Fibulin-5 Is a Novel Binding Protein for Extracellular Superoxide Dismutase

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**Abstract**—The extracellular superoxide dismutase (ecSOD) plays an important role in atherosclerosis and endothelial function by modulating levels of the superoxide anion (O$_2^-$) in the extracellular space. Although heparan sulfate proteoglycan is an important ligand for ecSOD, little is known about other biological binding partners of ecSOD. The goal of this study was to identify novel proteins that interact with ecSOD. A yeast two-hybrid screening of a human aorta cDNA library using ecSOD as bait identified fibulin-5 as a predominant binding protein for ecSOD. Further analysis showed that the binding domain of ecSOD within fibulin-5 mapped to its C-terminal domain. In vitro pulldown assays and coimmunoprecipitation analysis further confirmed that ecSOD interacts with fibulin-5 in vitro and in vivo. Studies using fibulin-5-/- mice indicated that fibulin-5 is required for binding of ecSOD to vascular tissue. Importantly, the decrease in tissue-bound ecSOD levels in aortas from fibulin-5-/- mice was associated with an increase in vascular O$_2^-$ levels. Furthermore, immunohistochemical analysis using ApoE-/- mice suggested a codistribution of ecSOD and fibulin-5 in atherosclerotic vessels. In summary, we provide in this study the first evidence that the ecSOD-fibulin-5 interaction is required for ecSOD binding to vascular tissues, thereby regulating vascular O$_2^-$ levels. This interaction may represent a novel mechanism for controlling vascular redox state in the extracellular space in various cardiovascular diseases such as atherosclerosis and hypertension in which oxidative stress is increased. *(Circ Res. 2004;95:1067-1074.)*

**Key Words:** superoxide dismutase ■ fibulin-5 ■ extracellular matrix ■ atherosclerosis ■ superoxide

Vascular production of the superoxide anion (O$_2^-$) is increased in many common cardiovascular diseases including atherosclerosis, hypercholesterolemia, hypertension, ischemic heart disease, diabetic cardiomyopathy, and heart failure.1 One of the major cellular defenses against O$_2^-$ and formation of peroxynitrite is the superoxide dismutases (SODs).2 In mammalian tissue, three isoforms of superoxide dismutase have been identified: Cu/ZnSOD, MnSOD, and extracellular superoxide dismutase (ecSOD). These isozymes differ in their location: Cu/ZnSOD is localized in the cytosol, MnSOD in the mitochondria, and ecSOD in the extracellular space. In the vessel wall, one-third to one-half of the total vascular SOD is ecSOD.3 In healthy vessels, ecSOD is produced predominately by vascular smooth muscle cells, but in atherosclerotic vessels, ecSOD is also generated by lipid-laden macrophages.4,5 Because of its extracellular location, ecSOD plays an important role in modulating nitric oxide bioactivity by protecting nitric oxide from O$_2^-$ in the vascular extracellular space, especially in pathological states, such as atherosclerosis and hypertension where O$_2^-$ is increased.2,6

The ecSOD is a secretory tetrameric glycoprotein with a heparin-binding domain.7 The protein is composed of an N-terminal signal peptide, which permits secretion from cell, an N-linked glycosylation site at Asn-89, which contributes to the solubility of the enzyme, an active site that binds copper and zinc, and a C-terminal region that corresponds to a heparin-binding domain.8 Approximately 99% of the total ecSOD is tissue-bound, whereas a small proportion circulates in the blood.8 Heparan sulfate proteoglycan is a well-known ligand for ecSOD on cell surfaces and in the extracellular matrix.9 However, only a small portion of the tissue-bound ecSOD is displaced by heparin injection,8 suggesting that other ligands for ecSOD may exist.7

The major goal of this study was to identify novel proteins that interact with ecSOD. Using the yeast two-hybrid system, we discovered that fibulin-5 is an important biological ligand for ecSOD. Fibulin-5 is also known by the acronym EVEC (Embryonic Vascular EGF-like repeat Containing protein)10 and by the acronym DANCE (Developmental Arteries and Neural Crest EGF-like).11 We further confirmed the interaction between ecSOD and fibulin-5 using in vitro pulldown assays and coimmunoprecipitation assays in mammalian cells as well as in insect cells. Furthermore, we found that ecSOD binds to the C-terminal domain of fibulin-5. Moreover, we...
examined functional significance of the interaction of ecSOD and fibulin-5 using fibulin-5−/− mice, and found that vascular O2− levels are markedly increased in fibulin-5−/− mice in which ecSOD binding to tissue is markedly decreased.

Materials and Methods

Animals Studied

C57BL/6J mice and ApoE−/− mice on a C57BL/6J background were obtained from the Jackson Laboratory (Bar Harbor, Maine). The fibulin-5−/− mice and control littersmates were generated as previously described.13

Yeast Two-Hybrid Library Screening

To identify novel proteins that interact with ecSOD, we screened a human aorta cDNA library using ecSOD as bait and the MATCH-MAKER GAL4 yeast two-hybrid system 3 (Clontech Laboratories Inc).

In Vitro Pulldown Assays and Generation of Recombinant ecSOD and Fibulin-5

For in vitro pulldown assays using recombinant ecSOD and fibulin-5, the human ecSOD and the human fibulin-5 were overexpressed in a Drosophila expression system (Invitrogen Corp). In vitro pulldown assays were performed as previously described.13

Coimmunoprecipitation of ecSOD and Fibulin-5 in Stably Transfected Drosophila Schneider Cells and CHO Cells

The Drosophila Schneider cells and CHO cells stably expressing ecSOD were generated according to the manufacturer’s instructions. Coimmunoprecipitation of ecSOD and fibulin-5 in those cells were performed as previously described.13

Immunohistochemical Studies

Immunohistochemical analysis for ecSOD and fibulin-5 were performed as previously described.4

Western Analysis of ecSOD and Fibulin-5 in Plasma and Aortas From Fibulin-5−/− Mice and Control Littersmates

The protein expression of ecSOD and fibulin-5 in plasma and aortas was determined by Western blotting analysis, as previously described.4

Real-Time PCR

RNA was isolated and amplified as described previously with minor modifications.9 Primer sequences and cycling conditions are listed in expanded Materials and Methods section in the online data supplement available at http://circres.ahajournals.org.

Dihydroethidium Labeling of Aortas From Fibulin-5−/− Mice and Control Littersmates

To measure ROS production in vessels in situ, frozen cross-sections of aortas were stained with dihydroethidium (Molecular Probes Inc) using a previously validated method.14

Materials

All chemicals and reagents were purchased from Sigma Chemical Company, unless otherwise specified.

Data Analysis

All data are expressed as mean±SEM. Comparisons between groups of animals or treatments were made by one-way ANOVA, followed by the Tukey-Kramer post hoc test. Values of P<0.05 were considered statistically significant.

Results

Fibulin-5 as a Novel Binding Protein for ecSOD

The yeast two-hybrid system was used to identify candidate proteins that interact with ecSOD. After an initial screening, 1257 independent clones grew as large colonies on the Trp−/Leu−/His− plates. Of these, 389 clones exhibited β-galactosidase activity, as tested by the filter assay. PCR was performed using the flanking primers specific for pACT2 plasmid to screen inserts ranging between 0.4 and 1.6 kb. Positive clones were sequenced, and DNA homology searches using the NCBI BLAST program identified six different partial clones of fibulin-5 (Δ1 through Δ6) (Figure 1A). Those cDNAs were in frame with the GAL-4 activation domain of the pACT2 plasmid. Importantly, perlecan, one of major heparan sulfate proteoglycan in the vessel wall and an established physiological ligand for ecSOD,8,15−18 was also included in our positive clones from the yeast two-hybrid screening, validating the yeast two-hybrid system in the present study.

To verify fibulin-5 as an ecSOD binding protein, we cloned the full coding sequence of fibulin-5 cDNA into the plasmids carrying either the GAL-4 DNA-binding domain (pGBK7T) or the GAL4 activation domain (pACT2) and adopted them as either prey or bait with ecSOD. As positive and negative controls, we used pGBK7T−53/pGADT7-T and pGBK7T−Lam/pGADT7-T, respectively. Figure 1B shows a robust growth of yeast coexpressing ecSOD and fibulin-5 (pGBK7T-ecSOD/pACT-fibulin-5) in Trp−/Leu−/His− media. Furthermore, yeasts coexpressing ecSOD and fibulin-5 showed a marked production of blue colonies in filter β-galactosidase assays and also showed a marked increase in β-galactosidase activity in liquid β-galactosidase assays (Figure 1B). These findings further confirmed the interaction of ecSOD with fibulin-5.

EcSOD Binds Specifically to the C-Terminal Domain of Fibulin-5

We next determined the ecSOD binding site in fibulin-5. As shown in Figure 1B, yeast coexpressing ecSOD and full-length fibulin-5 or one of its several deletion mutants (Δ1 through Δ6) exhibited a robust growth in Trp−/Leu−/His− media, a marked production of blue colonies in filter β-galactosidase assays, and a marked increase in β-galactosidase activity in liquid β-galactosidase assays. In contrast, the fibulin-5 deletion mutant (Δ7) lacking C-terminal domain (amino acids 320 to 448) showed neither growth nor β-galactosidase activity. These results indicate that ecSOD interacts specifically with the C-terminal domain of fibulin-5 (amino acids 320 to 448).

Interaction of ecSOD and Fibulin-5 in Cell-Free Systems

To determine whether fibulin-5 directly interacts with ecSOD, we performed in vitro pulldown assays using recombinant ecSOD with V5 tag (∼30 kDa) and recombinant fibulin-5 with Myc tag (∼66 kDa) proteins isolated and purified from the media of stably transfected Drosophila Schneider cells. As shown in Figure 2A, Myc-tagged fibulin-5 protein bound to V5-tagged ecSOD protein, but not
transiently transfected with Myc-tagged fibulin-5, ecSOD was coinmunoprecipitated with fibulin-5 in the conditioned media from these cells (Figure 2B and 2C). These data clearly suggest that both ecSOD and fibulin-5 interact in vivo.

Codistribution of ecSOD and Fibulin-5 in Control and Atherosclerotic Vessels
To determine whether ecSOD and fibulin-5 colocalize in intact vessels, we performed immunohistochemical analysis in control and atherosclerotic vessels. In control mouse aorta, ecSOD and fibulin-5 were codistributed in medial layer, whereas in atherosclerotic vessels from ApoE−/− mice, they were codistributed on the endothelial cell surface, in the extracellular matrix, and in the adventitia (Figure 2D and 2E). These results suggest that ecSOD binds to fibulin-5 in vivo in both control and atherosclerotic vessels. Of note, fibulin-5 protein is abundantly expressed in normal adult vessels, whereas fibulin-5 mRNA expression is markedly less in aortas of fibulin-5−/− mice compared with those from ApoE−/− mice (online Figure I).

Increase in Plasma ecSOD Level and Decrease in Tissue-Bound ecSOD Level in Fibulin-5−/− Mice
To examine whether ecSOD binds to vascular tissue through interaction with fibulin-5 in intact vessels, we used fibulin-5−/− mice and control littersmates. Plasma ecSOD levels in fibulin-5−/− mice were significantly increased by 2.4±0.2-fold as compared with levels in control littersmates (Figure 3A). To test whether the increase in plasma ecSOD in fibulin-5−/− mice was caused by a decrease in ecSOD binding to tissue, we next performed immunohistochemical analysis. EcSOD immunostaining was markedly decreased in aortas of fibulin-5−/− mice compared with controls (Figure 3B). Consistent with this, Western analysis demonstrated that protein levels of ecSOD were decreased in aortas from fibulin-5−/− mice (65±5%-fold decrease) compared with those from control littersmates (Figure 3C). In contrast, protein levels of Cu/ZnSOD were not different between aortas from fibulin-5−/− and control mice (Figure 3C).

To exclude the possibility that the decrease in ecSOD protein expression in aortas of fibulin-5−/− mice was caused by a decrease in ecSOD mRNA, we performed real-time PCR. As shown in Figure 4A, ecSOD mRNA levels in aortas from fibulin-5−/− mice and control littersmates did not differ.

It has been shown that heparan sulfate proteoglycan is an important ligand for ecSOD in the extracellular matrix. To eliminate the possibility that the decrease in ecSOD protein expression was caused by a decrease in the amount of heparan sulfate proteoglycan, we performed immunohistochemical analysis of perlecan, one of the major heparan sulfate proteoglycans in the vessel wall. Importantly, immunostaining of perlecan was not altered in aortas from fibulin-5−/− mice compared with aortas from control mice (Figure 4B). Moreover, it has been shown that the C-terminal heparin-binding domain of ecSOD is cleaved during intracellular proteolytic processing. In this study, Western blots consistently revealed a full-length (upper band) and a proteolyzed (lower band) ecSOD in mouse aortas (Figure 3C). However, the ratio of proteolyzed (lacking heparin-binding domain) to

**Figure 1.** Mapping the ecSOD binding site in fibulin-5 by the yeast two-hybrid system. A, Schematic representation of fibulin-5 and its various deletion mutants. Fibulin-5 has been subdivided as indicated at the top. Black boxes represent N-terminal signal sequences, and ovals represent calcium binding EGF-like (cbEGF) domains. An RGD motif is indicated in the first cbEGF domain. Position numbers of the amino acid residues are indicated. B, Semiquantitative assessment of growth in liquid assays (center) and liquid luminescent assays (right) of yeast cells coexpressing ecSOD and full-length fibulin-5 or its several deletion mutants (Δ1 through Δ7). Growth is designated by semiquantitative assessment with maximal growth at ++++. The quantitative values are presented as mean±SD (n=5) and are expressed as relative light units (RLU) normalized on cell content (OD600). pGBK7-T-Lam/pGAD-T and pGBK7-T-S53/pGAD-T were used as negative and positive controls, respectively.

Coimmunoprecipitation of ecSOD and Fibulin-5 in Stably Transfected Cells
To confirm further the interaction of ecSOD and fibulin-5 in vivo, we performed coimmunoprecipitation assays in Droso- phila Schneider 2 (Figure 2B) and CHO cells (Figure 2C) stably expressing ecSOD. These cells have been shown to allow proper glycosylation and secretion of a variety of mammalian proteins. When Droso phila Schneider cells or CHO cells stably expressing V5-tagged ecSOD were
nonproteolyzed ecSOD was not altered in aortas from fibulin-
5/H11002 mice compared with those from control mice (45±9% versus 54±13%, respectively) (Figure 3C). Taken together, these data suggest that the decrease in tissue-bound ecSOD in fibulin-5/H11002 mice is caused by the loss of ecSOD binding to fibulin-5, and not caused by altered posttranslational processing in these animals.

**Increased O$_{2}^{\cdot-}$ Production in Aortas From Fibulin-5$^{-/-}$ Mice**

To determine the functional significance of the decrease in tissue-bound ecSOD in fibulin-5$^{-/-}$ mice, we examined O$_{2}^{\cdot-}$ production in aortas from fibulin-5$^{-/-}$ mice and control littermates using the dihydroethidium (DHE) fluorescence method. DHE (2 µmol/L) is a fluorescent dye that has been shown to specifically detect O$_{2}^{\cdot-}$ in situ.$^{14}$ DHE staining clearly demonstrated that superoxide production was markedly increased in aortas from fibulin-5$^{-/-}$ mice as compared with those from control littermates (Figure 5A and 5B). Importantly, the fluorescence signal was markedly decreased by the addition of SOD, suggesting that DHE staining mainly reflects an increase in O$_{2}^{\cdot-}$. Furthermore, additional experiments using recombinant ecSOD and fibulin-5 proteins showed that fibulin-5 had no direct effect on ecSOD activity (data not shown). Taken together, these findings suggest that fibulin-5 plays an important role in ecSOD binding to the tissue, thereby modulating vascular O$_{2}^{\cdot-}$ levels.

**Discussion**

In the present study, using a yeast two-hybrid system, we discovered fibulin-5 as a novel binding protein for ecSOD. The functional significance of this interaction was demon-
strated by the observation that vascular O$_2^-$ levels are robustly increased in fibulin-5/−/− mice in which ecSOD binding to vascular tissue is markedly reduced. Moreover, we found a potential codistribution of ecSOD and fibulin-5 in both control and atherosclerotic vessels. Given that ecSOD plays an important role in scavenging O$_2^-$ in vascular extracellular space, ecSOD binding to fibulin-5 may represent a novel mechanism by which ecSOD regulates vascular redox state.

In vitro pulldown assays confirmed a direct interaction between ecSOD and fibulin-5. This interaction in vivo was further verified by coimmunoprecipitation of ecSOD and fibulin-5 in mammalian cells as well as in insect cells. Moreover, yeast two-hybrid mapping experiments identified that the binding site of ecSOD in fibulin-5 encompasses a globular cysteine-free C-terminal domain of fibulin-5, ie, residues 320 to 448. The C-terminal domain of fibulin-5 is a unique module for the fibulin family and shares a significant homology with that of fibulin-3 and fibulin-4 (human fibulin-5 versus fibulin-4, 53%; human fibulin-5 versus fibulin-3, 50%; fibulin-3 versus fibulin-4, 53%), but a weak homology with that of fibulin-1C, fibulin-1D, and fibulin-2.

Figure 3. A, Western blotting analysis of plasma ecSOD in control littermates and fibulin-5/−/− mice. Ten and twenty µg of protein from plasma of both control and fibulin-5/−/− mice were loaded in adjacent lanes and size separated by SDS-PAGE. After transferring to a nitrocellulose membrane, ecSOD protein was detected by immunoblotting with a polyclonal antibody against ecSOD as previously described. *P<0.01 compared with wild-type aortas. B, Immunohistochemical analysis of ecSOD expression in aortas of control littermates and fibulin-5/−/− mice. Immunostaining for ecSOD in aortas from control (A) and fibulin-5/−/− (B) mice was performed by identical methods as described for Figure 4. Brown, Immunoreactivity with ecSOD. NC (negative control), Absence of staining in aortas from control (C) and fibulin-5/−/− (D) mice when primary antibody was replaced with rabbit IgG.
lysyl oxidase-like 1 protein (LOXL1), a critical component for elastic fiber homeostasis. Thus, it is possible that ecSOD may be involved in regulating interactions between fibulin-5 and either Lp(a) or LOXL1. Of note, fibulin-5 has been shown to bind to \( \alpha\beta_5 \), \( \alpha\beta_3 \), and \( \alpha\beta_1 \) integrins and to mediate endothelial cell adhesion via its RGD motif. Moreover, it has been reported that integrin activities are regulated by extracellular redox state. As such, it is tempting to speculate that ecSOD, a potent superoxide scavenger in the extracellular space, may participate in regulating function of integrins via binding to fibulin-5.

In the present study, we determined the interaction of ecSOD with fibulin-5 in vivo using fibulin-5/- mice (Figure 6). Immunohistochemical analysis demonstrates that ecSOD codistributes with fibulin-5 in medial layer of aorta from control mice (Figure 2D) and a marked decrease in ecSOD staining in that from fibulin-5/- mice (Figure 3B). In parallel, fibulin-5/- mice showed a significant increase in plasma ecSOD levels, and a marked decrease in tissue-bound ecSOD levels in aorta, compared with control mice in which fibulin-5 protein is abundantly expressed (Figure 3C). Of note, the ecSOD mRNA levels were not changed in aortas from fibulin-5/- mice as assessed by real-time PCR and fibulin-5/- mice showed neither a decrease in immunostaining of perlecan, which is one of the major components of heparan sulfate proteoglycans in the vessel wall nor an increase in heparin-binding domain cleaved ecSOD. Furthermore, numerous studies have demonstrated that affinity for

![Graph](image1)

**Figure 4.** A, Real-time quantitative RT-PCR analysis to examine the ecSOD mRNA in aortas from control littermates and fibulin-5/- mice. GAPDH mRNA levels were used as internal control. Mean data for three separate experiments. B, Immunohistochemical analysis of perlecan expression in aortas of control littermates and fibulin-5/- mice. Immunostaining in aortas from control (A) and fibulin-5/- (B) mice was performed using the anti-perlecan antibody (1:200), followed by HRP-conjugated goat anti-rat IgG (1:200). Brown, Immunoreactivity with perlecan. NC (negative control), Absence of staining in aortas from control (C) and fibulin-5/- (D) mice when primary antibody was replaced with rat IgG.

![Image](image2)

**Figure 5.** In situ detection of superoxide production with dihydroethidium (DHE) in aortas from control littermates and fibulin-5/- mice. A, Fresh-frozen control (top left) and fibulin-5/- (top right) aortas were incubated with DHE for 30 minutes. SOD (500 U/mL) applied topically to the sequential cut sections of control (bottom left) and fibulin-5/- (bottom right) aortas during the 30 minute incubation with DHE. SOD abolished the fluorescence, confirming specificity of the fluorescent signal for \( O_2^- \). Data are representative of 3 separate experiments. e indicates endothelium; m, media; a, adventitia. B, Digital scans of DHE-stained aortas from control and fibulin-5/- mice were quantified using NIH Image software. Results shown are mean±SEM. *P<0.01 compared with control aortas.
heparan sulfate proteoglycan is important for localization of ecSOD in the extracellular matrix, and recent reports indicate that enhanced proteolysis of the heparin-binding region of ecSOD significantly alters its tissue localization during pathological processes, such as lung injury. In addition to our current findings, we have previously shown that the level of hydroxyproline, an indicator of collagen content, was not changed in fibulin-5−/− mice, although type I collagen is another ligand for ecSOD. Taken together, these findings strongly suggest that ecSOD binds to vascular extracellular matrix not only through the interaction with heparan sulfate proteoglycan but also with fibulin-5, which may explain why only a small portion (3%) of the tissue-bound ecSOD is displaced by heparin injection. Further studies will be required to investigate how the interaction of ecSOD and fibulin-5 are regulated and whether their interaction is observed in other tissues. However, because ecSOD protein expression is not completely abolished in aortas from fibulin-5−/− mice, it is possible that ecSOD may bind to other ligands.

It has been shown that ecSOD plays an important role in regulating basal O$_2^-$ level in vascular tissue. We therefore measured vascular O$_2^-$ levels in control and fibulin-5−/− mice to determine the functional significance of interaction of ecSOD and fibulin-5 in vivo. Figure 5 demonstrates that a marked decrease in tissue-bound ecSOD levels in fibulin-5−/− mice is associated with an increase in vascular O$_2^-$ level assessed by SOD inhibitable dihydroethidium (DHE) fluorescence signal. We also found that recombinant fibulin-5 has no effect on ecSOD activity. Taken together, these results suggest an essential role of ecSOD binding to fibulin-5 in modulating basal levels of O$_2^-$ in vascular tissue. Because SOD cannot enter the intracellular space, the detected SOD inhibitable DHE signal may mainly reflect the O$_2^-$ derived from extracellular space. Indeed, oxysteridium, a specific fluorescent product by the reaction of dihydroethidium and superoxide anion, is cell-permeable (unpublished observation, 2004). However, we cannot exclude the possibility that an increase in vascular O$_2^-$ level in fibulin-5−/− mice is caused by other mechanisms including enhanced superoxide generation system. This point requires further investigation.

To gain insight into the role of interaction of ecSOD with fibulin-5 in atherosclerosis, where O$_2^-$ is increased, we performed immunohistochromical analysis of ecSOD and fibulin-5 in atherosclerotic vessels from ApoE−/− mice. We demonstrated that ecSOD partially codistributes with fibulin-5 in endothelial surface, extracellular matrix, and adventitia (Figure 2E), which is different from their codistribution in medial layer of aorta observed in control mice. Of interest, previous studies have shown that both ecSOD and fibulin-5 are highly induced in balloon-injured arteries and in atherosclerosis. Thus, it is possible that an increase in protein expression of fibulin-5 enhances binding of ecSOD to vascular tissue, resulting in increased vascular ecSOD protein expression. Taken together, these findings most likely represent a novel feed forward protective mechanism whereby ecSOD modulates vascular O$_2^-$ levels through interaction with fibulin-5.

Of note, the present study shows that fibulin-5 protein is abundantly expressed in normal adult vessels, although original reports demonstrated that fibulin-5 mRNA is very low in normal adult cells, and is markedly increased after vascular injury or in atherosclerosis. Our results also show that fibulin-5 mRNA is markedly less expressed in aortas from control mice compared with those from ApoE−/− mice (online Figure 1). Because fibulin-5 is an elastin binding protein as well as a secretory protein, it is possible that fibulin-5 may be accumulated on the elastic fibers after secretion, which may contribute to increased protein expression in aorta.

Several lines of evidence suggest that ecSOD plays an important role in regulating blood pressure. Jung et al. reported that ecSOD deficiency enhanced an increase in blood pressure and O$_2^-$ in response to angiotensin II and in the two-kidney and one-clip model. Furthermore, Chu et al. showed that gene transfer of ecSOD reduces arterial pressure in a genetic model of hypertension. Of interest, fibulin-5−/− mice, in which tissue-bound ecSOD was markedly decreased in aorta, also show an increase in systolic blood pressure, pulse pressure, and aortic stiffness. In addition, it has been shown that aortic stiffness, which contributes to an increase in pulse pressure, is positively associated with oxidative stress through increasing elastin degradation, elastase activity via activation of increased MMP and phenotypic modulation of medial vascular smooth muscle cells from the contractile type to the synthetic one. Thus, it is conceivable that fibulin-5 may also participate in...
regulating aortic stiffness by modulating vascular redox state through binding to ecSOD, thereby controlling pulse pressure and aortic compliance.

In summary, we have demonstrated that the interaction of ecSOD with fibulin-5 is essential to ecSOD binding to vascular tissue, which modulates $O_2^-$ levels in the vasculature. This interaction may represent a novel mechanism for controlling vascular redox state in the extracellular space in various cardiovascular diseases such as hypertension and aortic compliance.

Acknowledgments

This research was supported by NIH R01 HL70187, Program Project Grant HL58000, AHA National Scientist Development Grant 0030180N, and AHA Grant In Aid 0455242B. We thank Dr David G. Harrison for helpful discussions and Suzanne Mertens and Shelby Hacker for excellent technical assistance.

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Circ Res. 2004;95:1067-1074; originally published online November 4, 2004;
doi: 10.1161/01.RES.0000149568.85071.FB

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Data Supplement

Expanded Materials and Methods

Animals studied: C57BL/6J mice and ApoE−/− mice on a C57BL/6J background were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). The fibulin-5−/− mice and control littermates were generated as previously described.1

Yeast two-hybrid library screening: To identify novel proteins that interact with ecSOD, we screened a human aorta cDNA library (Clontech Laboratories Inc., Palo Alto, California, USA) using ecSOD as bait and the MATCHMAKER GAL4 yeast two-hybrid system 3 (Clontech Laboratories Inc.). In this system, we used the yeast host strain AH109, which contains three different reporter genes (His, Ade, and β-galactosidase) that are tightly controlled by a GAL4 responsive region.

For generation of bait constructs, we amplified the full coding sequence of human ecSOD cDNA by primers from the 5' and 3' ends of the coding region (F1, R1) using human ecSOD cDNA as a template (Table 1). The resultant polymerase chain reaction product was subcloned into the EcoRI/SalI sites of the pGBK7 plasmid (Clontech Laboratories Inc.) to generate a fusion protein with the GAL4 DNA-binding domain (ecSOD-pGBK7), which contains a nutritional marker of TRP1. The human aorta cDNA library (Clontech Laboratories Inc.) was constructed in the pACT2 vector to generate fusion products with the GAL4 activation domain, which contains a nutritional marker of LEU2. Before library screening, the expression of GAL4-ecSOD fusion protein in AH109 from ecSOD-pGBK7 was confirmed by Western blotting (data not shown). Furthermore, we verified that this bait plasmid itself was not able to activate the prototrophic reporter genes HIS3 and β-galactosidase.
The yeast host strain AH109 was sequentially transformed with ecSOD-pGBKKT7 and human aorta cDNA library plasmids (Clontech Laboratories Inc.) using the lithium acetate method according to the manufacturer’s protocol. Interactions were identified by growth on Trp⁻/Leu⁻/His⁻ medium, and confirmed by β-galactosidase assays. For qualitative β-galactosidase activity assays, yeast cells grown in Trp⁻/Leu⁻ medium were transferred onto nitrocellulose filters, and were placed in liquid nitrogen for 30 s five times to break the yeast cell wall. After thawing, the filters were incubated for 8 hours in a buffer containing 4mM X-gal to visualize the appearance of blue colonies. For quantitative β-galactosidase activity assays, the Luminescent β-galactosidase Detection Kit II (Clontech Laboratories Inc.) was used. The cDNA library plasmid was extracted from positive yeast colonies, and sequenced by dideoxy nucleotide sequencing method.

In order to map the location of ecSOD binding site in fibulin-5, we amplified the full length of human fibulin-5 cDNA by using primers from the 5' and 3' ends of coding region (F2 and R2) (Table 1) with expressed sequence tag BC022280 including the full coding sequence of fibulin-5 cDNA as a template. For deletion ∆7 of fibulin-5, we used primers F2 and R3 (Table 1). The resultant polymerase chain reaction product was gel-purified, digested, and subcloned into the EcoRI/XhoI sites of the pACT2 plasmid (Clontech Laboratories Inc.) and sequenced by dideoxy nucleotide sequencing.

**Overexpression and purification of recombinant ecSOD and fibulin-5:** For in vitro pull-down assays using recombinant ecSOD and fibulin-5, the human ecSOD and the human fibulin-5 were overexpressed in a *Drosophila* expression system (Invitrogen Corp., Carlsbad, California, USA). The human ecSOD coding region without the terminal codon was amplified by PCR using primers F1 and R4 (Table 1), and subcloned into the EcoRI/XbaI sites of the pAc5.1/V5-His plasmid (Invitrogen Corp.) to add a C-terminal His₆ and V₅ tag (pAc5.1/ecSOD-V₅-His). Similarly, the pAc5.1/Myc-His plasmid (Invitrogen Corp.) containing human fibulin-5 was
generated using primers F2 and R5 (pAc5.1/fibulin-5-Myc-His) (Table 1). Schneider 2 cells (Invitrogen Corp) stably expressing ecSOD with V5-His6 tag or fibulin-5 with Myc-His6 tag was obtained following the manufacturer's instructions (Invitrogen Corp.). Each protein production was confirmed by immunoblotting.

For amplification and purification of recombinant proteins, cells were grown in serum-free media (Invitrogen Corp.) containing 300 µg/ml hygromycin B. Upon harvesting, cells were spun at 4000xg for 10 min at 4°C and the supernatant was collected. Recombinant ecSOD protein with V5-His6 tag and recombinant fibulin-5 protein with Myc-His6 tag were secreted into the media, and were purified by a nickel-charged chelating Ni-NTA chromatography (Qiagen Inc., Valencia, California, USA). The purity of proteins was confirmed by Coomassie blue staining of SDS-polyacrylamide gel and Western blot.

**In vitro pull-down assays:** One µg of recombinant ecSOD with V5 tag protein or IgG was incubated with 1 µg of recombinant fibulin-5 with Myc-tag protein for 2 h in a buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1.5 mM MgCl2 and 0.1% Tween 20). Two µg of anti-V5 antibody (Invitrogen Corp.) or anti-Myc antibody (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) were added, and the samples were incubated for 18 h with constant rocking. The immunocomplexes were captured with 20 µl of protein A/G agarose beads (Santa Cruz Biotechnology Inc.), and incubated at 4°C for 1.5 h. Immunoprecipitates were separated using SDS-PAGE and transferred to nitrocellulose membranes, and immunoblotted with anti-V5 antibody to detect ecSOD or anti-Myc antibody to detect fibulin-5.

**Generation of Chinese hamster ovary (CHO) cells stably expressing ecSOD:** Flp-In CHO cells (Invitrogen Corp.) were maintained in Ham's F-12 medium (Invitrogen Corp.) supplemented with 2 mM L-glutamine, 100 µg/ml Zeocin, and 10% FBS. The human ecSOD coding region without the terminal codon was amplified by PCR by using primers F3 and R6 (Table 1), and subcloned into the Nhel/Xhol sites of the pcDNA5/FRT/V5-His plasmid.
(Invitrogen Corp.). The expression plasmid encoding flp recombinase (pOG44; Invitrogen Corp.) and pcDNA5/FRT/ecSOD-V5-His were co-transfected into Flp-In-CHO cells by Lipofectamine 2000 (Invitrogen Corp.) and selected under hygromycin B (100 µg/ml) for 10 days. The expression and the activity of ecSOD were confirmed in the conditioned media from Flp-In-CHO cells stably expressing ecSOD by Western analysis and SOD activity assays as described previously.3

**Coimmunoprecipitation of ecSOD and fibulin-5 in stably transfected *Drosophila* Schneider cells and CHO cells:** First of all, to examine the interaction of ecSOD with fibulin-5 in vivo, *Drosophila* Schneider cells stably expressing ecSOD were transiently transfected with pAc5.1/fibulin-5-Myc-His, using Lipofectamine 2000 (Invitrogen Corp.). After 48 hours, the serum free conditioned media was collected and filtered, and a mixture of protease inhibitors (Roche Diagnostics Corp., Indianapolis, Indiana, USA) was added. Anti-V5 antibody (to immunoprecipitate ecSOD) or anti-Myc antibody (to immunoprecipitate fibulin-5) was added to 1 ml of conditioned media and the proteins were immunoprecipitated at 4°C overnight with continuous rocking. Immunoprecipitation and immunoblotting were performed as described previously.4

Second, we investigated the interaction of ecSOD with fibulin-5 in CHO cells stably expressing ecSOD. The fibulin-5 coding region without the termination codon was subcloned into the EcoR1/XbaI sites of pEF6-Myc-His plasmid (Invitrogen Corp.). CHO cells stably expressing ecSOD were transiently transfected with pEF6/fibulin-5-Myc-His, using Lipofectamine 2000 (Invitrogen Corp.). After 48 hours, coimmunoprecipitation studies were performed as described above.

**Immunohistochemical studies:** Tissues from aortic segments were embedded in OCT (Miles Laboratories Inc., Elkhart, Indiana, USA) and frozen in liquid nitrogen. Cryosections were stained with either a rabbit polyclonal antibody against murine ecSOD (1:10,000)3 or a rabbit
polyclonal antibody against rat fibulin-5 (1:80), followed by a biotin-conjugated goat anti-rabbit IgG (1:100; Bio-Rad Laboratories, Hercules, California, USA). In other experiments, cryosections were stained with a rat monoclonal antibody against murine perlecan (1:500; Chemicon International, Temecula, California, USA), followed by a biotin-conjugated rabbit anti-rat IgG (1:200; Bio-Rad Laboratories). Staining was developed using the ABC-AP Kit (Vector Laboratories, Burlingame, California, USA).

Dihydroethidium labeling of aortas from fibulin-5-/- mice and control littermates: To measure ROS production in vessels in situ, frozen cross-sections of aortas were stained with dihydroethidium (Molecular Probes Inc., Eugene, Oregon, USA) using a previously validated method. Three frozen 30-µm tissue sections from each of 3 matched pairs of aortas from fibulin-5-/- mice and control littermates were placed on glass slides. The sections were submerged in 2 µM dihydroethidium in Krebs/HEPES buffer and incubated at 37°C for 30 min in a dark, humidified container. Tissue sections were then visualized with a Bio-Rad MRC 1024 argon confocal microscope with fluorescence detected with a 585-nm long-pass filter, and images were collected and stored digitally. Paired aortas from fibulin-5-/- mice and control littermates were processed in parallel, and images were acquired with identical acquisition parameters.

Real-time PCR: Total RNA was isolated from mouse aortas using an RNeasy Mini Kit (Qiagen Inc.). Purified RNA was treated with RNase-free DNase to remove contaminating DNA according to the manufacturer’s protocol (Qiagen Inc.). RNA (200 ng) was reverse-transcribed using the RETROscript Kit (Ambion Inc., Austin, Texas, USA) with random hexamers as the primer.

Changes in mRNA levels in aortas from fibulin-5-/- and control mice were compared by quantitative real-time RT-PCR analysis, using the Light Cycler thermocycler (Roche Diagnostics Corp.). Reactions were prepared in the presence of the fluorescent dye SYBR green I (Roche
Diagnostics Corp.) for specific detection of double-stranded DNA. The PCR buffer composition was: 20 mM Tris-HCl pH 8.4 at 25°C, 50 mM KCl, 250 µg/mL BSA, 200 µM deoxynucleotides, 1:84,000 SYBR green I, 0.05 U/µL Taq DNA polymerase (Invitrogen Corp.). Amplification conditions included an initial denaturation at 95°C for 60 seconds, followed by 45 cycles at 65°C for 10 seconds, 72°C for 10 seconds. Cumulative fluorescence was measured at the end of the extension phase of each cycle. Primer sequences (F3 and R7 for ecSOD, F5 and F8 for GAPDH) are depicted in Table 1. Product-specific amplification was confirmed by melting curve and agarose gel electrophoresis analysis. Quantification was performed at the log-linear phase of the reaction, and cycle numbers obtained at this point were plotted against a standard curve prepared with serially -diluted control samples. Results were normalized by GAPDH expression levels.

**Western analysis of ecSOD and fibulin-5 in plasma and aortas from fibulin-5−/− mice and control littermates:** The protein expression of ecSOD and fibulin-5 in plasma and aortas was determined by Western blotting analysis, as previously described elsewhere.3.

**Materials:** All chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, Missouri, USA) unless otherwise specified.

**Data analysis:** All data are expressed as mean ± SEM. Comparisons between groups of animals or treatments were made by one-way ANOVA, followed by the Tukey-Kramer post hoc test. Values of $P<0.05$ were considered statistically significant.
Table I: Primers used for plasmid construction and real time PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position*</th>
<th>Sequence</th>
<th>Template</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>-15 to 4</td>
<td>5'-CCG <strong>GAATTC</strong> TGCCGAGCTCCAGCCATGC-3'</td>
<td>human ecSOD cDNA</td>
</tr>
<tr>
<td>F2</td>
<td>-15 to 8</td>
<td>5'-CCG <strong>GAATTC</strong> TCGCGGTCTTGGACATGCCAGG-3'</td>
<td>human fibulin-5 cDNA</td>
</tr>
<tr>
<td>F3</td>
<td>-15 to 4</td>
<td>5'-<strong>CTA GCTAGC</strong> TGCCGAGCTCCAGCCATGC-3'</td>
<td>human ecSOD cDNA</td>
</tr>
<tr>
<td>F4</td>
<td>10 to 31</td>
<td>5'-TTCTTTGTTCTACGGCTTGCTAC-3'</td>
<td>mouse aorta (ecSOD)</td>
</tr>
<tr>
<td>F5</td>
<td>298 to 317</td>
<td>5'-TTCACCACCATGGAGAGGC-3'</td>
<td>mouse aorta (GAPDH)</td>
</tr>
<tr>
<td>F6</td>
<td>753 to 776</td>
<td>5'-TGTGACCC AGGATATGAA CTTGAG-3'</td>
<td>mouse aorta (fibulin-5)</td>
</tr>
<tr>
<td>R1</td>
<td>710 to 729</td>
<td>5'-ACGC <strong>GTCGAC</strong> CCAGCGCTCAGGCGCCTTGAC-3'</td>
<td>human ecSOD cDNA</td>
</tr>
<tr>
<td>R2</td>
<td>1338 to 1356</td>
<td>5'-CCG <strong>CTCGAG</strong> GCGCGAGGCTCAGAATGGG-3'</td>
<td>human fibulin-5 cDNA</td>
</tr>
<tr>
<td>R3</td>
<td>925 to 942</td>
<td>5'-CCG <strong>CTCGAG</strong> TCA GCATTGGAAGCCCCTTG-3'</td>
<td>human fibulin-5 cDNA</td>
</tr>
<tr>
<td>R4</td>
<td>706 to 729</td>
<td>5'-<strong>CTAG TCTAGA</strong> CCGCGCGCCGCGCCTTGACACTC-3'</td>
<td>human ecSOD cDNA</td>
</tr>
<tr>
<td>R5</td>
<td>1332 to 1356</td>
<td>5'-<strong>CTAG TCTAGA</strong> GCGCGAGGCGAATGGGTAATGC-3'</td>
<td>human fibulin-5 cDNA</td>
</tr>
<tr>
<td>R6</td>
<td>706 to 729</td>
<td>5'-CCG <strong>CTCGAG</strong> CCGCGCGGCGGCGCCTTGACACTC-3'</td>
<td>human ecSOD cDNA</td>
</tr>
<tr>
<td>R7</td>
<td>162 to 190</td>
<td>5'-CTCCATCCAGATCTCCAGC-3'</td>
<td>mouse aorta (ecSOD)</td>
</tr>
<tr>
<td>R8</td>
<td>515 to 534</td>
<td>5'-GGCATGGACTGTGGTGGTCATGA-3'</td>
<td>mouse aorta (GAPDH)</td>
</tr>
<tr>
<td>R9</td>
<td>972 to 993</td>
<td>5'-AGCCCGGTCTTTGATATTGTACC -3'</td>
<td>mouse aorta (fibulin-5)</td>
</tr>
</tbody>
</table>

* Numbers indicate nucleotide positions from translation start site. Underlined bold italics in F1, F2, F3, R1, R2, R3 R4, R5, and R6 show the restriction site. Italics in R3 show additional stop codon. Italics in R3 show additional stop codon.
REFERENCES


Supplemental figure I: Panel A: Real-time quantitative RT-PCR analysis to examine the fibulin-5 mRNA in aortas from control littermates and fibulin-5−/− mice. GAPDH mRNA levels were used as internal control. Mean data for three separate experiments. Panel B: Western analysis of protein expression of fibulin-5 in aortas of control (C57Bl/6) and ApoE−/− mice. (upper panel) Representative Western blots for control and ApoE−/− mice. Five and ten µg of protein from tissue homogenates of aortas of control and ApoE−/− mice were loaded in adjacent lanes and size separated by SDS-PAGE. After transfer to a nitrocellulose membrane, fibulin-5 proteins were detected by immunoblotting with their respective antibodies. (lower panel) Densitometric analysis of western blots for fibulin-5 in aortas from control and ApoE−/− mice. Data are mean ± SEM (n = 3 for both groups). *p < 0.01; #p < 0.05 vs. control cells.
Supplemental Figure 1

A  Fibulin-5 mRNA

![Graph showing Fibulin-5 mRNA fold increase in Control vs. ApoE−/− mice. The bar for ApoE−/− is significantly higher than for Control, marked by "# p<0.01 vs. the WT".]

B  Fibulin-5 protein

![Western blot showing Fibulin-5 protein levels in Control vs. ApoE−/− mice. The bar for ApoE−/− is significantly higher than for Control, marked by "* p<0.05 vs. the WT".]

Control ApoE−/−

Fibulin-5 mRNA Fibulin-5 protein

# p<0.01 vs. the WT

* p<0.05 vs. the WT