

EUK-8, a Superoxide Dismutase and Catalase Mimetic, Reduces Cardiac Oxidative Stress and Ameliorates Pressure Overload-Induced Heart Failure in the Harlequin Mouse Mutant

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OBJECTIVES	The purpose of this study was to identify apoptosis-inducing factor (AIF) as a cardiac mitochondrial antioxidant and assess the efficacy of EUK-8, a salen-manganese catalytic free radical scavenger, to protect the AIF-deficient myocardium against pressure overload.
BACKGROUND	Oxidative stress has been postulated to provoke cell death and pathologic remodeling in heart failure. We recently characterized the apoptosis-inducing factor-deficient harlequin (Hq) mouse mutant to display excessive pressure overload-induced oxidative stress, cell death, accelerated progression to heart failure, and a reduced capacity of subsarcolemmal mitochondria to scavenge free radicals, suggesting a role for AIF as a novel mitochondrial antioxidant.
METHODS	Oxidative stress-sensitized Hq mutant mice and their wild-type (WT) counterparts were given low-dose EUK-8 (25 mg/kg/day), an antioxidant with superoxide dismutase, catalase, and oxyradical scavenging properties, or vehicle for 4 weeks, and subjected to pressure overload (transverse aortic constriction) for 4 weeks. Myocardial geometry and function was serially assessed by echocardiography.
RESULTS	EUK-8 ameliorated survival in Hq and WT mice subjected to pressure overload. EUK-8 also improved left ventricular end-systolic dimensions and fractional shortening, prevented myocardial oxidant stress, attenuated necrotic and apoptotic cell death, and attenuated cardiac hypertrophy and fibrosis in both mutant and WT mice.
CONCLUSIONS	The protection against pressure overload-induced heart failure in Hq mice by EUK-8 substantiates the notion that AIF functions as an important mitochondrial antioxidant in the heart. Furthermore, because antioxidant treatment protected both the oxidative stress-prone Hq mouse model and WT mice against pressure overload-induced maladaptive left ventricular remodeling and cardiac decompensation, it may be useful as a novel therapeutic tool in the treatment of human heart failure. (J Am Coll Cardiol 2006;48:824–32) © 2006 by the American College of Cardiology Foundation

Accumulating evidence suggests that oxidative stress triggers cardiac cell death and the pathogenesis of a number of cardiovascular diseases, such as ischemic heart disease, heart failure, and atherosclerosis (1–3). Production of reactive oxygen species (ROS), such as superoxide anions ($\cdot\text{O}_2^-$) and hydrogen peroxide (H_2O_2), from mitochondrial sources is enhanced under various pathologic stimuli (3,4). To

counteract oxidative stress, mammalian cells are equipped with elaborate antioxidant mechanisms, including superoxide dismutases (SODs) in mitochondria (MnSOD), the cytosol (CuZn-SOD), plasma membrane, and extracellular spaces (extracellular SOD), catalase and glutathione peroxidases, and other nonenzymatic antioxidants. Antioxidants exert their cellular protective effects by directly scavenging ROS or their precursors and by attenuating the catalysis of ROS generation via binding to metal ions.

Apoptosis-inducing factor (AIF) is a highly conserved flavoprotein with pyridine nucleotide-disulphide oxidoreductase and deoxyribonucleic acid (DNA) binding domains. The AIF precursor is synthesized in the cytosol and imported into mitochondria, where AIF localizes in the mitochondrial intermembrane space. Changes in mitochondrial permeability, secondary to loss of the mitochondrial membrane potential ($\Delta\Psi_m$), induces translocation of AIF into the cytosol and nucleus where it may participate in chromatinolysis (5,6). We and others have recently unveiled an additional role for AIF or programmed cell death 8

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Abbreviations and Acronyms

AIF	= apoptosis-inducing factor
DNA	= deoxyribonucleic acid
DHE	= dihydroethidium
FS	= fractional shortening
Hq	= harlequin
LVID	= left ventricular internal dimension
$\cdot\text{O}_2^-$	= superoxide anion
ROS	= reactive oxygen species
SOD	= superoxide dismutase
TAC	= transverse aortic banding
WT	= wild-type

(Pdc8) as a neuronal and cardiac antioxidant, using the harlequin (Hq) mutant mouse harboring a proviral insertion in the first intron of the AIF gene resulting in >90% decrement in AIF expression in brain and heart (7,8). The Hq mice have a decreased ability to clear oxygen radicals and display increased sensitivity to necrotic and apoptotic cell death, resulting in progressive degeneration of cerebellar and retinal neurons (7), increased sensitivity of the myocardium to ischemic insults, and accelerated progression to heart failure upon pressure overload (8).

The variety of phenotypes observed in Hq mice resembles those in experimental models with premature-aging syndromes: increased oxidative stress, accelerated progression to heart failure in response to stress, neurodegenerative changes, kyphosis, age-related skin changes, growth retardation, and short life span (9). In fact, intrinsic oxidative stress was demonstrated to increase with age in the heart, owing both to age-related impairment of transcriptional responses to oxidant stress (10), diminished expression of antioxidant defense enzymes such as catalase, glutathione peroxidase, and MnSOD (11), and increased myocardial expression of NADPH oxidases (12) and to age-dependent cardiac mitochondrial dysfunction (13). Moreover, endogenous cardiac AIF protein levels were found to decrease upon pressure overload and in a genetic model of severe heart failure (8), mimicking the decrease of other antioxidant enzymes in heart failure (14). These age-related effects may contribute to the higher incidence of cardiovascular disorders, such as heart failure, in the aged population. As such, the Hq mouse may serve as a relevant model to study premature aging-related cardiovascular disorders due to decreased antioxidant ability (8).

Accordingly, the aims of the present study were to establish the causality of antioxidant defects in the Hq mouse model by chronic treatment of pressure-overloaded Hq mice with EUK-8, an antioxidant with powerful superoxide dismutase (SOD), catalase, and oxyradical scavenging properties. By administering pressure-overloaded wild-type (WT) counterparts with the same compound, the preclinical efficacy of this synthetic antioxidant to protect the myocardium against the noxious effects of pressure overload was tested.

METHODS

Mice. Wild-type male and female mice on a B6CBACa-*A^{w-J/A}* (B6CBA) background and mice hemizygous or homozygous for the X-linked Hq mutation (*Pdc8^{Hq}*) on the same background were used. All protocols were performed according to institutional guidelines and approved by the local animal care and use committee.

Aortic banding. Transverse aortic banding (TAC) or sham surgery was performed in 2-month-old mice by subjecting the aorta to a defined 25-gauge constriction between the first and second truncus of the aortic arch as described previously in detail (8). Doppler echocardiography was used to calculate the pressure gradient between the proximal and distal sites of the transverse aortic constriction using the Doppler-estimated Bernoulli equation (15), and only mice with a pressure gradient >20 mm Hg were used in this study (8).

Transthoracic echocardiography. Noninvasive echocardiographic measurements were performed with a Hewlett-Packard Sonos 5500 instrument (Hewlett-Packard) using a 15-MHz transducer (15-6L linear probe, Philips Medical Systems, Eindhoven, the Netherlands) applied parasternally to the shaved chest wall of mice anesthetized with isoflurane as previously described (16). In M-mode, the following parameters were obtained: end-diastolic left ventricular internal diameter (LVIDd), end-systolic left ventricular internal diameter (LVIDs), and posterior wall and interventricular septum wall thickness. Fractional shortening (FS) was calculated as: $[(LVIDd - LVIDs)/LVIDd] \times 100$. Animals were subjected to serial weekly echocardiographic analyses after sham operation or aortic banding for the duration of the study (4 weeks).

Antioxidant treatment. The mice were randomized to treatment with either EUK-8 (intraperitoneal injections, 3 times a week, 25 mg/kg/day; Calbiochem EMD Biosciences, Darmstadt, Germany) or an equal volume of vehicle (phosphate-buffered saline) for the duration of the study.

Immunolabeling and histologic analysis. Heart tissue was fixed in 3.7% formaldehyde and embedded in paraffin, and sectioned at 6- μm thickness for visualization. Sections were stained with hematoxylin and eosin (H&E) or Sirius red or incubated with antibodies against cleaved caspase-3 (1:1000; Cell Signaling, Danvers, Massachusetts), cleaved PARP (1:200; Cell Signaling), or 8-OHdG (7.5 $\mu\text{g}/\mu\text{l}$; Oxis International Inc., Portland, Oregon). Envision+ kit (Dako Cytomation, Glostrup, Denmark) was used as a secondary reagent. Stainings were developed using DAB (brown precipitate), and slides counterstained with hematoxylin and visualized using a Nikon Eclipse E600 microscope (Nikon, Melville, New York).

In situ detection of reactive oxygen species. Reactive oxygen species production was determined using the fluorescent dye dihydroethidium (DHE). Cryosections (10 μm) were incubated with 5 $\mu\text{mol/l}$ DHE at 37°C for 30 min.

Images were captured on a Zeiss Axiovert 135M microscope (Carl Zeiss MicroImaging, Thornwood, New York) and pixel intensity of the nuclei was measured using SPOT-advanced software.

Statistical analysis. The results are presented as mean \pm SEM. Statistical analyses were performed by using INSTAT 3.0 software (GraphPad, San Diego, California) and Student *t* test or analysis of variance followed by Tukey post-test when appropriate. Kaplan-Meier survival curves were compared between groups with the use of log rank tests. Statistical significance was accepted at a *p* value of <0.05 .

RESULTS

EUK-8 prevents myocardial oxidant damage provoked by biomechanical stress.

Biomechanical stress activates signaling cascades including oxidative stress (17), predisposes cardiac muscle to late-onset apoptosis (8,18–20), and provokes progressive left ventricular remodeling and heart failure (18,19). First, we confirmed whether our TAC procedure provoked myocardial oxidative stress and if our EUK-8 treatment regimen could diminish this effect. The oxidative fluorescent dye DHE was used to evaluate the production of superoxide. Dihydroethidium is freely permeable to cells and in the presence of superoxide anions oxidizes to a fluorescent product that is intercalated into the DNA. Fluorescent product is therefore a marker of intracellular superoxide anion generation. All sham-operated groups showed low levels of superoxide. In response to pressure overload, however, both WT and Hq mice demonstrated an increased amount of superoxide. This increase was about 20% higher in Hq mice compared with their WT counterparts (Fig. 1A). EUK-8 treated TAC animals, of both genotypes, displayed decreased levels of oxidative stress, to levels similar to sham-operated animals. To evaluate a downstream marker for oxidative stress-related damage, we stained the myocardium of all genotypes for 8-OHdG, a marker for free radical DNA damage. Untreated sham-operated animals showed low amounts of 8-OHdG-positive nuclei. Aortic banding elevated oxidative damage in both groups, with Hq-TAC animals displaying more damage than their WT-TAC counterparts (Fig. 1B). Markedly reduced levels of oxidative DNA damage were evident in EUK-8-treated animals in all experimental groups. Conclusively, these data directly demonstrate that Hq mice, like their WT counterparts, at baseline have no signs of oxidative stress but display significantly more production of ROS and oxidative stress-induced damage after pressure overload than WT mice. Moreover, these data also indicate that TAC provokes oxidative stress and damages crucial structures in the heart and that treatment with EUK-8 effectively counters oxidative damage in pressure-overloaded animals of all experimental groups.

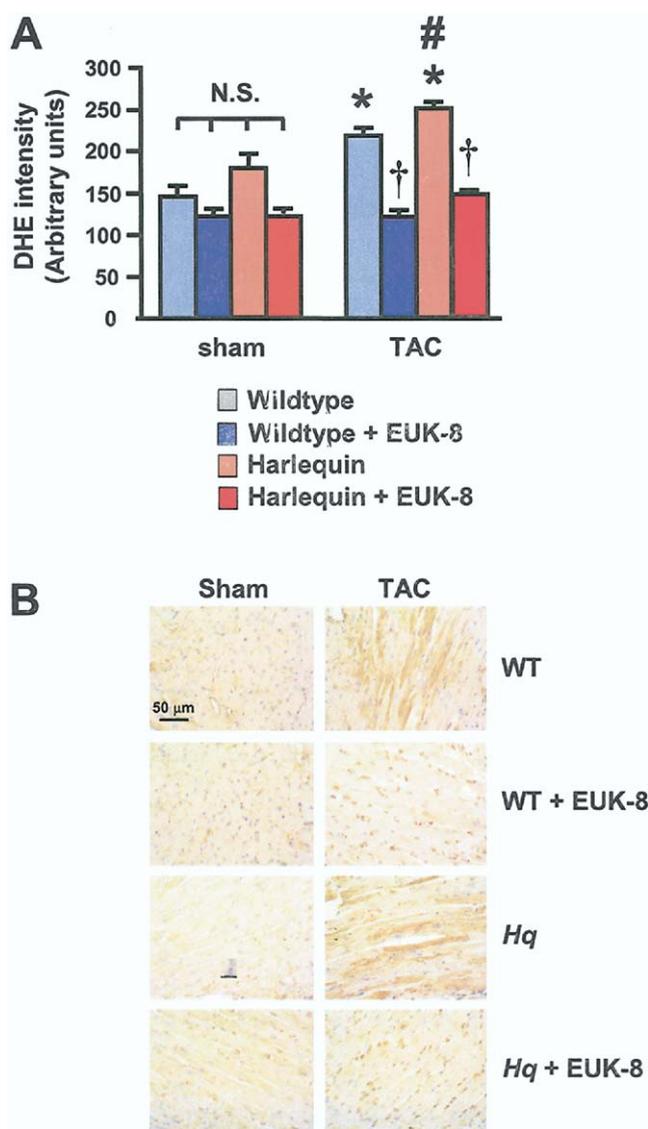


Figure 1. (A) Dihydroethidium (DHE) was used to visualize and quantify the production of superoxide in situ. Wild-type (WT) and harlequin (Hq) hearts demonstrated a significantly higher level of superoxide production after aortic banding; this increase was attenuated by administration of antioxidant EUK-8. (B) Left ventricular myocardial sections were subjected to immunostaining with 8-OHdG, a marker of oxidative DNA damage, which was increased in nontreated mice after aortic banding; however, EUK-8-treated mice had similar levels of 8-OHdG staining as sham-operated mice.

EUK-8 improves survival following biomechanical stress.

Survival rates were evaluated of WT and Hq mice subjected to sham operation or pressure overload, treated with EUK-8 or vehicle. Survival of the sham-operated mice in all experimental groups was 100% after 28 days of TAC. Survival of WT-TAC mice amounted to 68% at 4 weeks, and this was improved by the EUK-8 regimen, to 82% (Fig. 2). More dramatic differences in survival were evident in the Hq experimental groups: Survival of Hq-TAC mice at 28 days was 38% for the vehicle group and 69% for the EUK-8-treated group (Fig. 2). Overall, these observations in post-TAC survival correlate well with the blunted dete-

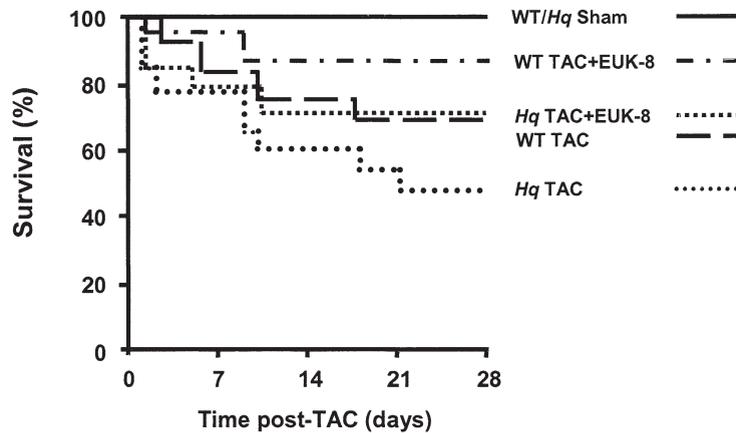


Figure 2. Kaplan-Meier survival curve demonstrates increased post-transverse aortic constriction (TAC) mortality in harlequin (Hq) mutant mice compared with wild-type (WT), which was attenuated by EUK-8 treatment.

rioration of cardiac function and geometry in vehicle-treated WT-TAC and Hq-TAC mice compared with WT-TAC and Hq-TAC mice treated with EUK-8.

EUK-8 prevents functional and geometrical deterioration in response to pressure overload. Cardiac geometry and function was assessed noninvasively by serial echocardiography at weekly intervals up to 28 days after TAC (Fig. 3A). After 7 days of pressure overload, WT mice demonstrated no deterioration in cardiac function (FS: sham $55.2 \pm 0.9\%$, 7 days after TAC $55.3 \pm 1.0\%$) (Table 1, Figs. 3A and 3B), whereas Hq mice already demonstrated a significant decline in cardiac function (8), indicated by a decrease of 14.7% in FS (sham $52.9 \pm 0.7\%$, 7 days after TAC $45.1 \pm 0.9\%$; $p < 0.05$) (Table 1, Figs. 3A and 3B). At 28 days of pressure overload, WT mice demonstrated a significant decline in FS (sham $55.2 \pm 0.9\%$, 7 days after TAC $55.3 \pm 1.0\%$, 28 days after TAC $43.3 \pm 0.9\%$; $p < 0.05$ [TAC vs. sham]) (Figs. 3A and 3B). The Hq mice showed a more pronounced deterioration in cardiac function in time after pressure overload, as demonstrated by a progressive decline in FS (28 days after TAC $31.4 \pm 1.0\%$; $p < 0.05$ [TAC vs. sham]) (Figs. 3A and 3B), and increasing LVIDs (sham 1.62 ± 0.09 mm, 7 days after TAC 1.63 ± 0.08 mm, 28 days after TAC 2.10 ± 0.09 mm; $p < 0.05$ [TAC vs. sham]) (Table 1).

Administration of EUK-8 for 28 days provoked no change in cardiac function or geometry in either sham-operated experimental group. In contrast, EUK-8 significantly improved FS after pressure overload in WT-TAC mice compared with the corresponding vehicle-treated group (WT-TAC + EUK-8: $51.2 \pm 1.1\%$; Hq-TAC + EUK-8: $55.2 \pm 1.8\%$; $p < 0.05$ [treated vs. nontreated]) (Fig. 3B). Similarly, EUK-8 attenuated the increase in left ventricular (LV) internal diameters in both WT and Hq mice subjected to biomechanical stress.

After 28 days of pressure overload, both WT and Hq mice demonstrated a dramatic increase in heart weight compared to sham-operated mice, as shown by heart weight (HW) to tibia length (TL) or to body weight (BW)

(HW/TL, HW/BW) ratio (Fig. 3C). This increase was more pronounced in Hq mice compared to WT. Treatment with the antioxidant EUK-8 attenuated the increase in these ratios for both genotypes. These data indicate that EUK-8 administration attenuates pressure overload-induced functional and geometrical deterioration.

Effect of EUK-8 on cell death and fibrosis. Because oxidative stress provokes apoptotic cell death, the incidence of that form of cell death was evaluated using immunohistochemical labeling to detect the presence of activated (cleaved) caspase-3 upon sacrifice of all experimental groups (28 days after TAC). A significant increase in apoptosis was found after TAC in all experimental groups relative to their respective sham-operated vehicle-treated groups. The increase in caspase-3-positive cardiac muscle was relatively mild in WT-TAC mice (3- to 4-fold), whereas Hq-TAC mice demonstrated a dramatic increase in apoptosis. Treatment with EUK-8 attenuated both the marked increase in apoptotic myocytes in the Hq-TAC as well as in the WT-TAC mice (WT-sham: $0.12 \pm 0.01\%$; WT-sham + EUK-8 $0.13 \pm 0.01\%$; Hq-sham: $0.19 \pm 0.02\%$; Hq-sham + EUK-8: $0.15 \pm 0.02\%$; WT-TAC: $0.38 \pm 0.04\%$; WT-TAC + EUK-8: $0.30 \pm 0.04\%$; Hq-TAC: $0.80 \pm 0.07\%$; Hq-TAC + EUK-8: $0.38 \pm 0.04\%$; $p < 0.05$) (Fig. 4A). Immunohistochemical analyses using an antibody to cleaved PARP, a late hallmark of apoptosis, confirmed the findings obtained with activated caspase-3, demonstrating the ability of EUK-8 to markedly reduce apoptotic cell death in pressure-overloaded WT and Hq mice (WT-sham: $0.30 \pm 0.04\%$; WT-sham + EUK-8: $0.28 \pm 0.04\%$; Hq-sham: $0.33 \pm 0.07\%$; Hq-sham + EUK-8: $0.33 \pm 0.04\%$; WT-TAC: $0.88 \pm 0.10\%$; WT-TAC + EUK-8: $0.61 \pm 0.04\%$; Hq-TAC: $1.92 \pm 0.18\%$; Hq-TAC + EUK-8: $0.63 \pm 1.0\%$; $p < 0.05$) (Fig. 4B).

To further analyze the histologic alterations in the experimental groups, cross-sectional myofiber area and the extent of fibrosis was assessed. The H&E staining confirmed an increase in cross-sectional myofiber size in response to mechanical load in both genotypes, although this was more

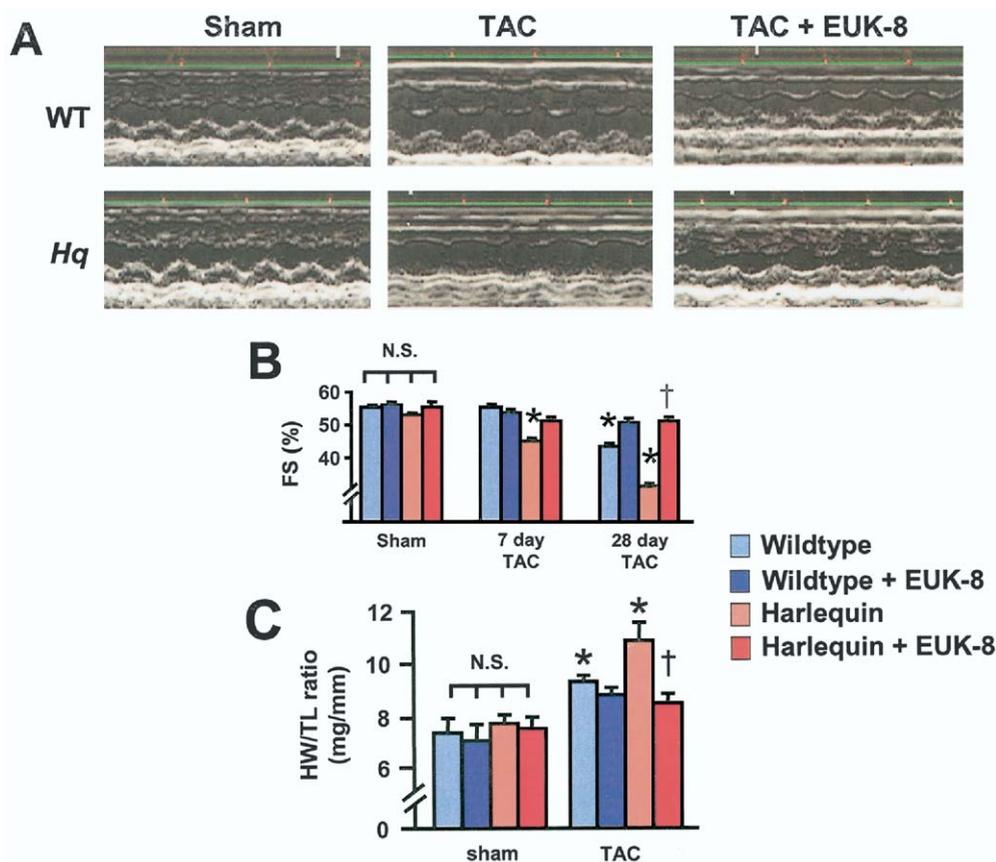


Figure 3. (A) Representative M-mode echocardiography images of wild-type (WT) and harlequin (Hq) mice, either treated with vehicle or with EUK-8, following sham operation or 7 or 14 days after transverse aortic constriction (TAC). (B) Fractional shortening (FS), a measure of systolic contractility, was similar in sham-operated mutant and WT mice but was progressively and significantly decreased in Hq mutant mice compared with WT mice after 4 weeks of TAC. (C) The heart weight (HW)/tibial length (TL) ratios increased in untreated WT and Hq mice after 4 weeks of TAC compared with corresponding sham-operated groups, although the percentage increase was more pronounced in Hq mutant mice. The WT and Hq mice treated with EUK-8 did not demonstrate a significant increase in HW/TL ratio after TAC compared with corresponding sham groups.

pronounced in Hq-TAC mice (WT-sham: $179 \pm 11 \mu\text{m}^2$; WT-sham + EUK-8: $182 \pm 9 \mu\text{m}^2$; Hq-sham: $154 \pm 7 \mu\text{m}^2$; Hq-sham + EUK-8: $163 \pm 9 \mu\text{m}^2$; WT-TAC: $358 \pm 10 \mu\text{m}^2$; WT-TAC + EUK-8: $237 \pm 8 \mu\text{m}^2$; Hq-TAC: $391 \pm 9 \mu\text{m}^2$; Hq-TAC + EUK-8: $242 \pm 13 \mu\text{m}^2$; $p < 0.01$) (Figs. 5A and 5B). Furthermore, pronounced interstitial fibrosis was evident in Hq-TAC but not in WT-TAC hearts. EUK-8 treatment resulted in a significant attenuation of interstitial fibrosis in both genotypes (Figs. 5C and 5D). Collectively, these data demonstrate a predisposition of AIF-deficient mice to develop LV dysfunction, dilation, and increased mortality, coupled with pathologic LV remodeling encompassing accentuated oxidative stress, cell death, and fibrosis in response to mechanical load. All these pathologic processes were attenuated by administration of the antioxidant EUK-8.

DISCUSSION

AIF has a mitochondrial antioxidant function in the heart. Although AIF has been implicated to play a role in the execution of the apoptotic pathways (reviewed in Joza et al. [6]), recent studies indicate an additional role for AIF as a factor conferring protection against peroxide-induced cell

death and neurodegeneration in specific subsets of neurons (7,21) and protecting the myocardium from oxidative stress under ischemic or biomechanical stress in response to aortic banding (8). Although AIF does not appear to physically associate with components of the respiratory complex (22), it has been proposed that the increased sensitivity to oxidative stress observed in Hq mice results from the participation of AIF in mitochondrial respiration by handling of reactive oxygen radicals that are normally released by the respiratory chain (22). Increased production of ROS can result from respiratory chain dysfunction and/or decreased antioxidant mechanisms. However, we and others failed to find alterations in respiratory complex activity in Hq hearts (8,22), nor did we observe an increase in mitochondrial number (8) which might compensate for respiratory deficiencies. In contrast, loss of complex I activity was evident in Hq brain and retina at 10 weeks of age (22), which may point to differences between tissues in assembly of respiratory complexes or handling free radical production.

Alternatively, our previous findings indicate a deficiency in the scavenging of exogenous H_2O_2 in isolated Hq mitochondria (8). Secondly, the data in the present study

Table 1. Echocardiographic Characteristics in Wild Type (WT) and Harlequin (Hq) Mice After Sham Operation and After Transverse Aortic Constriction (TAC)

	Sham				TAC			
	Vehicle		EUK-8		Vehicle		EUK-8	
	WT (n = 7)	Hq (n = 5)	WT (n = 6)	Hq (n = 4)	WT (n = 7)	Hq (n = 8)	WT (n = 7)	Hq (n = 8)
BW, g	26.9 ± 2.4	23.8 ± 1.3	22.5 ± 1.5	19.9 ± 2.8	29.9 ± 0.6	24.2 ± 0.7	28.4 ± 2.5	22.7 ± 1.5
IVS, mm	0.72 ± 0.08	0.83 ± 0.10	0.75 ± 0.04	0.86 ± 0.02	1.25 ± 0.03*	1.17 ± 0.06*	0.97 ± 0.04†	0.91 ± 0.05†
LVPWth, mm	1.19 ± 0.05	1.15 ± 0.05	0.96 ± 0.05	0.97 ± 0.06	1.63 ± 0.06*	1.62 ± 0.05*	1.28 ± 0.05*†	1.15 ± 0.05†
EDD, mm	3.25 ± 0.12	3.44 ± 0.16	2.93 ± 0.12	3.12 ± 0.10	3.04 ± 0.11	3.05 ± 0.09	3.05 ± 0.11	3.17 ± 0.16
ESD, mm	1.46 ± 0.08	1.62 ± 0.09	1.29 ± 0.06	1.40 ± 0.05	1.72 ± 0.05	2.10 ± 0.09*‡	1.50 ± 0.09	1.55 ± 0.10†
FS, %	55 ± 1	53 ± 1	56.0 ± 1.0	55.2 ± 1.8	43.4 ± 0.9*	31.4 ± 1.0*‡	50.8 ± 1.4†	51.2 ± 1.1*†
EF, %	90 ± 1	90 ± 1	92 ± 1	90 ± 1	82 ± 1	68 ± 1	88 ± 1	88 ± 1
HR, beats/min	479 ± 27	471 ± 31	452 ± 31	457 ± 17	494 ± 14	483 ± 18	525 ± 25	486 ± 14
AoPg, mm Hg	4 ± 1	5 ± 1	3 ± 1	3 ± 1	24 ± 5	23 ± 3	20 ± 2	25 ± 3

Data are expressed as mean ± SEM. *Indicates p < 0.05 vs. corresponding sham group. †Indicates p < 0.05 vs. vehicle-treated group with same genotype after TAC. ‡Indicates p < 0.05 Hq vs. WT after TAC.

AoPg = aortic pressure gradient; BW = body weight; EDD = end-diastolic diameter; EF = ejection fraction; ESD = end-systolic diameter; FS = left ventricular fractional shortening calculated as (left ventricular internal diameter in diastole [LVIDd] – LVID in systole)/LVIDd; HR = heart rate; IVS = intraventricular septal thickness; LVPWth = left ventricular posterior wall thickness.

demonstrate that cardiac dysfunction in the AIF-deficient myocardium could be repaired by exogenous antioxidant delivery. Thirdly, AIF-negative cells under stress conditions were found to deplete nonoxidized glutathione more rapidly than cells that expressed AIF (23), suggesting that AIF is necessary for the maintenance of glutathione levels, at least under some conditions. Finally, crystal structures of AIF reveal 2 functionally distinct regions: a domain required for DNA fragmentation and a domain with homology to NADH-dependent ferredoxin reductase (24), suggesting that AIF may function as a NADH oxidase, accepting electrons from NADH and transferring them to molecular oxygen to form the O₂⁻-free radical, which undergoes dismutation to H₂O₂ (25). In this light it is of interest to note the similarity between the oxidoreductase AIF and the thioredoxin system, a powerful antioxidant protein-disulfide oxidoreductase system consisting of thioredoxin 1, thioredoxin reductase, and NADPH (26). In fact, lowering the antioxidant function of the thioredoxin system in the heart provokes oxidative stress in the cardiac muscle (27) and predisposes the heart, as does the Hq mutation, to cardiomyocyte apoptosis and cardiac decompensation in response to mechanical load. Our findings are in line with earlier studies that indicate that biomechanical stress activates signaling cascades including oxidative stress (17), predisposes cardiac muscle to late-onset apoptosis (8,18–20), and provokes progressive left ventricular remodeling and heart failure (18,19). Taken together, the combined observations strongly suggest that AIF itself, or as part of a yet to be determined complex, has direct free radical scavenging properties.

Antioxidant treatment in heart failure. Reactive oxygen species have been implicated in a wide range of disorders, including Alzheimer's disease, Parkinson's disease, myocardial arrhythmias, and heart failure, regardless of etiology (21,28). Proof of principle in animal models has been

obtained with antioxidants, notably vitamins C and E (29,30), albeit at higher doses than used in clinical trials (31,32). Consequently, many studies have attempted to ameliorate or attenuate the progression of cardiovascular disease by chronic or acute antioxidant treatment. Such

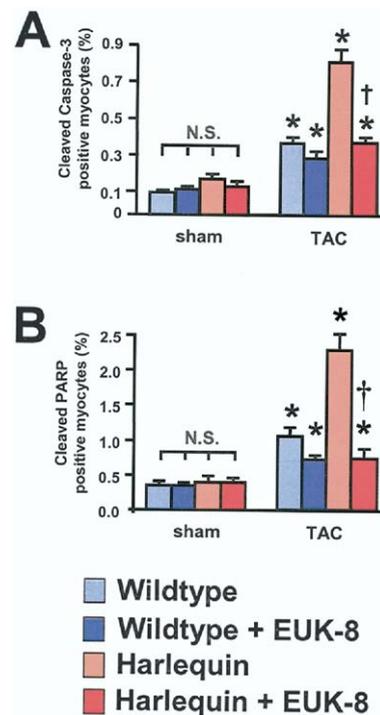


Figure 4. (A) Wild-type (WT) and harlequin (Hq) hearts, subjected to 28 days of mechanical load, displayed increased myocyte apoptosis as scored by cleaved caspase-3-positive myocytes. The percentage increase in Hq mutants was more pronounced and was attenuated by treatment with EUK-8. (B) The WT and Hq hearts demonstrated increased cleaved PARP-positive myocytes after transverse aortic constriction (TAC), with the percentage increase in Hq mutants being more pronounced than in WT. Treatment with EUK-8 significantly reduced the amount of PARP-positive myocytes in Hq-TAC hearts.

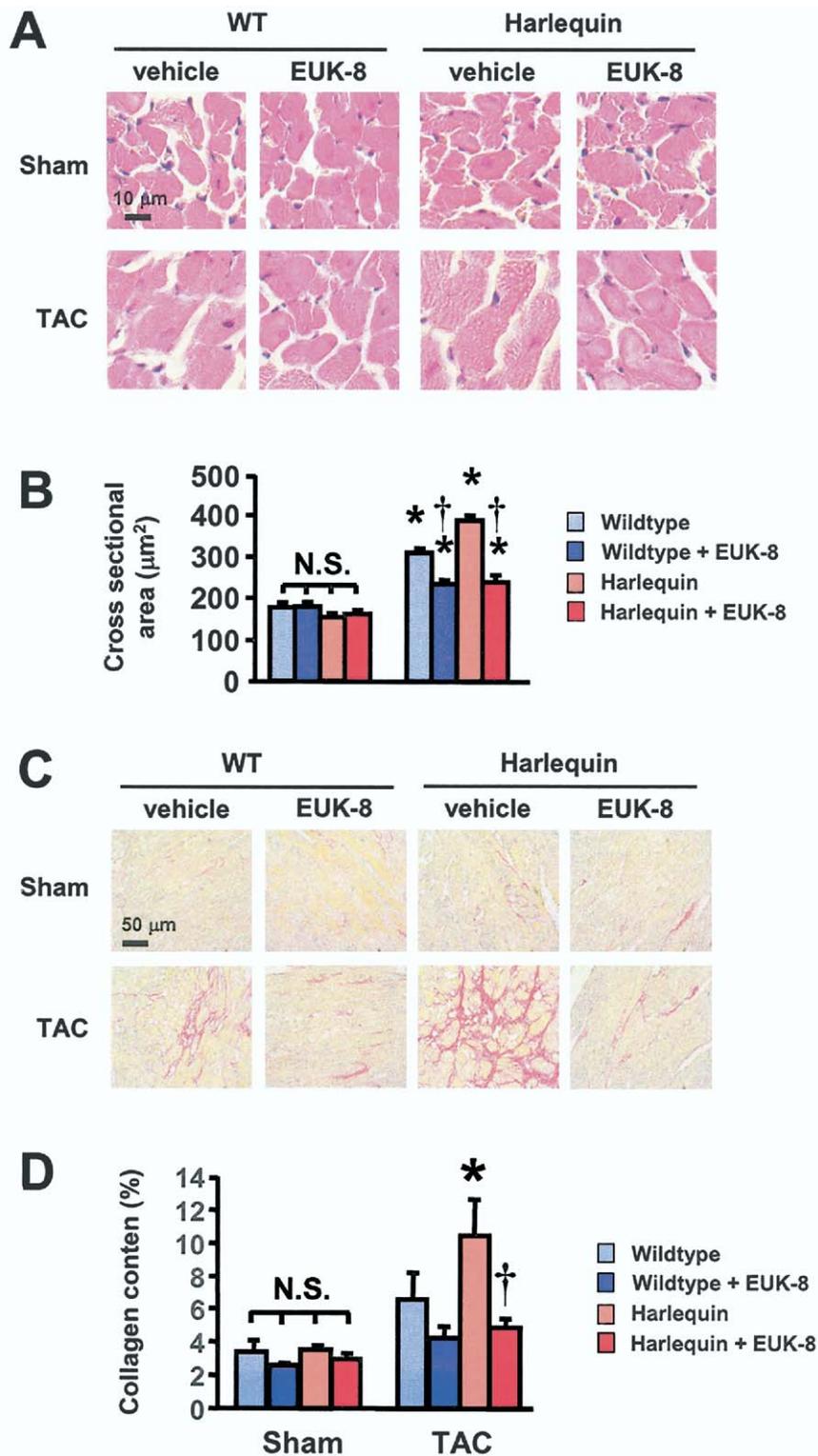


Figure 5. (A) Representative hematoxylin and eosin (H&E) sections at 400 \times magnification of wild-type (WT) and harlequin (Hq) hearts, treated with vehicle or EUK-8, either after sham treatment or 28 days of transverse aortic constriction (TAC). The Hq-TAC mice demonstrated a marked increase in myocyte fiber diameter compared with other experimental groups; this increase was attenuated by treatment with EUK-8. (B) Quantification of cross-sectional area of myofibers from indicated groups shows an accentuated myocyte hypertrophy response in Hq-TAC mice. (C, D) Sirius red staining demonstrates significant fibrosis in Hq mice after TAC, which is diminished by EUK-8 administration.

studies have yielded at best equivocal or mildly beneficial results with regard to slowing disease progression or improving specific indicators (2,31,33,34).

Those studies seem to cast doubt on the oxidative stress theory of cardiovascular disease, but there are several reasons to believe that this is not justified. Although vitamin E effectively scavenges lipid peroxyl radicals, it has limited activity against other oxidants, such as superoxide, peroxy-nitrite, and hypochlorous acid. The rate constant for the reaction of vitamin E with O_2^- is 5 orders of magnitude slower than the rate of reaction of O_2^- with endogenous antioxidant enzymes and molecules such as superoxide dismutase and nitric oxide (35). Further, oral intake of vitamin E only modestly increases its plasma and tissue levels, making it unlikely that these vitamins may affect biologic processes. Finally, many of the oxidative reactions that contribute to heart failure occur in the cytoplasm, nucleus, and interstitial space. Vitamin E is concentrated in lipid layers and in the low-density lipoprotein particle and is therefore unlikely to affect these events. Also, vitamin E may have adverse pro-oxidant effects, because the tocopheroxyl radical generated when vitamin E reacts with a radical can promote lipid peroxidation by attacking polyunsaturated fatty acids (36). In theory, efficacy should be improved by using catalytic antioxidants that can destroy multiple toxic mitochondrial ROS without themselves becoming inactivated.

The compounds used in this study, synthetic mimetics of superoxide dismutase and catalase, are examples of such agents. At the cellular level, EUK-8 is known to dismutate O_2^- to H_2O_2 and then further catalyze its breakdown to H_2O and O_2 , and it is capable of nitrosative species breakdown, such as reducing $ONOO^-$ to NO_2^- or oxidizing NO to NO_3^- (37). Our data show that the compound, administered at 25 mg/kg, extended the life span of pressure-overloaded WT and AIF-deficient mice. Our data are in line with beneficial effects of EUK-8 in protecting cultured cardiomyocytes against excessive free radical injury provoked by H_2O_2 treatment (8), providing cardioprotection in aged rats (38), dampening adrenergic hypertrophy signaling in cultured cardiomyocytes (39,40), and attenuating ischemia/reperfusion injury (41) and postischemic reperfusion arrhythmias (42) in the rat heart.

It has been argued (43) that manganese-salens may participate in a much wider range of cellular reactions. Given their chemical structure, these compounds may also remove radical species that are produced as a part of normal metabolic processes and, as such, could act as antimetabolites, slowing down metabolism and indirectly preventing oxidative damage (43). These considerations warrant future investigation of the precise mode of action of manganese-salens before ascribing its protective properties exclusively to removal of superoxide. Despite this cautionary note, the protective effect of EUK-8 supplementation in the AIF hypomorphic mutant is likely more related to removal of excess superoxide, because pressure-overloaded Hq heart

muscle demonstrates increased oxidative stress which was reduced to baseline levels by EUK-8 supplementation (Fig. 1).

Fundamental and clinical perspectives. The ramifications of the present study are several. First, because Hq mutant mice display numerous aspects of premature-aging syndrome, this mouse model may serve as a new and unique genetic model to study the degenerative processes of cardiac aging. Second, using a pharmacologic antioxidant approach, we “corrected” the genetic defect underlying the susceptibility of the Hq mouse heart to cope with injury in the form of sustained pressure overload, unveiling the precise nature of function of AIF (free radical scavenging) in cardiac tissue and, possibly, in other cell types. Finally, these observations in AIF hypomorphic mice suggest that the class of synthetic catalytic “mitoprotective” antioxidants exemplified by EUK-8 can permeate the myocardium, gain access to the mitochondria, and attenuate the mitochondrial damage attributable to oxidative stress as well as the secondary reactions resulting from oxidative stress after pressure overload, including DNA damage, cell death, fibrosis, unfavorable left ventricular remodeling, and mortality. Because EUK-8 also protected pressure-overloaded WT mice, the present study provides the first preclinical data suggesting the potential efficacy of a complete new class of drugs for heart disease for the general population. Given the ambiguous reports of the efficacy of tested antioxidants in heart failure, our findings with EUK-8 open the avenue for this class of membrane-permeable drug for therapeutic clinical use in patients.

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