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Extracellular Superoxide Dismutase Regulates Cardiac Function and Fibrosis

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Abstract

Aims—Extracellular superoxide dismutase (EC-SOD) is an antioxidant that protects the heart from ischemia and the lung from inflammation and fibrosis. The role of cardiac EC-SOD under normal conditions and injury remains unclear. Cardiac toxicity, a common side effect of doxorubicin, involves oxidative stress. We hypothesize that EC-SOD is critical for normal cardiac function and protects the heart from oxidant-induced fibrosis and loss of function.

Methods—C57BL/6 and EC-SOD-null mice were treated with doxorubicin, 15 mg/kg (i.p.). After 15 days, echocardiography was used to assess cardiac function. Left ventricle (LV) tissue was used to assess fibrosis and inflammation by staining, western blot, and hydroxyproline analysis.

Results—At baseline EC-SOD-null mice have LV wall thinning and increases in LV end diastolic dimensions compared to wild type mice, but have normal cardiac function. After doxorubicin, EC-SOD-null mice have decreases in fractional shortening not apparent in WT mice. Lack of EC-SOD also leads to increases in myocardial apoptosis and significantly more LV fibrosis and inflammatory cell infiltration. Administration of the metalloporphyrin AEOL 10150 abrogates the loss of cardiac function, and potentially fibrosis, associated with doxorubicin treatment in both wild type and EC-SOD KO mice.

Conclusions—EC-SOD is critical for normal cardiac morphology and protects the heart from oxidant-induced fibrosis, apoptosis and loss of function. The antioxidant metalloporphyrin, AEOL 10150 effectively protects cardiac function from doxorubicin-induced oxidative stress, *in vivo*. These findings identify targets for the use of antioxidant agents in oxidant-induced cardiac fibrosis.

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Disclosures: Dr. Day is a consultant for and holds equity in Aeolus Pharmaceuticals which is commercially developing metalloporphyrins as therapeutic agents.

Keywords

Extracellular superoxide dismutase (EC-SOD); oxygen free radicals; fibrosis; cardiomyopathy; apoptosis; doxorubicin; cardiac fibrosis

Introduction

Extracellular superoxide dismutase (EC-SOD) or SOD3 is an antioxidant enzyme that has been shown to protect the heart from ischemic damage and inflammation[1,2]. The specific mechanisms by which EC-SOD protects against fibrosis and tissue damage in various organs, including the lung and heart, have been investigated, but remain unclear. As an antioxidant enzyme, EC-SOD scavenges superoxide and modifies oxidant balances within tissues, is important throughout the cardiovascular system[3] and may be important in cardiac morphology. EC-SOD contains a matrix-binding domain that localizes to heparan sulfate proteoglycans like syndecan-1, and is expressed on epithelial, endothelial cell surfaces, and in the heart[3-5]. This localization is critical for the ability of EC-SOD to protect against oxidants [6-9]. A percentage of the population has a mutation in the matrix-binding domain of EC-SOD [10,11], which decreases its matrix affinity, and increases the risk for development of cardiovascular and ischemic heart disease[12].

Studies in the heart suggest that EC-SOD is important for preventing oxidative injury after myocardial infarction and may contribute to cardiac remodeling[13,14]. In the lung, the loss of EC-SOD results in increased inflammation and pulmonary fibrosis[15-17]. Our laboratory has shown that one mechanism through which EC-SOD protects the lung is in part by preventing oxidative fragmentation of extracellular matrix components, syndecan-1 and hyaluronan[6,7].

This study aimed to better understand how EC-SOD regulates normal morphology of the heart in the un-injured state and its role in preventing fibrosis and loss of cardiac function. To investigate the direct role and functional significance of EC-SOD in normal heart morphology and oxidant-induced fibrosis, we utilized wild type and EC-SOD null mice (EC-SOD KO) under normal conditions and in a doxorubicin-induced injury model. Doxorubicin is an anthracycline chemotherapeutic agent, which can lead to cardiomyopathy and heart failure [18]. The primary mechanism of doxorubicin-induced toxicity involves the generation of cellular free radical species[19]. Antioxidant metalloporphyrin compounds have shown potential efficacy in reducing doxorubicin-induced oxidative stress and apoptosis, *in vitro* [19]. This study shows that EC-SOD regulates baseline cardiac morphology and protects the heart from fibrosis, apoptosis and loss of function after oxidative injury. Furthermore, this is the first report that *in vivo* administration of the superoxide dismutase-like metalloporphyrin, AEOL10150[20], protects against oxidant-induced loss of cardiac function. This new information provides a better understanding of the role of preventative and therapeutic antioxidant agents in fibrotic/ischemic cardiac injuries.

Materials and Methods

Animal Treatments

The University of Pittsburgh IACUC approved all animal protocols and the investigation conforms with the National Institutes of Health Guidelines. Female wild type C57BL/6 and EC-SOD-null mice (EC-SOD KO)[21] were treated with a single intraperitoneal injection of saline or 15 mg/kg doxorubicin (Adriamycin®, Bedford Laboratories, Bedford, OH). Echocardiography was performed at day 15 after treatment, as described below. Animals were euthanized for the collection of serum and hearts. Whole heart weights were determined

gravitimetricaly. The left ventricle with intraventricular septum was dissected out, dried for hydroxyproline analysis or sectioned for protein homogenization (see subsequent sections for details).

For select experiments, the antioxidant metalloporphyrin AEOL 10150 (Aeolus Pharmaceuticals, Inc.) [20] was administered. WT and EC-SOD KO female mice, 8-10 weeks old were randomized to 4 groups per strain, n=6-8 mice per group (treatment 1 (single i.p.) + treatment 2 (multiple, s.c.)): doxorubicin + vehicle saline; doxorubicin + AEOL10150; control saline + vehicle saline; control saline + AEOL10150. The compound AEOL 10150 was administered by sub-cutaneous injection twice daily at 5mg/kg/injection. Vehicle injections contained sterile saline only. Injections started on day 0 and continued to day 14. Doxorubicin (15 mg/kg, i.p.) or control saline was administered on day 0 after one dose of AEOL 10150. Echocardiography and tissue collection was completed on day 15, as described below.

Echocardiography

Echocardiography was completed using a Visual Sonics 770 machine with a 25-MHz linear transducer, as previously described [22,23]. Briefly, mice were anesthetized with 2.5% Tribromoethanol (Avertin®, Sigma). Parasternal short axis B-mode and M-mode images and heart rate (bpm) were collected. Measurements were performed on 5 beats and averaged. Left ventricular end diastolic dimension (LVEDD, mm) and diastolic posterior wall thickness (LVPWT, mm) were determined at maximal ventricular relaxation and LV end systolic dimension (LVESD, mm) was determined at the maximal posterior-wall motion. The relative posterior wall thickness is a ratio of LVPWT/LVEDD, as previously described [24]. Echocardiography measurements and data are provided in table 1. Percent fractional shortening and ejection fraction were calculated using the following equations from M-mode image measurements:

$$\text{Fractional Shortening } \% = ((\text{LVEDD} - \text{LVESD}) / \text{LVEDD}) \times 100$$

$$\text{Ejection Fraction } \% = ((\text{LV Vol}_{\text{diastolic}} - \text{LV Vol}_{\text{systolic}}) / \text{LV Vol}_{\text{diastolic}}) \times 100$$

Left ventricular volume, μl :

$$\text{LV Vol}_{\text{diastolic}} = (7 / (2.4 + \text{LVEDD})) \times \text{LVEDD}^3 \times 1000$$

$$\text{LV Vol}_{\text{systolic}} = (7 / (2.4 + \text{LVESD})) \times \text{LVESD}^3 \times 1000$$

Hydroxyproline Analysis

Cardiac LV tissue was dried in glass vacuoles in a 110°C oven for 48 hours. Acid hydrolysis was completed by adding 6M HCl. Vials were vacuumed to remove oxygen, sealed and incubated under anoxic conditions for 24 hours at 110°C, dried and analyzed for hydroxyproline using chloramine-T, as previously described [25,26]. Briefly, 4-hydroxy-L-proline standards and the processed tissue samples were incubated with chloramine-T for 20 minutes, followed by 3.15M perchloric acid for 5 minutes. P-dimethylaminobenzaldehyde solution was added and each were incubated for 20 minutes at 60°C for color development.

Standards and samples were read in a 96-well plate at 557nm on a spectrophotometer (SpectraMax plus, Molecular Devices, Sunnyvale, CA).

Blood Analysis

Blood smears were created for every sample, dried for 24 hours and stained with Diff Quick. Slides were cover-slipped with Permount and inflammatory cell differentials were determined on de-identified samples. Blood samples were processed by incubation at room temperature for 1 hour followed by 2 hours on ice and centrifuged at 14,000 xg. Serum concentrations were determined by the Bradford Protein Assay (Pierce, Rockford, IL) and analyzed by western blot analysis for EC-SOD. The serum was also assayed for necrotic cell death by lactate dehydrogenase (LDH) using a QuantiChrom™ LDH Assay Kit, according to the manufacturers instructions (BioAssay Systems, Hayward, CA).

Hydroxyproline Analysis—This assay has been previously described[25,26] and modifications were made. The left ventricle and intraventricular septum were dried in glass vacuoles (Thermo-Fisher) in a 110°C oven for 48 hours. Acid hydrolysis was completed by adding 2 ml of 6M HCl to each sample. The vials were vacuumed to remove oxygen, which was replaced with nitrogen gas, sealed and incubated under anoxic conditions for 24 hours at 110°C. The vials were opened and the acid was allowed to evaporate for 24 hours at 110°C. Each sample was reconstituted with 2 ml of sterile PBS, sealed with parafilm and incubated for 1 hour in a 60°C water bath. Each sample was centrifuged and the supernatants were analyzed for hydroxyproline.

4-hydroxy-L-proline standards (0-5 µg/ml) were created from a 10 µg/ml hydroxyproline stock solution. All of the standards and sample dilutions (1:4) were created using PBS in a final volume of 1ml. Samples were incubated with 0.5 ml of chloramine-T solution (50mM chloramine-T, 30% v/v Ethylene glycol monomethyl ether, 50% (v/v) hydroxyproline buffer (0.26M citric acid, 1.46M sodium acetate, 0.85M sodium hydroxide, 1.2% v/v glacial acetic acid), distilled H₂O for the remaining volume) for 20 minutes at room temperature, followed by 0.5ml of 3.15M perchloric acid for 5 minutes at room temperature. Samples were mixed well after each addition. P-dimethylaminobenzaldehyde solution (1.34M pdimethylaminobenzaldehyde dissolved in Ethylene glycol monomethyl ether) was added to each (0.5ml) and incubated for 20 minutes at 60°C for color development. The standards and samples were read in a 96-well plate at 557nm on a spectrophotometer (SpectraMax plus, Molecular Devices, Sunnyvale, CA).

Tissue Homogenization

To obtain soluble protein fractions, the dissected left ventricular tissue was homogenized on ice in KBr buffer (50mmol/L potassium phosphate, pH7.4, 0.3 mol/L potassium bromide) with protease inhibitors (10 µmol/L Dichloroisocoumarin, 100 µmol/L E-64 (trans-Epoxy succinyl-leucylamido-[4-guanidino]butane), and 3 mM EDTA, Sigma). Samples were centrifuged at 14,000 rpm for 10 minutes at 4°C and the supernatant (soluble protein fraction) was collected. CHAPS detergent buffer (50mmol/L Tris-HCl, pH7.4, 150 mmol/L NaCl, 10 mmol/L CHAPS) with the same protease inhibitors was used to collect the membrane protein fraction from the remaining tissue pellet. CHAPS samples were rotated for 2 hours at 4°C, followed by sonication and centrifugation using a Sorvall RC-5C centrifuge at 17,200 xg for 10 minutes at 4°C, and the supernatants were collected. All samples were stored at -80°C until use.

Western Blotting

Serum (10µg protein) and soluble membrane fraction LV homogenate samples (40µg protein) were separated by SDS-PAGE, transferred to PVDF membranes at 500mA for 90 minutes and blocked overnight in 5% dry milk in PBS-tween at 4°C. Membranes were probed for EC-SOD

[27], CuZnSOD (1 μ g/ml, anti-SOD1, Abcam ab13498), MnSOD, caspase-3 (anti-mouse caspase-3, recognizes 37kDa pro-form and 12-17 kDa active form), or nitrotyrosine (polyclonal antibody, Cayman Chemical, Ann Arbor, MI) followed by a peroxidase (HRP)-conjugated antibody, donkey anti-rabbit IgG or donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). ECL chemiluminescence reagents were used for band visualization (ECL-plus, GE Healthcare). Ponceau red or Coomassie blue membrane staining and β -actin were used to normalize protein loading. Images were captured with a Gel Logic 2200 system (Kodak) for densitometry analysis of bands. Protein specific bands were normalized to the protein loading staining or β -actin. Data are reported as mean normalized net intensities $\pm$ SEM.

Carbonyl Modification Detection for Oxidative Stress

Carbonyl oxidative modifications of proteins were detected using the Oxyblot Assay (Chemicon). Briefly, equal amounts of LV homogenate samples (membrane protein fractions) were derivatized to 2,4-dinitrophenylhydrazones by reacting each with dinitrophenylhydrazine (DNPH). Non-derivatized samples were used as assay controls. Samples were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was probed with an antibody against DNP, according to the manufacturers instructions.

Immunohistochemistry

Select heart tissues were perfused and fixed in 10% buffered formalin overnight at 4°C for paraffin embedding. Tissues were then sectioned at 4 μ m, placed on slides and heated at 60°C overnight, and deparaffinized in xylene. The sections were then rehydrated in an ethanol series and stained with Hematoxylin and Eosin, Masson's Trichrome kit (Sigma), or Picro-Sirius Red [28,29]. For Sirius Red staining, nuclei were stained for 1 minute with Weigert's hematoxylin, washed then stained for 1 hour in 0.1% Sirius Red stain (Direct Red 80, Sigma) in saturated picric acid. Slides were washed twice in acidified water (5% acetic acid), dehydrated in an ethanol series, and cleared with xylene. Sections were cover-slipped with Permount (Sigma). Images were collected using bright field microscopy at 40-100X magnification. H&E sections were imaged at 2X magnification on a macro-dissecting scope (Olympus Inc., Center Valley, PA).

To determine the localization of EC-SOD in the heart, 4 μ m sections of paraffin embedded hearts were heated for 30 minutes at 60°C followed by deparaffinizing with xylene and rehydration in an ethanol series. Slides were stained for EC-SOD by traditional streptavidin-biotin immunohistochemical staining. Briefly, the antibodies used were an rabbit anti-mouse EC-SOD antibody, as previously described[7], and an anti-rabbit biotin-conjugated IgG (Jackson ImmunoResearch). The Vectastain ABC Elite Kit was used, including the Avidin DH and biotinylated HRP reagents (Vector, Burlingame, CA) and the Vector NovaRed Substrate. After staining, slides were dehydrated in an ethanol series, and cleared with xylene. Sections were cover-slipped with Permount and images were collected using bright field microscopy at 40X magnification.

For the analysis of inflammatory cell influx into left ventricle tissues, cardiac sections were stained for CD45 positive cells, which is a marker of inflammatory cells. Paraffin embedded hearts were sectioned at 4 μ m and were adhered to slides for 30 minutes to overnight at 60°C followed by deparaffinizing with xylene and rehydration in an ethanol series. The sections were treated with sodium citrate buffer for 20 minutes at 95°C for antigen retrieval. Sections were stained with an anti-mouse CD45 antibody (1 μ g/ml, ab25386, Abcam, Cambridge, MA) and a secondary antibody labeled with Cy3 (1:5000, Jackson immunoresearch, Westgrove, PA). Hoechst stain was used for nuclear localization. Stained sections were imaged at 40X using an Olympus IX7 microscope (Olympus). Control sections were stained with non-immune mouse IgG.

Using the TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling) method, cardiac sections were stained for the detection of fragmented DNA, which is indicative of apoptotic cells. Sections were incubated with TdT-reaction solution and nuclei were visualized using a TUNEL kit (Promega, Madison, WI) and DAPI nuclear stain. Fluorescence images were obtained by using a Nikon Microphot-FXA fluorescence microscope (Nikon Instruments, Inc, Melville, NY), as described[30]. Quantification of tunel-positive cells was completed by determining the percentage of tunel-positive (red) cells in multiple high power fields (n=3 sections per mouse strain and treatment group). Data are reported as mean percentage of tunel-positive cells per left ventricle section $\pm$ SEM.

Statistical Analysis

Graphpad Prism Software was used for statistical analyses (Graphpad, San Diego, CA). Cardiac functional and anatomical data are reported as means $\pm$ SEM and are analyzed by 2-way ANOVA for the comparison of the wild type and EC-SOD KO mice strains. Mean densitometry for western blotting and other quantitative data, such as hydroxyproline, are reported as mean $\pm$ SEM and were also analyzed by 2-way ANOVA. Bonferroni post-tests were also performed for all ANOVA data.

Results

EC-SOD localizes to the myocardium and lack of EC-SOD results in changes in left ventricular dimensions

As the primary extracellular antioxidant enzyme, the localization of extracellular superoxide dismutase (EC-SOD) was determined using immunohistochemistry on cardiac tissue sections from normal wild type mice. EC-SOD is localized to myocardial cells, as well as, the endothelium of the vasculature of the heart (Figure 1A). The significance of EC-SOD in normal cardiac morphology was determined utilizing echocardiography in control (saline)-treated wild type (WT) and EC-SOD KO mice. Control EC-SOD KO mice have a significant decrease in left ventricular posterior wall thickness (LVPWT, mm) compared to control WT mice (Figure 1B and 1C). They also have a significant increase in intraventricular end diastolic dimension (LVEDD, Figure 1D, * p <0.05). While significant changes occur in the morphology of the LV, EC-SOD KO mice appear to have compensated, as they have similar cardiac function compared to control WT mice (Figure 2A, percent fractional shortening; Fig. 2B, percent ejection fraction). It is evident in the H&E sections of the heart that control-treated EC-SOD KO mice have a decrease in the posterior wall thickness compared to control WT (Figure 2C, H&E sections).

EC-SOD protects the heart from loss of cardiac function

The functional significance of cardiovascular EC-SOD after oxidant injury was determined by utilizing echocardiography to assess cardiac dimensions and function after doxorubicin treatment. By day 15, doxorubicin causes a significant decrease in the posterior wall thickness of the left ventricle (LVPWT) in WT mice (Figure 1B, * p <0.05). No additional decreases were seen in the LVPWT of doxorubicin-treated EC-SOD KO mice, however functional impairment was evident. EC-SOD KO mice had a significant decrease in LV fractional shortening (Figure 2A, * p <0.05; EC-SOD KO control 33.5% $\pm$ 2.9; doxorubicin 18.1% $\pm$ 2.1) and ejection fraction (Figure 2B, * p <0.05; EC-SOD KO control 62.7% $\pm$ 4.3; doxorubicin 38.1% $\pm$ 3.9). Doxorubicin-treated WT mice had a trend toward decreases in FS and EF but did not reach significance. Figure 2C shows representative images for WT and EC-SOD KO groups, including H&E staining of cardiac sections, and B-mode and M-mode images from echocardiography. Doxorubicin causes an increase in the intraventricular dimension in both WT and EC-SOD KO mice. These functional findings suggest that lack of EC-SOD exacerbates systolic dysfunction associated with doxorubicin-induced left ventricular injury.

Lack of EC-SOD results in increased ventricular fibrosis and oxidative stress after doxorubicin

Lack of cardiac EC-SOD results in increased fibrosis in left ventricular tissue, as shown by increased Sirius red staining of collagen fibers (Figure 3 B, D versus A, C) and increased collagen via Trichrome staining (Figure 3 F, H versus E, G; blue fibers). Deposition of acellular collagen is present between cardiomyocytes within the left ventricle (Figure 3-I), as well as, typical doxorubicin-induced cellular pathology including cytoplasmic vacuolization (Figure 3J, 3-I, arrows). Doxorubicin exposure causes a significant increase in LV hydroxyproline in wild type mice (Figure 3K, control 0.8 ± 0.04 $\mu\text{g}/\text{mg}$ LV tissue; doxorubicin 0.9 ± 0.02 $\mu\text{g}/\text{mg}$, $*p < 0.05$). EC-SOD KO mice treated with doxorubicin had a significant increase in hydroxyproline compared to KO controls (Figure 3K, EC-SOD KO control: 0.8 ± 0.02 $\mu\text{g}/\text{mg}$ LV tissue; EC-SOD KO doxorubicin 1.0 ± 0.04 $\mu\text{g}/\text{mg}$, $*p < 0.05$) and a significant increase compared to similarly treated WT mice (WT doxorubicin: 0.9 ± 0.03 $\mu\text{g}/\text{mg}$ LV tissue; EC-SOD KO doxorubicin 1.0 ± 0.04 $\mu\text{g}/\text{mg}$, $*p < 0.05$). Control WT and EC-SOD KO mice had similar hydroxyproline levels, while control EC-SOD KO mice show slight increases in LV collagen deposition by Sirius red staining compared to control WT mice (Figure 3B versus 3A).

The lack of EC-SOD results in increased oxidative stress, shown by increases in carbonyl modifications to proteins in the membrane-fractions of cardiac homogenates (Figure 4A, $*p < 0.05$). Doxorubicin also increases the carbonyl content in WT mice. These data suggest there are increased levels of cardiac free radical species in the absence of EC-SOD. No changes were seen in nitrotyrosine oxidative modifications in membrane or soluble protein fractions of left ventricle cardiac homogenates (data not illustrated). In WT mice, EC-SOD protein expression significantly increases in left ventricle membrane fraction homogenates (Figure 4B, $*p < 0.05$) and proteolyzed EC-SOD (lacking the matrix binding domain) significantly increases in the serum (Figure 4C, $*p < 0.05$) in response to doxorubicin. Furthermore, responses in intracellular CuZn SOD trend toward increasing but do not reach statistical significance ($p = 0.12$, Figure 4D) and no changes are seen in mitochondrial MnSOD (Figure 4E) at day 15 after doxorubicin treatment.

Weight loss was used as a marker of overall injury status. In both WT and EC-SOD KO mice, doxorubicin led to a significant weight loss compared to control mice ($*p < 0.05$, Percent weight loss in WT doxorubicin: $1.9\% \pm 1.5$ and EC-SOD KO doxorubicin: $11.4\% \pm 2.0$; Table 1). Notably, EC-SOD KO mice lost significantly more weight (9.5% , $*p < 0.05$) compared to wild type mice after doxorubicin administration.

Increased apoptosis in EC-SOD KO mice after oxidative injury

After doxorubicin treatment, WT and EC-SOD KO mice have significant decreases in the total heart mass by day 15 (Gravimetric weights – WT doxorubicin: 113.8 ± 0.004 mg; EC-SOD KO doxorubicin: 105.2 ± 0.004 mg.; $*p < 0.05$; Table 1). No significant differences were seen in serum lactate dehydrogenase levels (LDH), which were used as a marker of cellular necrosis.

Lack of EC-SOD resulted in increased apoptosis in the LV after oxidative injury, as evident by a significant increase in active caspase-3 in soluble heart homogenates after doxorubicin (Figure 5A, activated caspase-3, 12-17 kDa form; reported as mean normalized net band intensity $\pm$ SEM - EC-SOD KO control: 0.1 ± 0.02 ; EC-SOD KO doxorubicin: 0.6 ± 0.10 ; $p < 0.05$). The ratio of active to inactive caspase-3 is significantly higher in doxorubicin-treated EC-SOD KO mice compared to control KO mice and both WT treatment groups (Figure 5B, EC-SOD KO controls ratio: 0.1 ± 0.01 and EC-SOD KO doxorubicin ratio 0.6 ± 0.13 , $p < 0.05$; WT control: 0.1 ± 0.04 ; WT doxorubicin: 0.2 ± 0.05 ; $p < 0.05$ versus KO doxorubicin). There were no changes in the inactive pro-form of caspase-3 (Figure 5B, 37kDa). By TUNEL

analysis, doxorubicin injury causes a significant increase in the percentage of tunel-positive, or apoptotic, cells in wild type and EC-SOD KO mice (Figure 5C and D, $*p<0.05$; mean percent tunel-positive cells $\pm$ SEM; control WT: 0.0%, dox WT: 18.2% $\pm$ 1.0; control EC-SOD KO: 16.4% $\pm$ 2.1, dox EC-SOD KO: 49.6% $\pm$ 6.3). In addition, the lack of EC-SOD causes a further increase in the percentage of apoptotic cell in both control and doxorubicin-treated groups, as shown by an increase in the percentage of TUNEL-positive cells in the doxorubicin-treated EC-SOD KO group compared to wild type mice.

AEOL 10150 prevents doxorubicin-induced loss of cardiac function

The antioxidant metalloporphyrin, AEOL 10150, was administered to doxorubicin and control-treated WT and EC-SOD KO mice for 15 days. AEOL 10150 prevented the loss of cardiac function due to doxorubicin. WT and EC-SOD KO mice both displayed a decrease in fractional shortening and ejection fraction, as previously seen. AEOL 10150 administration prevented the loss of cardiac function, as WT and EC-SOD KO mice had significant increases in LV fractional shortening (Figure 6A, $*p<0.05$) and ejection fraction (Figure 6B, $*p<0.05$). AEOL10150 treatment may also alter fibrosis development, as there is a strong trend ($p=0.09$) toward a decrease in hydroxyproline content in left ventricle tissues of wild type mice after doxorubicin and AEOL10150 treatment (Figure 6C).

Increased inflammation in EC-SOD KO mice after oxidative injury—Paraffin embedded heart sections were stained for CD45, a marker of inflammatory cells, to determine the burden of these cells in the myocardium of the left ventricle with a lack of EC-SOD and after doxorubicin. EC-SOD KO mice have a significant influx of CD45+ inflammatory cells into the myocardium of the left ventricle after doxorubicin injury compared to wild type mice (Figure 7A). In addition, by day 15 after doxorubicin exposure, both wild type and EC-SOD KO mice have an increased number of circulating inflammatory cells including macrophages and neutrophils on blood smear analysis ($*p<0.05$, Figure 7B and C). AEOL10150 also appears to decrease macrophages within circulating blood, as trends exist in the blood smear cell differential data (Figure 7B), suggesting that it may lower the number of circulating macrophages in EC-SOD KO mice.

Discussion

EC-SOD is an important antioxidant in the cardiovascular system for its protective role against oxidative stress and superoxide[3,31]. This study shows that myocardial EC-SOD regulates the normal morphology of the left ventricle and acts to protect against oxidative injury to the heart by limiting fibrosis, apoptosis, and loss of cardiac function. Control EC-SOD KO mice had significant decreases in the thickness of the left ventricle posterior wall and increases in the intraventricular area, emphasizing the importance of EC-SOD in maintaining normal cardiac morphology. While changes in the left ventricle structure of control EC-SOD KO animals are subtle but statistically significant, the lack of EC-SOD leaves the heart more susceptible to oxidative injury and enhanced functional impairment in response to doxorubicin injury. EC-SOD KO mice have normal cardiac function by fractional shortening and ejection fraction until they are challenged by doxorubicin, which produces a greater functional decline than seen in doxorubicin-challenged wild type mice.

A primary pathologic feature of doxorubicin cardio-toxicity is cardiac fibrosis that leads to non-ischemic dilated cardiomyopathy and congestive heart failure[18]. Doxorubicin induces apoptosis through the release of superoxide and hydrogen peroxide[32]. The *in vivo* data show that the lack of EC-SOD leads to significantly more fibrotic remodeling in the left ventricle by collagen deposition. The lack of EC-SOD results in significantly more fibrosis, which is the reparative response of the myocardium to a loss of cells after oxidative injury. The cardiac

injury seen in both wild type and EC-SOD KO mice after doxorubicin treatment involves a significant loss in myocardial mass through cell death. No changes in LDH release were seen in the serum suggesting that necrotic cell death is not occurring. However, EC-SOD KO mice have a significant increase in apoptosis in LV tissue after doxorubicin, shown by both increases in caspase-3 activation and in the percentage of TUNEL-positive myocardial cells.

Control EC-SOD KO had 16.4% $\pm$ 2.1 apoptotic cells, suggesting that EC-SOD alone is important for myocardial cell survival under normal conditions. Doxorubicin injury caused a significant increase in apoptosis in LV tissue of both wild type and EC-SOD KO mice, with a significant further increase in the KO mice (mean percent tunel-positive cells $\pm$ SEM; dox WT: 18.2% $\pm$ 1.0 compared to dox EC-SOD KO: 49.6% $\pm$ 6.3). Over the course of 15 days this apoptotic loss of myocardial cells can result in a significant decrease in myocardial mass and thinning of the left ventricle wall. This suggests that, mechanistically, EC-SOD functions to oppose apoptosis after exposure to doxorubicin. Thus, EC-SOD protects against cardiomyocyte death, replacement of tissue with collagen, and subsequent loss of cardiac function, in part, by regulating apoptosis. Both of these pathological responses, cellular apoptosis and remodeling by fibrosis, promote the loss of cardiac function.

EC-SOD has been previously shown to have an anti-inflammatory role in the models of lung fibrosis[15,17,33,34] and the SOD-like antioxidant, AEOL10150, has been shown to decrease macrophage accumulation in a model of radiation induced lung injury[35]. The current study shows that EC-SOD may also play an anti-inflammatory role in the heart, as EC-SOD KO mice have significantly more CD45-positive cells that infiltrate into the myocardium after doxorubicin compared to wild type mice. Furthermore, doxorubicin causes a significant increase in macrophages that are circulating in the blood of wild type mice, which may be decreased by treatment with the antioxidant AEOL10150.

Interestingly, on blood smear analysis, EC-SOD KO mice have lower relative numbers of inflammatory cells such as macrophages, neutrophils, and lymphocytes. This finding may be due to increased movement of inflammatory cells from the circulating blood into tissues, such as the heart and lung, resulting in a higher number of inflammatory cells in the left ventricle and lower numbers in the blood stream. While the relative numbers of circulating inflammatory cells is lower in EC-SOD KO mice, similar increases appear to occur with doxorubicin injury and potential decreases in inflammation with AEOL10150.

The significant impairment and damage caused by a lack of EC-SOD are interesting in the context of the oxidative model used. Doxorubicin is known to cause increased levels of intracellular oxidative stress[36,37] and superoxide[38]. Our data supports the presence of extracellular superoxide because the EC-SOD KO mice more severe cardiac injury and there are oxidative modifications that occur to membrane bound proteins in the heart after doxorubicin. This suggests that extracellular antioxidants such as EC-SOD play an important role in inhibiting oxidative stress even if it is initiated inside the cell.

A similar role for EC-SOD is described in lung fibrosis, where the chemotherapeutic Bleomycin, generates increased levels of intracellular superoxide and free radicals[39] and results in more severe fibrosis in EC-SOD KO mice[33]. It has also been shown that superoxide can be transported outside of the cell through a bicarbonate-chloride anion exchange protein (AE2)[40], where superoxide could then be scavenged by EC-SOD. While not the focus of this study, one potential mechanism through which doxorubicin may lead to increased production of intracellular and extracellular oxidants is through the conversion of nitric oxide synthase (NOS) into a preferential superoxide generator. The primary role of NOS is to produce nitric oxide, however uncoupling of the NOS enzyme can switch this role to superoxide production inside the cell. The superoxide can then be transported outside of the cell through the AE2

exchange protein, thus increasing the levels of extracellular superoxide and additional free radical products by one-electron reduction reactions. EC-SOD is the primary extracellular antioxidant that would protect the myocardium from this increase in superoxide outside of the cell. Endothelial NOS (eNOS) uncoupling has been shown to be important in myocardial oxidative stress and tissue remodeling in a model of chronic pressure overload[41]. It is known that doxorubicin converts NOS3 into a superoxide generator[42,43], as does a lack of arginine and tetrahydrabioppterin[44], any of which would subsume an important role for each of the SOD isoforms in cardio-protection, including EC-SOD.

While the lack of EC-SOD is detrimental to cardiac function after oxidative injury, our data show that the superoxide dismutase-like metalloporphyrin, AEOL 10150[20], effectively prevents the loss of cardiac function (percent fractional shortening and ejection fraction) after doxorubicin in both wild type and EC-SOD KO mice. Furthermore, hydroxyproline quantification of left ventricle tissue samples suggests that AEOL10150 may reduce fibrosis development. AEOL 10150 has both intracellular and extracellular action, as it can readily cross the plasma membrane. We utilized EC-SOD KO animals as a tool to target extracellular oxidative stress. The protective findings with AEOL suggest that exogenous superoxide dismutase activity can, in part, prevent the oxidative injury and loss of function, providing a potential new therapeutic avenue for cardiac injuries involving oxidative stress.

There are cardiovascular clinical implications through genetic differences that are known in the EC-SOD matrix-binding domain sequence. The EC-SOD gene variant (EC-SOD^{R213G}) is an arginine-213 to glycine substitution in the matrix-binding domain, which results in decreased binding affinity of EC-SOD for the tissue matrix[11,45,46]. Clinically, 2-6% of the population has this polymorphism/mutation[47,48] and these individuals are at increased risk for developing cardiovascular disease. A study from Denmark reports a 2.3 fold increase in the risk of ischemic heart disease in heterozygous individuals for EC-SOD^{R213G} [49]. Our present data show that EC-SOD is critical in protecting the heart against oxidant-induced damage resulting in cardiac fibrosis, cardiomyocyte apoptosis, and loss of function. These findings raise interesting questions about the potential effects of the EC-SOD^{R213G} gene variant on an individual's susceptibility to oxidative insults to the heart and the development of progressive fibrosis.

In summary, we have demonstrated that EC-SOD is important in the regulation of normal cardiac morphology and loss of function associated with oxidative injury by doxorubicin in a mouse model. Mechanistically, EC-SOD opposes caspase-3-mediated apoptosis and is important in limiting extracellular oxidative stress and inflammation in the heart. In addition, we report the novel finding that the superoxide dismutase-like metalloporphyrin, AEOL 10150 can prevent doxorubicin-induced loss of cardiac function, potentially through reducing fibrosis development. These findings warrant extension to new studies on the utilization of antioxidants, potentially superoxide dismutase-like compounds, in order to prevent oxidant-induced cardiac injury.

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Abbreviations

Dox, Doxorubicin; ECM, Extracellular Matrix; EC-SOD, Extracellular Superoxide Dismutase; EF%, Ejection Fraction; FS%, Fractional Shortening; KO, Knock-out Mouse; LV, Left Ventricle; WT, Wild type.

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Figure 1.

EC-SOD localization was determined by immunohistochemical staining of paraffin-embedded heart sections using a Nova Red substrate. Images were captured at 40X magnification. A) EC-SOD localizes to myocardial cells and endothelial cells of the vasculature. The lack of EC-SOD results in changes in left ventricular morphology/dimensions in un-injured EC-SOD-null (KO) animals compared to wild type (decreases posterior wall thickness and increased intraventricular area) and after doxorubicin injury. B) LV posterior wall thickness determined by echocardiography (LV PWT, mm), * $p < 0.05$ compared to Control WT; ** $p < 0.05$ compared to both WT treatment groups. C) Relative posterior wall thickness (LV PWT/ LVEDD), * $p < 0.05$ compared to both WT treatment groups. D) LV end diastolic dimensions (LVEDD, mm), * $p < 0.05$ compared to both WT treatment groups. All data are reported as mean $\pm$ SEM and analyzed by 2-way ANOVA with Bonferroni post-test.

Figure 2.

Lack of EC-SOD results in a significant decrease in cardiac function after doxorubicin treatment. A) Percent fractional shortening in wild type and EC-SOD KO mice. * $p < 0.05$ compared to control treatment; $n = 7-8$; B) Percent ejection fraction of the left ventricle, * $p < 0.05$ compared to control treatment; $n = 7-8$; C) Representative images for wild type and EC-SOD KO groups depicting 4 μ m H&E-stained cardiac sections at 2X magnification and B- and M-mode images of the left ventricle from echocardiography, $n = 7-8$.

Figure 3.

EC-SOD KO mice have significantly more left ventricular fibrosis after doxorubicin. Fibrosis is evident in paraffin tissue sections by increased Sirius Red within the LV wall tissue of doxorubicin treated wild type and EC-SOD KO mice. Sirius Red staining: A) Control WT mouse; B) Control EC-SOD KO; C) Doxorubicin-treated WT; D) Doxorubicin-treated EC-SOD KO; Increased collagen content is also evident by Trichrome staining (blue staining) in the LV posterior wall: E) Control WT; F) Control EC-SOD KO; G) Doxorubicin-treated WT; H) Doxorubicin-treated EC-SOD KO; I) Collagen deposition (blue staining) at higher magnification in the LV tissue of a doxorubicin-treated EC-SOD KO mouse. J) Cellular ultra-structural pathology can be appreciated by cytoplasmic vacuolization (arrows) in cardiac sections from a doxorubicin-treated EC-SOD KO mouse. These are also noted in section I. Hydroxyproline: K) Significant increase in hydroxyproline content after doxorubicin injury (μg/mg LV tissue); *p<0.05 WT dox or KO dox compared to respective control mice. Bar with *p<0.05, EC-SOD KO doxorubicin compared to WT doxorubicin, n=6-8.

Figure 4.

Lack of EC-SOD and doxorubicin treatment results in increased markers of oxidative stress and causes changes in antioxidant responses. Carbonyl modifications and CuZnSOD (SOD1), MnSOD (SOD2) and EC-SOD (SOD3) proteins were detected by western blot analysis. Lane densities were determined and reported as mean $\pm$ SEM. A) Increased carbonyl content in the membrane protein fractions of left ventricle tissue homogenate by Oxyblot analysis. A non-derivatized sample (ND) was included as a negative control. Densitometry was completed on each lane, * $p < 0.05$. Superoxide dismutase proteins were analyzed in the soluble protein fractions of left ventricle (LV) homogenates or serum (indicated) after doxorubicin treatment, * $p < 0.05$: B) Serum EC-SOD; C) LV homogenate EC-SOD; D) LV homogenate CuZnSOD;

p=0.12 for EC-SOD KO control compared to KO doxorubicin; E) LV homogenate MnSOD. Band net intensity was normalized to ponceau red stain or β -actin.

Figure 5.

Lack of EC-SOD exacerbates apoptotic cell death in left ventricular tissue. Apoptosis was analyzed in the soluble protein fraction of LV homogenate samples by western blot for caspase-3 and TUNEL staining on LV tissue sections, 4 μ m thick. A) Caspase-3, inactive (37kDa pro-form) and active (12-17kDa form), in the soluble protein fractions of LV homogenates. Grey bars: Control group, Black bars: doxorubicin group. Normalized to coomassie blue staining, * $p < 0.05$, $n = 3-5$. B) Relative active caspase-3 (active to inactive form). * $p < 0.05$. C) Quantification of TUNEL positive cells from left ventricle tissue sections ($n = 3$ per group and strain), * $p < 0.05$. Data are reported as the mean percent TUNEL-positive cells $\pm$ SEM. D) Representative TUNEL staining of LV sections from each treatment group. Top panel: TUNEL (red), middle panel: DAPI nuclear stain (blue), bottom panel: overlay of TUNEL and DAPI.

Figure 6.

Antioxidant AEOL 10150 prevents decreases in fractional shortening and ejection fraction due to doxorubicin-induced oxidative injury. AEOL was administered by twice daily subcutaneous injections at 5mg/kg/injection for 15 days after doxorubicin. Female wild type and EC-SOD KO mice were used at 8-10 weeks old (n=6-8 per treatment group). Cardiac function was assessed by echocardiography. A) Percent fractional shortening, $*p < 0.05$. B) Percent ejection fraction, $*p < 0.05$. 2-way statistical analysis with Bonferroni post-test was used. C) Hydroxyproline quantification of left ventricle/intraventricular septum tissues. $*p < 0.05$, mean hydroxyproline $\pm$ SEM.

Figure 7.

Lack of EC-SOD and doxorubicin treatment results in increased inflammation. Left ventricle tissue sections were stained for CD45, a marker of inflammatory cells, and DAPI nuclear stain. A) Doxorubicin treatment causes an increase in CD45-positive inflammatory cells in the left ventricle of both wild type and EC-SOD KO mice, with enhanced inflammatory cell infiltration in EC-SOD KO mice. (B-C) Blood smears, from wild type and EC-SOD KO mice treated with doxorubicin with or without the antioxidant agent AEOL10150, were stained with Diff Quick. Inflammatory cell differentials (macrophages, neutrophils and lymphocytes) were counted for twenty high power fields (HPFs) at 40x magnification on a bright field microscope, n=6-9 mice per treatment group. Blood smear cell counts: mean cell count $\pm$ SEM B) Macrophages, *p<0.05; C) Neutrophils, *p<0.05, ^p=0.09; D) Lymphocytes, *p<0.05.

Table 1

Anatomic and Functional Data for Wild type and EC-SOD KO mice

Parameters	Strain	Control (day 15)	Doxorubicin (day 15)
Body Weight (g)	WT	20.5 ± 0.4	18.8 ± 0.2 [*]
	EC-SOD KO	20.4 ± 0.4	18.1 ± 0.5 ^{**}
% Weight Change	WT		-1.9 ± 1.5 [*]
	EC-SOD KO		-11.4 ± 2.0 ^{**§}
Heart Mass (mg)	WT	126 ± 0.01	114 ± 0.004 [*]
	EC-SOD KO	121 ± 0.01	105 ± 0.004 ^{**}
Heart rate (bpm)	WT	397 ± 25.7	380 ± 27.6
	EC-SOD KO	416 ± 29.1	420 ± 22.2
Fractional Shortening (%)	WT	28.5 ± 2.7	23.5 ± 2.4
	EC-SOD KO	33.5 ± 2.9	18.1 ± 2.1 ^{**}
LVEDD (mm)	WT	3.20 ± 0.2	3.1 ± 0.08
	EC-SOD KO	3.77 ± 0.1 [*]	3.6 ± 0.06 [§]
LVESD (mm)	WT	2.37 ± 0.2	2.4 ± 0.12
	EC-SOD KO	2.39 ± 0.3	2.9 ± 0.11
LVPWT (mm)	WT	0.48 ± 0.02	0.34 ± 0.02 [*]
	EC-SOD KO	0.35 ± 0.03 [*]	0.36 ± 0.02 [*]
Relative PWT (LVEDD/LVPWT)	WT	0.15 ± 0.01	0.13 ± 0.01
	EC-SOD KO	0.11 ± 0.01 [*]	0.11 ± 0.01 [*]

Data shown as mean ± SEM

^{*} p<0.05 versus WT control^{**} p<0.05 versus EC-SOD KO control[§] p<0.05 versus WT Doxorubicin