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Cardiovascular Research 55 (2002) 239–249

Cardiovascular Research

www.elsevier.com/locate/cardiores

Review

Extracellular superoxide dismutase and cardiovascular disease

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Received 7 November 2001; accepted 17 January 2002

Abstract

Excessive production and/or inadequate removal of reactive oxygen species, especially superoxide anion ($O_2^{\bullet -}$), have been implicated in the pathogenesis of many cardiovascular diseases, including atherosclerosis, hypertension, diabetes, and in endothelial dysfunction by decreasing nitric oxide (NO) bioactivity. Since the vascular levels of $O_2^{\bullet -}$ are regulated by the superoxide dismutase (SOD) enzymes, a role of SOD in the cardiovascular disease is of substantial interest. Particularly, a major form of SOD in the vessel wall is the extracellular SOD (ecSOD). This review will discuss the characteristics of ecSOD and the role of ecSOD in cardiovascular diseases.

Keywords: Atherosclerosis; Endothelial function; Free radicals; Hypertension; Ischemia; Nitric oxide; Reperfusion

1. Introduction

Aerobic organisms possess antioxidant defense systems that deal with reactive oxygen species (ROS) produced as a consequence of aerobic respiration and substrate oxidation. Low levels of ROS are indispensable in many biochemical processes, including intracellular signaling, defense against microorganisms and cell function. In contrast high dose and/or inadequate removal of ROS, especially superoxide anion ($O_2^{\bullet -}$), results in 'oxidative stress', which has been implicated in the pathogenesis of many cardiovascular diseases, including hypercholesterolemia, atherosclerosis, hypertension, diabetes, and heart failure.

Importantly, nitric oxide (NO) reacts with the superoxide anion at an extremely rapid rate. Therefore, the balance between the ambient levels of the superoxide anion and cellular antioxidant capacity, especially, superoxide dismutase (SOD) importantly regulate the bioactivity of nitric oxide. In the vessel wall, a major isoform of SOD is the extracellular superoxide dismutase (ecSOD). Because of its location, ecSOD has been hailed as the principal regulator of endothelium-derived nitric oxide bioactivity. In this review, we will focus on the importance of a balance among ecSOD, superoxide anion, nitric oxide and on the role of ecSOD in cardiovascular diseases. We will not discuss other mechanisms for altering NO bioactivity and the role of other antioxidants in cardiovascular disease, since there are numerous reviews.

2. Oxygen-derived radicals and their role in vascular disease

In the process of normal cellular metabolism, oxygen undergoes a series of univalent reductions, leading sequentially to the production of $O_2^{\bullet -}$, hydrogen peroxide, and H_2O [1]. Enzymes that may generate reactive oxygen species include components of the mitochondrial electron transport chain, xanthine oxidase, the cytochrome p450 monooxygenases, lipoxygenase, NO synthase, and the NADPH oxidase [2,3]. It has become apparent that a major source of reactive oxygen species in vascular tissues is a membrane-bound NADPH oxidase [3].

Reactive oxygen species are not simply toxic by-products of cellular metabolism, but participate in cellular

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Time for primary review 28 days.

0008-6363/02/$ – see front matter
PII: S0008-6363(02)00328-0

signaling and cell function. Hypertrophy and growth of vascular smooth muscle is stimulated by hydrogen peroxide, which is dependent on activation of c-fos and c-jun expression [4]. Furthermore, reactive oxygen species have been implicated as important in Ras-mediated signaling [5]. Likewise, activation of NF-κB, via dissociation of IκB from the heterodimeric p50p65 complex, is stimulated by reactive oxygen species [6]. This phenomenon seems to play a critical role in initiating expression of proinflammatory molecules such as VCAM-1 and MCP-1, early steps in atherogenesis [7].

A particularly important pathophysiological event related to reactive oxygen species is oxidation of lipids, and in particular low-density lipoprotein (LDL) [8], a process that is central to atherosclerotic lesion formation.

3. Superoxide anion and nitric oxide (Fig. 1)

Nitric oxide (NO) is produced by a variety of mammalian cells including the endothelium, neurons, macrophages, and VSMC from L-arginine via the enzyme family known as NO synthase (NOS) [9]. Release of NO stimulates soluble guanylyl cyclase, leading to an increase of intracellular cyclic guanosine monophosphate (cGMP) [10]. NO dilates blood vessels and inhibits proliferation of VSMC and platelet aggregation [10]. These properties are anti-atherogenic and loss of NO bioactivity is believed be an important feature in the early atherogenic process.

During the past several years, it has become clear that several common pathophysiological conditions, including hypercholesterolemia, hypertension, diabetes and cigarette smoking are associated with an increase in vascular production of $O_2^{\bullet-}$. This increased production of $O_2^{\bullet-}$ markedly influences the bioactivity of $NO^{\bullet}$ produced by the endothelium, and thus can have major effects on vascular tone. Because $O_2^{\bullet-}$ and $NO^{\bullet}$ are both radicals, and contain unpaired electrons in their outer orbitals, they undergo an extremely rapid, diffusion limited radical/radical reaction, leading to the formation of nitrite, nitrate, and very importantly, the peroxynitrite anion [11]. This reaction occurs at a rate of 6.7×10^{9} M^{-1} s^{-1}. This rate is approximately three-times faster than the reaction between $O_2^{\bullet-}$ and the superoxide dismutases, and 10,000 times faster than reactions between $O_2^{\bullet-}$ and the common antioxidant enzymes such as vitamin A, E and C [12]. The rapidity of the reactions between $O_2^{\bullet-}$ and $NO^{\bullet}$, and between $O_2^{\bullet-}$ and the superoxide dismutases, would suggest that in compartments where these three entities coexist, there could be interactions such that alterations in the amounts of either $O_2^{\bullet-}$ or superoxide dismutase could markedly alter levels of $NO^{\bullet}$.

The reaction between $O_2^{\bullet-}$ and $NO^{\bullet}$ not only results in loss of NO bioactivity, but in formation of the peroxynitrite anion ($ONOO^{-}$) [13–15]. Peroxynitrite, a potent oxidant, is likely involved in numerous pathophysiological processes. At physiological pH, peroxynitrite is protonated to form peroxynitrous acid, which can yield nitrogen dioxide and a hydroxyl-like radical [16]. Nitrogen dioxide is highly reactive and capable of participating in nitrosating reactions. In the vessel wall, peroxynitrite and peroxynitrous acid may contribute to lipid peroxidation and membrane damage [17]. In the past, it was unclear how scavenging of $O_2^{\bullet-}$ by superoxide dismutase could be beneficial in biological experiments, because the product of this reaction was hydrogen peroxide, a more potent oxidant than $O_2^{\bullet-}$. In fact, superoxide is a more potent reducing agent than an oxidizing agent [18]. One currently accepted explanation for this paradox is that superoxide dismutase prevents the formation of peroxynitrite, which is potentially a much more offending oxidizing agent than hydrogen peroxide [19].

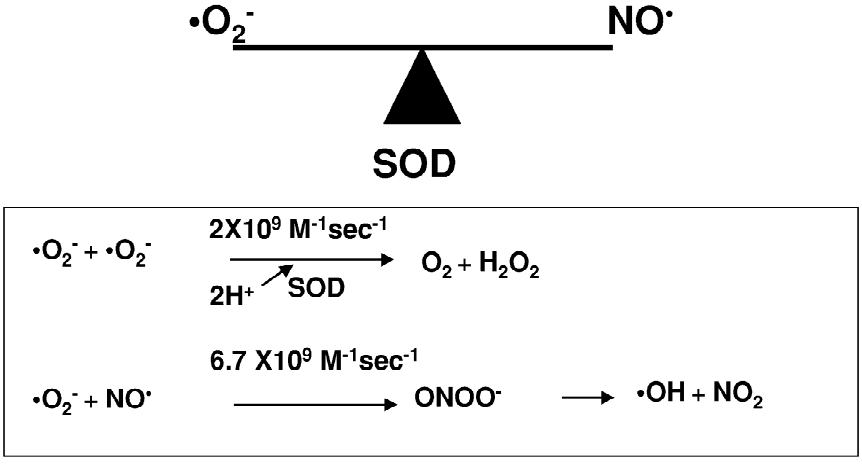

Fig. 1. Balance between $O_2^{\bullet-}$ and $NO^{\bullet}$: the role of SOD. Because $O_2^{\bullet-}$ and $NO^{\bullet}$ are both radicals, and contain unpaired electrons in their outer orbitals, they undergo an extremely rapid, diffusion limited radical/radical reaction, leading to the formation of nitrite, nitrate, and very importantly, the peroxynitrite anion. This reaction occurs at a rate of 6.7×10^{9} M^{-1} s^{-1}. This rate is approximately three-times faster than the reaction between $O_2^{\bullet-}$ and the superoxide dismutases.

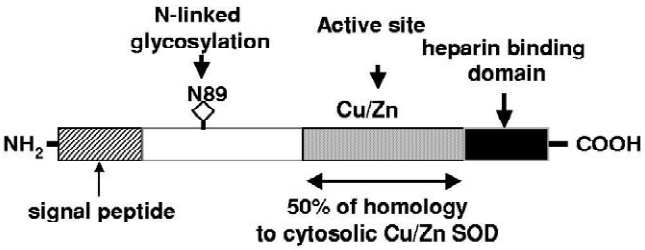

Fig. 2. EcSOD protein structure. ecSOD protein is composed of four domains. Firstly, an amino-terminal signal peptide permits secretion from the cell. Secondly, an N-linked glycosylation site at Asn-89 is useful in the separation of ecSOD from cytosolic Cu/ZnSOD and greatly increases the solubility of the protein. Thirdly, the domain containing active site (amino acid residues 96–193) shows about 50% homology to Cu/ZnSOD. All the ligands to Cu and Zn and the arginine in the entrance to the active site in Cu/ZnSOD can be identified in this domain of ecSOD. Finally, a C-terminal region corresponding to heparin-binding domain has a cluster of positively charged residues. This region is critical for binding to heparin sulfate glycosaminoglycans.

4. Extracellular superoxide dismutases (ecSOD)

All mammalian tissues contain three forms of superoxide dismutase [20]: Cu/ZnSOD (SOD1), MnSOD (SOD2) and extracellular superoxide dismutase (ecSOD or SOD3). What differs among these isoforms is the location: Cu/ZnSOD is localized in cytosol, MnSOD in mitochondria, and ecSOD in extracellular space. Importantly, ecSOD activity is ~10-fold higher in the vessel wall than in other tissues, where Cu/ZnSOD and MnSOD constitute the majority of SOD activity [21,22]. Thus, in the vessel wall, ecSOD will play a critical role in regulating the vascular redox state in the extracellular space.

The ecSOD is a secretory extracellular Cu/Zn-containing SOD discovered by Marklund et al. [23], and it is present on the surface of many cells. It has recently been demonstrated that the ecSOD is a very important component of the total superoxide dismutase in the vessel wall, comprising one-third to one-half of the total vascular SOD activity [21]. The predominant site of production of ecSOD is the smooth muscle cell in healthy vessels [21]. Studies from other laboratories, and our own, indicate that endothelial cells do not produce ecSOD [21,24]. Despite the fact that the ecSOD is predominantly made by vascular smooth muscle rather than endothelial cells, it binds to the heparan sulfates on the endothelial cell surface and can be internalized by endothelial cells [25]. Thus, this potent antioxidant enzyme can be made by vascular smooth muscle cells and end up inside adjacent endothelial cells.

In most species, ecSOD is a tetramer composed of two disulfide-linked dimers [26]. Each subunit has a molecular weight of ~34,000 and is composed of an amino-terminal signal peptide which permits secretion from the cell, an active domain which binds copper and zinc, and a carboxy-terminal region which is involved in binding to sulfated proteoglycat (Fig. 2). In vivo, both circulating (type A) and tissue bound ecSOD (type C) are present, with the tissue bound being ~99% of the total ecSOD (Fig. 3) [27]. Nonproteolysed subunits are classified as type C subunits,

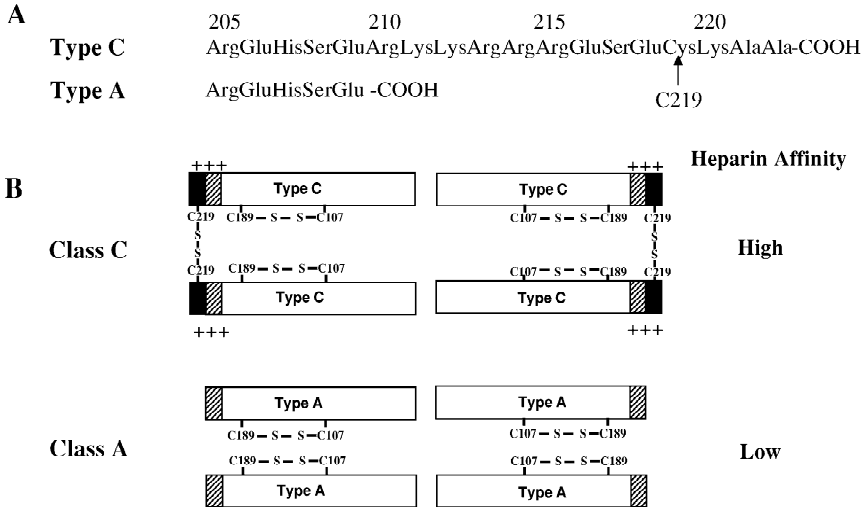

Fig. 3. Heparin binding affinity patterns of ecSOD in vivo. (A) The sequence of the heparin-binding domain is present in the full-length type C subunit and confers the heparin-binding affinity to the protein. The truncated type A subunit has no affinity to heparin. Proteolytic processing leading to the appearance of truncated ecSOD in vivo can occur both intracellularly and in the extracellular space [51]. (B) The subunit compositions of the two classes of ecSOD and their relative affinities to heparin are shown. In most species, ecSOD exists as a tetramer composed of two disulfide-linked dimers. Two of these dimers are held together noncovalently. In vivo, both circulating (class A) and tissue bound (class C) are present, with the tissue bound (class C) being ~99% of the total ecSOD. Class B (not shown in the figure) of ecSOD reveals partial C-terminal truncation and medium heparin affinity. Class C of ecSOD consists of all four C-terminal intact subunits (type C) that contain two disulfide bonds linking two pairs of heparin-binding domains together. In contrast, class A of ecSOD consists of all four C-terminal truncated subunits (type A) that do not contain a disulfide bond to link two heparin-binding domains. Truncation of the C-terminal region does not affect the nonconvalent protein–protein interactions stabilizing the tetramer, but affects the heparin binding properties significantly.

whereas the proteolysed subunits are classified as type A subunits (Fig. 3) [22]. A small portion of the ecSOD bound to the extracellular matrix can be displaced with heparin, increasing circulating levels of the enzyme.

Several mutations of the ecSOD have been described. In the rat, there is a single substitution of valine by aspartate at position 24, which results in the enzyme being a dimer and markedly decreases its affinity for heparan sulfate [28]. Thus, rat ecSOD does not bind to the vessel and these animals lack vascular ecSOD.

Because of its location, ecSOD may play a critical role in preventing destruction of $NO^{\bullet}$ released from the endothelium. Mice made deficient in the ecSOD display enhanced lung injury in response to hyperoxia [29]. It has been shown that ecSOD added to vessels can prevent the impairment of 'EDRF' activity in isolated vessels caused by $O_2^{\bullet -}$ released by pyrogallol [30]. Likewise, human recombinant ecSOD, when administered exogenously, can reduce infarct size in pigs [31]. A chimeric Cu/ZnSOD to which a heparin binding-domain has been added to form a mimic of ecSOD reduces blood pressure in rats made hypertensive by angiotensin II [32].

Characterization of the human ecSOD gene has revealed several potential transcriptional regulatory sites [33]. They include a cAMP-responsive element (nucleotides −438), an AP-1 binding site (nucleotides −397), a glucocorticoid response element (nucleotides −189), a xenobiotic response element (nucleotides +26), and antioxidant response elements (nucleotides +93 and +4464). The presence of these possible regulatory elements suggests that ecSOD expression may be highly regulated by multiple stimuli. Indeed, in cultured fibroblasts, ecSOD expression is regulated by cytokines, such that gamma interferon increases and TGF-beta decreases its expression [34]. Furthermore, as discussed below, we have found that ecSOD expression is substantially regulated in atherosclerosis [35], hypertension [36] and importantly by nitric oxide [37]. Thus, it appears that expression of ecSOD is highly regulated by multiple stimuli, and these stimuli may therefore markedly alter antioxidant defense status in the vessel wall.

5. ecSOD and nitric oxide

Immunohistochemical studies have shown that vascular ecSOD is localized in high concentrations between the endothelium and the smooth muscle, where endothelium-derived NO must transverse to stimulate smooth muscle relaxation [22]. Thus, ecSOD potentially plays an important role in protecting NO from superoxide anion in the extracellular space. Interestingly, we recently found that exogenous NO upregulates ecSOD expression in human aortic smooth muscle cells [37].

To gain further insight into the role of endogenous NO in ecSOD expression in vivo, we studied both normal and eNOS-deficient mice [37]. At baseline, the eNOS-deficient mice demonstrated a markedly depressed expression of ecSOD, which was unlikely due to an artifact caused by genetic manipulation, as it was observed in two separate strains of eNOS-deficient mice, produced by deletion of different eNOS exons. These findings strongly suggest that endothelium-derived NO modulates ecSOD expression in vivo.

Numerous conditions, including atherosclerosis, aging, cigarette smoking, and diabetes are associated with a decline in the production and/or biological activity of endothelium derived NO [2]. In these conditions, the loss of NO may lead to a decline in ecSOD expression, as we observed in eNOS-deficient mice. Indeed, in apo(E)-deficient mice, we have previously found that the vascular smooth muscle cell expression of ecSOD declines as the severity of atherosclerosis increases (Fig. 4) [35]. Landmesser et al. have recently shown that in patients with coronary artery disease, activity of endothelium-bound ecSOD, released by heparin bolus injection was positively correlated with flow-dependent, endothelium-mediated dilation (Fig. 5) [38]. It is interesting to speculate that other conditions associated with a long-term loss of NO would also decrease expression of ecSOD, adversely impacting the vascular redox state.

Physical exercise has been associated with a reduction in cardiovascular morbidity and mortality [39,40]. One mechanism that may underlie this beneficial effect involves an upregulation of the endothelial NO synthase (eNOS), increasing local production of NO [41]. Paradoxically, exercise also increases total body oxygen uptake, increasing production of reactive oxygen species [42], increasing the susceptibility of plasma LDL to oxidation [43] and increasing conjugated diene formation [44]. Although the manner in which the vasculature adapts to this oxidant stress remains unclear, we hypothesized that exercise would increase not only eNOS, but also ecSOD expression through the mechanisms dependent on endothelium-derived nitric oxide, since endogenous NO modulates ecSOD expression. To test this, we further examined the effect of exercise training on modulating vascular ecSOD (Fig. 6). Three weeks of treadmill exercise training dramatically increased eNOS protein level by about threefold in aortas of control mice [37]. Interestingly, in parallel with the increase in eNOS expression, we found that exercise training also increased ecSOD expression by about threefold. Importantly, this upregulation of ecSOD expression was not observed in aortas from eNOS-deficient mice, proving that NO was the stimulus for increased ecSOD expression as a result of exercise training. An increase in expression of ecSOD would represent an important physiological adaptation that would counteract the increase in oxidative stress in response to exercise training. Thus, the beneficial effect of exercise training on endothelium-dependent vasodilatation observed in several studies [45–47] may not only be due to an increase in expression of eNOS,

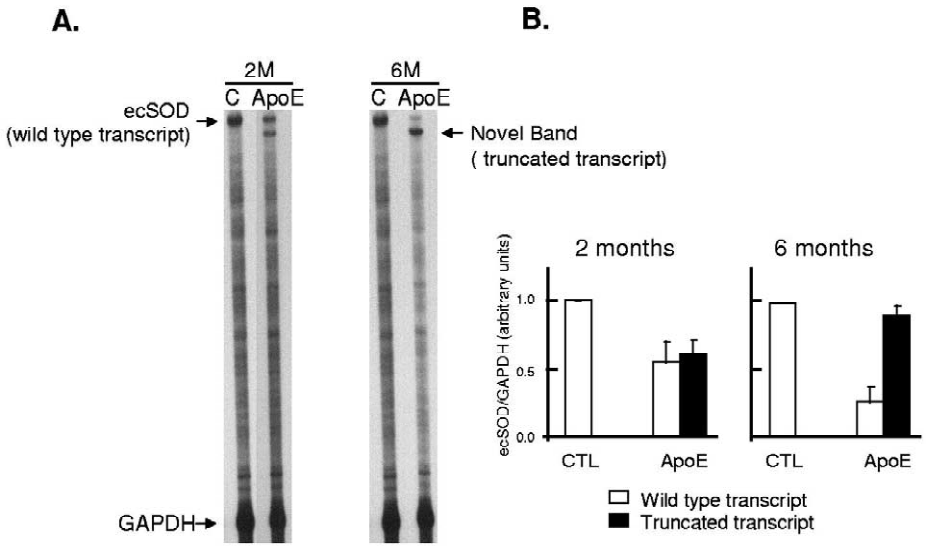

Fig. 4. Panel A, expression of ecSOD mRNA in aortas of control and apo(E)-deficient mice at 2 and 6 months of age. For each of these experiments, the control mice were of the same age as the apo(E)-deficient mice. RNase protection assay was performed using a riboprobe for mouse ecSOD and a riboprobe for GAPDH as an internal control for loading. Wild type transcript was derived from vascular smooth muscle cell, while truncated transcript was derived from lipid-laden macrophages. Of note, as apo-E-deficient mice aged, atherosclerosis progressed and macrophages accumulated in vessels of those. The expression of the truncated transcript of ecSOD by lipid-laden macrophages increased, while the expression of wild type ecSOD transcript by vascular smooth muscle cell declined. Panel B, densitometric analysis of ecSOD mRNA in 2 and 6-month-old mice for the wild type (open bars) and truncated ecSOD mRNA (closed bars). Data are $n=4$ for the 2-month-old mice and $n=9$ for the 6-month-old mice.

but also due to an increase in ecSOD expression, which serves to increase ambient levels of NO by decreasing its reaction with $O_2^{\bullet-}$ (Fig. 7). It remains to be determined whether a similar regulation of ecSOD expression could be obtained in vivo by various other stimulants such as oxidized low-density lipoprotein, statins, and hypoxia which regulate eNOS expression [48]. These responses may have an important implication in the pathogenesis of cardiovascular disease.

6. ecSOD and atherosclerosis

We and others have demonstrated that enzymatic activity and protein expression of ecSOD was markedly increased in macrophage-rich atherosclerotic lesion [35,49]. Indeed, immunohistochemical studies showed that ecSOD staining colocalized with lipid-laden macrophages in atherosclerotic vessels of apo(E)-deficient mice. Interestingly, in aortas from apo(E)-deficient mice, this was

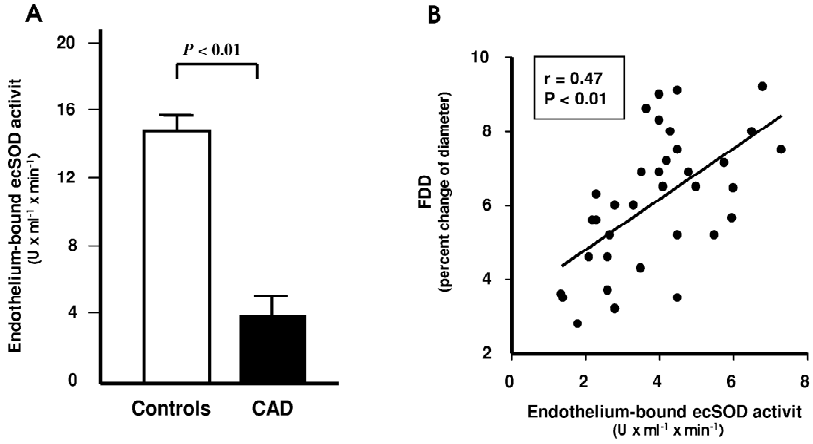

Fig. 5. (A) Endothelium-bound ecSOD activity in patients with coronary artery disease (CAD) ($n=35$) and age-matched control subjects ($n=15$). Endothelium-bound ecSOD activity was reduced in patients with CAD. Endothelium-bound ecSOD activity was calculated as the increase of plasma SOD activity by heparin bolus injection [38]. (B) Relation between endothelium-bound ecSOD activity and flow-dependent dilation (FDD) in 35 patients with CAD. Endothelium-bound ecSOD activity was positively correlated with FDD in patients with CAD. The FDD was measured, as previously described [45–47].

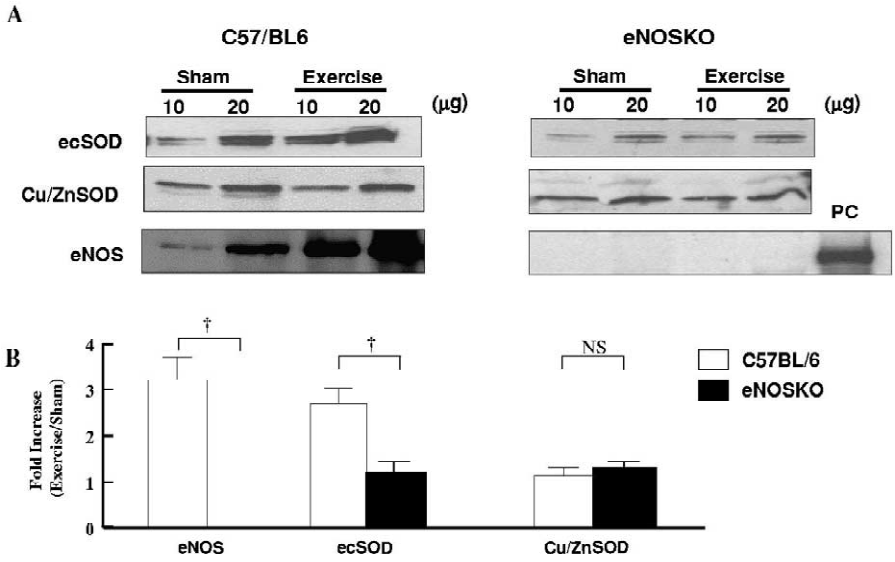

Fig. 6. Effect of 3 weeks exercise training on ecSOD, Cu/ZnSOD, ecNOS protein expression in aortas of C57BL/6 mice and eNOS-deficient mice. Panel A, Western analysis of ecSOD protein, Cu/ZnSOD protein, and eNOS protein in aortas of C57BL/6 and eNOS-deficient mice. Both untrained (sham) mice and mice exposed to 3 weeks exercise training were studied. Twenty μg of mouse aorta from C57BL/6 was used as a positive control (PC). Panel B, densitometric analysis of panel A. Data are mean±S.E.M. for $n=6$ (C57BL/6), $n=3$ (eNOS-deficient mice (strain A)). The data from eNOS deficient mice (strain B) ($n=3$) showed similar results. $^{\dagger}P<0.01$ versus control mice.

associated with the appearance of a novel, truncated ecSOD transcript, as determined by RNase protection assay (Fig. 4). Studies of isolated macrophages suggested that this novel form of ecSOD was a product of lipid-laden, but not normal macrophages. Consistent with this, as atherosclerosis progressed and macrophages accumulated in vessels from apo(E)-deficient mice, expression of the novel form of ecSOD derived from lipid-laden macrophages increased, while the wild type ecSOD expression derived from vascular smooth muscle cell declined (Fig. 4). The decrease in wild type ecSOD expression in atherosclerotic vessels from apo(E)-deficient mice is consistent with the decrease in ecSOD activity in connective tissue-rich human atherosclerotic lesions [38,49]. Thus, in atherosclerosis, ecSOD expression is increased in lipid-laden macrophages, whereas it is decreased in vascular smooth muscle cells.

There are several potential mechanisms by which ecSOD expression is regulated in atherosclerotic vessels. Firstly, ecSOD expression is regulated by nitric oxide bioactivity, as mentioned in the previous section [37,38]. Secondly, the expression level and distribution of ecSOD

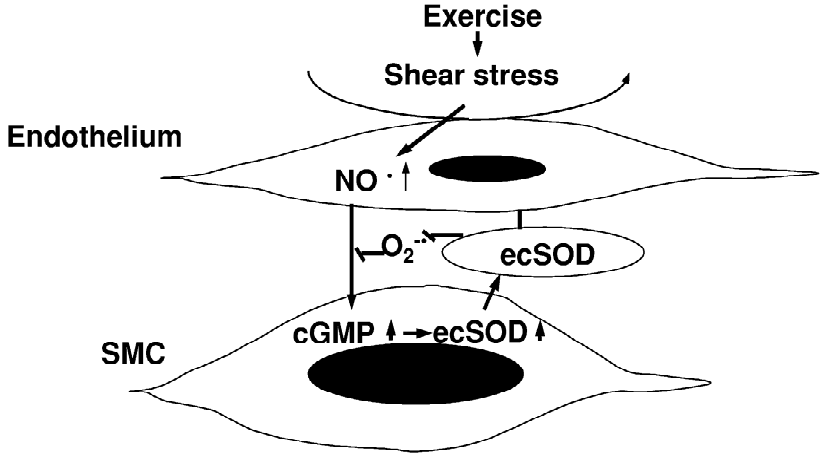

Fig. 7. Feed forward mechanism of nitric oxide bioactivity by ecSOD. Exercise increases shear stress, which results in increase in NO derived from eNOS. NO released from the endothelium acts in a paracrine fashion to stimulate ecSOD release from the vascular smooth muscle. This ecSOD is then secreted and adheres to extracellular matrix and endothelial surface. In this space, the ecSOD can protect NO from degradation by superoxide generated extracellularly. This phenomenon would represent an important feed-forward mechanism allowing preservation of NO and importantly modulating the extracellular redox state.

is regulated by proteolytic removal of heparin binding domain. Adachi et al. [50] reported that the level of low heparin affinity forms of ecSOD was increased in atherosclerotic patients, whereas that of high heparin affinity form of ecSOD was decreased. It has also been shown that proteolytic processing leading to the appearance of truncated ecSOD can occur both intracellularly and in the extracellular space [51]. In this context, it is interesting to note that the coronary artery concentration of heparan sulfate, the physiological ligand for ecSOD, tends to decrease with the atherosclerotic lesions, whereas the content of chondroitin sulfate increases [52]. Thirdly, ecSOD activity could be modulated by hydrogen peroxide produced by lipid-laden macrophages due to its peroxidase activity, because lipid laden macrophages produce a large amount of both superoxide anion and ecSOD, resulting in the accumulation of hydrogen peroxide. This peroxidase-like activity of Cu/ZnSODs has been shown to inactivate the enzyme [53]. Fourthly, ecSOD expression is regulated by inflammatory cytokines, including TNFα, INFγ, and IL-4 in the vascular smooth muscle cells, such that TNFα decreases ecSOD expression, while interferon γ and interleukin 4 increase its expression [54]. Finally, ecSOD expression is regulated by homocysteine, an independent risk factor for atherosclerosis [55,55a]. Homocysteine decreased ecSOD expression and the binding of ecSOD to vascular endothelial surface, resulting in a loss of the ability to protect endothelial surfaces from oxidative stress. Thus, in atherosclerotic vessels, ecSOD seems to be regulated in multifaceted ways.

The functional significance of ecSOD activity in the development of atherosclerosis remains unclear. Recently, Sentman et al. observed that genetic deletion of ecSOD paradoxically caused a slight decrease in atherosclerotic lesions in apo(E)-deficient mice after 1-month atherogenic diet, while having no effect on the amount of atherosclerosis in these animals after 3 months on the atherogenic diet or after 8 months on standard chow [56]. The authors concluded that ecSOD may enhance or have little effect on the development of atherosclerotic lesions. Care must be taken in interpretating studies of mice with a life-long deficiency in an enzyme, because multiple adaptations may occur. For example, in the case of the ecSOD-deficient mouse, there may be downregulation of enzymes that produce $O_2^{\bullet -}$ or upregulation of other antioxidant defenses.

7. ecSOD, angiotensin II, and hypertension

Angiotensin II has been shown to activate a membrane-bound NADPH oxidase, the major source of reactive oxygen species in blood vessels [57]. This contributes to impaired EDRF/NO-dependent relaxation [58], and the hypertension caused by angiotensin II is ameliorated by treatment with membrane-targeted forms of SOD [32]. These findings suggest that in hypertension associated with activation of the renin–angiotensin II system, SOD expression and activity may play a critical role in modulating hemodynamics, vascular responsiveness and vascular redox state. Indeed, a 7-day infusion of angiotensin II increased ecSOD expression, but not expression of the cytosolic Cu/ZnSOD, in a dose-dependent fashion [36] (Fig. 8). This was associated with an increase in ecSOD activity. In contrast, ecSOD protein expression was not changed in aortas of mice made hypertensive by norepinephrine, which increased blood pressure to a similar extent as angiotensin II. Losartan, a specific antagonist of the AT1 receptor, completely abrogated the effect of angiotensin II on expression of ecSOD (Fig. 8). In addition, this effect of angiotensin II could be mimicked in vessels placed in culture (organoid cultures) and human vascular smooth muscle cells [36,59]. These findings suggest that increased ecSOD expression in angiotensin II-induced hypertension was not due to the direct effect of hypertension, but likely due to the direct effect of angiotensin II through activation of AT_1 receptor.

We also found that this effect of angiotensin II on

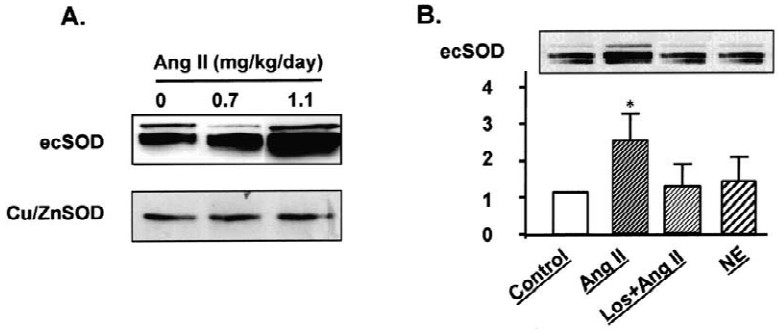

Fig. 8. Panel A, Western blots comparing the effect of angiotensin II on expression of Cu/ZnSOD and ecSOD in vivo. Mouse aortas were harvested following a 7-day infusion of angiotensin II (1.1 mg/kg/day×7 days), homogenized and subjected to Western analysis using antibodies specific to either the cytosolic Cu/ZnSOD or the murine ecSOD. Data are representative of three separate experiments. Panel B, ecSOD expression in control mice, mice treated with either angiotensin II, angiotensin II and Losartan, or norepinephrine. Upper panel shows a representative Western blot. Lower panel depicts mean densitometric data for three separate experiments. *$P<0.05$ compared with sham-operated mice.

ecSOD expression was not due to oxidative stress induced by angiotensin II, because neither DPI, a flavoprotein inhibitor which blocks NADH/NADPH oxidase activity, nor tiron, a superoxide scavenger, affected the response to angiotensin II. In contrast, the MAP kinase kinase inhibitor PD98059, completely prevented the effect of angiotensin II on ecSOD expression. Furthermore, this effect of angiotensin II could be observed in human aortic smooth muscle cells and was due not only to an increase in ecSOD transcriptional rate, but also due to a prolongation of the half-life of ecSOD mRNA.

There are several potential implications for the increase in ecSOD activity in angiotensin II-induced hypertension. Firstly, increased ecSOD expression may represent an important adaptive mechanism that allows increased scavenging of $O_2^{\bullet -}$ in hypertension in which superoxide production is increased. Secondly, as discussed below, there are several known polymorphisms of ecSOD in humans [60,61]. While none of these are associated with an obvious phenotype, it is unclear how they may respond to hypertension and activation of the renin–angiotensin system. Thus, ecSOD may play a critical role in modulating the oxidative state of vessel wall in pathological processes in which the renin–angiotensin II is activated.

8. ecSOD and ischemia–reperfusion injury

Numerous studies have implicated reactive oxygen and nitrogen species (ROS and RNS) as playing important roles in the pathogenesis of ischemia–reperfusion injury (for review see Ref. [62]). In a tissue undergoing ischemia–reperfusion, there remain a number of unanswered questions. Which cell type or types are generating the enhanced production of ROS/RNS? Which cell type(s) are sensitive to the resulting oxidative/nitrosative stress? How do the tissue and/or cells deal with the enhanced production of these reactive species? Tissues that undergo ischemia–reperfusion injury contain multiple antioxidant systems, including both enzymatic and nonenzymatic antioxidants and intracellular and extracellular antioxidants. Enzymatic antioxidants include superoxide dismutase, catalase, and glutathione peroxidase, whereas nonenzymatic antioxidants include vitamin C and vitamin E. It is now becoming clear that these endogenous antioxidants play significant roles in preserving function following ischemia–reperfusion [62].

Two of the more extensively studied models of ischemia–reperfusion injury involve analysis of myocardial function and myocardial infarct size. Prior studies have investigated the effect of endogenous antioxidants such as SOD, catalase, glutathione peroxidase, and vitamin E on myocardial function and infarct size (for review see Ref. [62]). Variable protective effects have been observed with administration of exogenous Cu/ZnSOD (SOD1), leading to the hypothesis that the efficacy of Cu/ZnSOD treatment may be limited by the inability to access intracellular region or its short plasma half-lives. Interestingly, ecSOD has much longer half-life than that of Cu/ZnSOD: the half-life of Cu/ZnSOD in circulation is about 7 min, whereas that of ecSOD is about 20 h [63]. Furthermore, ecSOD is concentrated in the interstitial space due to its heparin-binding region, whereas exogenous Cu/ZnSOD is freely distributed across the extracellular space. Of note, careful examination of the distribution kinetics of Cu/ZnSOD indicates that the interstitial levels of this enzyme rather than the plasma levels are primarily responsible for protection against myocardial ischemia–reperfusion injury [64]. Several studies have now demonstrated beneficial effects of ecSOD in preserving cardiac function and in limiting myocardial infarct size following ischemia–reperfusion. Initial studies utilized recombinant human ecSOD to demonstrate preserved cardiac function following ischemia–reperfusion in isolated rat hearts [65–67]. Subsequently, recombinant ecSOD was also shown to reduce the infarct size when given just prior to coronary reperfusion in pigs [31]. We have demonstrated that overexpressing human ecSOD in the heart of transgenic mice greatly improves preservation of myocardial function after global normothermic ischemia–reperfusion [68,69]. More recently, Li et al. have utilized myocardial gene transfer techniques for increasing ecSOD levels in rabbits and have demonstrated both protection against myocardial stunning [70] and reduction in infarct size [71] following ischemia–reperfusion. Thus, a variety of approaches have shown that ecSOD reduces cardiac ischemia–reperfusion injury. Whether similar protection will be afforded in other tissue models of ischemia–reperfusion injury remains to be determined.

9. ecSOD and polymorphisms

In humans, identification of individuals with elevations in their serum ecSOD level [72] led to the discovery of a single base pair mutation resulting in an Arg to Gly substitution at position 213 [61,73]. This R213G polymorphism, which occurs in 2.2–6% of the population [74], results in an 8–10-fold increase in serum levels in the heterozygous state and up to a 30-fold increase in homozygous persons [73]. Whereas the specific pathologic and/or protective effects, if any, of this mutation are not known, biochemical studies demonstrate the R213G mutation to have reduced susceptibility to trypsin-like proteinases [72] and impaired binding to endothelial cell surfaces [75]. However, some recent data suggests that diabetic patients on hemodialysis who have the R213G polymorphism demonstrate increased risk for ischemic cardiovascular and cerebrovascular disease [76]. Two additional polymorphisms in ecSOD have been described. One is an AB>G substitution resulting in an amino acid change from Thr to Ala at position 40 (T40A). A second is

a silent CBT substitution at cDNA position 280. Neither of these has been linked to any pathologic condition [60].

10. Future directions

Recently, Schachinger et al. showed that impaired endothelium-dependent vasodilation in the coronary circulation of humans has profound prognostic implications in that it predicts adverse cardiovascular events and long-term outcome [77]. One of the major mechanisms for loss of NO bioactivity involves a rapid reaction with the superoxide anion. Accordingly, the concept of antioxidant therapies, i.e. reinforcement of endogenous antioxidant defenses to protect more effectively against oxidative stress, is of substantial interest.

A large number of studies have demonstrated that a variety of antioxidants, including enzymatic and nonenzymatic antioxidants, improved the bioactivity of NO and/or reduced the extent of atherosclerosis (see review in Refs. [8,78,79]). Furthermore, abundant data from epidemiological observational studies have shown an inverse association between antioxidant intake or body status and the risk of cardiovascular diseases [8,79]. By contrast, recent clinical trials, such as the recent GISSI and HOPE trials, have found no benefits of vitamin E supplementation on cardiovascular disease risk [8,79]. Although increased vascular levels of ecSOD would be 10,000 times more potent than administration of commonly employed antioxidant vitamins in scavenging superoxide anion in the aqueous milieu, elevated tissue levels of ecSOD may enhance extracellular levels of hydrogen peroxide, which may have proatherogenic properties. Thus, it is clear that additional studies of ecSOD and other vascular antioxidant defense systems are needed.

In the case of ecSOD, the analysis of its enzymatic properties, such as possible peroxidase activity is needed. Studies of mice with ecSOD conditionally 'knocked-out' will be very revealing. Studies of the role of ecSOD in vascular diseases other than atherosclerosis, such as diabetes will be important to pursue. Overall, understanding the role of ecSOD in the tenuous balance between the superoxide anion and NO will continue to provide increasing insight into the vascular redox state as a major regulator for cardiovascular disease.

Acknowledgements

This work was supported by the American Heart Association Scientist Development Grant award (0030180N), NIH RO-1 HL39006, NIH RO-1 HL59248, Program Project Grant HL5800, and a VA Merit Grant. We thank Dr. Masuko Ushio-Fukai for helpful discussion and support.

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