Antioxidant-based therapies for angiotensin II-associated cardiovascular diseases

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1Department of Cellular and Integrative Physiology, Nebraska Center for Nanomedicine, University of Nebraska Medical Center, Omaha, Nebraska; and 2Division of Molecular Pharmaceutics and Center for Nanotechnology in Drug Delivery, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

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Rosenbaugh EG, Savalia KK, Manickam DS, Zimmerman MC. Antioxidant-based therapies for ANG II-associated cardiovascular diseases. Am J Physiol Regul Integr Comp Physiol 304: R917–R928, 2013. First published April 3, 2013; doi:10.1152/ajpregu.00395.2012.—Cardiovascular diseases, including hypertension and heart failure, are associated with activation of the renin-angiotensin system (RAS) and increased circulating and tissue levels of ANG II, a primary effector peptide of the RAS. Through its actions on various cell types and organ systems, ANG II contributes to the pathogenesis of cardiovascular diseases by inducing cardiac and vascular hypertrophy, vasoconstriction, sodium and water reabsorption in kidneys, sympathoexcitation, and activation of the immune system. Cardiovascular research over the past 15–20 years has clearly implicated an important role for elevated levels of reactive oxygen species (ROS) in mediating these pathophysiological actions of ANG II. As such, the use of antioxidants, to reduce the elevated levels of ROS, as potential therapies for various ANG II-associated cardiovascular diseases has been intensely investigated. Although some antioxidant-based therapies have shown therapeutic impact in animal models of cardiovascular disease and in human patients, others have failed. In this review, we discuss the benefits and limitations of recent strategies, including gene therapy, dietary sources, low-molecular-weight free radical scavengers, polyethylene glycol conjugation, and nanomedicine-based technologies, which are designed to deliver antioxidants for the improved treatment of cardiovascular diseases. Although much work has been completed, additional research focusing on developing specific antioxidant molecules or proteins and identifying the ideal in vivo delivery system for such antioxidants is necessary before the use of antioxidant-based therapies for cardiovascular diseases become a clinical reality.

antioxidants; free radical scavengers; oxidative stress; cardiovascular disease; angiotensin; therapy

CARDIOVASCULAR DISEASES RANK high among the most common and devastating disorders in adults. The World Health Organization reports that cardiovascular diseases are the number-one cause of death globally, claiming 17.1 million lives per year. According to the American Heart Association and the Centers for Disease Control and Prevention (CDC), ~67 million adults in the United States have high blood pressure, that is, one in every three American adults. As reported by the National Heart, Lung, and Blood Institute, heart failure affects about 5.7 million people in the United States and is the cause of nearly 300,000 deaths per year. Cardiovascular diseases, like hypertension and heart failure, are commonly associated with increases in circulating and tissue levels of ANG II, which contributes to the cardiac and vascular hypertrophy, inflammation, sympatheexcitation, and oxidative stress observed in these diseases (36, 43, 46, 111). Although numerous therapies have been developed and are used clinically, many have undesirable side effects and are not 100% effective in all patients (40, 80, 104). Recent research efforts using various animal models of cardiovascular disorders, as well as clinical trials, have focused on antioxidant-based therapeutics to alleviate the oxidative stress involved in these diseases. Strategies to deliver antioxidants include gene therapy, dietary sources, low-molecular-weight free radical scavengers, polyethylene glycol (PEG) conjugation, and nanomedicine-based technologies (Table 1). The use of these antioxidant-based therapeutic strategies and their limitations in cardiovascular diseases are discussed in this review.

Renin-Angiotensin System, Reactive Oxygen Species, and Cardiovascular Disease

The renin-angiotensin system (RAS) plays an important role in regulating body fluid and cardiovascular homeosta-
Table 1. Summary of the advantages and disadvantages of the antioxidant strategies used to decrease levels of free radicals and/or ROS and improve cardiovascular function in experimental animal models of cardiovascular disease and/or cardiovascular disease patients

<table>
<thead>
<tr>
<th>Antioxidant Strategy</th>
<th>Free Radical or ROS Target</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene therapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonviral vectors</td>
<td>Depends on the gene expressed</td>
<td>Excellent experimental tools</td>
<td>Potential for toxicity</td>
<td>2, 23, 27, 42, 69, 70, 73, 74, 149, 155, 160, 161</td>
</tr>
<tr>
<td>Viral vectors</td>
<td>by the vector</td>
<td>Can overexpress antioxidant of interest in CV disease model of choice</td>
<td>Induce host immune response</td>
<td>15, 33, 39, 49, 53, 56, 75, 83, 88, 90, 123, 134, 147, 157</td>
</tr>
<tr>
<td>Dietary Sources</td>
<td></td>
<td>Improve CV function in a large collection of CV disease animal models</td>
<td>Poor targeting following IV administration to specific tissue/cells</td>
<td>7, 9, 19, 21, 22, 26, 72, 76, 96, 99, 103, 126, 127, 145</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>Lipid hydroperoxide</td>
<td>Readily available</td>
<td>High doses may be harmful</td>
<td>16, 57, 143, 156, 159</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>α-tocopherol radicals</td>
<td>Administered orally</td>
<td>Not specific for one free radical or ROS</td>
<td>54, 58, 62, 82, 91, 92, 114, 119, 125, 128, 129, 138, 139, 152, 153</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Lipid radicals</td>
<td>Protect lipid membranes</td>
<td>May alter off-target redox signaling by producing/ scavenging additional ROS</td>
<td>103, 126, 127, 145</td>
</tr>
<tr>
<td>Carotenoids</td>
<td></td>
<td>Used extensively in animal models and patients to improve CV function</td>
<td>High doses may be harmful</td>
<td>16, 57, 143, 156, 159</td>
</tr>
<tr>
<td>Low-molecular-weight free radical scavengers</td>
<td>Depends on the scavenger</td>
<td>Administered orally</td>
<td>Not specific for one free radical or ROS</td>
<td>16, 57, 143, 156, 159</td>
</tr>
<tr>
<td>Nitroxides (tempol)</td>
<td>To date, most CV studies have utilized antioxidant mimetics to target O$_2^-$ and H$_2$O$_2$</td>
<td>Cell permeable</td>
<td>May alter off-target redox signaling by producing/ scavenging additional ROS</td>
<td>16, 57, 143, 156, 159</td>
</tr>
<tr>
<td>Porphyrins</td>
<td></td>
<td>Can be chemically modified to target subcellular organelles (i.e., mitochondria)</td>
<td>High doses may be harmful</td>
<td>16, 57, 143, 156, 159</td>
</tr>
<tr>
<td>Nanotechnology</td>
<td></td>
<td>Improve CV function in a large collection of CV disease animal models</td>
<td>Not specific for one free radical or ROS</td>
<td>16, 57, 143, 156, 159</td>
</tr>
<tr>
<td>Polymers</td>
<td>Depends on the conjugated antioxidant protein</td>
<td>PEG is a FDA-approved compound</td>
<td>Ability of PEG to deliver proteins across the cell membrane of all cell types remains unclear</td>
<td>16, 57, 143, 156, 159</td>
</tr>
<tr>
<td>PEG conjugation</td>
<td>To date, most CV studies have used PEG-SOD (to target O$_2^-$) and PEG-Catalase (to target H$_2$O$_2$)</td>
<td>In vivo PEG improves circulation time of protein, delays protein clearance, and slows proteolytic degradation</td>
<td>High doses of polymers may be toxic</td>
<td>16, 57, 143, 156, 159</td>
</tr>
<tr>
<td>PEG-SOD</td>
<td></td>
<td>Improves CV function in various CV disease animal models</td>
<td>Incorporation of antioxidant enzyme into complex may reduce activity</td>
<td>16, 57, 143, 156, 159</td>
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<tr>
<td>PEG-catalase</td>
<td></td>
<td>PEG decreases permeability of proteins across the blood-brain barrier</td>
<td>To date, the reported use of nanotechnology to deliver antioxidant proteins in CV disease animal models is limited</td>
<td>16, 57, 143, 156, 159</td>
</tr>
<tr>
<td>Nanotechnology</td>
<td></td>
<td>Protect their cargo (i.e., antioxidant protein) in vivo from clearance and degradation</td>
<td>Ideal route of administration (i.e., oral vs. intravenous) remains unclear</td>
<td>16, 57, 143, 156, 159</td>
</tr>
<tr>
<td>Polymers</td>
<td>To date, most CV studies have delivered SOD protein (to target O$_2^-$)</td>
<td>Improve stability of antioxidant protein</td>
<td>High doses of polymers may be toxic</td>
<td>16, 57, 143, 156, 159</td>
</tr>
<tr>
<td>Block ionomer complexes</td>
<td></td>
<td>Can penetrate plasma membranes</td>
<td>Incorporation of antioxidant enzyme into complex may reduce activity</td>
<td>16, 57, 143, 156, 159</td>
</tr>
</tbody>
</table>

References (Ref.) are listed in the far right column. CV, cardiovascular; FDA, Food and Drug Administration; IV, intravenous; PEG, poly(ethylene glycol); ROS, reactive oxygen species; SOD, superoxide dismutase.

sis, and dysregulation of the RAS is associated with the pathogenesis of cardiovascular disease. The RAS and one of its primary effector peptides, ANG II, is known to exist not only in systemic circulation, but also locally within numerous organs, including the brain (4, 89), heart (4), vasculature (4), adipose tissue (35), gonads (132), pancreas (124), placenta (95), and kidney (4, 65). ANG II binds to its specific G protein-coupled receptors, ANG II type 1 receptor (AT1R) and ANG II type II receptor (AT2R), to induce its (patho)physiological actions. These actions, primarily carried out by AT1R activation, include increased sympathetic output, vasoconstriction, vasopressin and aldosterone release, cellular growth and migration, and sodium and water reabsorption in kidneys. More recently, AT1R signaling has also been shown to cause upregulation of the immune system, particularly T-cell activation, which participates in the development and maintenance of hypertension (79). The AT1R has a wide tissue distribution with high expression in the vasculature, kidney, cardiovascular control brain regions, adrenal gland, and heart. In general, AT2R-mediated actions are thought to oppose those of AT1R.

Production of reactive oxygen species (ROS), such as superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), and modulation of redox-sensitive signaling pathways are involved in the intracellular signaling pathway(s) of ANG II. It is well documented that increased ROS and the resulting oxidative stress in
ANG II-associated cardiovascular diseases result from the activation of NAPDH oxidases (112, 131), mitochondrial dysfunction (5, 18, 32, 154), and inflammation (38, 47, 78, 79, 122), as well as the reduction of endogenous antioxidant enzymes (17, 42). Nitric oxide synthase (NOS) is another source of O$_2^-$ under conditions where its substrate arginine or cofactor tetrahydrobiopterin are limited, a phenomenon termed NOS uncoupling, which commonly occurs in ANG II-related pathological conditions, such as hypertension, atherosclerosis, and diabetes (87). Regardless of source, O$_2^-$ is removed in the cell by SOD enzymes yielding H$_2$O$_2$ and oxygen, while various other antioxidant enzymes, such as catalase and glutathione peroxidases, work to scavenge H$_2$O$_2$.

The major ROS targets or downstream effectors in the ANG II signaling cascade include transcription factors, ion channels, matrix metalloproteinases (MMPs), kinases, and phosphatases. Redox-sensitive transcription factors, such as NF-κB and activator protein-1, can perpetuate the proinflammatory environment associated with ANG II-related cardiovascular diseases by inducing the expression of proinflammatory cytokines (44, 55, 71). ANG II-mediated increase in ROS, particularly O$_2^-$, has been shown to modulate ion channel activity, including potassium and calcium channels, which, in turn, regulate neuronal firing and vascular contraction (135, 154, 162). Two potential redox-sensitive targets that may mediate these ion channel activity changes are PKC and CaMKII, which are known to contribute to ANG II-induced modulation of neuronal potassium and calcium currents (135–137). ANG II-induced ROS also have a stimulatory effect on MMPs that regulate the extracellular matrix and vascular remodeling (68). Furthermore, ANG II-induced ROS can affect cell growth and apoptosis through its interactions with MAPK proteins, tyrosine kinases, and tyrosine phosphatases (121, 142). Collectively, these different ROS-sensitive effectors in various cell types work in concert to mediate systemic cardiovascular dysfunction.

Under normal conditions, the rate of ROS generation is balanced by the rate of elimination through endogenous and dietary antioxidants such as SOD, catalase, thioredoxin, glutathione, and vitamins. However, with dysregulation of ANG II signaling, this balance is tilted in favor of elevated ROS, which contributes to cardiovascular dysfunction leading to diseases, including hypertension, heart failure, atherosclerosis, stroke, ischemia-reperfusion (I/R) injury, and diabetes (17, 42, 94, 151, 158). In many cardiovascular diseases, ANG II and ROS induce vascular damage by promoting endothelial dysfunction, structural remodeling, and vascular inflammation (140–142).

Current pharmacological therapies for ANG II-associated cardiovascular diseases include angiotensin-converting enzyme inhibitors (ACEI), angiotensin receptor blockers (ARBs), β-blockers, calcium channel blockers, renin inhibitors, statins, and diuretics. ACEI decrease the formation of ANG II, whereas ARBs prevent ANG II from binding to the AT1R. Although they are among the most commonly prescribed drugs for cardiovascular diseases, ACEI and ARBs can induce potential side effects, such as cough, hyperkalemia, and angioedema, and some patients fail to respond favorably to these treatments (52, 100, 110). In fact, according to the CDC, 23.9% of hypertensive patients in the United States (~16 million people) have uncontrolled hypertension despite being on currently available prescription medications. β-blockers that reduce heart rate (HR) and blood pressure by blocking the effects of norepinephrine and epinephrine are another common therapy for cardiovascular disorders; however, their benefits are often constrained by poor outcomes in human patients due to an exaggerated decrease in HR (29).

To improve upon the current cardiovascular therapies, recent focus has been placed on using antioxidants and free radical scavenging molecules to target the ROS associated with ANG II-related cardiovascular diseases. Antioxidant therapies may have an advantage over current therapies, such as ACEI and ARBs, because of their ability to scavenge ROS generated not only by ANG II, but also by the proinflammatory cytokines that coincide with cardiovascular disease. As discussed below, scavenging ROS in numerous animal models has shown promise as a therapeutic strategy to prevent the development and exacerbation of ANG II-based cardiovascular diseases, but certain challenges must be overcome before antioxidant therapies become a clinical reality.

**Gene Therapy and Viral Overexpression of Antioxidant Enzymes**

The overall goal of gene therapy is to treat diseases by introducing new genes into a given population of somatic cells for the purpose of changing their cellular function. Typically, a segment of DNA is introduced into cells using a delivery system such as a viral vector. A wide variety of viral and nonviral delivery systems have been developed for the alteration of gene expression in experimental systems, each approach having strengths and weaknesses. Generally, nonviral gene delivery has lower toxicity and immunogenicity, unlimited transgene insertion size, and the ability to transfect both dividing and nondividing cells. However, low transfection efficiencies and lysosomal degradation of nonviral vectors equate to poor nuclear uptake and transgene expression. In contrast, viral vectors efficiently enter the nucleus leading to robust transgene expression; however, they can induce host immune responses and toxicity. Examples of viral delivery systems include recombinant adenovirus, adeno-associated virus, and retrovirus.

Numerous experimental models using adenoviral-mediated overexpression of endogenous antioxidant enzymes clearly indicate the therapeutic potential of such antioxidants. Adenovirally mediated overexpression of SOD (AdSOD) and catalase in the heart provides protection to the myocardium following I/R injury (69, 70, 149). Similarly, intravenous administration of adenovirus encoding CuZnSOD in rats minimizes I/R-induced acute renal failure (155). AdSOD injected directly into the heart normalizes chemoreflex sensitivity and favorably reduces sympathectomization in response to hypoxia (30, 31). Studies have also shown that AdSOD delivery to the brain in a rabbit model improves cardiac function and prolongs survival (42, 73, 74). Although adenoviral vectors are excellent experimental tools in cardiovascular disease models, limitations hinder their potential clinical use. For example, adenoviruses...
may elicit immune responses, limiting their duration of expression in cardiovascular tissues to days or weeks (23, 27). Furthermore, viral vectors tend to accumulate in the liver following systemic administration (i.e., intravenous injection); thus, limiting their uptake and therapeutic potential in cardiovascular tissues (2, 23, 27).

Dietary Sources of Antioxidants

Several clinical studies have reported a correlation between consumption of fruits and vegetables rich in natural antioxidants and decreased risk of cardiovascular disease (123). One such natural antioxidant is water-soluble ascorbic acid (also known as vitamin C) that is present in numerous fruits and vegetables. Ascorbic acid is critical in the prevention of atherosclerotic plaque (83, 130) due to its ability to prevent lipid hydroperoxide formation in plasma lipoproteins, such as low-density lipoprotein (LDL), by reducing α-tocopherol radicals formed upon reaction with lipid peroxyl radicals. Supplemental ascorbic acid, typically administered at supraphysiologic concentrations, has been widely studied as an antioxidant in a variety of pathologic conditions linked to elevated oxidative stress. In animal models of myocardial infarction, orally administered or locally delivered ascorbic acid reduced the area of infarction and extent of myocardial damage (15, 84). Ascorbic acid has been shown to lower blood pressure in hypertensive (33, 39) and diabetic patients (90). When administered to individuals with chronic lower extremity vasculocnstriction, two independent studies have shown marked improvements in lower extremity blood flow of postmenopausal women (88) and healthy older men (53) to levels comparable to younger control individuals. Furthermore, ascorbic acid has been shown to exert protective effects in a rat model of renal I/R injury (63). In contrast, several other studies did not report beneficial effects of ascorbic acid for stroke (64) or endothelial dysfunction (34, 147).

Tocopherol (vitamin E) is a fat-soluble radical-chain breaker found in vegetable oils, nuts, seeds, and leafy green vegetables. Because of its hydrophobic nature, tocopherol operates in a lipid environment providing antioxidant protection in membranes and lipoprotein domains. Data from several cardiovascular studies suggest that tocopherol reduces the risk of cardiovascular diseases, such as coronary heart disease (118, 133, 134). In several animal models and patients with heart failure or myocardial infarction, tocopherol stores are diminished and tocopherol supplementation improves cardiac performance, decreases infarct size, and delays progression to heart failure (28, 49, 102, 107). Tocopherol has also been shown to improve endothelial function and reduce blood pressure (13, 14). Despite its observed benefits in cardiovascular diseases, there have been several unsuccessful clinical trials with tocopherol treatment (56, 75, 144, 157). One limitation with tocopherol as an antioxidant-based therapeutic strategy is that low doses are ineffective but high doses are harmful (123). Another potential problem with tocopherol is that it concentrates in lipoproteins and thus does not interact with cytoplasmic ROS. As discussed above, ANG II and cytokines increase levels of intracellular ROS, which, in turn, mediate signaling pathways in various cell types, leading to cardiovascular dysfunction. To address the affinity for lipophilic environments, a water-soluble analog of tocopherol, trolox, was formulated. In a canine model of myocardial infarction, trolox protected myocyte cell cultures from free radical damage induced by hypoxanthine and xanthine oxidase and reduced myocardial damage (84). In addition, trolox prevented renal failure and improved the glomerular filtration rate following renal I/R injury in rats (148).

Dietary flavonoids, such as resveratrol, naringenin, quercetin, and catechins, are polyphenolic compounds with antioxidant properties that may also work to prevent and/or treat cardiovascular disease. These dietary antioxidants are found in tea, coffee, soy, fruit, vegetables, olive oil, cocoa, cinnamon, oregano, and red wine. Resveratrol was reported to have protective effects in diabetic nephropathy (60), coronary heart disease (150), myocardial infarction (20), hypertension (11), I/R injury (106), and stroke (120). Quercetin is another polyphenol with identified protective effects in a wide range of cardiovascular diseases, including atherosclerosis (61), myocardial infarction (109), cardiac hypertrophy (77), and hypertension (41). In addition to their antioxidant properties, polyphenols have been reported to have antiviral, antiallergic, antiplatelet, anti-inflammatory, and antitumor activities (24, 146). Given the abundance of studies that illustrate the beneficial effects of polyphenols in oxidative stress-related diseases, they appear to be a promising therapeutic strategy; however, more studies must be conducted to decipher the mechanisms and specific site of action and possible harmful effects before validating them as a clinically relevant therapeutic strategy for cardiovascular disorders.

Polyunsaturated hydrocarbon carotenoids are a group of phytochemicals that give many fruits and vegetables their characteristic pigment. They are recognized as critical players in the prevention of human diseases and maintenance of good health. Carotenes (vitamin A) and lycopene are types of carotenoids present in fruits, vegetables, liver, and eggs. Natural carotenones and lycopenes are free radical scavengers in lipophilic environments, such as membranes and lipoproteins (101, 113). Adequate consumption of carotenoids helps prevent cardiovascular diseases, such as atherosclerosis, ventricular remodeling following myocardial infarction, diabetes, and stroke (85, 113, 116). Lycopene supplementation has been shown to be beneficial for I/R injury (6, 48), atherosclerosis (115), long-term diabetic complications (93), and controlling cholesterol and blood pressure (117). β-carotene was reported to protect renal tissues against I/R-induced oxidative damage (50, 51). In spontaneously hypertensive rats, astaxanthin (a water-dispersible synthetic carotenoid)-enriched diet for 8 wk decreased oxidative stress, lowered systolic blood pressure, attenuated left ventricular hypertrophy, and improved endothelial function (86). In renal and hepatic I/R models, astaxanthin treatment reduced I/R injury (25). No adverse effects have been reported for astaxanthin, and it can be administered either orally or intravenously (37). In contrast to these carotenoid studies, many other carotenoid treatment trials have failed to demonstrate cardiovascular benefits and may even show deleterious effects (1, 3, 12, 56). Similar to tocopherol, carotenoids preferentially accumulate in lipophilic membranes; thus, it is tempting to speculate that these dietary antioxidants do not reliably provide protection in cardiovascular diseases because the ROS driving a particular pathology is likely produced in the cytoplasm and is able to exert its detrimental effects even when tocopherol and carotenoid levels are elevated.
Low-Molecular-Weight Free Radical Scavengers

Other antioxidants commonly used in experimental models of cardiovascular diseases are low-molecular-weight free radical scavengers. These are synthetic chemical compounds that often mimic naturally occurring antioxidants. In cardiovascular studies, the most commonly used compound from this group is the cell-permeable nitroxide 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (tempol). Nitroxides like tempol are classified as nonmetal catalytic antioxidants that undergo one- or two-electron reduction reactions, yielding hydroxylamines or ox-ammonium cations, respectively, which themselves are inter-convertible. Although tempol is often called an SOD mimic because of its ability to catalytically scavenge $\text{O}_2^{-}$, this nomenclature is misleading because tempol also facilitates $\text{H}_2\text{O}_2$ metabolism through its catalase-like action (99). Nevertheless, several studies have reported that tempol decreases blood pressure in various hypertensive animal models, including high-fat diet-induced hypertension, spontaneously hyper-tensive rats, salt-induced hypertension, and ANG II-infused rats (9, 21, 76, 126). One major advantage of tempol for clinical application is that it can be administered orally. In fact, in ANG II-infused rats, orally administered tempol attenuated the ANG II-induced hypertension and improved vascular function (145). In rats with myocardial infarction caused by left descending coronary artery ligation, orally administered tempol initiated 4 h after infarction and continued for 6 wk prevented increases in left ventricular end-diastolic pressure, volume, and decreases in ejection fraction. Furthermore, orally administered tempol normalized the increased renal sympathetic nerve activity, plasma norepinephrine levels, and AT1R expression, while favorably attenuating the cardiac sympathetic afferent reflex in these myocardially infarcted rats (127). In a rabbit model of chronic heart failure, tempol reversed the ANG II-mediated NADPH oxidase-driven $\text{O}_2^{-}$ signaling associated with enhanced carotid body chemoreceptor sensitivity to hypoxia (72). In two-kidney, one-clip Goldblatt hypertensive (2K-1C) rats, administration of tempol reduced the mean arterial blood pressure, prevented vascular dysfunction, and improved renal blood perfusion and oxygenation to a greater extent than administration of the ARB candesartan (22, 103). In a rat model of renal I/R injury, tempol treatment was also reported to reduce oxidative stress and cell death in kidneys (19).

Porphyrins, a second class of low-molecular-weight anti-oxidants, are metal-based catalytic antioxidants that can perform both one- and two-electron transfers and are often referred to as SOD and catalase mimetics. Metalloporphyrins have been shown to possess at least four distinct antioxidant properties, which include scavenging $\text{O}_2^{-}$, $\text{H}_2\text{O}_2$, peroxynitrite (ONOO$^-$), and lipid radicals. Most metalloporphyrins contain either an iron or manganese moiety that is coordinated by four nitrogen axial ligands. Porphyrins have been successful in preventing the deleterious effects of cardiomypathy, stroke, diabetes, I/R injury, and other oxidative stress conditions (7, 26). In addition, mangenese(III) tetraakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP) prevented mitochondrrial dysfunction and apoptosis in a rat model of I/R injury (96). A major drawback of porphyrins is that they exert toxicity at high doses (7).

PEG is an FDA-approved compound, composed of repeating ethyleneglycol units, which is commonly used in industrial manufacturing and drug modifications. PEG has several favorable chemical properties that make it useful in various biological, chemical, and pharmaceutical applications. PEGylation delays protein clearance from the body by kidney excretion, phagocytosis by the reticulo-endothelial system, and proteolytic degradation. Furthermore, PEG is nontoxic and nonimmunogenic (143). The structure of PEG is highly flexible, which allows for increased solubility and decreased aggregation. Given the short half-life of many antioxidant proteins in circulation (98, 105), PEGylation has been shown to improve the circulation time of such proteins, including CuZnSOD (16, 57, 159). PEGylated CuZnSOD (PEG-SOD) has been shown to protect the heart from I/R-induced arrhythmias and reduce the myocardium infarct size following ischemic injury (143). In addition, numerous studies have used direct injection of PEG-SOD into the brain to increase SOD activity and inhibit the pathophysiological actions of ANG II-induced $\text{O}_2^{-}$ (16, 57, 159). Although these direct brain injection studies support the concept that $\text{O}_2^{-}$ contributes to brain angiotensinergic signaling, the clinical impact of PEG-SOD as it relates to neurocardiovascular diseases is quite limited as PEGylation decreases the permeability of proteins across the blood-brain barrier (143, 156), and peripheral administration of PEG-SOD does not significantly increase brain CuZnSOD activity (156). In addition to limited permeability across the blood-brain barrier, the ability of PEGylation to deliver proteins across the plasma membrane of neurons remains unclear. However, PEGylation may enhance delivery of antioxidant proteins to other cell types, such as endothelial cells (10, 81).

Nanoformulated Antioxidant Enzymes

Nanotechnology and nanomedicine-based therapeutics have become hot topics in scientific research. Nanotechnology is the science of changing the intrinsic and extrinsic properties of substances (i.e., shape, charge, hydrophobicity) that are present in both atomic and subatomic sizes for biomedical and engineering applications. The field of nanomedicine uses nanotechnology to address the pharmacodynamic and pharmacokinetic obstacles of pharmacological agents and to improve drug delivery by precise drug release into specified targets. Nanomaterials can be formulated to entrap, encapsulate, or bind agents, such as DNA, proteins, peptides, and low-molecular-weight compounds. As suggested by their name, one of the major advantages of nanomaterials is their size, which is typically in the 1–100-nm range. Because of their small size, nanocarriers can penetrate many physiological barriers, such as plasma membranes. Bound nanomaterials also improve the solubility, stability, and absorption of pharmacological agents. In addition, nanof ormulations protect their cargo from clearance by the reticulo-endothelial system, renal system, and proteolytic degradation. Classes of nanomaterials include liposomes, nanoparticles, polymeric micelles, block ionomer complexes, nanogels, quantum dots, dendrimers, nanotubes, and nanofibers. Among these nanocarriers, liposomes and polymer-based nanomaterials are the most widely used systems in drug delivery by precise drug release into specified targets.
delivery because these compounds are generally biodegradable, do not accumulate in the body, and have low toxicity and immunogenicity (82).

Several antioxidant nanoformulations have been constructed and investigated for therapeutic impact in cardiovascular diseases, including I/R injury and stroke (58). For example, CuZnSOD protein encapsulated in biodegradable poly(D,L-lactide coglycolide), or PLGA nanoparticles (PLGA-SOD), has shown potential as a therapeutic agent for I/R injury. In a rat focal cerebral I/R injury model, PLGA-SOD administered at the time of reperfusion through intracarotid injection significantly reduced the infarct volume, maintained blood-brain barrier integrity, prevented edema, decreased levels of ROS, and protected neurons from apoptosis (114). Furthermore, the protection from I/R injury afforded by PLGA-SOD increased survival and improved the neurological function of rats exposed to the cerebral ischemia (114). Other nanoformulated antioxidants studied in models of I/R injury are peroxalate nanoparticles, which instantaneously and specifically decompose H2O2, the most abundant ROS generated during I/R injury (66, 67). However, clinical application of peroxalate nanoparticles is hindered by their instability in vivo (66, 67). More recent peroxalate nanoparticle formulations that were generated with p-hydroxybenzyl alcohol (HBA) incorporated into the hydrophobic polymer are more stable in vivo and retain high specificity for scavenging H2O2 (59). Polyoxalate is a commonly used scaffold for the polymeric HBA because of its ease of synthesis, biocompatibility, and complete biodegradation. These HBA-modified peroxalate nanoparticles have strong antioxidant and anti-inflammatory functions (59), and thus, they may have therapeutic potential in various cardiovascular diseases associated with oxidative stress and inflammation.

There have also been studies delivering antioxidants to the heart using nanotechnology for treatment of myocardial oxidative stress and dysfunction. In a monocye chemotactic protein-1 transgenic murine model of cardiomyopathy, cerium oxide (CeO2) nanoparticles administered intravenously twice a week for 2 wk markedly inhibited progression of left ventricular dysfunction and remodeling. Furthermore, CeO2 nanoparticle treatment decreased myocardial inflammation, oxidative stress, and endoplasmic reticulum stress (97). In other studies, poly(cyclohexane-1,4-diy acetone dimethylene ketal) polymer was used to deliver CuZnSOD to the heart. These CuZnSOD polyketal particle-encapsulated microparticles (PKSOD) injected into the perimeter of the cyanotic ischemic zone scavenged excess O2·−, prevented myocyte apoptosis, and improved cardiac function in a rat model of myocardial I/R injury (125). Although these PKSOD microparticles were not in the nanometer-size range, they could potentially be reformulated to afford the benefits of nanomedicine, including a more clinically relevant and noninvasive route of administration than direct myocardial injection. Another study using polyketal polymers for sustained release of drugs with self-assembling nanofibers reported that these multifunctional scaffolds could be used to deliver antioxidants to rat models of myocardial infarction (139).

Another focus of nanomedicine research is the delivery of antioxidants to the central nervous system for the treatment of ANG II-dependent neurocardiovascular diseases associated with oxidative stress, including hypertension and heart failure. Our laboratory is currently investigating the therapeutic potential of a polyethyleneimine-polyethylene glycol, PEI-PEG, polymer that electrostatically binds to CuZnSOD (so-called CuZnSOD nanozyme) to deliver CuZnSOD protein to the brain for treatment of neurogenic hypertension. We recently reported that CuZnSOD nanozyme delivers functional CuZnSOD protein to neurons resulting in an attenuation of ANG II-mediated O2·− production and potassium channel inhibition without inducing significant cytotoxicity (119). In the same study, we also reported that a single intracarotid injection of CuZnSOD nanozyme inhibits the central ANG II-induced pressor response for up to 3 days (119). More recently, various polymers have been developed to increase the bioavailability of CuZnSOD nanozyme and extend the therapeutic impact beyond 3 days. Specifically, our second-generation nanozymes are composed of a highly positively charged polymer, poly-l-lysine (PLL). PLL is covalently bound to PEG to form a cationic block copolymer that is capable of electrostatically binding to negatively charged carboxyl groups on CuZnSOD protein. The second-generation nanozyme is synthesized, such that the complex retains a collective neutral charge at physiological pH. Furthermore, second-generation nanozymes are synthesized with increased levels of stability due to cross-link bonds between the PLL polymer chains (Fig. 1). Klyachko et al. (62) identified an advantage for cross-linking polymers when delivering nanoformalized complexes in vivo. Data from this study showed that mice that were given cross-linked nanozyme had significantly higher levels of CuZnSOD in the brain and blood 1 h following intravenous injection compared with non-cross-linked complexes. Thus, there has been a movement in the nanomedicine industry to synthesize cross-linked polymers to enhance protein delivery to the brain and increase circulation time of polymers in the blood. Importantly, cross-linking our second-generation nanozyme does not alter CuZnSOD activity, and complexes with either reducible or nonreducible cross-linked

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**Fig. 1.** Schematic of CuZnSOD nanozyme in which negatively charged CuZnSOD protein (green) electrostatically interacts with positively charged poly-l-lysine-poly-ethylene glycol (PLL-PEG; blue/red lines). CuZnSOD nanozyme retains neutral charge at physiological pH and, as depicted here, our second-generation nanozyme is synthesized with increased stability due to either reducible or nonreducible cross-link bonds (orange circle) between PLL polymer chains.
bonds are able to scavenge O$_2^-$ to the same extent as non-cross-linked CuZnSOD nanzymes and free CuZnSOD protein (Fig. 2).

In addition to our nanzyme formulations, CuZnSOD protein conjugated to poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymer (Pluronic-SOD) has also been shown to scavenge intracellular O$_2^-$ (153). Notably, it has been demonstrated that pluronic-modified proteins are able to cross the blood-brain barrier and may subsequently become a primary therapeutic candidate for cardiovascular diseases associated with elevated O$_2^-$ levels in the brain (8, 54, 108, 152).

While our group has focused on the delivery of nanoforulated antioxidants to the brain, others have targeted the vascular endothelium for treatment of the vascular oxidative stress and inflammation implicated in the pathogenesis of many cardiovascular diseases. Polystyrene or PGLA polymer nano-
carriers have been used to deliver SOD and catalase to the vascular endothelium (91). To improve vascular specificity, SOD and catalase were conjugated to antibodies directed against constituently expressed endothelial antigens, such as intercellular adhesion molecule-1 (ICAM-1) and platelet endothelial cell adhesion molecule-1 (PECAM-1), so that these enzymes would bind to the endothelium following intravascular administration. In addition to augmenting antioxidant defenses, Muzykantov and colleagues propose that this immune-targeting strategy may have the added benefit of reducing inflammation and leukocyte-mediated vascular damage by blocking ICAM-1 and PECAM-1 uptake (92, 129, 138). PECAM-targeted SOD nanocarriers were successful in reducing vascular inflammation (128), while both the immunity-targeted SOD and catalase nanocarriers prevented ROS-induced vascular hyperpermeability (45). The advantageous properties substantiate the therapeutic potential of immune-targeting strategies for tissue edema. Although nanotechnology is a seemingly promising field, it is still a relatively new and developing area of study, especially in the field of cardiovascular research and requires further investigation and several improvements. Nanomaterials not only act on cells to produce biologically favorable effects but may also generate adverse effects. The risks and benefits of exposure to nanomaterials, routes of entry, molecular mechanisms of action, and potential cytotoxicity need to be further studied and better understood, especially with regard to clinically relevant treatments of cardiovascular diseases.

Perspectives and Significance

Cardiovascular diseases, particularly involving enhanced ANG II signaling, are associated with oxidative stress, resulting from overproduction of ROS and reduced levels of antioxidant activity. Several studies have investigated the ability of various antioxidants to treat and lower the risk of cardiovascular diseases. Gene therapy, dietary antioxidants, low-molecular-weight free radical scavengers, PEFlipation of antioxidant proteins, and nanoformulated antioxidant delivery systems have all been examined for the treatment of ANG II-associated cardiovascular diseases (Fig. 3). Although many studies have shown significant therapeutic potential for antioxidant formulations, others have failed to reveal any beneficial effects. Lack of interaction between the therapeutic antioxidant and specific ROS driving the pathological condition may account for the inability of some antioxidant strategies to improve cardiovascular function. Specifically targeting tissues, cells, and subcellular locations where the oxidative stress occurs will likely improve the efficacy of these antioxidant treatments. Another possible reason for the ineffectiveness of antioxidant therapy could be that the antioxidants are administered too late for successful intervention when extensive pathology is present, whereas successful trials involve antioxidants administered early in the disease progression or as a preventative therapy. Alternatively, antioxidant interventions may fail due to incorrect dosing or inappropriate biomarkers of oxidative damage. Given the recent finding that ANG II signaling activates the immune system, which further fuels the oxidant fire, it is reasonable to continue promoting investigation of antioxidant-based therapeutic strategies for cardiovascular diseases.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: E.G.R., K.K.S., and M.C.Z. conception and design of research; E.G.R., K.K.S., and M.C.Z. interpreted results of experiments; E.G.R., K.K.S., and M.C.Z. prepared figures; E.G.R. drafted manuscript; E.G.R., K.K.S., and M.C.Z. edited and revised manuscript; E.G.R., K.K.S., and M.C.Z. approved final version of manuscript; K.K.S. analyzed data; D.S.M. synthesized and characterized the CuZnSOD nanozymes used in Figure 2.

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