Antioxidant, EUK-8, Prevents Murine Dilated Cardiomyopathy

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Background: Mice lacking manganese-superoxide dismutase (Mn-SOD) activity exhibit the typical pathology of dilated cardiomyopathy (DCM). In the present study, presymptomatic and symptomatic mutant mice were treated with the SOD/catalase mimetic, EUK-8.

Methods and Results: Presymptomatic heart/muscle-specific Mn-SOD-deficient mice (H/M-Sod2−/−) were treated with EUK-8 (30 mg·kg−1·day−1) for 4 weeks, and then cardiac function and the reactive oxygen species (ROS) production in their heart mitochondria were assessed. EUK-8 treatment suppressed the progression of cardiac dysfunction and diminished ROS production and oxidative damage. Furthermore, EUK-8 treatment effectively reversed the cardiac dilatation and dysfunction observed in symptomatic H/M-Sod2−/− mice. Interestingly, EUK-8 treatment repaired a molecular defect in connexin43.

Conclusions: EUK-8 treatment can prevent and cure murine DCM, so SOD/catalase mimetic treatment is proposed as a potential therapy for DCM. (Circ J 2009; 73: 2125–2134)

Key Words: Connexin43 (Cx43); Dilated cardiomyopathy (DCM); EUK-8; Manganese-superoxide dismutase (Mn-SOD); Reactive oxygen species (ROS)

The findings of several clinical studies support the hypothesis that reactive oxygen species (ROS) play an important role in the pathogenesis of cardiovascular diseases such as ischemic heart disease, atherosclerosis, and heart failure.1 ROS are mainly generated in mitochondria as a byproduct of normal cellular aerobic metabolism. Excess production of ROS, such as superoxide anions (O2−) and hydrogen peroxide (H2O2), by mitochondria is thought to be associated with increased morbidity. To protect cells from oxidative stress, mammalian cells contain antioxidant molecules, including manganese-superoxide dismutase (Mn-SOD) (in mitochondria), copper/zinc-SOD (in the cytosol), extracellular-SOD (in the extracellular space),2 glutathione peroxidases, catalase, and other nonenzymatic antioxidants. SOD detoxifies O2− radicals via dismutation to produce H2O2 and H2O. Because Mn-SOD is a superoxide scavenger enzyme located in the mitochondrial matrix, it plays a pivotal role in protecting cells from mitochondrial ROS. Two laboratories have independently generated Mn-SOD-deficient mice by deletion of different segments of the Sod2 gene. These mutant mice suffer neonatal death within 18 days because of dilated cardiomyopathy (DCM), metabolic abnormalities such as ketosis and lactic acidosis, steatosis, and neurodegeneration in the brain, in a strain-dependent manner.3,4

In our previous studies, we established several types of tissue-specific Mn-SOD-deficient mice to define the phenotypes observed in systemic Mn-SOD KO mice.5–7 We reported that heart/muscle-specific Mn-SOD-deficient (H/M-Sod2−/−) mice showed DCM involving an excess generation of ROS by mitochondria.6 Moreover, A16V polymorphism of the Sod2 gene, which is associated with a 30–40% reduction in enzymatic activity, affects idiopathic DCM.8 These findings suggest that Mn-SOD plays an important role in the susceptibility to DCM onset.

The synthetic salen-manganese complex, EUK-8, possesses both SOD and catalase mimetic and antioxidant.9–11 EUK-8 dismutates O2− to H2O2 and then further catalyzes its breakdown to O2 and H2O. It has been reported that EUK-8 treatment has a protective effects in model organisms associated with ROS-induced pathologies. In vivo studies have shown that EUK-8 improves cardiac mitochondrial dysfunction,12 delays aging,13 and prevents adrenergic hypertrophy,14 ischemia/reperfusion injury,11 and postischemic reperfusion arrhythmias.15 Interestingly, EUK-8 treatment extended the lifespan of wild-type nematodes16 and Sod2-null mutant mice.17 These results suggest that EUK-8 scavenges ROS generated in the cytoplasm or organelles, including mitochondria.
EUK-8 treatment also reduces ROS generation in heart mitochondria, as well as oxidative DNA and protein damage. Furthermore, EUK-8 dramatically reverses established cardiac dilatation and pump failure. In the present study, we administered EUK-8 to H/M-Sod2−/− mice to evaluate its effect on cardiac dilatation and dysfunction. We present the first preclinical data concerning salen-manganese compounds, suggesting the possibility of a new class of drugs for the treatment of DCM.

Methods

Animals
We used H/M-Sod2−/− mice, produced by crossbreeding Mn-SODhomo mice with muscle creatine kinase-Cre transgenic mice using in vitro fertilization techniques. Genotyping was performed by tail DNA polymerase chain reaction as described previously. All mice were housed in plastic cages (5 animals/cage) in a pathogen-free barrier facility and were kept under a 12-h light/dark cycle. The mice were maintained and studied according to protocols approved by the Animal Care Committee of the Tokyo Metropolitan Institute of Gerontology.

Isolated Tissue
Images of isolated hearts were obtained with a VHX-100 digital microscope (KEYENCE, Osaka, Japan).

Histological Studies
For histological analysis, heart tissues were immersed in 10% buffered formalin. Fixed tissues were dehydrated, embedded in paraffin, sectioned into 4-μm slices, and stained with hematoxylin–eosin (H&E). Myocardial sections were stained with Azan to evaluate the degree of fibrosis and cardiomyocyte diameter. Images were obtained using a Pixera Pro600EX camera attached to a VANOX-S microscope (Olympus, Tokyo, Japan). The fibrotic area and cardiomyocyte diameter (>30 cells) were quantitatively analyzed with Qwin Plus V3 (Leica Co Ltd). The collagen volume percentage was expressed as the mean of all fields examined for each animal.

Determination of Oxidative DNA Damage
Nuclear DNA was isolated as described previously. Measurement of 8-oxo-2′-deoxyguanosine (8-oxodG) was performed by the electrochemical detection-high-performance liquid chromatography method as described previously. The 8-oxodG content was expressed as the molar ratio of 8-oxodG to 10^6 dG. The amount of dG was calculated from the absorption at 260 nm in the same sample.

Administration of EUK-8
EUK-8 (Calbiochem, San Diego, CA, USA) suspended in saline at 1.5 mg/ml was injected intraperitoneally (30 mg/kg body weight) into the mutant mice once daily. The preventive administration began from 4 weeks of age and continued for 4 weeks. The therapeutic administration began from 16 weeks of age and continued for 2 weeks. Saline was injected as a control.

Echocardiography
The mice were anesthetized with 2.5% avertin (20 μl/g body weight), and echocardiography was performed via ultrasoundography (using an EnVisor equipped with a 12-5MHz Sector transducer; Philips Medical Systems, Andover, MA, USA and an MA or Xario, equipped with a 12-MHz transducer, Toshiba Medical Systems, Tokyo, Japan). The heart was imaged in the 2-dimensional parasternal short-axis view, and an M-mode echocardiogram of the midventricle was recorded at the level of the papillary muscle. Heart rate, anterior and posterior wall thicknesses, and the end-diastolic and systolic internal dimensions of the left ventricle were obtained from the M-mode image.

Measurement of O$_2^-$ and H$_2$O$_2$ Production
The mitochondrial fraction was isolated as described previously. O$_2^-$ formation was measured using the chemiluminescent probe 2-methyl-6-p-methoxyphenylethylnil-imidazopyrazinone (ATTO, Tokyo, Japan). Heart mitochondria (4 μg mitochondria protein/well) were incubated with a respiratory substrate (5 mmol/L succinate), 1 μmol/L Amplex Red, 0.2 units/ml horseradish peroxidase, and 0.1% bovine serum albumin. The reactions were performed in triplicate in 100 μl volumes in a 96-well plate. H$_2$O$_2$ formation was measured fluorometrically (excitation: 530 nm, emission: 590 nm) over 30 min at 37°C in a SPECTraMax Gemini XS (Molecular Devices, Sunnyvale, CA, USA). Fluorescence units were converted using the standard curve for a known concentration of H$_2$O$_2$. The results are expressed as pmol H$_2$O$_2$/μg mitochondria/min.

Measurement of Protein Carbonylation and ATP Content
For the quantitation of cardiac protein carbonylation, the nuclear and mitochondrial fractions of the heart were separated. To prepare the nuclear fractions, crude nuclear fractions were sedimented from tissue homogenates at 1,000 g for 5 min. The nuclear fractions were then washed at 1,000 g for 5 min. The mitochondrial fractions were described before. Nuclear and mitochondrial protein carbonylation was determined by Oxyblot (Chemicon, Billerica, MA, USA) according to the manufacturer’s protocol. Immunoreactive spots were visualized with ECL (GE Healthcare, Buckinghamshire, UK) and quantitated using an LAS-3000 (Fujifilm, Tokyo, Japan). ATP content was measured using an ATP measurement kit (TOYO B-Net, Tokyo, Japan) according to the manufacturer’s protocol.

Western Blot Analysis
Protein extracts were prepared from heart tissues using extraction buffer composed of 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton-X 100, and Complete Protease Inhibitor (Roche Diagnostics, Penzberg, Germany). The protein concentration of the samples was measured with a DC protein assay kit (Bio-Rad, Richmond, CA, USA). The proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and detected with specific antibodies for connexin43 (Cx43) (1:1,000; Cell Signaling, Danvers, MA, USA), Mn-SOD (1:10,000; catalog number SOD-111; StressGen, Victoria, Canada), and actin (1:2,500; Sigma, St Louis, MO, USA). The blots were incubated with horseradish peroxidase-conjugated secondary antibodies, and immunoreactive bands were visualized with ECL and LAS-3000.

Statistical Analysis
Data are expressed as means±SD. Differences among groups
were tested by ANOVA. Comparisons between 2 groups were performed using the paired Student’s t-test. P<0.05 was considered to be significant.

Results

Mn-SOD Deficiency Causes DCM Associated With Fibrosis

To address the pathological effects of mitochondrial oxidative stress on cardiac function, we generated H/M–Sod2−/− mice using a Cre-loxp system. At 4 weeks of age, these mice showed normal heart weights (Figure 1A). From 8 weeks of age, however, all H/M–Sod2−/− mice showed heart dilatation and progressively increasing heart weight to body weight (HW/BW) ratios (Figure 1A). On the other hand, the mice had no obvious abnormalities in their skeletal muscles (data not shown).

In the H/M–Sod2−/− mice, H&E staining showed that the left ventricular (LV) wall was characterized by myocardial degeneration, myocyte disarray, and myocardial cells containing irregular myofilaments (Figures 1B, C). With Azan staining, there were diffuse fibrotic scars surrounding the myocardial cells (Figures 1D, E). Some of the thickened fibrotic foci were caused by myocardial remodeling. Quantitative assessment of the fibrotic area (the ratio of collagen area to total area) showed that the H/M–Sod2−/− mice had a 12.3-fold larger fibrotic area compared with age-matched WT mice (Figure 1F).
EUK-8 Prevents Cardiac Enlargement in H/M-Sod2−/− Mice

To investigate whether Mn-SOD deficiency facilitates oxidative DNA damage in the heart, we next measured the level of 8-oxodG, which is a biomarker of oxidative DNA damage, as quantified by electrochemical detection-HPLC.19 In heart nuclear DNA, the ratio of 8-oxodG/106 dG was increased by 2.2-fold in the H/M-Sod2−/− mice compared with that in the wild-type (WT) mice (P<0.05), although there were no differences between the livers of the mutant and WT mice (Figure 1G). These results suggest that the accumulation of oxidative damage, such as oxidized DNA, causes DCM in H/M-Sod2−/− mice. During aging, the ratio of 8-oxodG/106 dG in heart nuclear DNA remained stable from 2 to 24 months, but there was a significant 2-fold increase in the amount of 8-oxodG in the heart nuclear DNA of the C57BL/6 mice and rats >24 months of age.18,21 Interestingly, the increased amount of 8-oxodG in the nuclear DNA of these aged hearts was similar to that observed in the H/M-Sod2−/− mice, suggesting that oxidative stress modifies cardiac nuclear DNA in aged and Sod2-deficient mice.

EUK-8 Prevents Cardiac Enlargement in H/M-Sod2−/− Mice

It has been reported that the antioxidant EUK-8 significantly extended the lifespan of sod2 nullzygous mice,17 and the survival of apoptosis-inducing factor-deficient harlequin mice subjected to pressure overload.22 EUK-8 also ameliorates the cardiac phenotype of WT mice that have undergone pressure overload.22 To determine whether treatment with EUK-8 prevents the progression of DCM in H/M-Sod2−/− mice, we administered EUK-8 daily, beginning at 4 weeks of age and continuing for 4 weeks. The H/M-Sod2−/− and WT mice that were treated with EUK-8 had similar body weight gains to those of the saline-treated groups throughout the experimental period (Figure 2A). We found no deleterious effects on growth or general health from the EUK-8 regimen (data not shown). After the administration period, we evaluated the HW/BW ratio, fibrotic area, and cardiomyocyte diameter. Interestingly, EUK-8 treatment prevented macroscopic cardiac enlargement and elevation of the HW/BW ratio in H/M-Sod2−/− mice (Figure 2B). Azan staining, however, showed that interstitial fibrosis increased by 6.4-fold after EUK-8 treatment, indicating that EUK-8 failed to suppress cardiac fibrosis in the H/M-Sod2−/− mice (Figures 2C, D). After saline treatment, the fibrotic area increased 9.6-fold in the mutant mice (Figures 2C, D). On the other hand, EUK-8 treatment significantly prevented cardiac hypertrophy in the H/M-Sod2−/− mice although cardiomyocyte diameter was slightly increased by 1.3-fold in the saline-treated mutant mice compared with WT mice (Figure 2E). These findings are significant and indicate that EUK-8 effectively prevented the development of cardiac enlargement, but not fibrosis, in H/M-Sod2−/− mice.

To examine whether EUK-8 has any preventive effects on the progression of cardiac dysfunction in H/M-Sod2−/− mice, we next performed echocardiographic examinations. Cardiac performance was evaluated at 4 weeks of age (pre-treatment). Despite their normal HW/BW ratio (Figure 1A), H/M-Sod2−/− mice showed deterioration of echocardiographic parameters, such as the LV end-diastolic internal...
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Figure 3. Echocardiographic analysis before and after the treatment period. Echocardiographic parameters include left ventricular end-diastolic internal dimension (LVIDd) and left ventricular end-systolic internal dimension (LVIDs), interventricular septum (IVS), and the percentage of fractional shortening (%FS) (Figure 3). At 8 weeks of age, the saline-treated mutant mice had significantly dilated LV internal dimension (P<0.01), thickened IVS (P<0.01), and impaired %FS (P<0.01) (Figure 3). After the administration of EUK-8 for 4 weeks, cardiac dilation (LVIDd: P<0.01, LVIDs: P<0.01) and pump failure (%FS: P<0.05) were markedly compared with the saline treatment group (Figure 3). These results demonstrate that EUK-8 significantly delays the progression of cardiac dysfunction caused by the loss of Mn-SOD expression.

EUK-8 Reduces ROS Generation and Oxidative Damage

We then examined the generation of O$_2^-$ and H$_2$O$_2$, using isolated cardiac mitochondria. When succinate was added as a respiratory substrate, the heart mitochondria in the saline-treated mutant mice generated elevated O$_2^-$ levels (147%, P<0.01) relative to those of the WT mice (P<0.01) (Figure 4A). In contrast, EUK-8 treatment significantly decreased O$_2^-$ generation (28%, P<0.05) compared with saline treatment in the mutant mice (Figure 4A). H$_2$O$_2$ generation in the cardiac mitochondria of the saline-treated mutant mice was reduced by 46% compared with the WT mice (Figure 4B), indicating that Mn-SOD deficiency decreases mitochondrial H$_2$O$_2$ generation because of a lack of Mn-SOD-mediated dismutation reactions. Furthermore, H$_2$O$_2$ generation was also decreased in the EUK-8 treatment group because EUK-8 also possesses catalase activity. Both O$_2^-$ and H$_2$O$_2$ generation were decreased in mitochondria isolated from the EUK-8-treated mutant mice.

Because EUK-8 treatment had suppressed excess ROS generation in the cardiac mitochondria of mutant mice, we next analyzed oxidative DNA damage in nuclear DNA isolated from the heart and liver. As shown in Figure 1G, there was a 2.2-fold increase in the amount of 8-oxodG...
in the nuclear DNA of the H/M-Sod2−/− mice. As expected, EUK-8 treatment caused a 2-fold decrease in the ratio of 8-oxodG/106 dG in the heart nuclear DNA (P<0.05) of the H/M-Sod2−/− mice (Figure 4C), but did not affect the nuclear DNA in the liver (Figure 4C). Next, to investigate oxidative protein damage in nuclei and mitochondria, we analyzed the protein carbonylation of fractionated nuclear and mitochondrial proteins in the H/M-Sod2−/− mice. Protein carbonylation was elevated in the nuclear and mitochondrial fractions, but the difference was not statistically significant (Figures 4D,E). EUK-8 treatment significantly reduced both nuclear and mitochondrial protein carbonylation in H/M-Sod2−/− mice (Figures 4D,E). Interestingly, EUK-8 also decreased the expression of oxidative proteins in both fractions in the WT mice (Figures 4D,E). These results indicate that excess ROS generation, as well as oxidative DNA and protein damage, are significantly suppressed by EUK-8.

It has been reported that the ATP content of failing human hearts is 25–30% lower than that of healthy hearts. Moreover, we have previously reported that the ATP content in H/M-Sod2−/− hearts is approximately 50% lower than that of control mice. Because EUK-8 treatment improved cardiac contractility in mutant mice (Figure 3), we examined the heart’s ATP content. In the hearts of mutants that...
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had been treated with saline it was significantly decreased to 39.9% of the level in the WT mice. In contrast, EUK-8 treatment recovered 22.7% more ATP content than did saline treatment in the mutant mice, but the difference was not statistically significant (Figure 4D).

EUK-8 Reverses Cardiac Enlargement and Dysfunction in H/M-Sod2−/− Mice

To examine the potential of EUK-8 as a therapeutic agent for DCM, we analyzed whether EUK-8 treatment was able to reverse established DCM in 16-week-old H/M-Sod2−/− mice. As previously described, the HW/BW ratio and cardiac contractility of 16-week-old H/M-Sod2−/− mice were significantly exacerbated. Interestingly, after administration of EUK-8 for 2 weeks, cardiac dilatation was dramatically improved in the H/M-Sod2−/− mice compared with saline-treated mutant mice, and EUK-8 also significantly ameliorated the HW/BW ratio compared with pretreatment and after saline treatment (Figure 5A). In the echocardiographic analysis, cardiac dilatation (LVIDd: P<0.05, LVIDs: P<0.01) and contractility (%FS: P<0.05) were also improved by administration of EUK-8 compared with their pretreatment levels in mutant mice (Figure 5B). These results indicate that EUK-8 dramatically reversed established DCM caused
by mitochondrial oxidative stress related to Mn-SOD deficiency.

**EUK-8 Recovers Cx43 Expression in H/M-Sod2−/− Mice**

In preliminary experiments, we detected a drastic downregulation of Cx43 in the hearts of H/M-Sod2−/− mice. Cx43 is a major gap junction protein that regulates cardiac conduction. It is speculated that Cx43 downregulation causes the development or progression of heart failure in H/M-Sod2−/− mice, so to test this, we investigated levels of Cx43 in H/M-Sod2−/− mice. As shown in Figure 6, the levels of Cx43 in these mice were markedly reduced in the hearts of saline-treated H/M-Sod2−/− mice (P<0.01). Interestingly, EUK-8 treatment significantly improved the Cx43 levels compared with saline-treated (P<0.05), indicating that EUK-8 treatment restores the downregulated expression of Cx43 or suppresses the enhanced degradation of Cx43. Microarray analysis showed that Cx43 expression was altered by Mn-SOD deficiency (0.50-fold, compared with WT mice). However, no obvious change was observed in Cx43 expression after EUK-8 treatment (0.60-fold, compared with WT mice). Therefore, the results of the immunoblot analysis suggest that EUK-8 treatment suppressed the enhanced degradation of Cx43 in the hearts of mutant mice (Figure 6).

**Discussion**

In the present study, we have shown that the synthetic antioxidant EUK-8 prevents cardiac enlargement and dysfunction in H/M-Sod2−/− mice (Figures 2, 3). Our findings suggest that the class of synthetic catalytic “mitoprotective” antioxidants exemplified by EUK-8 can permeate through the plasma membrane of cardiomyocytes, gain access to mitochondria, and attenuate excess ROS generation. These protective effects of EUK-8 lead to the suppression of oxidative DNA and mitochondrial protein damage (Figures 4C, E). EUK-8 treatment also restored the cardiac ATP level, as well as cardiac contractility, and induced improvements in LV dilation and heart weight. Melov et al reported that EUK-8 prevented the loss of mitochondrial aconitase activity in the brains of Sod2-null mice, and restored decreased mitochondrial succinate dehydrogenase activity in the hearts of Sod2-null mice via its antioxidant effect. Those reports suggest that EUK-8 protects mitochondrial enzymes involved in the Krebs cycle and respiratory chain, indicating that it improves mitochondrial function via mitoprotective effects. Taken together, the results suggest that attenuation of oxidative damage improves impaired cardiac function in H/M-Sod2−/− mice.

ROS are associated with a wide range of age-related disorders, including Alzheimer’s disease, Parkinson’s disease, myocardial arrhythmias, and heart failure. These disorders commonly show increased levels of 8-oxodG in serum. Kono et al reported that the levels of 8-oxodG were elevated in the serum and myocardium of patients with DCM. In the present study, we showed a 2.2-fold increase in 8-oxodG in the cardiac nuclear DNA of H/M-Sod2−/− mice (Figure 1G), and EUK-8 treatment significantly suppressed 8-oxodG formation in nuclear DNA (Figure 4C). Although we failed to show the molecular mechanisms through which mitochondrial Mn-SOD-deficiency causes the oxidation of nuclear DNA, we speculate that peroxynitrite mediates oxidative modification of nuclear DNA. 

Further studies are needed to prove whether mitochondrial NO synthase (nNOS) overexpression reduces the formation of cytoplasmic peroxynitrite via a reaction with cellular nitric oxide (NO). In our microarray analysis, we showed that neuronal NO synthase (nNOS) was upregulated by 9.1-fold in the hearts of the H/M-Sod2−/− mice (data not shown), suggesting that increased nNOS enhances NO production in the cardiomyocytes of these mice. Analysis of cardiac NO and peroxynitrite levels could shed light on the molecular mechanisms of oxidative modification in cardiac DNA and proteins in H/M-Sod2−/− mice. Although we did not measure the level of mitochondrial 8-oxodG, we showed that EUK-8 treatment reduced the level of mitochondrial protein carbonylation in the hearts of H/M-Sod2−/− mice (Figure 4F). These results permit us to speculate that EUK-8 treatment also suppresses oxidative mitochondrial DNA damage.

Digitalis, β-blockers, angiotensin-converting enzyme inhibitors, and diuretic drugs are the currently used therapeutic agents for DCM. The β-blocker, carvedilol, reduces the morbidity and mortality of patients with congestive heart failure, and more importantly, carvedilol and several of its metabolites possess potent antioxidant activity. We examined whether other antioxidants, MnTBAP and CoQ10 (data not shown), would improve the symptoms of H/M-Sod2−/− mice and found that their protective or therapeutic effects were weaker than those of EUK-8, a result that implies antioxidant therapy may be effective for the treatment of DCM, particularly EUK-8. On the other hand, we can not exclude the possibility that the observed protective effects of EUK-8 in the hearts of the H/M-Sod2−/− mice might have been caused by factors other than its antioxidant capability. Barandier et al reported that EUK-8 has significant vasodilatory effects. EUK-8 might maintain LV func-
tion in H/M-Sod2−/− mice through vasodilatory rather than antioxidant activity. Further studies are needed to clarify the protective effects of EUK-8 in vivo.

During histochemical analysis, we found no reduction in fibrosis after EUK-8 treatment in the H/M-Sod2−/− mice (Figure 2D), whereas van Empel et al reported that EUK-8 treatment significantly reduced cardiac fibrosis in harlequin mice.23 The differences in the preventive effects of EUK-8 on fibrogenesis are still unexplained. One possibility is that the cardiac fibrosis caused by Mn-SOD deficiency is not the same as that caused by pressure overload. Another possibility is that the antioxidant effects of EUK-8 might be limited to fibrogenesis induced by Mn-SOD deficiency.

Connexins are transmembrane proteins, the best known function of which is to form gap junction channels.25,26,34 In human studies, downregulation of the principal gap junction protein, Cx43, was implicated in arrhythmia in ischemic heart disease and heart failure caused by DCM.25,35 In this study, we showed that Cx43 was downregulated in H/M-Sod2−/− hearts and when EUK-8 was administered to the mutant mice, the Cx43 level significantly improved without transcriptional restoration. These results suggest that EUK-8 protects against the downregulation of Cx43 caused by posttranslational mechanisms such as degradation and modification. Several studies have focused on oxidative stress and protein downregulation. Mitochondrial aconitase and succinate dehydrogenase activity were found to be reduced in Mn-SOD-deficient mice. Moreover, the IRP1 and ApoB proteins are also downregulated in the livers of Cu/Zn-SOD-deficient mice.36,37 Excess ROS present during SOD-deficiency may attack specific target molecules in cells, leading to the degradation of proteins. Our data suggest that Cx43 is a target molecule of mitochondrial ROS in the heart.

In conclusion, we demonstrated the efficacy and limitations of the antioxidant EUK-8 in murine DCM. We also indicate the possibility that antioxidants are powerful tools for preventing age-related and/or oxidative stress-dependent heart diseases. Further studies with our model mice will provide opportunities to develop novel medicines for the treatment of heart diseases.

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