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Targeting Cellular Antioxidant Enzymes for Treating Atherosclerotic Vascular Disease

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Abstract

Atherosclerotic vascular dysfunction is a chronic inflammatory process that spreads from the fatty streak and foam cells through lesion progression. Therefore, its early diagnosis and prevention is unfeasible. Reactive oxygen species (ROS) play important roles in the pathogenesis of atherosclerotic vascular disease. Intracellular redox status is tightly regulated by oxidant and antioxidant systems. Imbalance in these systems causes oxidative or reductive stress which triggers cellular damage or aberrant signaling, and leads to dysregulation. Paradoxically, large clinical trials have shown that non-specific ROS scavenging by antioxidant vitamins is ineffective or sometimes harmful. ROS production can be locally regulated by cellular antioxidant enzymes, such as superoxide dismutases, catalase, glutathione peroxidases and peroxiredoxins. Therapeutic approach targeting these antioxidant enzymes might prove beneficial for prevention of ROS-related atherosclerotic vascular disease. Conversely, the development of specific antioxidant enzyme-mimetics could contribute to the clinical effectiveness.

Key Words: Vascular disease, Atherosclerosis, Reactive oxygen species, Antioxidant enzymes, Antioxidant therapeutics

INTRODUCTION

Despite considerable advances over the past 50 years, cardiovascular disease (CVD) remains the major cause of global mortality. The etiology and pathophysiology of CVDs are complex, but the major risk factors include unhealthy lifestyle and behaviors coupled with a multifactorial complex interaction between environmental and genetic factors (McCord, 2004). Growing evidence suggests that highly reactive oxygen species (ROS) of endogenous or environmental origin play a cognitive role in the genesis and progression of various CVDs.

The primary cause of CVD is atherosclerosis, which is characterized by thickening of the walls of the arteries. Atherosclerosis is a chronic inflammatory disease that progresses slowly during a lifetime and typically begins before adulthood. Among the initiating causes of atherosclerosis, the oxidative modification hypothesis has been confirmed in numerous studies and especially, ROS stimulate oxidation of low density lipoprotein (LDL), cholesterol, cholesterol derived species, and protein modifications which can lead to foam cell formation and atherosclerotic plaques (Sauer *et al.*, 2010).

The production and effect of ROS depend on the expression and proper function of enzymes involved in ROS regulation in vascular cells. Moreover, cells contain numerous antioxidant defenses that detoxify ROS or reduce their effects. The site of ROS generators and distribution of antioxidant enzymes are highly localized within the cell. The imbalance between ROS generator and eliminator occurs due to the change of overall redox balance and modification of target molecules (Fig. 1) (Day, 2004). Allopurinol, xanthine oxidase inhibitor, as a potential antioxidant reverses endothelial dysfunction in heavy smokers, type-2 diabetics with mild hypertension, and in patients of chronic heart failure. Moreover, no deleterious effects were observed with this therapy, thereby clearly indicating that antioxidants decrease atherosclerotic progression (Traber and Atkinson, 2007).

In this article, we review current and potential approaches targeting antioxidant enzyme that could be employed to suppress atherosclerotic cardiovascular disease.

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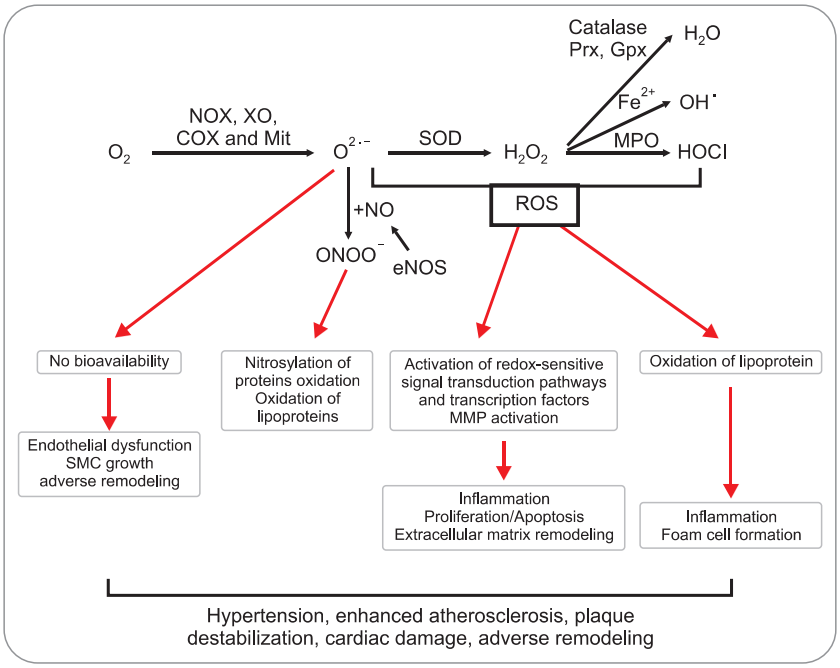

Fig. 1. Cellular antioxidant systems and their biological consequences in cardiovascular system. Superoxide anion ($O_2^{\bullet-}$) can be produced by numerous oxidoreductases [NADPH oxidase (NOX), xanthine oxidase (XO), cyclooxygenase (COX) and mitochondrial enzymes (Mit)]. Superoxide can react with nitric oxide (NO), forming peroxynitrite ($ONOO^-$) and leading to loss of NO bioavailability. Superoxide dismutase (SOD) can convert superoxide to hydrogen peroxide (H_2O_2). ROS can stimulate redox-sensitive signaling pathways, such as tyrosine kinases, phosphatases, and transcription factors, by oxidizing redox-sensitive target proteins. $O_2^{\bullet-}$ and H_2O_2 can also increase expression of matrix metalloproteinases, promote endothelial cell apoptosis and contribute to lipid oxidation.

CELLULAR ROS CHEMISTRY

A single electron addition to O_2 forms the superoxide anion radical ($O_2^{\bullet-}$). Addition of a second electron, as occurs during superoxide dismutation, forms hydrogen peroxide (H_2O_2). If a third electron is donated to O_2, the highly reactive hydroxyl radical (OH•) is formed, which occurs when superoxide reacts with iron (Fe^{2+}) via Fenton chemistry or during peroxynitrite ($OONO^-$) decomposition. $OONO^-$ is generated when $O_2^{\bullet-}$ reacts with nitric oxide (NO), and mediates both oxidant and nitrating reactions. ROS have distinct biological properties, which include chemical reactivity, half-life and lipid solubility. OH• has irreversible reactivity towards biological molecules, whereas $O_2^{\bullet-}$ and H_2O_2 are mild oxidants and reversibly oxidize the preferred biological targets (Sauer *et al.*, 2001).

CELLULAR ROS GENERATOR IN MAMMALIAN SYSTEM

The major cellular sources of vascular superoxide include NADPH-dependent oxidases (Rajagopalan *et al.*, 1996), xanthine oxidases (Ohara *et al.*, 1993), lipoxygenases, mitochondrial oxidases and NO synthases (Watts and Staels, 2004). The evidence from the *in vivo* studies using animal models suggests that membrane-bound NADPH oxidases, xanthine oxidases and dysfunctional eNOS are the major sources of the superoxide anion in various preatherogenic conditions (Ohara *et al.*, 1993). Other sources of superoxide include enzymes involved in the metabolism of arachidonic acid and the mitochondrial electron transport chain.

NADPH oxidase

The prototype of NADPH oxidase complex (NOX) contains membrane subunits (p22phox, gp91phox/Nox2), cytosolic regulatory subunits (p47phox, p67phox) and G protein Rac. Nox catalytic subunits possess flavin- and heme-binding regions and generate $O_2^{\bullet-}$ via one electron transfer from NADH or NADPH to oxygen. Of the various Nox isoforms, Nox1, Nox2 and Nox4 are the most important in vascular cells (Table 1) (Lambeth, 2004). With the exception of Nox5 all the Nox isoforms require $p22^{phox}$ as a docking subunit. Nox4 functions constitutively and does not require cytosolic subunits. Interestingly, Nox isoforms can be differentially associated with various vascular disease phenotypes. Nox1 expression directly alter cell proliferation (Suh *et al.*, 1999) and treatment of vascular smooth muscle cells (VSMCs) with platelet-derived growth factor (PDGF) upregulates Nox1, at the same time downregulating Nox4 (Lassegue *et al.*, 2001). Expression of Nox2 and $p22^{phox}$ is greatly increased with the progression of human atherosclerosis (Guzik *et al.*, 2006), whereas Nox4 is increased in early lesions but rather decreased in severe lesions (Sorescu *et al.*, 2002).

Xanthine oxidase

Xanthine oxidase (XO) can be an additional source of vascular superoxide. Various stimuli, such as hypoxia and reoxygenation, cytokines, and oscillatory shear-stress, increase endothelial XO activity (Griendling, 2005). In CVD patients, the

Table 1. NOX isoenzymes in mammalian cells

Type	Domain structure	Distribution	Regulatory factors	Functions
Nox1 (Mox-1)	Inducible, Flavo-protein, transmembrane cluster	Colon, VSMC, prostate	NOXO, NOXA, and p22phox	Proliferation response (Lee *et al.*, 2009)
Nox2 (gp91*phox*)	Flavo-protein, transmembrane cluster	Phagocyte	P47phox, p67phox, p40phox, Rac1/2	Host defense (Bedard and Krause, 2007)
Nox3	Flavo-protein, transmembrane cluster	Fetal kidney	Not determined	Unclear (Bedard and Krause, 2007)
Nox4 (Renox)	Flavo-protein, transmembrane cluster	Kidney, osteoclasts, ovary, eye, widespread	Not determined	Oxygen sensing, iron transport, host defense (Bedard and Krause, 2007)
Nox5	EF hands, Flavo-protein, transmembrane cluster	Lymph nodes, testis, mammary gland, cerebrum	Calcium	Fertilization (Musset *et al.*, 2012)
Duox1, Duox2 (p138Tox)	Peroxidase, EF hands, Flavo-protein, transmembrane cluster	Thyroid, cerebellum, colon, lung, prostate, pancreatic islets	Calcium	Hormone synthesis (Milenkovic *et al.*, 2007)

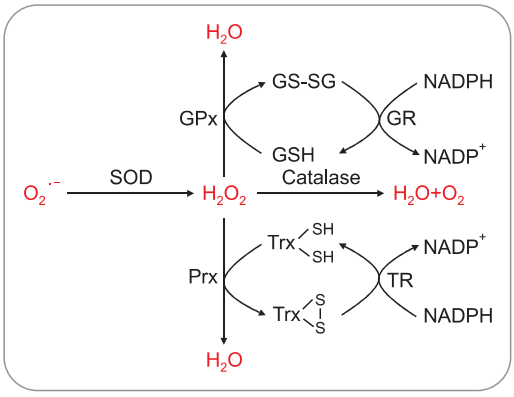

Fig. 2. Cellular antioxidant enzymes system. Superoxide anion can be converted to H_2O_2 by the reaction of SOD. Catalase is a H_2O_2 dismutase that contains a heme group and is exclusively present in the peroxisome. GPx catalyzes the reduction of the hydroperoxides by utilizing the electrons transferred from NADPH via glutathione reductase (GR) and glutathione (GSH). 2-Cys Prx reduces hydroperoxides to water by utilizing electrons transferred from NADPH via thioredoxin (Trx) and thioredoxin reductase (TR).

endothelial level of XO is increased and correlates with the degree of endothelial vasodilatation (Landmesser *et al.*, 2002).

Endothelial NO synthase

In the absence of its cofactor (BH_4) or its substract (L-arginine), the endothelial NO synthase (eNOS) generates $O_2^{\cdot-}$ instead of NO. BH_4 plays a role in stabilizing the dimeric conformation of eNOS, crucial for NO production (Alp and Channon, 2004). BH_4 oxidation and NOS uncoupling has been demonstrated in hypertension and hypercholesterolemia (Landmesser *et al.*, 2003).

CELLULAR ANTIOXIDANT ENZYMES IN MAMMALIAN SYSTEM

Superoxide dismutases

Cells constantly produce $O_2^{\cdot-}$ as a by-product of normal aerobic metabolism. Superoxide dismutase (SOD) is the main defense against $O_2^{\cdot-}$, catalyzing its dismutation to H_2O_2 and O_2 (Fig. 2) (Abreu and Cabelli, 2010). Based on the metal cofactor they harbor, human SODs can be classified into four groups: copper-zinc SOD (Cu/ZnSOD), manganese SOD (MnSOD), and extracellular SOD (EC-SOD). MnSOD is the SOD typically found in mitochondria and peroxisomes, whereas Cu/ZnSOD is usually the most abundant SOD in the cytosol. The EC-SOD is the secreted form of Cu/ZnSOD (Table 2). These enzymes are thus fairly ubiquitous in aerobic organisms (Reddi *et al.*, 2009).

Glutathione peroxidases

Glutathione peroxidases (GPxs) were the first selenocysteine-containing proteins discovered in mammals. The "classical" glutathione peroxidase, now called GPx1, was first described as an erythrocyte enzyme that specifically reduces H_2O_2 by GSH, but later shown to reduce a broad scope of organic hydroperoxides (Toppo *et al.*, 2009). In mammals, up to eight distinct GPxs have been detected. Most of them are selenoproteins (mammalian GPx1, GPx2, GPx3, GPx4 and, depending on species, GPx6), while in the remaining two or three variants the active site selenocysteine residue is replaced by cysteines. Only GPx1, 3 and 4 have been functionally characterized to some extent (Table 3).

Catalase

Catalases are enzymes that catalyse the conversion of H_2O_2 to water and oxygen using either an iron or manganese cofactor with high catalytic rate. Catalase is encoded by a single gene, which is highly conserved among species. Mammals, including humans and mice, express catalase in all tissues, and a high concentration of catalase can be found in the liver, kidneys and erythrocytes. A study of catalase activity in mice reported high catalase activity in the liver (66,100 units/g tissue), lung (2,390 units/g tissue) and erythrocytes (6,340 units/ml blood) (Nishikawa *et al.*, 2002). The expression is regulated at the transcription, post-transcription and post-translation levels. High catalase activity is detected in peroxisomes. Catalase is also found in the cytosol in erythrocytes (Nishikawa *et al.*, 2009). The crystal structure of tetrameric human erythro-

Table 2. SOD isoenzymes in mammalian cells

Type	Structure	Distribution	Function
SOD1 (Cu, Zn SOD)	Homodimer; non-disulfide linked	Cytosol	Familial amyotrophic lateral sclerosis (ALS) by mutated SOD1 (Zhang *et al*., 2007)
SOD2 (MnSOD)	Tetramer, contains a Mn ion bound to one aspartate and three histidine residues	Mitochondria	Protect mitochondria form ROS damage (Kokoszka *et al.*, 2001)
SOD3 (extracellular SOD;EC-SOD)	Tetramer composed of two disulfide-linked dimers	Extracellular space, ~10 fold higher in the essel wall than in other tissues	Regulating the vascular redox state in extracellular space (van Deel *et al*., 2008)

Table 3. GPx isoenzymes in mammalian cells

Type	Structure	Distribution	Function
GPx1 (cytosolic GPx;cGPx)	Homotetramer; contains a single selenocysteine residue in each of four identical subunits	Abundant in cytosol of erythrocytes, kidney, liver or lung	Selenium-dependent, Ubiquitously distributed (Chu *et al.*, 2004)
GPx2 (gastrointestinal GPx; GI-GPx)	Homotetramer; selenocysteine at active site 40 of the protein sequence	Abundant in the epithelium of the whole gastrointestinal tract	Selenium-dependent (Yan and Chen, 2006)
GPx3 (plasma/extracellualar GPx;pGPx)	A glycosylated homotetramer of 23 kDa subunits	The only extracellular isoform of GPxs; a secretrd protein into blood plasma; also expressed in the kidney, lung, heart, placenta	Selenium-dependent, Extracellular peroxidase (Olson *et al.*, 2010)
GPx4 (phospholipid hydro-peroxide GPx;PHGPx)	Monomer; selenocysteine at active site 73)	In most tissue both in cytosol and associated with membranes	Selenium-dependent, protect phospholipid, inactive structural capsule of epididymal spermatozoa (Imai and Nakagawa, 2003)
GPx5 (epididymal androgerelated protein or secretory GPx)	221 amino acids	In epididymis; secreted protein.	Selenium-independent (Vernet *et al.*, 1999)

cyte catalase is very similar to those of bovine liver catalase with functionally important amino acid sequences conserved (Safo *et al.*, 2001).

Heme oxygenase

Humans and rodents have two heme oxygenase (HO) isoenzymes, HO-1 and HO-2 encoded by the HMOX-1 and HMOX-2 genes, respectively. HO-1 expression is induced ubiquitously in response to oxidative stress whereas HO-2 is constitutively expressed. HO are evolutionarily conserved enzymes that catabolize hemes, iron (Fe) protoporphyrin (IX), into equimolar amounts of labile Fe, carbon monoxide (CO), and biliverdin (Gozzelino *et al*., 2010).

Peroxiredoxins

Peroxiredoxins (Prx) are a group of ubiquitous peroxidase enzymes in which redox-active cysteine residues participate in the reduction of H_2O_2 (Kang *et al*., 2005). Based on their catalytic mechanism, Prx have been separated into three classes: typical 2-Cys, atypical 2-Cys, and 1-Cys Prx. Typical 2-Cys Prxs are the largest subfamily of Prxs and contain two catalytic cysteine residues. This group includes PrxI, PrxII, PrxIII, and PrxIV (Table 4). The peroxidatic cysteine is oxidized directly by H_2O_2, generating a sulfenic derivative that is stabilized by the formation of a disulfide bond with the other resolving cysteine in a neighboring Prx molecule (Wood *et al*., 2003). The atypical 2-Cys Prxs including PrxV are functionally monomeric and both the peroxidatic cysteine and its corresponding resolving cysteine are contained within the same polypeptide. The 1-Cys Prxs including PrxVI conserve only the peroxidatic cysteine and do not contain the resolving cysteine (Choi *et al*., 1998).

THERAPEUTIC USE OF ANTIOXIDANT ENZYMES IN ATHEROSCLEROTIC VASCULAR DISEASE

An increased amount of superoxide radicals was reported in the arteries of spontaneously hypertensive rats (Fig. 3) (Chu *et al*., 2003). In this case, genetic transfer of EC-SOD amelio-

rated endothelium function and decreased the arterial pressure. The involvement of SOD in atherosclerosis has been suggested indirectly by the observation that the activity and content of EC-SOD are increased in the aorta of ApoE$^{-/-}$ mice compared with control mice (Fukai *et al.*, 1998). However, neither the absence nor overexpression of EC-SOD did affect atherosclerosis in ApoE$^{-/-}$ and LDLR$^{-/-}$ mouse (Laukkanen *et al.*, 2001; Sentman *et al.*, 2001) and SOD1 overexpression had no effect on progression of atherosclerosis in ApoE$^{-/-}$ mice (Yang *et al.*, 2004). Therefore, we must need a more careful investigation for the role of SOD in atherosclerosis. Increased catalase activity has been identified in foam cells from rabbit aortic lesions (Chen *et al.*, 2012). Overexpression of catalase was reported to retard atherosclerosis progression and to decrease the aortic content of F2-isoprostanes in ApoE$^{-/-}$ mice (Yang *et al.*, 2004). However, the underlying mechanisms for this protective effect remain to be established unambiguously. Despite its apparent importance in H_2O_2 removal, humans with inherent deficiency of catalase called "acatalasemia" or catalase KO mice suffer few ill effects (Bliznakov, 1999). Overexpression of catalase or catalase together with SOD-1 in ApoE$^{-/-}$ mice inhibited development of atherosclerosis in this model (Yang *et al.*, 2004). The synthetic compound mimicking both SOD and catalase activities via selenium and manganese, EUK-8 protects against remodeling of the left ventricle and cardiac decompensation in mice model developing heart failure (van Empel *et al.*, 2006).

Low levels of both GPx1 and GPx3 are associated with the development of vascular disease. For example, in the Athero Gene study of patients with a history of CVD, those with low erythrocyte GPx1 activities had increased recurrent events (Blankenberg *et al.*, 2003). Individuals with both low high density lipoprotein cholesterol and GPx3 activity are at markedly increased risk for death from CVD. In animal study, GPx1 deficiency resulted in impaired endothelium-dependent vasodilatation and an increase in the aortic content of F2-iso-

Table 4. Prx isoenzymes in mammalian cells

Type	Structure	Distribution	Functions
PrxI (2-Cys)	Dimer	Cytosol, nucleus	Signal regulation (c-Abl, c-Myc, GDE2, p38, etc) (Rhee *et al.*, 2012)
PrxII (2-Cys)	Decamer (Basic unit: dimer)	Cytosol, nucleus	Signal regulation (PDGF, VEGF, LPS, etc) (Choi *et al.*, 2005)
PrxIII (2-Cys)	Dimer	Mitochondria	Apoptosis (Chang *et al.*, 2004)
PrxIV (2-Cys)	Dimer	ER, extracellular	ER foldase, Epididymal spermatozoa (Nguyen *et al.*, 2011)
PrxV (atypical 2-Cys)	Dimer	Mainly peroxisome, some in cytosol and mitochondria	Unclear (Wood *et al.*, 2003)
PrxVI (1-Cys)	Monomer	Cytosol	Unclear (lung phospholipid metabolism and cellular invasive/metastatic potential) (Wood *et al.*, 2003)

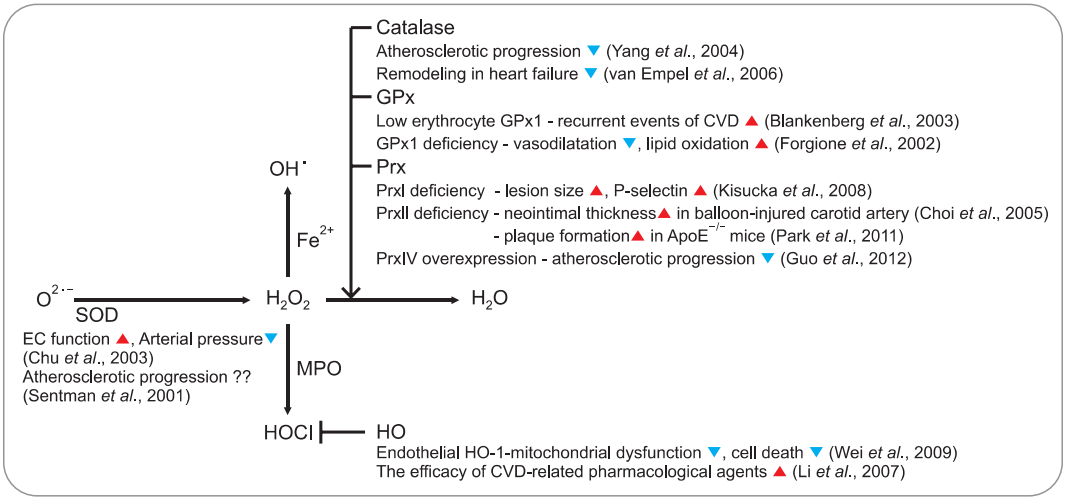

Fig. 3. Involvement of cellular antioxidant enzymes in cardiovascular diseases. The positive and negative effects are indicated by red and blue arrowheads, respectively. The related references are also indicated in parentheses.

prostanes, indicative of increased lipid oxidation in the vessel wall of these animals (Forgione *et al.*, 2002). The size of atherosclerotic lesions in the aortic sinus decreased significantly after 20 weeks of high-fat feeding in mice lacking GPx1 as compared with control mice (Stocker and Keaney, 2004).

The role of HO-1 was shown to include protection against cellular oxidative stress and pathological conditions, including atherosclerosis and other CVDs (Ryter *et al.*, 2006). In endothelial cells, expression of HO-1 has been suggested to protect against HOCl-mediated mitochondrial dysfunction, caspase-3 activation, and cell death via enzymatic activity and the generation of biliverdin and CO (Wei *et al.*, 2009). HO-1 induction is thought to contribute to the efficacy of pharmacological agents used in the treatment of CVDs, including statins, rapamycin, aspirin, and probucol (Li *et al.*, 2007).

Although a limited number of studies have suggested an involvement of Prxs in atherosclerosis, some of the evidence is interesting. For example, Prx II was shown to suppress the proliferation and migration of smooth muscle cells (SMCs) with the site-selective phosphorylation of the PDGF receptor and increased the neointimal thickness of SMCs in a balloon-injured carotid artery (Choi *et al.*, 2005). Deficiency of Prx II in the ApoE$^{-/-}$ background mice fed a high-cholesterol diet accelerated plaque formation through increased expression of adhesion molecules, leading to increased immune cell adhesion and infiltration into the aortic intima (Park *et al.*, 2011). Prx IV overexpression suppressed the development of atherosclerosis in ApoE$^{-/-}$ mice fed a high-cholesterol diet (Guo *et al.*, 2012). Increased expression of the Prx I has been reported in advanced lesions in ApoE$^{-/-}$ mice (Mayr *et al.*, 2005), and the lack of Prx I in ApoE$^{-/-}$ mice has been associated with increases in both lesion size and endothelial expression of the adhesion molecule P-selectin (Kisucka *et al.*, 2008).

Although numerous experimental studies have indicated that antioxidants and scavenging ROS could prevent pathological events leading to atherosclerosis, translating this concept into the treatment of human disease has been problematic. ROS have important signaling properties, and the nonselective approach of scavenging all ROS could have deleterious effects. Distinguishing between pathologic radical and signaling ROS is currently difficult. It is generally accepted that the profound changes of ROS are observed in advanced stages of CVD. However, in the early stages that involve the initiation of disease, the alteration in the ROS level may be a highly localized event within individual cellular compartments or even by individual antioxidant enzymes without affecting overall cellular redox status. Such local changes of ROS by certain antioxidant enzyme systems result in the disturbance of redox signaling leading to the pathological consequences. Therapeutic interventions on the level of global redox status inside cells might not be sufficient to correct these disturbances. Novel strategies should instead target a specific cellular antioxidant enzyme by either inhibiting or mimicking the activity following an in-depth study for selective function of each antioxidant enzyme.

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