axis regulates Nox2-dependent ROS production in macrophages. Deletion of TWEAK in *ApoE*<sup>-/-</sup> mice reduces ROS production in macrophages within atherosclerotic plaques, suggesting a possible role of TWEAK/Fn14 and downstream Nox2-derived ROS in atherosclerosis.<sup>91</sup> Another study shows that Rac2 can modulate atherosclerotic calcification through regulating interleukin-1 $\beta$  (IL-1 $\beta$ ) production in macrophages.<sup>92</sup> Rac2 can modulate Rac1 activity, which in turn promotes ROS production via Nox,<sup>93</sup> leading to the production of macrophage IL-1 $\beta$  and subsequent vascular SMC calcium deposition in atherosclerotic lesions.<sup>92</sup> These studies suggest that a role

of Nox regulated by different signaling pathways may contribute to the development of atherosclerosis, although more cell-specific experiments are still needed to provide further evidence.

Although numerous studies have focused on Nox, mitochondria are also an important source of oxidative stress in macrophages.<sup>94</sup> Early studies have observed an increase in mitochondrial ROS and damage in human atherosclerosis.<sup>95</sup> Recent studies indicate an important role of macrophage mitochondrial oxidative stress in atherosclerosis.<sup>96–98</sup> Wang et al<sup>96</sup> used a mitochondrial catalase transgenic mouse, which can quench mitochondrial ROS and protect against mitochondrial ROS-induced damage in vivo. Both mouse models, including transplantation of mitochondrial catalase transgenic bone marrow cells into *Ldlr*<sup>-/-</sup> mice and macrophage-specific mitochondrial catalase transgenic mice in *Ldlr*<sup>-/-</sup> background, showed decreased inflammatory monocyte infiltration, mitochondrial ROS in lesional macrophages, and attenuation of atherosclerosis lesions. Further studies suggest that macrophage mitochondrial ROS promotes MCP-1 production, which affects monocyte infiltration and lesional inflammation. Another recent study in human monocyte/macrophage provides further evidence of mitochondrial ROS regulation in human macrophages.<sup>97</sup> Shirai et al<sup>97</sup> isolated monocytes from patients with atherosclerotic coronary artery disease and showed that monocyte-derived macrophages from the patients produced more IL-6 and IL-1 $\beta$ , which was highly dependent on mitochondrial ROS but not Nox2. Neither *Nox2* siRNA knockdown by nor Nox2 inhibition by gp91dstat had any effects on cytokine production. However, mitoTEMPO, a mitochondria-target ROS scavenger, significantly reduced IL-6 and IL-1 $\beta$  production in macrophages from the patients, indicating a critical role of ROS from mitochondria, but not Nox2, in regulating cytokine production in macrophages derived from atherosclerotic patients.<sup>97</sup> Besides, Tumurkhuu et al<sup>98</sup> demonstrated 8-oxoguanine glycosylase, a major DNA glycosylase responsible for removing mitochondrial oxidative stress-induced DNA damage, plays a protective role in atherosclerosis by preventing excessive inflammasome activation in macrophages, further supporting the critical role of macrophage mitochondrial oxidative stress in promoting atherosclerosis.

Another potential source of cellular oxidative stress is the XO. XO inhibitors have been reported to inhibit macrophage ROS formation, inflammatory cytokine release, and atherosclerosis.<sup>99–101</sup> However, XO breaks down hypoxanthine and xanthine to uric acid and produces ROS, both of which may affect the function of macrophages. A recent report has elucidated that XO-dependent generation of ROS, rather than uric acid, mediates inflammatory cytokine production in macrophages.<sup>102</sup> However, there is still a lack of solid evidence demonstrating the role of macrophage XO in atherosclerosis.

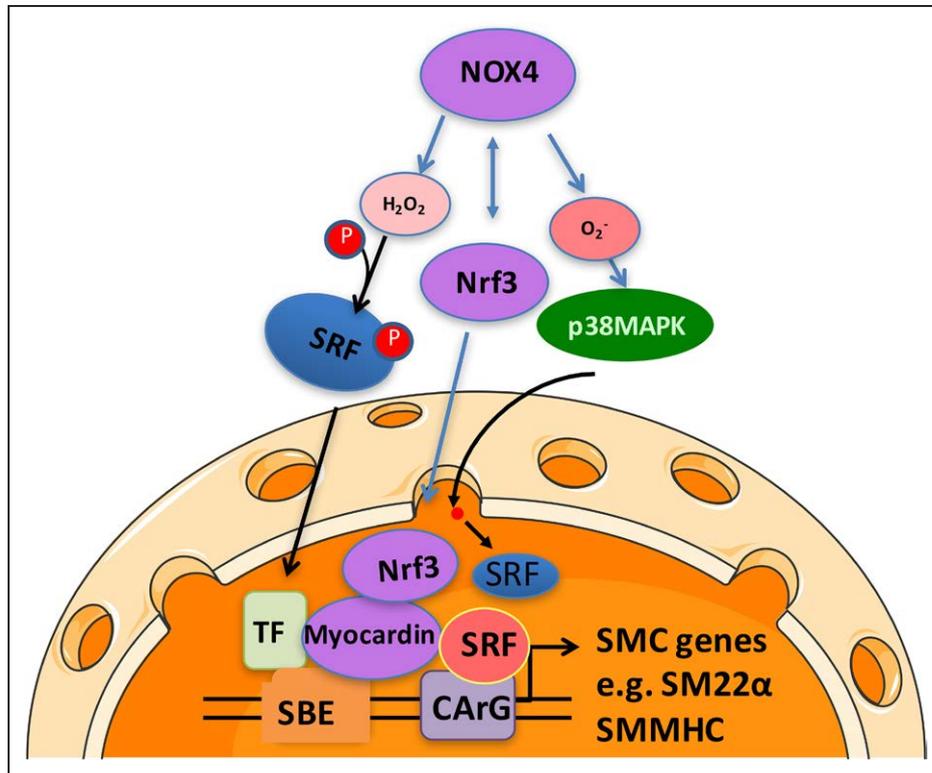
Macrophage not only serves as a source of oxidative stress in atherosclerosis but also itself can modulate or be affected by extracellular oxidative stress. Hemopexin, a hemoglobin scavenger protein, can transport heme into macrophages, thereby, inhibiting heme-mediated ROS production and ROS-mediated oxidative damage. Mehta et al<sup>103</sup> have recently reported a role

of hemopexin and macrophage in the regulation of oxidative stress in atherosclerosis. *ApoE*<sup>-/-</sup> mice deficient in hemopexin had higher oxidative stress, more macrophage infiltration, and atherosclerotic plaque. Hemopexin deficiency results in the dysfunction of uptake and metabolism of heme in macrophages. The accumulation of heme causes oxidative stress, leading to dysfunctional HDL, abnormal macrophage function, and atherosclerosis aggravation.<sup>103</sup> Recent research by Korytowski et al<sup>104</sup> showed that steroidogenic acute regulatory family protein D1, which transport both cholesterol and 7-hydroperoxycholesterol to mitochondria of macrophages under oxidative stress, induces mitochondrial lipid peroxidative damage that impairs reverse cholesterol transport in macrophages. Once cholesterol import exceeds export in macrophages, lipid-overloaded macrophages accumulate in atherosclerotic plaques, which obstruct blood flow and advance the progress of atherosclerosis,<sup>104</sup> in which ROS exerts its effects on the most events, if not all.

### Stem/Progenitor Cells

Stem/progenitor cells are characterized by the unique capacity for unlimited growth and self-renewal while maintaining the potential to differentiate into specialized cells.<sup>105</sup> Vascular tissue-resident or adult stem cells have been discovered and display variable capacities for differentiation.<sup>106–108</sup> Stem/progenitor cells can differentiate into vascular cell lineages, which may contribute to the regenerative process and could be useful for the treatment of atherosclerosis.<sup>109</sup> Recently, several publications in *ATVB* and other journals have demonstrated the progress in research on the role of stem/progenitor cells in atherosclerosis and oxidative stress.<sup>13,110–114</sup> As mentioned earlier, oxidative stress response is a key event in the development of atherosclerosis, in which stem/progenitor cells sense the signal of ROS and other related species. One of the primary roles of ROS is to promote stem cell differentiation into SMCs important for both of neointimal formation after angioplasty and plaque stability.<sup>115</sup> Thus, it would be crucial to understand the mechanisms of stem cell differentiation.

There is evidence demonstrating the factors responsible for stem cell differentiation. For example, Wang et al<sup>116</sup> revealed that shear stress induced and suppressed angiogenic growth factors and SMC-associated growth factors, respectively. In addition to shear stress, growth factors and cytokines have been shown to directly regulate SMC differentiation, and the expression levels of cytokines and growth factors are likewise altered during differentiation of mesenchymal stem cells, for example.<sup>117</sup> Importantly, it was found that oxidative stress is essential for stem cells to differentiate into SMCs.<sup>118</sup> ROS are highly reactive molecules that are generated, for example, after interaction of integrins, extracellular matrix, and cytokines. They act as second messengers and mediate a host of cellular processes, including vascular physiology and pathogenesis, including hypertension, restenosis, and atherosclerosis. Xiao et al<sup>119</sup> demonstrated that Nox4-derived H<sub>2</sub>O<sub>2</sub> is integral to the differentiation of stem cells into SMCs. Silencing of *Nox4* suppressed differentiation, while sustained Nox4 signaling enhanced differentiation of SMC gene markers. Nox4 translocation from the cytoplasm to the nucleus resulted in upregulation of H<sub>2</sub>O<sub>2</sub>, which in turn led to



**Figure 4.** Regulation of Nox4/Nrf3-derived reactive oxygen species (ROS) in smooth muscle cell (SMC) differentiation. Activated Nox4 can lead to ROS ( $\text{H}_2\text{O}_2$ ,  $\text{O}_2^-$ ) production.  $\text{H}_2\text{O}_2$  stimulation results in upregulation and phosphorylation of SRF. The phosphorylated SRF is mostly localized in the nucleus that has an ability to bind to the CArG recruiting myocardin to the promoter. Meanwhile,  $\text{O}_2^-$  generated by Nox4 can also activate SMC gene expression via p38MAPK pathway. Nrf indicates nuclear factor erythroid 2-related factor.

induction and phosphorylation of SRF (serum response factor) and its translocation into the nucleus.<sup>119</sup> Phosphorylated SRF binds to the CArG element on the promoter–enhancer regions of SMC-specific genes, recruiting myocardin to the promoter to form a SRF/myocardin complex. This complex was shown to be essential for regulating early-stage Nox4-mediated stem cell differentiation (Figure 4). Furthermore, Nrf3, a member of the cap N collar family of transcription factors, is now considered to be a crucial transcription factor in regulating SMC differentiation by modulating the balance of ROS generation. Pepe et al<sup>46</sup> recently demonstrated that Nrf3 is indispensable for stem cell differentiation toward SMCs. Usually, Nrf3 resides in the ER. During the early stages of SMC differentiation, Nrf3 can directly bind to the promoter region of SMC-specific genes (ie,  $\alpha\text{SMA}$ , and  $\text{SM22}\alpha$ ) that promotes the formation of the SRF/myocardin complex. Cytoplasmic Nrf3, on the other hand, is able to promote Nox4-mediated ROS production, which drives SMC differentiation.<sup>46</sup> Together, signal pathways mediated by Nox/Nrf3 affect stem/progenitor differentiation into SMCs that influence neointimal formation and plaque stability.

### Summary

In pathophysiology of the vessel wall, excessive concentrations of lipids result in free radical formation, and interaction of these molecules with the endothelial wall of the arteries leads to endothelial activation, an early sign of vascular inflammation.<sup>120</sup> An inflamed endothelium recruits inflammatory

cells, such as monocytes, via the expression of various mediators and chemokines.<sup>121</sup> This, in addition to the imbalance of ROS generation, leads to the disabling of monocytes into foam cells, which consume dead cells and lipids. This debris eventually develops into a sclerotic, fibrofatty plaque, which decreases the compliance of the vessel, increases the possibility of embolus or thrombus development, through plaque rupture, and finally increases the risk of multiple comorbidities.<sup>122</sup> In all of these processes, ROS plays a significant role in homeostasis of vascular cells and the pathogenesis of atherosclerosis.

Regulation of antioxidant response is complex and, therefore, difficult to target. While high levels of factors responsible for the resolution of the excessive oxidative stress can be beneficial in one cell type, they may be detrimental to the others. The best example shown was Nrf2, whose high levels in SMCs were protective,<sup>38</sup> while its deficiency in myeloid cells caused attenuation of atherosclerosis<sup>35,36</sup> or its aggravation.<sup>37</sup> On the other hand, while glutathione is a potent antioxidant, its high levels in SMCs caused attenuated inactivation of ROS1 kinase and led to increasing in SMC proliferation and migration.<sup>81</sup> Furthermore, as evidenced in stem cells, ROS were crucial for their differentiation to SMC.<sup>46,47,119</sup> ROS and oxidative stress are involved in regulation of many pathways, for example, stem cell differentiation, response to ER stress,<sup>44</sup> and control of inflammation. Therefore, potential atherosclerosis therapies involving the regulation of oxidative stress levels would require precise

targeting of certain types of ROS in particular cells and what is more at the specified stage of the disease.

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## Disclosures

None.

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KEY WORDS: atherosclerosis ■ endothelial cells ■ reactive oxygen species ■ smooth muscle cells ■ stem cells

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