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Targeted interception of signaling reactive oxygen species in the vascular endothelium

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Abstract

Reactive oxygen species (ROS) are implicated as injurious and as signaling agents in human maladies including inflammation, hyperoxia, ischemia-reperfusion and acute lung injury. ROS produced by the endothelium play an important role in vascular pathology. They quench, for example, nitric oxide, and mediate pro-inflammatory signaling. Antioxidant interventions targeted for the vascular endothelium may help to control these mechanisms. Animal studies have demonstrated superiority of targeting ROS-quenching enzymes catalase and superoxide dismutase to endothelial cells over nontargeted formulations. A diverse arsenal of targeted antioxidant formulations devised in the last decade shows promising results for specific quenching of endothelial ROS. In addition to alleviation of toxic effects of excessive ROS, these targeted interventions suppress pro-inflammatory mechanisms, including endothelial cytokine activation and barrier disruption. These interventions may prove useful in experimental biomedicine and, perhaps, in translational medicine.

Reactive oxygen species & vascular pathology

Reactive oxygen species (ROS) superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are small molecules implicated as injurious and signaling agents in human maladies including inflammation, hyperoxia, ischemia-reperfusion (I/R) and acute lung injury (ALI) [1]. Activated phagocytes release ROS, causing tissue damage. Endothelial cells (ECs) lining the luminal surface of blood vessels also produce ROS [2] using the mitochondrial respiratory chain [3], membrane-bound **NADPH oxidases** (NOX) [4], xanthine oxidase [5], uncoupled nitric oxide synthase (NOS) [6] and other enzymatic systems (Figure 1). The mitochondrial respiratory chain is the major producer of injurious ROS that play an important role in apoptosis and cell pathology [7]. ECs play key regulatory functions in the vascular system and, therefore, effects of endothelial ROS, both endogenous and exogenous, are of great biomedical importance [8].

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Antioxidants, including antioxidant enzymes (AOEs) catalase and superoxide dismutase (SOD), inhibit the effects of ROS in cell culture, animals and, to a limited extent, clinical studies [8,9]. Some forms of chronic mild oxidative stress seem amenable to preventative or prolonged treatment with antioxidants, antioxidant inducers, AOEs (including their polyethylene glycol [PEG] conjugated variants that have enhanced bioavailability) or, in a more distant future, gene therapy [9]. However, effective and specific treatment of acute vascular oxidative stress remains a significant and challenging goal [10]. In acute conditions, such as lung inflammation, I/R and ALI, expedient quenching of ROS in given compartments of target cells is needed. Nontargeted antioxidants do not afford the required spatiotemporal precision of action.

In particular, precise interventions are needed to correct local aberrations of ROS involved in pathological signaling. Inflammatory agents (e.g., cytokines TNF and interleukin-1 β) cause abnormal endothelial activation, which manifests, among other signs, by the expression of molecules mediating leukocyte migration (e.g., vascular cell adhesion molecule-1 [VCAM]) [2]. In activated endothelium, NOX releases O₂^{•−} in the milieu and cellular organelles including endosomes [11]. O₂^{•−} spontaneously transforms into H₂O₂ and O₂ in a fast reaction, which is further accelerated by SOD. Thus, extracellular SOD rapidly quenches O₂^{•−} in the milieu [12]. O₂^{•−} can cross cell membranes via the chloride channel ClC3 [13]. In turn, H₂O₂, a more stable molecule, can:

- Further react directly with cellular components, such as sulfhydryl groups of cell proteins;
- In the presence of free transition metals, produce extremely reactive hydroxyl radical ·OH;
- Be degraded by catalase or peroxidases.

Reactions of ROS (in particular O₂^{•−}) are compartmentalized within nanometers of the generation site. O₂^{•−} released by NOX into endosomes [14] (inaccessible for mitochondrial, cytosolic and extracellular SOD) has been implicated in NF κ B-mediated signaling leading to inflammatory changes [14–16].

In order to control these effects of ROS in ECs (and, presumably, other cell types), at least two key intertwined aims must be achieved. First, we need to understand signaling and injurious mechanisms of ROS at a subcellular level. Second, we need means to interfere in these mechanisms at this level in selected cell types and phenotypes; for example, in the signaling endosomes of pathologically activated ECs. This article reviews these two aspects of targeted antioxidant interventions.

ROS pathological signaling in vasculature

General mechanisms of ROS signaling

Many agents including growth factors, cytokines, hormones and neurotransmitters are able to cause transient ROS generation by nonphagocytic cells [17]. In many cases, ROS-mediated signaling in nonphagocytic cells requires endocytosis of a ligand–receptor complex and formation of a signaling endosome that contains activated NOX generating

ROS. This mechanism has been demonstrated for pro-inflammatory signaling induced by cytokines, hypoxia-reoxygenation, platelet-derived growth factor, epidermal growth factor and angiotensin II [14]. Recent studies of the role of mitochondrial ROS suggest that the regulated production of ROS by respiratory chain complexes I and III may also be involved in numerous signal transduction paths including metabolic signaling, inflammation, apoptosis and autophagy [18,19].

Spatially confined production of ROS allows tuned regulation of redox-mediated signal transduction. ROS, in particular H_2O_2 , oxidize sensitive cysteine residues in target proteins (phosphatases, kinases, small GTPases, transcription factors and ion channels (Figure 2) [2]). Oxidation of specific cysteine in phosphatases can inactivate the enzyme. Blocking homeostatic dephosphorylation can cause activation of corresponding kinases and turn on the specific signaling event. On the other hand, it was demonstrated that some kinases are more active when regulatory cysteine is oxidized by H_2O_2 , which thereby activates signaling.

ROS in hypertension

Superoxide generated in activated endothelium by NOX or the mitochondrial respiratory chain quenches a key vasodilating mediator NO and produces damaging reactive nitrogen species peroxynitrite, $ONOO^-$ (Figure 3). Moreover, peroxynitrite oxidizes tetrahydrobiopterin, a key cofactor of endothelial NOS, which in the absence of this cofactor generates superoxide instead of NO [6]. Thus, ROS cause vasoconstriction and, therefore, oxidative stress is a pathological factor in hypertension [20]; for example, in response to activation of the rennin–angiotensin system [21]. The key agent of this system, angiotensin II, boosts superoxide production in the vasculature, which leads to vasospasm [22]. Excessive ROS also cause endothelial dysfunction, inflammation, vascular hypertrophy, apoptosis, vascular remodeling and fibrosis [23]. *In vivo* studies detected high ROS production in hypertensive conditions [24–26]. In contrast, reduction of ROS or inhibition of ROS-generated enzymes in experimental conditions has vasodilatory effect. Nevertheless, clinical trials of antioxidants in hypertensive patients did not produce significant improvements, perhaps due to failure to interfere effectively in ROS signaling [20].

Vascular oxidative stress & thrombosis

Thrombosis is the leading cause of morbidity and mortality in cardiovascular pathology and stroke. Thrombus formation is initiated by activation of a coagulation cascade leading to thrombin formation, and by activation of platelets, leading to their adhesion and aggregation [27]. Several *in vivo* studies showing the inhibitory effect of antioxidants on platelet activation and recruitment to growing thrombus provide evidence for the involvement of ROS in thrombosis (Figure 4) [28–30]. Exposure of platelets to ROS can decrease the threshold for agonist-induced platelet activation and, therefore, promote thrombosis [31,32]. Several lines of evidence suggest that ROS modulate activity of platelet-signaling molecules, including Ca^{2+} -ATPase and inositol 1,3,5-trisphosphate receptors [33,34]. The $\alpha IIb\beta 3$ integrin receptor, a key mediator of platelet aggregation and further coagulation, is also regulated by ROS [35]. In addition, vascular ROS can regulate platelet function indirectly; for example, via quenching NO, a known inhibitor of platelet aggregation [36]. ROS can

also regulate platelet function via oxidation of low-density lipoproteins (LDL), lipids and their derivatives. Oxidized LDL (oxLDL) induce platelet aggregation and secretion [37]. The oxLDL play a key role in the development and progression of atherosclerosis [38]. Within atherosclerotic lesions, oxLDL inhibit anticoagulant β_2 -glycoprotein 1 [39–41]. The auto-antibodies against oxLDL/ β_2 -glycoprotein 1 complexes correlate with arterial thrombosis in autoimmune patients [42]. Furthermore, hypercholesterolemia aggravates platelet adhesiveness via ROS formed by the NOX [43].

In turn, activated platelets secrete platelet-derived growth factor, transforming growth factor- β_1 , EGF and VEGF, all known to stimulate ROS generation by vascular NOX [44,45]. Therefore, platelet activation and vascular ROS flux form a vicious cycle. In addition, oxidants inactivate anti-thrombotic endothelial protein thrombomodulin and cause ECs to release inhibitors of fibrinolysis [46]. Vascular ROS also contribute to thrombosis through regulation of the extrinsic coagulation cascade initiated by the tissue factor [47], via upregulation of tissue factor expression in vascular cells [48].

ROS signaling in inflammation

Pathological inflammation is an important factor in the mechanisms of many diseases including cancer, diabetes and atherosclerosis [49]. ROS produced by activated phagocytes have long been implicated in inflammation. However, ROS produced by nonphagocytic cells, first of all ECs, play an important role as well [50].

Inhibitory effects of antioxidants on cell stimulation by cytokines were observed decades ago [51]. Subsequent studies revealed that ROS are important elements of signaling pathways and involved in cell proliferation, differentiation and migration [52]. NF- κ B transcription factor plays a central role in inflammation and innate immunity. Canonical pathways for NF- κ B activation are mediated by Toll-like receptors, a family of TNF receptors and other cytokine receptors. NF- κ B, a dimer of p50 and p65 subunits, is normally restricted from entering the nucleus by inhibitory proteins. NF- κ B-induced transcription leads to synthesis of pro-inflammatory proteins including adhesion molecules mediating migration of leukocytes releasing ROS. In turn, ROS modulate NF- κ B signaling in several ways including endosomal signaling (Figure 5). Thus, ROS and NF- κ B positively affect each other, forming a vicious cycle of pro-inflammatory activation [53]. However, excessive ROS may also inhibit NF- κ B signaling [54].

Oxidative impairment of endothelial barrier function

A surplus of vascular ROS has been implicated in endothelial barrier dysfunction [55]. An increased EC permeability is the hallmark of many disorders including inflammation [56]. Extracellular ROS (in particular, H_2O_2) cause endothelial barrier dysfunction [57,58]. Furthermore, in response to thrombin, histamine, VEGF, TNF and inflammatory cytokines, ECs generate ROS that serve as signaling molecules mediating endothelial barrier regulation (Figure 6) [59].

A balance between EC adhesion and actin–myosin-based contractile forces within cells controls the structural integrity of endothelial barrier [60]. ROS cause cytoskeletal remodeling through signaling, including increase in cytosolic Ca^{2+} level, activation of Rho

GTPases and enhanced phosphorylation of the myosin light chain, all of which mediate stress fiber formation and cell contractility, leading to increased vascular permeability [60–62]. Thus, through stimulation of cytoskeletal remodeling, ROS destabilize junctions between ECs, including **tight junctions** and **adherens junctions**, which are bound to actin cytoskeleton. H₂O₂ exposure of ECs results in internalization of VE-cadherin, a key molecule of adherens junctions essential for the stability of EC contacts [58,63]. In addition, vascular ROS cause phosphorylation and redistribution of the tight junction molecules [64] and activate matrix metalloproteinases, leading to disassembly of cell junctions and increased endothelial permeability [65].

The need to specifically intercept selected ROS effects

ROS signaling plays an important role in many vascular diseases including hypertension, thrombosis, inflammation and edema. In theory, ROS quenching may provide beneficial effects in management of these conditions. However, the effect of the antioxidants depends on their delivery to the sites of ROS action. For example, ROS interception in signaling endosomes is needed to alleviate endothelial pro-inflammatory activation caused by cytokines, whereas quenching extracellular ROS may alleviate tissue damage caused by activated leukocytes. Furthermore, quenching of selected ROS species may be required. For example, hypertension is mediated by superoxide depleting NO and, thus, quenching of superoxide is needed. On the other hand, in I/R injury, H₂O₂ seems to be the most damaging ROS and its removal may attenuate the cellular damage. AOE is able to remove specific ROS in recurrent manner and, if delivered to the right place, might block an undesired ROS-mediated processes. In the following sections we review approaches to intercept pathological ROS and demonstrate some examples of protective properties of the delivery of AOE to vascular endothelium.

Means to intercept ROS

AOEs & antioxidants

Cells have several systems of antioxidants that control the level of ROS. AOE includes SOD, enzymes that destroy superoxide anions [66]. Cytosolic SOD1 or Cu, Zn-SOD, controls superoxide produced by NOX, xanthine oxidase, cyclooxygenase and other sources. Mitochondrial SOD2 destroys superoxide produced by the mitochondrial respiratory chain. SOD3, or extracellular SOD secreted by cells, binds to extracellular matrix via its heparin-binding domain and destroys extracellular superoxide. Superoxide dismutation yields H₂O₂ that in turn gets destroyed by catalase, peroxiredoxins and peroxidases [2].

In addition to AOE, cells contain significant amounts of nonenzymatic antioxidants including glutathione, ascorbate, tocopherols, bilirubin, uric acid and other molecules, which scavenge ROS, hydroxyl radical and other free radicals [23]. Antioxidant molecules have been extensively tested in models of oxidative stress, but most of the well-controlled studies failed to demonstrate their beneficial effects [67]. Such disappointing results may be explained in part by their relatively slow reaction with ROS and limited availability in the proper cellular compartments [20]. In the context of the latter challenge, significant progress is being reported in mitochondrial targeting of quinones for degradation of excessive ROS

generated by an imbalanced respiratory chain [68]. Recent studies also demonstrated the therapeutic antioxidant potential of NOS cofactor tetrahydrobiopterin and folic acid [69]. Significant efforts are being invested in the last decade in antioxidant gene therapy. Delivery of viral and nonviral genes encoding SOD, catalase, glutathione peroxidase and other antioxidant proteins has been designed and effects of these interventions are being tested *in vitro* and in animal studies. Although out of the focus of this article, antioxidant gene therapy may eventually improve treatment of chronic conditions involving oxidative stress [70]. In the following sections we describe the potentials of targeted AOE for vascular delivery.

Advanced formulations for vascular delivery

Most antioxidant agents have no specific affinity to the endothelium and, therefore, despite being exposed to circulation, ECs take up a minor fraction (in most cases <1%) of the drug. However, conjugation of drugs and their carriers (e.g., liposomes) with antibodies and antibody fragments that bind to EC surface determinants including angiotensin-converting enzyme (ACE), **platelet–EC adhesion molecule (PECAM)** and **intercellular adhesion molecule (ICAM)** permits effective delivery of 10–30% of the dose to the endothelium [8]. ECs internalize conjugates anchored to ACEs, ICAMs and PECAMs. This enables quenching of ROS in endosomes [16]. Of note, ICAM molecules delivering anchored anti-ICAM–catalase conjugates into the endosomes recycle to the cell surface, thereby allowing sustained intracellular delivery [71].

Within a few hours, internalized conjugates get degraded in the lysosomes, which limits duration of the effect [72]. To resolve this issue, methods to encapsulate AOE into polymeric and nonpolymeric nanocarriers permeable for ROS, but not proteases, have been developed [73,74]. Using PEG–catalase instead of native enzyme further enhances the encapsulation efficacy [75]. Modulating the formulations allows production of catalase-loaded nanocarriers of spherical or filamentous shape [76]. Coating of catalase-loaded nanocarriers by anti-PECAM provides endothelial targeting of the cargo *in vitro* and *in vivo* and prolonged antioxidant protection of the endothelium [77].

Of note, the PEG–poly(lactic-co-glycolic acid) polymer matrix is readily diffusible for H₂O₂, but not superoxide; hence encapsulation into PEG–poly(lactic-co-glycolic acid) nanocarriers obliterates enzymatic activity of SOD [77]. In contrast, encapsulation of either catalase or SOD into micelles formed by controlled precipitation of magnetic nanoparticles using calcium and oleate provides composite nanocarriers (200–300 nm diameter) containing active catalase or SOD accessible for either H₂O₂ or superoxide and protected from proteases [74]. Targeting this formulation to ECs using magnetic delivery or anti-PECAM conjugated to the surface of the micelles confers endothelial targeting and further boosts antioxidant protection [74].

Targeted interception of ROS signaling in vascular pathology

Antioxidants including SOD mimetics [78], mutant SOD that binds to the glycocalyx [79,80], AOE delivery using membrane-permeating peptides [81] and cell transfection by AOE genes [82] exert variable protective effects in cells and, at lesser rate of success, in

animals [83,84]. PEG-based ‘stealth’ AOE delivery has been extensively studied. PEG chains (2–10 kDa) coupled to a protein, form a hydrated shell that:

- Enhances its hydrodynamic radius and water solubility;
- Inhibits its interactions with cells and proteins [85].

This prolongs circulation, enhances bioavailability and effects of PEG–proteins [86]. Several PEG–protein conjugates are in the clinical testing and in use [87]. Indeed, conjugation with PEG [88] or PEG–pluronic [89] and loading in PEG-coated carriers [90,91] prolongs AOE circulation, enhancing their bioavailability and protective effects in animal models including stroke (likely due to better diffusion in to the CNS [92,93]) and some forms of chronic oxidative stress [94–96]. Quenching of extracellular ROS (e.g., by PEG–AOE) is an important axis of management of oxidative stress.

However, keeping in mind that ROS act within nanometer range of the flux sites, targeting is the key requirement. For example, intratracheal AOE delivery and transfection alleviated oxidative stress in the airways, but not in lung vasculature [97,98]. However, endothelial ROS play specific and important functions in conditions including I/R, ALI and inflammation [99–101]. As discussed above, endothelial ROS produced in response to pathological factors [102] cause vascular abnormalities, including:

- Exposure of adhesion molecules facilitating white blood cell transmigration (e.g., VCAM) [103];
- Loss of thrombomodulin, which unleashes thrombin, causing thrombosis and inflammation [104];
- Endothelial barrier disruption leading to vascular leakage and edema [55].

Admittedly, our knowledge of the role of endothelial ROS in pathology remains limited [105,106], in part due to lack of site-specific antioxidant interventions [107]. Nevertheless, endothelial uptake and effects of PEG–AOE are no better than those of naked AOE *in vitro* and *in vivo* [16]. As a result, despite high blood levels, PEG–AOE had no effect on pro-inflammatory endothelial activation [16].

Therefore, nontargeted approaches provide neither endothelial AOE delivery nor address specific cellular compartments. To achieve this goal, several groups devised ‘vascular immunotargeting’ strategies by conjugating cargoes with ligands of specific endothelial surface epitopes [108–115]. The general scheme of the AOE delivery action is depicted on Figure 7. Epitopes tested for this goal include ACE [116], other peptidases such as aminopeptidase P [117] and aminopeptidase N [118], cell adhesion and other molecules [8,110,119,120]. Thus, conjugation of AOE with antibodies to ACE, PECAM and ICAM, and using AOE-loaded nanocarriers targeted by these antibodies, have recently been shown to provide highly effective and specific antioxidant effects [74,77,112,115,121]. Anti-ACE/AOE, anti-ICAM/AOE and anti-PECAM/AOE conjugates provided protective antioxidant effects superior to nontargeted AOE formulations such as PEG–AOE in models of acute pulmonary vascular oxidative stress [122–126]. In particular, success in protecting lungs against oxidative stress (e.g., I/R injury in lung transplantation) by anti-ICAM/AOE, anti-

ACE/AOE [127] and anti-PECAM/AOE [125,126] has recently been reproduced by several laboratories in diverse animal models [128–130].

Antihypertensive effect of SOD delivery

Superoxide produced by ECs inactivates NO, thereby causing hypertension [131]. In addition, the peroxynitrite formed inactivates enzymes required for synthesis of vasodilator prostacyclin [132] and NO synthases [133]. Several studies demonstrated feasibility of SOD delivery to the endothelium as antihypertensive intervention. Heparin-binding fusion protein of SOD1 binds to the endothelial glycocalyx and decreases blood pressure in hypertensive rats [134]. Similar effect on blood pressure normalization was demonstrated after administration of SOD mimetic tempol [135]. SOD encapsulated into liposomes also showed hypotensive effects in Ang II-induced hypertension in mice [136]. SOD specifically targeted to the endothelium using carrier antibodies directed to endothelial adhesion molecules prevented oxidation of tetrahydrobiopterin and normalized vasoreactivity of large vessels in Ang II-treated hypertensive mice [126]. It is noteworthy that H₂O₂ may function as a vasodilator in some types of vessels [137] and the delivery of catalase to the endothelium did not show vasodilatory effects in Ang II-induced hypertension model [126].

Anti-inflammatory properties of targeted SOD

In theory, antioxidants can provide antiinflammatory effects [8,138]. Anti-inflammatory properties of SOD have been noticed a while ago [139] and SOD modifications having prolonged life time in the bloodstream (e.g., conjugation with PEG) have been tested in inflammation models [140,141]. SOD mimetics and superoxide quenchers inhibit VCAM expression in TNF-treated EC cultures [94,142]. Liposomal delivery of *N*-acetylcysteine was protective against lipopolysaccharide-mediated inflammation [143].

However, these interventions lack specific enzyme delivery and, despite three decades of intense research, the initial enthusiasm towards PEG–SOD has not been translated yet into human therapy [141]. Hybrid chimeric SOD (called SOD2/3) was genetically engineered by combination of catalytic unit of SOD2 and heparin-binding domain of SOD3. It binds to the endothelium and exerts anti-inflammatory activity [80,144]. Targeted anti-PECAM/SOD provided effective protection in oxidative stresses caused by both extracellular and intracellular superoxide radical in cell culture [115,145]. Importantly, ECs internalize these conjugates via a specific CAM-mediated endocytosis pathway [146]. This provides an ideal delivery mechanism to the key destination, the redox-active signaling endosomes [106]. As a result, anti-PECAM/SOD, but not PEG–SOD or other antioxidants, attenuated VCAM expression by ECs in response to diverse cytokines [16] and attenuated pro-inflammatory effects of lipopolysaccharide in mice [16]. Thus, specific targeting of AOE may inhibit redox-sensitive inflammatory signaling and attenuate harmful consequences of excessive inflammatory response (Figure 7).

Normalization of pathological abnormalities of endothelial permeability

Vascular ROS play an important role in regulation of endothelial barrier function. Therefore, SOD and catalase have been tested to treat ROS-mediated endothelial barrier dysfunction [57,147]. For example, a recombinant SOD fusion protein that can bind to heparin-like

proteoglycans on the EC surface exhibited promising protective effect on I/R-induced vascular permeability [144]. Furthermore, both *in vivo* and *in vitro* studies showed the alleviation of abnormal endothelial permeability by anti-PECAM/AOE formulations but not by untargeted AOE (Figure 8) [58,126]. Endothelial targeting or expression of catalase, but not SOD, inhibited endothelial permeability in response to xanthine/xanthine oxidase-generated extracellular ROS, suggesting that extracellular H₂O₂ is the key disruptor of endothelial barrier function in this model [58].

Furthermore, ECs produce ROS by NOX in response to vasoactive pro-inflammatory agents including VEGF and thrombin [148] that disrupt endothelial layer integrity and cause edema [148]. Interestingly, anti-PECAM/SOD, but not anti-PECAM/catalase, attenuated VEGF-induced endothelial barrier dysfunction, implicating O₂^{•-} in this type of pathological redox signaling [58]. It is noteworthy that nontargeted catalase and SOD including PEG-conjugated enzymes provided no effect, due to lack of delivery to the site of ROS influx and effect. Therefore, AOE targeted to ECs provide versatile molecular tools for identifying the roles of specific ROS in vascular pathology and may be translated into remedies for these ROS-mediated vascular abnormalities.

Future perspective

In the last three decades, antioxidant interventions with optimized pharmacokinetics and specific delivery to endothelium have been devised and tested *in vitro* and in animal studies in early preclinical research. Some of these approaches demonstrated impressive superiority versus nontargeted interventions in animal models of human pathologies involving ROS signaling in the vasculature. The immediate goals are now:

- To refine delivery of antioxidants to selected subcellular compartments where signaling ROS are produced (specific types of endosomes and cellular vesicles, mitochondria and endoplasmic reticulum);
- To define optimal regimens of administration, therapeutic dose and time windows for the interventions in animal models;
- To translate most promising prototypes showing milestone achievements in these animal studies into a format applicable in clinical studies (i.e., replacement of targeting and enzymatic moieties by proteins and peptides that can be used in humans).

Fulfillment of these objectives will set a stage for the key steps of the subsequent industrial development of these targeted therapeutic interventions including scaling up and quality control of production, rigorous pharmacokinetic and toxicological studies in large animals and, eventually, clinical testing. Taking into account the current pace of the progress in the field, it is not overly optimistic to expect that many of these objectives will be achieved within less than a decade. These, in turn, can provide a solid basis for future clinical development and application of a new generation of targeted antioxidant interventions for treatment prevalent human maladies involving pathological ROS signaling.

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Key Term

NADPH oxidases

Membrane enzyme complexes that produce superoxide and, in some cases, hydrogen peroxide when activated by agonists including cytokines, one of the major sources of signaling reactive oxygen species.

Tight junction

Intercellular junctional complex that is particularly enriched in the brain microvasculature of the blood–brain barrier. Tight junctions (TJs) are responsible for the maintenance of functional endothelial barriers. Distinct from adhesion junctions, TJs do not form a continuous seal around the cells, but contain discontinuities or pores, allowing selective molecular sieving. Claudins, occludin and zona occludens are essential components of TJs and have been implicated in regulating the permeability properties of TJs.

Adherens junctions

Cellular membrane contacts formed by vascular endothelial–cadherins complexes with catenins. Adherens junctions are critical in regulation of transendothelial migration of blood cells, as well as paracellular permeability. Disruption of vascular endothelial–cadherin distribution or the homophilic interaction leads to increased endothelial permeability and is associated with such pathological processes as inflammation and acute lung injury.

Platelet-endothelial cell adhesion molecule

This 130-kDa glycoprotein belongs to the Ig-like superfamily of adhesion molecules. Platelet–endothelial cell adhesion molecule-1 is particularly abundant on endothelial cells, where it is localized on cell–cell borders and facilitates leukocyte endothelial transmigration. Platelet–endothelial cell adhesion molecule-1 is a good target for endothelial drug delivery, either to the cell surface or intracellularly, depending on the design of targeting system.

Intercellular adhesion molecule

Another member of Ig-like superfamily of adhesion molecules. It serves as a receptor for leukocyte adhesion to inflamed endothelium. Intercellular cell adhesion molecule-1 is upregulated in response to inflammatory signals and participates in the recruitment of leukocytes to the sites of inflammation. Intercellular cell adhesion molecule-1 is a good candidate for endothelial targeting, in some aspects similar to platelet-endothelial cell adhesion molecule-1.

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Executive summary

- Reactive oxygen species (ROS) pathological signaling in vasculature causes endothelial dysfunction. Pathological changes in the vasculature caused by excessive ROS, in particular produced by the endothelium, are increasingly recognized as important mechanisms of human diseases, in particular, hypertension, thrombosis and inflammation.
- Means to intercept ROS include antioxidant enzymes and antioxidants. Antioxidant interventions targeting to the vascular endothelium may help to control these mechanisms. Animal studies have demonstrated superiority of targeting catalase and superoxide dismutase to endothelial markers including angiotensin-converting enzyme and cell-adhesion molecules over nontargeted formulations. These new means will help to dissect mechanisms of vascular oxidative stress and may eventually be translated into the clinical domain, thereby improving management of disease conditions involving this pathological mechanism.
- Targeted interception of ROS signaling in vascular pathology combines a diverse arsenal of targeted antioxidant formulations devised in the last decade, and shows highly promising results of specific quenching of endothelial ROS *in vitro* and in animal models. In addition to direct alleviation of toxic and injurious effects of excessive ROS, these novel targeted interventions provide suppression of specific pro-inflammatory mechanisms, including endothelial cytokine activation and barrier disruption.
- Advanced formulations for vascular delivery, including new generations of antioxidant nanocarriers will find use in experimental biomedicine and, perhaps, in translational medicine.

Figure 1. The metabolism and role of reactive oxygen species in signaling and vascular oxidative stress

COX: Cyclooxygenase; GSHPx: Glutathione peroxidase; MPO: Myeloperoxidase; SOD: Superoxide dismutase; XO: Xanthine oxidase.

Figure 2. Molecular mechanisms of reactive oxygen species signaling

ROS produced by a family of NADPH oxidase enzymes or by mitochondria (complexes I and III of the respiratory chain) are able to oxidize cysteine residues of several components of signaling pathways. This can cause an increase in ion-channel activity or activation of kinase activity. The most studied ROS signaling mechanism is the inhibition of phosphatase activity by H_2O_2 , which in turn leads to kinase activation. ROS can also increase DNA binding to the transcription factor.

NOX: NADPH oxidase; ROS: Reactive oxygen species.

Figure 3. Mechanism of AngII-induced hypertension

AngII binds to its receptor AT₁, which activates NOX2 and results in superoxide generation. Other systems that produce superoxide in an AngII responsive manner include the mitochondrial respiration chain and nitric oxide synthase uncoupling and NOX1, induced in pathological conditions. Superoxide scavenges NO, a critical vasodilator. Consequent depletion of NO causes development of hypertension.

NOX: NADPH oxidase; ROS: Reactive oxygen species.

Figure 4. The involvement of vascular reactive oxygen species in thrombosis

ROS in vascular lumen are derived from various vascular cells, including (A) activated leukocytes, (B) adherent platelets and (C,D) endothelial cells. ROS themselves can directly stimulate platelet activation and aggregation (1). ROS can also indirectly regulate formation of thrombosis by decreasing bioavailability of NO (2), which is generated by endothelial cells (E), increasing the levels of ONOO⁻ (3), oxidized LDL (4) and TF that initiates explosive coagulation cascade (5).

LDL: Low-density lipoprotein; NOX: NADPH oxidase; Ox-LDL: Oxidized LDL; P: Platelet; PMN: Polymorphonuclear leukocyte; ROS: Reactive oxygen species; TF: Tissue factor.

Figure 5. Relationship between reactive oxygen species and inflammatory NF-κB signaling

The activation of NF-κB produces cell-specific set of target proteins, including cell adhesion molecules (specific for endothelial cells, top box), antioxidant proteins (middle box) and pro-oxidant proteins (bottom box). ROS may modulate NF-κB function by either its activation or, in some cases, by inhibition. Outcome of NF-κB regulation by ROS will depend on cell type and its redox status, localization, production time and type of ROS. COX: Cyclooxygenase; ICAM: Intercellular adhesion molecule; NOX: NADPH oxidase; ROS: Reactive oxygen species; TRADD: Tumor necrosis factor receptor type 1-associated death domain protein; TRAF: Tumor necrosis factor receptor-associated factor; VCAM: Vascular cell adhesion molecule-1.

Figure 6. Involvement of reactive oxygen species in the regulation of endothelial barrier function
 ROS derived from activated PMNs and endothelial cells in response to permeability enhancers (thrombin and VEGF) are involved in multiple signaling pathways leading to endothelial barrier disruption. Vascular ROS stimulate intracellular Ca²⁺, RhoA activity and phosphorylation of MLC, resulting in stress-fiber formation and increased contractility. ROS increase several kinase activities (e.g., p38 MAP kinase, protein kinase C and tyrosine kinase Pyk2) that are implicated in cytoskeletal remodeling and destabilization of intercellular junctions.

MLC: Myosin light chain; MMP: Matrix metalloproteinase; NOX: NADPH oxidase; PMN: Polymorphonuclear leukocyte; ROS: Reactive oxygen species; ZO: Zona occludens.

Figure 7. Targeted antioxidant interventions

SOD or CAT conjugated with antibody to the endothelial target, specifically binds to endothelial cells and efficiently degrades the superoxide anion and hydrogen peroxide, respectively. Reactive oxygen species removal protects cells against oxidative stress or inhibits unwanted signaling.

CAT: Catalase; COX: Cyclooxygenase; GSHPx: Glutathione peroxidase; MPO: Myeloperoxidase; NOX: NADPH oxidase; SOD: Superoxide dismutase; XO: Xanthine oxidase.

Figure 8. Endothelial delivery of antioxidant enzymes alleviates reactive oxygen species-mediated endothelial barrier dysfunction

AOEs including catalase and superoxide dismutase targeted to endothelials can quench extracellular and intracellular ROS that are generated in response to stimulation of permeability enhancers (thrombin and VEGF) or released from activated PMNs. Thus, endothelial delivery of AOEs can inhibit ROS-induced cytoskeletal remodeling and disassembly of intercellular junctions, protecting endothelial barrier function.

AOE: Antioxidant enzyme; NOX: NADPH oxidase; PMN: Polymorphonuclear leukocyte; ROS: Reactive oxygen species.