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CYP2J2-derived EETs Suppress ER Stress in Heart failure

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Running title: CYP2J2-EETs attenuate ER stress in heart

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Non-standard abbreviations:

14,15-DHET: 14,15-dihydroxyeicosatrienoic acid; AA: arachidonic acid; ATF6: activation of transcription factor 6; Ang II: angiotensin II; CHOP: C/EBP homologous protein; CYP2J2: Cytochrome P450 epoxygenase 2J2; DHE: Dihydroethidium; DMEM: Dulbecco's modified Eagle's medium; dP/dt max: maximal slope of systolic pressure increment; dP/dt min: diastolic pressure decrement; Ea: arterial elastance; EET: epoxyeicosatrienoic acids; ER:

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endoplasmic reticulum; FBS: fetal bovine serum; GRP78: glucose-regulated protein 78 kDa; IRE1α: inositol-requiring transmembrane kinase and endonuclease 1α; ISO: isoproterenol; JNK: c-JUN NH2-terminal kinase; LV: left ventricular; LVEDP: LV end diastolic pressure; LVESP: left ventricular end systolic pressure; PERK: RNA-dependent protein kinase–like ER kinase; PI: propidium iodide; PLB: phospholamban; SERCA2a: sarcoplasmic/endoplasmic reticulum calcium ATPase; TG: Thapsigargin; TM: tunicamycin;

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Abstract

Prolonged endoplasmic reticulum (ER) stress causes apoptosis and is associated with heart failure. Whether CYP2J2 and its arachidonic acid metabolites (epoxyeicosatrienoic acids or EETs) have protective influence on ER stress and heart failure has not been studied. Assays of myocardial samples from patients with end-stage heart failure showed evidence of ER stress. Chronic infusion of isoproterenol (ISO) or angiotensin II (Ang II) by osmotic mini-pump induced cardiac hypertrophy and heart failure in mice as evaluated by hemodynamic measurements and echocardiography. Interestingly, transgenic (Tr) mice with cardiomyocyte-specific CYP2J2 expression were protected against heart failure compared to wild-type mice. ISO or Ang II administration induced ER stress and apoptosis, and increased levels of intracellular Ca^{2+}. These phenotypes were abolished by CYP2J2 overexpression *in vivo* or exogenous EETs treatment of cardiomyocytes *in vitro*. ISO or Ang II reduced SERCA2a expression in hearts or isolated cardiomyocytes; however, loss of SERCA2a expression was prevented in CYP2J2 Tr hearts *in vivo* or in cardiomyocytes treated with EETs *in vitro*. The reduction of SERCA2a activity was concomitant with increased oxidation of SERCA2a. EETs reversed SERCA2a oxidation through increased expression of antioxidant enzymes and reduced reactive oxygen species levels. Tempol, a membrane-permeable radical scavenger, similarly decreased oxidized SERCA2a levels, restored SERCA2a activity, and markedly reduced ER stress response in the mice treated with ISO. In conclusion, CYP2J2-derived EETs suppress ER stress

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response in the heart and protect against cardiac failure by maintaining intracellular Ca^{2+} homeostasis and SERCA2a expression and activity.

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Introduction

The endoplasmic reticulum (ER) is a central organelle of eukaryotic cells that participates in lipid synthesis, protein folding and maturation, and calcium storage (Lin JH et al., 2008). Various cellular stresses, such as ischemia, hypoxia, oxidative stress, reactive oxygen species, Ca^{2+} depletion of ER stores, and excessive accumulation of unfolded protein can lead to impairment of ER function (Marciniak SJ and Ron, 2006; Xu C et al., 2005). The accumulation of unfolded protein causes activation of transmembrane sensors/transducers, including inositol-requiring transmembrane kinase and endonuclease 1α (IRE1α), RNA-dependent protein kinase–like ER kinase (PERK), and activation of transcription factor 6 (ATF6) (Kaufman, 2002). Activation of these sensors induces a marked upregulation of ER chaperones such as glucose-regulated protein 78 kDa (GRP78) and GRP94. When ER stress is excessive and/or prolonged, initiation of the apoptotic pathways are triggered by induction of C/EBP homologous protein (CHOP), activation of c-JUN NH2-terminal kinase (JNK), or cleavage of caspase-12 (Kaufman, 2002; Mori, 2000). Increasing evidence suggests that ER stress-mediated apoptosis is involved in numerous diseases including heart failure (Lindholm et al., 2006; Ozcan, 2004; Wellen, 2005; Wencker, 2003).

Ca^{2+} storage and signaling, as well as the folding, modifying and packaging of newly synthesized proteins, are among the main functions of the ER in mammalian cells (Orrenius et al., 2003). It has long been known that Ca^{2+}

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signaling governs vital cell functions which are necessary for cell survival. More recently it has become clear that cellular Ca^{2+} overload, perturbation of intracellular Ca^{2+} compartmentalization, or depletion of the ER Ca^{2+} pool can result in ER stress and ER stress-mediated apoptosis (Orrenius et al., 2003). The cardiac isoform of the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA2a), which transfers calcium ions from the cytosol of the cardiomyocyte to the lumen of the endoplasmic reticulum during muscle relaxation, is a Ca^{2+} pump powered by ATP hydrolysis (Kawase and Hajjar, 2008). The pivotal role of SERCA2a in cardiomyocyte Ca^{2+} homeostasis has been confirmed. Several reports indicate that decreased SERCA2a protein levels and activity are a hallmark of heart failure in both human and experimental animal models (Meyer et al., 1995; Minamisawa et al., 1999). Restoration of SERCA2a expression by gene transfer effectively improves cardiac function in animal models (Adachi et al., 2004), as well as in heart failure patients (Jessup et al., 2011). Previous studies demonstrated that the activity of SERCA2a is decreased by reactive oxygen species (ROS) which are increased in heart failure (Kaneko et al., 1989a; Kaneko et al., 1989b; Liu et al., 2010). Indeed, several thiol residues in SERCA2a are potential targets for oxidation (Adachi et al., 2004; Cohen and Adachi, 2006; Dremina et al., 2007; Gutierrez-Martin et al., 2004). Cys674, the most important thiol residue required for SERCA2a activity, is known to be oxidized during oxidative stress (Adachi et al., 2004).

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Cytochrome P450 epoxygenase 2J2 (CYP2J2) is abundantly expressed in the human myocardium and metabolizes arachidonic acid (AA) to four biologically active epoxyeicosatrienoic acid (EET) regioisomers (5,6-, 8,9-, 11,12-, and 14,15-EET) (Zhao et al., 2012). Over the past 2 decades, it has become increasingly evident that CYP2J2-derived EETs exert many protective effects on the cardiovascular system. These effects include inhibition of cytokine-induced vascular cell adhesion molecule expression and leukocyte adhesion to the vascular wall (Node et al., 1999), upregulation of eNOS in endothelial cells (Jiang et al., 2007), protection of endothelial cells from apoptosis (Yang et al., 2007), and promotion of endothelial cell proliferation and angiogenesis via activation of MAP kinases and PI3 kinase/Akt signaling (Jiang et al., 2007). Importantly, 11,12-EET can induce antioxidant enzymes such as SODs and catalase which can reduce intracellular ROS level and attenuate several major apoptotic signaling events (Liu et al., 2011). Recently, Xu et al reported that CYP2J3 overexpression and increased EETs prevented high fat diet-induced ER stress in adipose tissue of rats (Xu et al., 2013). However, the role of EETs in ER stress and heart failure remain unknown. The goal of the present study was to establish whether EETs function as physiological inhibitors of aberrant ER stress and heart failure via their antioxidant effects.

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Materials and Methods

Antibodies and Reagents

Thapsigargin (TG), tunicamycin (TM), isoproterenol (ISO), angiotensin II (Ang II), N-Ethylmaleimide, Diethylenetriaminepentaacetic acid and MES were purchased from Sigma-Aldrich (St. Louis, MO).Antibodies for p-PERK, ATF6, GRP78, p-JNK, CHOP, p-CaMK II and β-actin were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibody for p-IRE1 was from Thermo Scientific Pierce Antibodies (Rockford, IL). Antibody for GRP94 was from Cell Signaling Technology (Beverly, MA). Antibody for caspase-12 was from Chemicon (Millipore, Billerica, MA). Dihydroethidium (DHE) was from Invitrogen (Carlsbad, CA). DeadEnd Fluorometric TUNEL System was from Promega (Madison, WI). 8,9-EET, 11,12-EET and 14,15-EET were from Cayman Chemical Company (Ann Arbor, MI). pCMV6-SERCA2a was from Origene Inc. All other reagents were purchased from commercial suppliers unless otherwise indicated.

Preparation of Human Heart Samples

Samples of human cardiac tissue were obtained under a protocol approved by the Institutional Review Board of Tongji Hospital and Tongji Medical College. The investigation conforms to the principles outlined in the Declaration of Helsinki. Subjects recruited to the study provided written informed consent. Human heart failure left ventricular (LV) specimens were obtained at the time of heart transplantation. Normal human heart tissue was

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obtained from traffic accident victims. Tissue samples were frozen in liquid nitrogen and then stored at -80°C until use.

Animals

Cardiac-specific CYP2J2 transgenic (Tr) mice on a pure C57BL/6 genetic background with transgene expression driven by the αMHC promoter were obtained from Dr. Darryl C. Zeldin at the National Institute of Environmental Health Sciences, NIH and were bred in the Experimental Animal Center of Tongji Medical College (Wuhan, China). All animal experimental protocols complied with the *NIH Guide for the Care and Use of Laboratory Animals*. The study was approved by the Institutional Animal Research Committee of Tongji Medical College. Mice were housed at the animal care facility of Tongji Medical College at 25°C with 12/12-h light/dark cycles and allowed free access to normal mice chow and water throughout the study period. Mice were randomly assigned to different treatment groups.

Animal treatment

C57BL/6 (WT) (n=15) and αMHC-2J2 Tr (n=15) mice were implanted with mini-osmotic pumps (Alzet model 1007D; DURECT Corp., Cupertino, California) as described previously (Son et al., 2010). Pumps were filled with isoproterenol (ISO) dissolved in 0.002% ascorbic acid or angiotensin II (Ang II) dissolved in saline to deliver at rates of 30ug/g/d or 1.5ug/kg/min, respectively, for 14 days. Control mice were implanted with pumps that delivered 0.002% ascorbic acid or saline. In separate experiments, C57BL/6 mice were

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implanted with mini-osmotic pumps with vehicle or ISO (30ug/g/d) for 14 days. Half the mice from each group were treated for 14 days with normal drinking water or water containing Tempol (Sigma, St. Louis, MO), a membrane-permeable radical scavenger, at a concentration of 400 mg/l.

Hemodynamic Measurements

Mice were anesthetized with pentobarbital (100 mg/kg i.p.) and placed on heating pads to maintain a core temperature of 37°C. A microtip pressure-volume catheter (SPR-839; Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced into the LV under pressure control as described (Pacher P et al., 2003). After stabilization for 20 minutes, the signals were recorded continuously at a sampling rate of 1,000/s using an ARIA pressure-volume conductance system (Millar Instruments) coupled to a Powerlab/4SP analog-to-digital converter (ADinstruments, Mountain View, CA) and then stored and displayed on a personal computer. Heart rate, LV end diastolic pressure (LVEDP), left ventricular end systolic pressure (LVESP), arterial elastance (Ea), tauWeiss, maximal slope of systolic pressure increment (dP/dt max) and diastolic pressure decrement (dP/dt min) were analyzed and corrected according to *in vitro* and *in vivo* volume calibrations with a cardiac pressure-volume analysis program (PVAN3.6; Millar Instruments).

Analysis of Cardiac Function by Echocardiography

Cardiac function was assessed by echocardiography, using a Visual sonic Vevo 2100 ultrasound machine equipped with a 30-Mhz transducer

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applied to the chest wall. Ventricular dimensions and ejection fraction were assessed using short axis view in two-dimensional and three-dimensional modes using the onboard Visual Sonics cardiac analysis package (He et al., 2012; Oikonomopoulos et al., 2011). Abbreviations: LVEDP, left ventricular end diastolic pressure; IVSd, interventricular septal thickness in diastole; IVSs, interventricular septal thickness in systole; LVIDd, left ventricular internal dimension in diastole; LVIDs, left ventricular internal dimension in systole; LVPWd, left ventricular posterior wall thickness in diastole; LVPWs, left ventricular posterior wall thickness in systole; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening.

Cell Culture

H9c2 cells, a subclone of the original clonal cell line derived from embryonic BD1X rat heart tissue, were obtained from American Type Culture Collection (CRL-1446). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 IU/ml) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Western Blot Analysis

Proteins were analyzed by SDS-PAGE and blotted using standard protocols (Duan et al., 2012). Expression was quantified by densitometry and normalized to β-actin expression. All groups were then normalized to their respective controls.

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Hoechst Staining.

Cultured cells were suspended at approximately 1-2×10^6/ml in buffered media (pH 7.2) and incubated with 10 mM Hoechst33258 dye at 37°C for 30 minutes. The cells then were observed under fluorescence microscope. Hoechst33258 stained cells were illuminated with an argon laser tuned for UV (346–352 nm) and the resulting fluorescence was detected at 460 nm.

Annexin V-FITC Apoptosis Assay.

Cultured cells were harvested with trypsin and resuspended in binding buffer. Five ul of Annexin V-FITC and 5 ul of propidium iodide (PI, 50 mg/ml) were added according to the manufacturer's protocol. Cells were then analyzed with a FACStar-Plus flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

TUNELAssay

Apoptosis in hearts was assayed with the DeadEnd Fluorometric TUNEL System (ProMega, Madison, Wis; No. G3250), following the manufacturer's instructions.

Measurement of Intracellular Ca^{2+}

H9C2 cells were cultured and pretreated with 14,15-EET one hour before treatment with ISO (10 uM) or Ang II (100nM). 24 hours later, intracellular Ca^{2+} concentration was measured using a Fluo-3 AM kit from Invitrogen according to instructions from the supplier , as previously described (Gao et al., 2011). Relative intracellular Ca^{2+} was expressed as percent of control.

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Microsome Preparation and SERCA2a Activity Measurements

Preparation of microsomes from H9C2 cells and heart tissues from mice or humans was performed as described previously (Dong et al., 2010). SERCA2a activity was measured using a Ca^{2+}-ATPase assay kit (Jiancheng Bio-engineering Institute, Nanjing, China) according to the manufacturer's instructions (Zhang et al., 2010). SERCA2a activity was normalized to protein concentration.

Biotinlated-iodoacetamide (b-IAM) Labeling of SERCA2a Cysteine-674.

Oxidation of Cys674 in SERCA2a in H9C2 cells and heart tissues of mice was assessed using the b-IAM labeling technique. b-IAM labeling of SERCA2a cysteine-674 was performed as previously described (Tong et al., 2008). Proteins were separated by SDS-PAGE and b-IAM labeled Cys674-SERCA2a was detected using SERCA2a specific antibody.

Detection of Reactive Oxygen Species

DCFH-DA was used as an ROS detection reagent using methods described previously (Liu et al., 2011). Cells were incubated with 10 μM DCFH-DA at 37°C for 20 min. Fluorescence was detected by flow cytometry (Liu et al., 2011). To detect superoxide, frozen heart tissue sections were incubated with dihydroethidium (D23107; Invitrogen) according to manufacturer's instructions (Son et al., 2010).

Evaluation of urine 14,15-DHET by ELISA

The stable 14,15-EET metabolite, 14,15-dihydroxyeicosatrienoic acid

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(14,15-DHET), was detected in the urine of mice using an ELISA kit (Detroit R&D) according to the manufacturer's instructions as previously described (Jiang et al., 2005).

Statistical Analysis

Continuous data are expressed as mean ± SEM. Group differences of continuous variables were compared by 1-way ANOVA or Student's t test as appropriate. Relationships between variables were determined by the Pearson correlation coefficient. $P<0.05$ was accepted as statistically significant.

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Results

Induction of ER stress in Failing Human Hearts

Similar to previous reports (Fu et al., 2010; Ni et al., 2011; Okada et al., 2004), ER stress and its associated apoptosis signaling pathways were a common occurrence in failing human hearts. Importantly, the expression of SERCA2a protein was significantly decreased in failing human hearts, which is consistent with previous studies (Meyer et al., 1995; Minamisawa et al., 1999; Zarain-Herzberg et al., 1996). The decrease in SERCA2a protein levels in failing hearts was accompanied by a reduction in SERCA2a activity (Fig. 1). pCMV6-SERCA2a was transfected into HEK293 cells as a positive control (Supplemental Figure 1).

Attenuation of Cardiac Hypertrophy and Dysfunction Induced by ISO or Ang II in αMHC-2J2 Tr Mice

CYP2J2 protein levels were determined in the heart tissues of WT and αMHC-2J2Tr mice. As showed in Fig. 2A, CYP2J2 protein was abundant in αMHC-2J2 Tr mice. Some cross-reactivity of the antibody with endogenous murine CYP2J proteins can be observed in WT mouse hearts. Here, cardiomyocytes were transfected with rAAV-CYP2J2 or rAAV-GFP, and the expression of CYP2J2 was determined as a positive control (Supplemental Figure 2). CYP2J2 functionality was demonstrated by the nearly four-fold increase in urinary 14,15-DHET levels in CYP2J2 Tr mice compared to WT mice (Fig. 2B).

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To investigate the effect of cardiomyocyte-specific CYP2J2 expression on ER stress signaling of failing hearts *in vivo*, we induced heart failure by continuous infusion of isoproterenol (ISO) for 2 weeks in WT and CYP2J2 Tr mice. There were no significant differences in baseline body weight and hemodynamic parameters between WT and CYP2J2 Tr mice (data not shown). On gross examination, CYP2J2 Tr mice showed less enlargement of the heart compared with WT mice after ISO treatment for 2 weeks (Fig. 2C, Supplemental Figure 3A). Consistent with this observation, the ratio of heart weight to body weight (HW/BW) at 2 weeks after ISO treatment was smaller in CYP2J2 Tr mice than in WT mice (Fig. 2D).

Heart function was examined by echocardiography and invasive pressure-volume analysis. As shown in Table 2, cardiac function in WT mice treated with ISO was decreased in comparison to that of CYP2J2 Tr mice. Thus, both LVIDd and IVSd were increased in WT mice treated with ISO compared with CYP2J2 Tr mice. WT mice also showed markedly reduced LVEF, LVFS, dP/dtmax and dP/dtmin, and increased left ventricular end diastolic pressure (LVEDP) compared to CYP2J2 Tr mice (Table 2).

A second animal model of heart failure compared WT and CYP2J2 Tr mice after continuous infusion of angiotensin II (Ang II) for 2 weeks. Results were very similar to those observed with ISO. CYP2J2 Tr mice showed reduced heart failure compared to WT mice (Figs. 2E and F, Table 2, Supplemental Figure 3B). Taken together, these data demonstrate that

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CYP2J2 Tr mice are protected against cardiac hypertrophy and decline of cardiac function in models of heart failure.

ER Stress Signaling in Failing Hearts of WT and CYP2J2 Tr Mice

We investigated ER stress signaling in failing hearts of WT and CYP2J2 Tr mice. Western blots showed that expression levels of ER stress molecules, glucose-regulated protein 78 kDa (GRP78), C/EBP homologous protein (CHOP) and cleaved ATF6 (c-ATF6) were markedly elevated, and PERK (p-PERK) and IRE-1 (p-IRE-1) as well as c-JUN NH2-terminal kinase (JNK) were markedly activated in failing hearts of WT mice after ISO or Ang II treatment (Figs. 3A-D). Interestingly, induction of these ER stress initiated signaling markers was suppressed in hearts from CYP2J2 Tr mice (Figs. 3A-D). These data provides compelling evidence that the activation of ER stress-mediated apoptotic signaling pathways in the failing hearts is attenuated by CYP2J2 expression. We also employed TUNEL-staining to quantify the number of cardiomyocytes undergoing apoptosis in the two groups. Compared to vehicle-treated controls, ISO- or Ang II-treated hearts from WT mice exhibited an increase in the percentage of cardiomyocyte-shaped, TUNEL-positive cells. In comparison, CYP2J2 Tr mice displayed fewer TUNEL-positive cardiomyocytes after ISO or Ang II treatment (Fig. 3E). These data suggest that ER stress and ER stress-mediated apoptosis are inhibited in CYP2J2 Tr mice.

Restoration of SERCA2a Expression and activity in CYP2J2 Tr Mice

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Elevation of intracellular Ca^{2+} is a common mechanism for aberrant ER stress and ER Stress-mediated apoptosis (Biagioli et al., 2008; Deniaud et al., 2008; Orrenius et al., 2003). Therefore, we assessed the activation of Calmodulin kinase II (CaMKII), a Ca^{2+}-dependent kinase, which can elevate intracellular Ca^{2+} levels, in the failing hearts of mice (Dzhura et al., 2000). CaMKII was significantly activated in the failing hearts of WT mice, as determined by an increase in phosphorylated CaMKII (p-CaMKII), relative to normal hearts (Fig. 4A). CaMKII phosphorylation was attenuated in CYP2J2 Tr Mice treated with ISO or Ang II compared with WT mice (Fig. 4A). These results suggest that CYP2J2 decreases activation of CaMKII and subsequent increase in the intracellular Ca^{2+} levels during ER stress.

SERCA2a plays an important role in maintaining intracellular Ca^{2+} homeostasis through its ability to pump cytosolic Ca^{2+} into the SR/ER stores (Cohen et al., 1999; Tong et al., 2008). Because the intracellular Ca^{2+} levels were elevated in failing mouse hearts, we reasoned that SERCA2a activity may have been inhibited. Indeed, the expression of SERCA2a was downregulated in WT mice exposed to ISO or Ang II. Interestingly, this effect was reversed by expression of CYP2J2 in transgenic mice (Fig. 4B). We further examined SERCA2a activity by measuring the inorganic phosphate (Pi) liberated from ATP hydrolysis. Both ISO and Ang II markedly reduced SERCA2a activity in WT mice compared to CYP2J2 Tr mice (Fig. 4C). Thus, expression and activity of SERCA2a were decreased in failing mouse hearts,

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while CYP2J2 expression attenuated this decline.

Oxidation of Cys674 in SERCA2a inhibits SERCA2a activity (Adachi et al., 2004). Therefore, we determined whether the oxidation of SERCA2a at Cys674 was increased in failing hearts using the b-IAM labeling technique (Tong et al., 2008). b-IAM labeling suggests non-oxidized Cys674, which is conducive to normal SERCA2a function. Failing hearts in WT mice exhibited decreased levels of b-IAM-SERCA2a compared with normal hearts, which indicates both increased Cys674 oxidation and decreased SERCA2A function. CYP2J2 expression in transgenic mice significantly attenuated the decline in b-IAM-SERCA2a in both ISO and Ang II models of heart failure (Fig. 4B). Thus, CYP2J2 at least partially prevented SERCA2a oxidation which is normally increased in failing WT mouse hearts.

A growing body of evidence suggest that ROS is increased and plays an important role in heart failure (Liu et al., 2010; Seddon et al., 2007), and that ROS depresses the activity of SERCA2a (Kaneko et al., 1989a; Kaneko et al., 1989b). In addition, CYP2J2 overexpression has been shown to suppress ROS levels (Liu et al., 2011). Therefore, we sought to determine whether CYP2J2 Tr hearts restored SERCA2a activation through reduction of ROS levels. As shown in Figure 4D, CYP2J2 expression significantly decreased the cellular levels of ROS compared with WT mice treated with ISO or Ang II. Tempol, a potent antioxidant, also significantly increased SERCA2a activity and the amount of b-IAM–SERCA2a in ISO-infused mice. Tempol did not affect

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SERCA2a protein levels but reduced the expression of ER stress markers (Figs. 4E-G). Taken together, this data suggests that the reduced oxidation of SERCA2a and protection against heart failure in CYP2J2 Tr hearts may occur at least partly through suppression of ROS levels.

The reduction in ROS levels in CYP2J2 Tr mice appears to occur through upregulation of anti-oxidant enzymes. As seen in Figure 4H, expression of ZnCu-SOD, Mn-SOD and catalase are suppressed in WT mice treated with ISO or Ang II. CYP2J2 expression protected against the decline in each of these enzymes. Therefore, attenuation in the reduction of ZnCu-SOD, Mn-SOD and catalase protein levels may be involved in the maintenance of SERCA2a activity in CYP2J2 Tr hearts during models of heart failure.

14,15-EET Reduced ER Stress Signaling and Apoptosis in Cultured Cardiomyocytes

EETs are the major metabolites of CYP2J2.To investigate the role of EETs on ER stress signaling *in vitro*, H9C2 cells were cultured with 8,9-EET, 11,12-EET, or 14,15-EET (1 uM each) for one hour before treatment with tunicamycin (TM) for 24 hours. Cell lysates were assayed by Western blotting. Each of the EETs decreased ER stress, and 14,15-EET exhibiting the greatest effect (Supplemental Figure 4A). We also treated H9C2 cells with 14,15-EET at different concentrations (50-1000 nM). The results showed that 14,15-EET significantly decreased GRP78 expression in a dose-dependent manner (Supplemental Figure 4B).

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We investigated the inhibition of 14,15-EET on ER stress signaling in cultured cells. H9C2 cells were treated with thapsigargin (TG) or TM (as positive controls for ER stress), ISO or Ang II and14,15-EET. Similarly to the results in *in vivo* study in CYP2J2 Tr mice (Fig. 3A-D), addition of exogenous 14,15-EET *in vitro* significantly decreased activation of ER stress pathway and ER stress-mediated apoptosis induced by these pharmacological agents TG (Fig. 5A), TM (Fig. 5), ISO (Fig. 5C) and Ang II, respectively (Fig. 5D). To confirm the importance of EETs in our models, we used 14,15-EEZE, a selective EET signaling antagonist (Gauthier et al., 2004; Seubert et al., 2006). 14,15-EEZE abolished the benefits of 14,15-EET on ER stress signaling (Figs. 5A-D). ER stress-mediated apoptosis, Hoechst staining and Annexin V-FITC binding showed similar results (Figs. 5E-H). Also, results showed that the inhibitory effects of CYP-2J2 overexpression on ER stress were attenuated by 14,15-EEZE (Supplemental Figure 2). Thus, EETs inhibit *in vitro* ER stress signaling and apoptosis in cardiomyocytes induced by a variety of stressors.

14,15-EET Upregulated the Expression and Activity of SERCA2a in Cardiomyocytes

Loss of Ca^{2+} homeostasis is a hallmark of cardiac failure. We examined the effect of EETs to restore Ca^{2+} levels in ER-stressed cardiomyocytes. Indeed, 14,15-EET reduced intracellular Ca^{2+} levels in H9C2 cells exposed to ISO or Ang II (Fig. 6A). 14,15-EET also attenuated ISO- or Ang II-induced the overexpression of p-CaMKII (Fig. 6B). These data suggest that 14,15-EET

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suppresses the elevation of intracellular [Ca^{2+}] induced by pharmacological stress agents *in vitro*. We next determined whether the upregulation of intracellular Ca^{2+} levels triggered an aberrant ER stress response in H9C2 cells. We assessed whether BAPTA (20uM), an intracellular Ca^{2+} chelator (Harrison and Bers, 1987), inhibited the ER stress response in H9C2 cells. Results showed that the addition of BAPTA attenuated the ER stress response and ER stress-mediated apoptosis in H9C2 cells exposed with ISO or Ang II (Fig. 6C).

The effect of EETs on the expression and activation of SERCA2a was assessed in H9C2 cells exposed to ISO or Ang II. 14,15-EET significantly alleviated the changes in expression and activity of SERCA2a in H9C2 cells treated with ISO or Ang II (Figs. 7A and B). A growing body of evidence suggested that ROS can depress the activity of SERCA2a (Kaneko et al., 1989a; Kaneko et al., 1989b). Treatment of H9C2 cells with ISO or Ang II for 24 hours led to a significant increase in ROS production. Interestingly, preincubation with 14,15-EET significantly attenuated ISO- or Ang II-induced ROS production in cardiomyocytes measured by flow cytometry (Fig. 7C).

We further determined whether oxidation of SERCA2aCys674 was reduced in H9C2 cells treated with 14,15-EET. The data showed that 14,15-EET markedly reduced the oxidation of SERCA2a at Cys674 induced by ISO or Ang II in these cells using the b-IAM labeling technique (Fig. 7A) (Tong et al., 2008). We also determined the expression of antioxidant enzymes in

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H9C2 cells. Similar to that observed in CYP2J2 transgenic hearts, 14,15-EET increased ZnCu-SOD, Mn-SOD and catalase protein levels (Fig. 7D).

These data suggest that intracellular Ca^{2+} overload triggers ER stress. CYP2J2 expression or EET treatment markedly attenuate the loss of antioxidant enzymes, intracellular Ca^{2+} overload and ER stress via maintenance of SERCA2a expression and function.

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Discussion

This study provides evidence for CYP2J2-derived EETs as important regulators of intracellular Ca^{2+} levels and ER homeostasis *in vivo* and *in vitro*. These actions are mediated through maintenance of SERCA2a expression and suppression of SERCA2a oxidation. We found that the levels of ER stress and associated apoptosis were significantly increased in hearts of mice treated with ISO or Ang II, as well as in cardiac tissue from heart failure patients. Consistent with previous studies, our data suggest that ER stress-initiated apoptosis plays an important role in cardiomyocyte apoptosis in failing hearts (Fu et al., 2010; Ni et al., 2011; Okada et al., 2004). CYP2J2 expression significantly reduced ER stress and associated apoptosis and attenuated the development of heart failure *in vivo*. Interestingly, administration of tauroursodeoxycholic acid, a chemical chaperone with clinical potential (Ozcan et al., 2006), significantly reduced the expression of ER stress markers and suppressed the development of heart failure in a manner similar to EETs (data not published). 14,15-EEZE, as selective EET antagonist, abolished these benefits. These results suggested that CYP2J2-derived EETs might confer their protective effects against heart failure by inhibiting the ER stress initiated apoptosis *in vivo* and *in vitro*.

Elevation of intracellular Ca^{2+} is a common mechanism for aberrant ER stress and ER Stress-mediated apoptosis (Biagioli et al., 2008; Deniaud et al., 2008; Orrenius et al., 2003). Intracellular Ca^{2+} chelator BAPTA inhibited

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aberrant ER stress induced apoptosis, which is consistently with other reports (Hajjar et al., 2008; Minamisawa et al., 1999). The pivotal role of SERCA2a in Ca^{2+} homeostasis has been described. Reports consistently show decreased levels of SERCA2a expression and activity in failing human hearts (Meyer et al., 1995; Minamisawa et al., 1999; Zarain-Herzberg et al., 1996). Accordingly, restoration of SERCA2a expression has proved to be effective in improving cardiac function in heart failure patients (Jessup et al., 2011) and in animal models of heart failure (Hajjar et al., 2008). In this study, we showed that SERCA2a protein levels and activity were reduced, levels of intracellular Ca^{2+} were increased and severe contractile dysfunction was present. CYP2J2 expression *in vivo* improved both systolic and diastolic function. *In vitro*, exogenous EETs supplementation attenuated the rise in intracellular Ca^{2+} and increased SERCA2a protein levels and activity. Thus, CYP2J2-derived EETs could maintain Ca^{2+} homeostasis and SERCA2a protein expression and activity.

A growing body of evidence suggested that ROS is increased and plays an important role in heart failure (Liu et al., 2010; Seddon et al., 2007), and ROS could depress the activity of SERCA2a (Kaneko et al., 1989a; Kaneko et al., 1989b). Indeed, we observed increased ROS generation in cardiac tissue or cardiomyocytes exposed to ISO or Ang II. Both CYP2J2 expression and exogenous EETs supplementation increased the expression of antioxidant enzymes (ZnCu-SOD, Mn-SOD and catalase) and attenuated the rise in ROS

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levels after ISO or Ang II. This may be, at least in part, the underlying mechanism by which EETs reduces ROS levels. However, there are also other possible mechanisms through which EETs may induce ROS scavenging. For example, EETs are reported to up-regulate heme oxygenase-1 (HO-1) in endothelial cells which results in decreased oxidative stress and a lower rate of apoptosis (Jozkowicz et al., 2007; Sacerdoti et al., 2007). The direct relationship between EETs and ROS has not been reported, but we could not exclude the possibility. CYP2J2-derived EETs thus limited oxidation of SERCA2a Cys674 *in vivo* and *in vitro.* Taken together, we conclude that CYP2J2-derived EET mediated inhibition of ER stress response is likely to occur through inhibition of oxidant-mediated SERCA2a.

SERCA2a activity can be affected by several processes, such as oxidation of the enzyme, a lower level of enzyme expression, reduced PKA activity, or reduced phosphorylation of phospholamban (PLB) (Muller and Simonides, 2005). In our study, we found the restoration of SERCA2a activity was mediated by inhibition of oxidant-mediated SERCA2a or upregulation of SERCA2a expression via CYP2J2-derived EETs; however, the relative contributions of these two mechanisms was not examined in this study. Many reports have demonstrated that EETs can exert effects through PI3-kinase-Akt signaling (Jiang et al., 2007; Node et al., 2006). Kim et al reported that upregulation of the SERCA2a protein can be mediated by the PI3-kinase-Akt-SERCA2a signaling cascade (Kim et al., 2008). Yu et al also

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reported enhancement of SERCA2a activity involved the PI3-kinase-Akt-eNOS pathway (Yu et al., 2006). Exactly how CYP2J2-derived EETs influence SERCA2a expression and activity warrants further investigation.

In summary, the present study provides insight on the protective role of CYP2J2-derived EETs in heart failure. The effects of CYP2J2 expression and EETs on cardiac protection are likely mediated through upregulation of antioxidant enzymes, suppression of ROS and inhibition of ER stress. These effects maintain SERCA2a activity and intracellular Ca^{2+} homeostasis. Our results suggest that CYP2J2-derived EETs may be a logical target for the development of drugs to prevent cardiac hypertrophy and cardiomyocyte apoptosis in failing hearts.

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Authership contributions

Participated in research design: Wang, Ni, Chen, D.W.Wang

Conducted experiments: Wang, Yang

Contributed new reagents or analytic tools: Wang, Duan

Performed data analysis: Wang

Wrote or contributed to the writing of the manuscript: Wang, Edin, Zeldin, D.W.Wang

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Footnotes

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Figure legends

Figure 1. SERCA2a expression and activity were reduced in failing human hearts. A, the expression ofSERCA2a in normal and failing human hearts is shown. N1 and N2, normal human hearts; P1-P6, failing human hearts. Corresponding clinical characteristics of the 6 patients with heart failure are shown in Table 1. B, SERCA2a activity was decreased in failing human hearts. Proteins were normalized to β-actin. N=4, P=6 (N, normal human hearts; P, failing human hearts); ***P*<0.05 vs. normal hearts.

Figure 2. Attenuation of cardiac hypertrophy and dysfunction induced by isoproterenol (ISO) or angiotensin II (Ang II) in CYP2J2 Tr mice. A, CYP2J2 protein level was increased in CYP2J2 Tr mice compared with WT mice. B, urinary 14,15-DHET levels were increased in CYP2J2 Tr mice compared with WT mice. N=10 per group; ***P*<0.05 vs. WT mice. C and E, representative gross appearance of hearts (left, scale bars=5mm) from CYP2J2 Tr and WT mice treated with ISO or Ang II, respectively. D and F, the ratio of HW/BW of CYP2J2 Tr and WT mice treated with ISO or Ang II, respectively. N=5; ***P*<0.05 vs. WT mice treated with saline, #*P*<0.05 vs. WT mice treated with ISO or Ang II.

Figure 3. ER stress signaling was suppressed in CYP2J2 Tr mice. A, ISO--induced ER stress and apoptosis was alleviated in CYP2J2 Tr mice. B,

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densitometric analysis of ER stress markers was shown. N = 5 per group. C, Ang II-induced ER stress and apoptosis were also inhibited in CYP2J2 Tr mice. D, densitometric analysis of ER stress markers was shown. N = 5 per group. E, representative images of TUNEL (original magnification ×400) showing cardiac myocytes apoptosis and quantitative analysis of TUNEL-positive myocardial cells in mice. Nuclei of normal cells are blue, and nuclei of apoptosis cells (TUNEL positive cells) from the same fields are identified by green fluorescence (scale bars=50um). N=5 ;***P*<0.05 vs. WT mice treated with saline, #*P*<0.05 vs. WT mice treated with ISO or Ang II.

Figure 4. CYP2J2 overexpression restored CaMKII and SERCA2a expression and activity in mice failing hearts. A, elevated CaMKII activation induced by ISO or Ang II was inhibited in CYP2J2 Tr mice. N = 5 per group. B, changes in b-IAM-SERCA2a and expression of SERCA2a were attenuated in CYP2J2 Tr mice exposed to ISO or Ang II. N = 5 per group. C, the activity of SERCA2a was restored in CYP2J2 Tr mice exposed to ISO or Ang II. N=5. D, the detection of ROS using dihydroethidium (DHE) staining (original magnification ×400) in mouse hearts (scale bars=50um). Quantification of ROS from 3 random fields per mouse. N=5 per group; ***P*<0.05 vs WT mice treated with saline, #*P*<0.05 vs. WT mice treated with ISO or Ang II. E-G, the effect of Tempol on SERCA2a expression and activity, b-IAM-SERCA2a levels, and expression of ER stress markers. N=5; ***P*<0.05 vs. control, #*P*<0.05 vs.

ISO. H, antioxidant enzyme expression in CYP2J2 Tr mice. N=5 per group; ***P*<0.05 vs. WT mice treated with saline, #*P*<0.05 vs. WT mice treated with ISO or Ang II.

Figure 5. 14,15-EET reduced ER stress signaling and apoptosis in cultured cardiomyocytes. A, effects of 14,15-EET and/or 14,15-EEZEon ER stress signaling induced by Thapsigargin (TG). B, effects of 14,15-EET and/or 14,15-EEZE on ER stress signaling induced by tunicamycin (TM). C, effects of 14,15-EET and/or 14,15-EEZE on ER stress signaling induced by ISO. D, effects of 14,15-EET and/or 14,15-EEZE on ER stress signaling induced by Ang II. E-H, Hoechst staining and Annexin V-FITC binding with flow cytometry analysis showed 14,15-EET inhibited apoptosis induced by ISO or Ang II, respectively. N=3 for each experiment; ***P*<0.05 vs. control, #*P*<0.05 vs. ISO or Ang II, ^*P*<0.05 vs. ISO+EET or Ang II+EET.

Figure 6. 14, 15-EET attenuates ER stress-induced rise of intracellular Ca^{2+} in cardiomyocytes. A and B, 14,15-EET attenuated the rise in intracellular Ca^{2+} levels and overexpression of p-CaMKII in H9C2 cells exposed to ISO or Ang II, respectively. N=3 for each experiment; ***P*<0.05 vs. control, #*P*<0.05 vs. ISO or Ang II. C, BAPTA attenuated the ER stress signaling in H9C2 cells exposed with ISO. N=3 for each experiment; ***P*<0.05 vs. control, #*P*<0.05 vs. ISO.

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Figure 7. 14, 15-EET preserved the expression and activity of SERCA2a in cardiomyocytes after ER stress. A, 14,15-EET attenuated the reduction of SERCA2a expression and prevented its oxidation in H9C2 cells treated with ISO or Ang II, respectively. B, 14,15-EET restored the activity of SERCA2a. N=3 for each experiment. C, 14,15-EETattenuated ISO- or Ang II-induced ROS production in cardiomyocytes. N=3 for each experiment. D, 14,15-EET cotreatment attenuated the loss of ZnCu-SOD, Mn-SOD and catalase protein levels observed in ISO or Ang II treated cells. N=3 for each experiment; ***P*<0.05 vs. control, #*P*<0.05 vs. Ang II.

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Table 1. Clinical characteristics of patients with heart failure

Patient	**1**	**2**	**3**	**4**	**5**	**6**
Age (y)	39	19	29	42	42	38
Gender	F	F	M	M	M	M
Diagnosis	DCM	DCM	DCM	DCM	DCM	DCM
Diabetes	N	N	N	N	N	N
Hypertension	N	N	N	N	N	N
NYHA Class	3	3	3	3	4	3
LVEF(%)	21	26	12	29	28	20

NYHA, New York Heart Association; LVEF, left ventricular ejection fraction; DCM, dilated cardiomyopathy

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Table 2. Hemodynamic and Echocardiographic parameters in CYP2J2 Tr and WT mice treated with ISO or Ang II.

	Saline		ISO		AngII	
	WT	Tr	WT	Tr	WT	Tr
N	5	5	5	5	5	5
dP/dtmax(mmHg/s)	11261±938.35	11376±1171.89	7164±777.67*	10104±981.64#	6941±977.32*	10591±1375.54&
dP/dtmin(mmHg/s)	-8439±1053.16	-8739±1025.11	-4915±782.48*	-6807±690.78#	-4584±554.01*	-6931.67±577.32&
LVEDP(mmHg)	4.68±1.28	5.09±1.39	12.83±3.00*	7.26±1.36#	12.78±2.30*	7.39±1.51&
IVSd(mm)	1.10±0.22	1.05±0.10	1.72±0.13*	1.17±0.18#	1.66±0.54*	1.10±0.27
IVSs(mm)	1.99±0.13	1.59±0.34	1.75±0.25	1.92±0.18	2.05±0.38	1.49±0.20
LVIDd(mm)	2.87±0.54	2.67±0.30	3.42±0.16	2.97±0.34	3.71±0.29	3.10±0.71&
LVIDs(mm)	1.07±0.41	1.05±0.34	2.23±0.29*	1.25±0.37#	2.77±0.43*	1.70±0.39&
LVPWd(mm)	1.10±0.52	1.00±0.40	1.65±0.46	1.38±0.34	1.74±0.29	1.25±0.35
LVPWs(mm)	1.71±0.80	1.33±0.29	2.10±0.36	1.90±0.08	2.11±0.58	1.46±0.40
LVEF(%)	90.55±5.69	91.18±3.98	63.29±6.97*	80.65±6.19#	61.87±8.30*	78.79±6.28&
LVFS(%)	68.19±10.61	72.47±12.26	41.28±4.98*	61.36±13.37#	40.99±16.06*	60.88±7.49&

dP/dtmax, maximal slope of systolic pressure increment; dP/dt min, maximal slope of diastolic pressure decrement; LVEDP, left ventricular end diastolic pressure; IVSd, interventricular septal thickness in diastole; IVSs, interventricular septal thickness in systole; LVIDd, left ventricular internal

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dimension in diastole; LVIDs, left ventricular internal dimension in systole; LVPWd, left ventricular posterior wall thickness in diastole; LVPWs, left ventricular posterior wall thickness in systole; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening. Values are mean ± SEM.*****P<0.05 vs. WT mice treated with saline, #P<0.05 vs. WT mice treated with ISO, &P<0.05 vs. WT mice treated with Ang II.

Figure 1

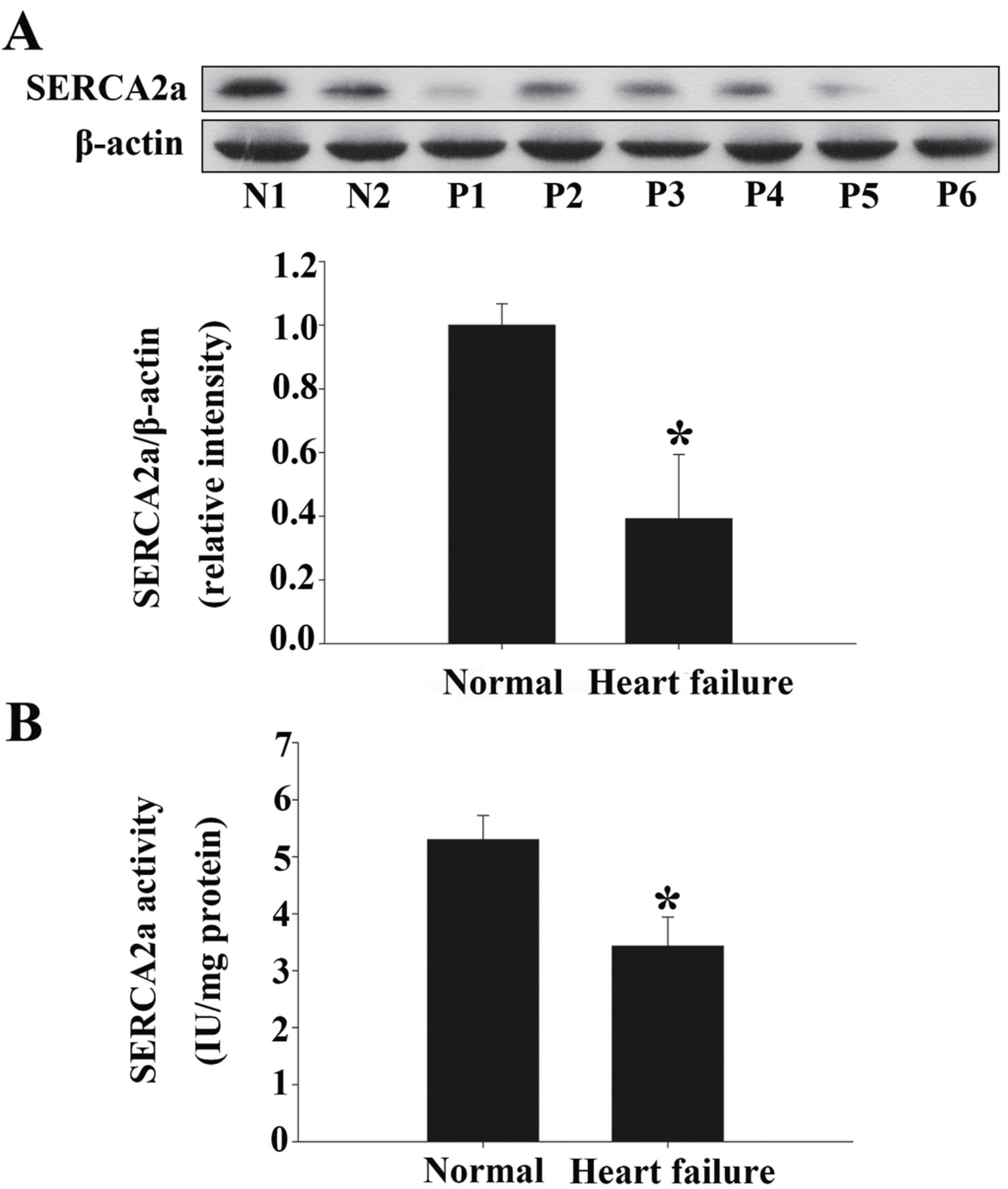

A
SERCA2a
β-actin
N1
N2
P1
P2
P3
P4
P5
P6
SERCA2a/β-actin
(relative intensity)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Normal
Heart failure
B
SERCA2a activity
(IU/mg protein)
7
6
5
4
3
2
1
0
*
Normal
Heart failure

Figure 2

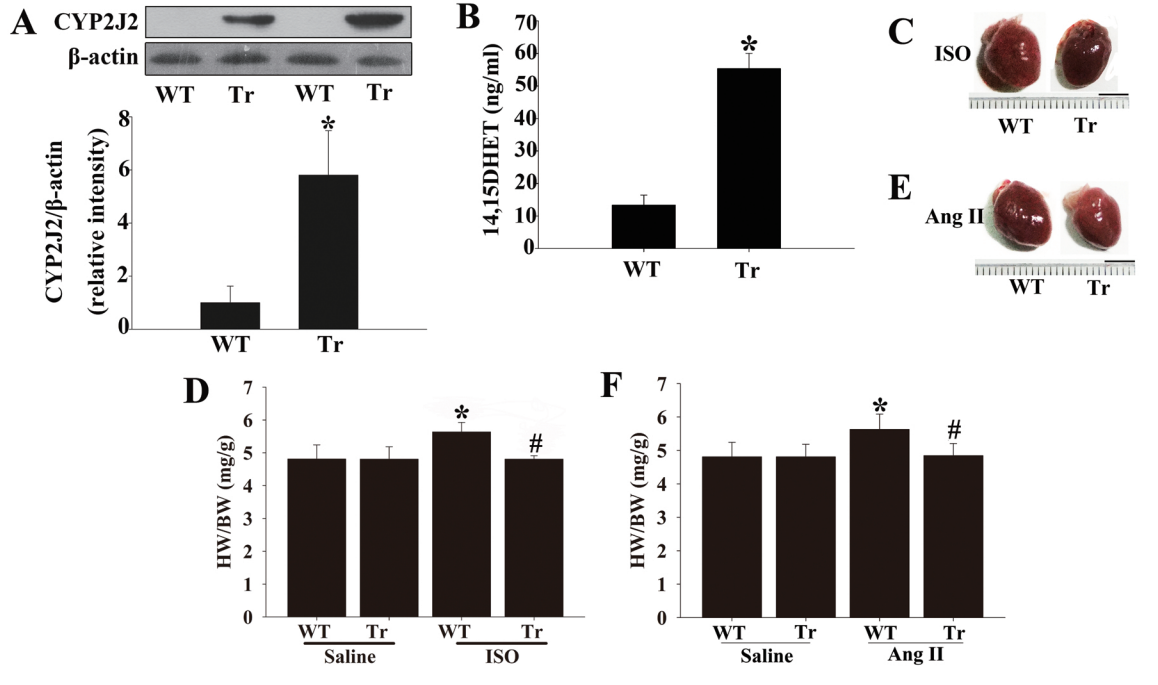

A
CYP2J2
β-actin
WT
Tr
WT
Tr
CYP2J2/β-actin
(relative intensity)
8
6
4
2
0
*
WT
Tr
B
14,15DHET (ng/ml)
70
60
50
40
30
20
10
0
*
WT
Tr
C
ISO
WT
Tr
E
Ang II
WT
Tr
D
HW/BW (mg/g)
7
6
5
4
3
2
1
0
*
#
WT
Tr
WT
Tr
Saline
ISO
F
HW/BW (mg/g)
7
6
5
4
3
2
1
0
*
#
WT
Tr
WT
Tr
Saline
Ang II

Figure 3

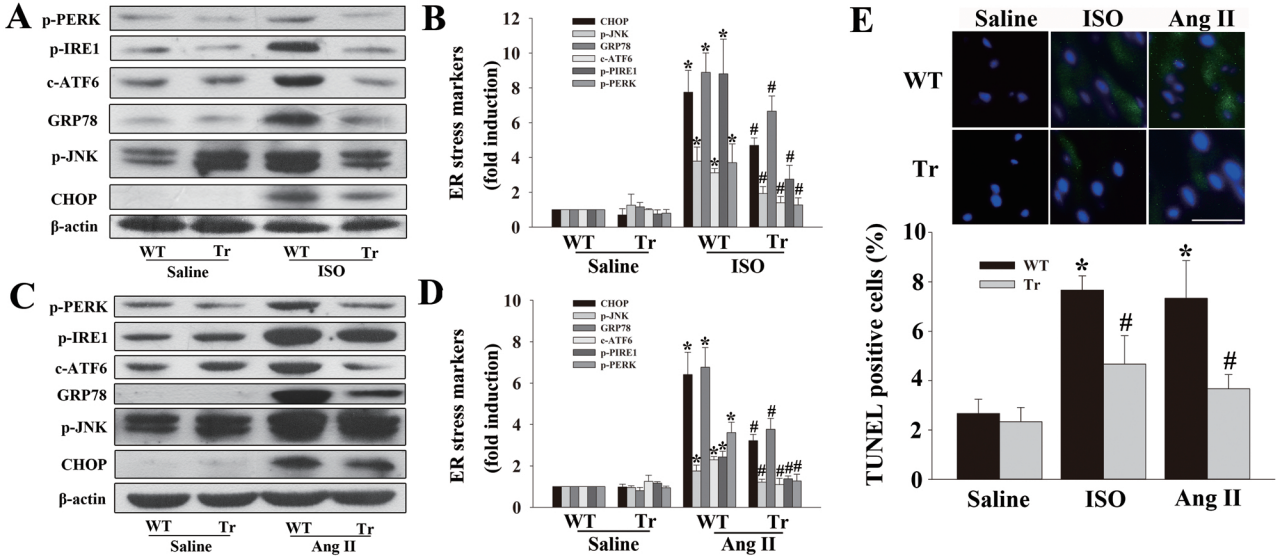

A
p-PERK
p-IRE1
c-ATF6
GRP78
p-JNK
CHOP
β-actin
WT Tr WT Tr
Saline ISO
B
CHOP
p-JNK
GRP78
c-ATF6
p-PIRE1
p-PERK
ER stress markers (fold induction)
WT Tr WT Tr
Saline ISO
C
p-PERK
p-IRE1
c-ATF6
GRP78
p-JNK
CHOP
β-actin
WT Tr WT Tr
Saline Ang II
D
CHOP
p-JNK
GRP78
c-ATF6
p-PIRE1
p-PERK
ER stress markers (fold induction)
WT Tr WT Tr
Saline Ang II
E
Saline ISO Ang II
WT
Tr
TUNEL positive cells (%)
WT
Tr
Saline ISO Ang II

Figure 4

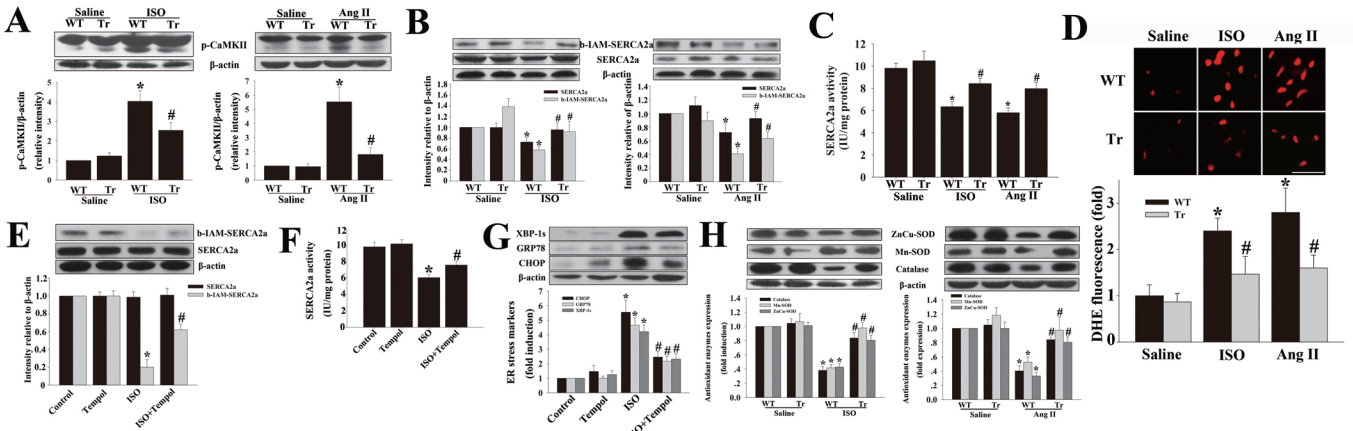

A
Saline
ISO
WT
Tr
p-CaMKII
β-actin
p-CaMKII/β-actin (relative intensity)
Ang II
B
b-IAM-SERCA2a
SERCA2a
Intensity relative to β-actin
C
SERCA2a avtivity (IU/mg protein)
D
DHE fluorescence (fold)
E
F
SERCA2a activity (IU/mg protein)
Control
Tempol
ISO+Tempol
G
XBP-1s
GRP78
CHOP
ER stress markers (fold induction)
H
ZnCu-SOD
Mn-SOD
Catalase
Antioxidant enzymes expression (fold induction)

Figure 5

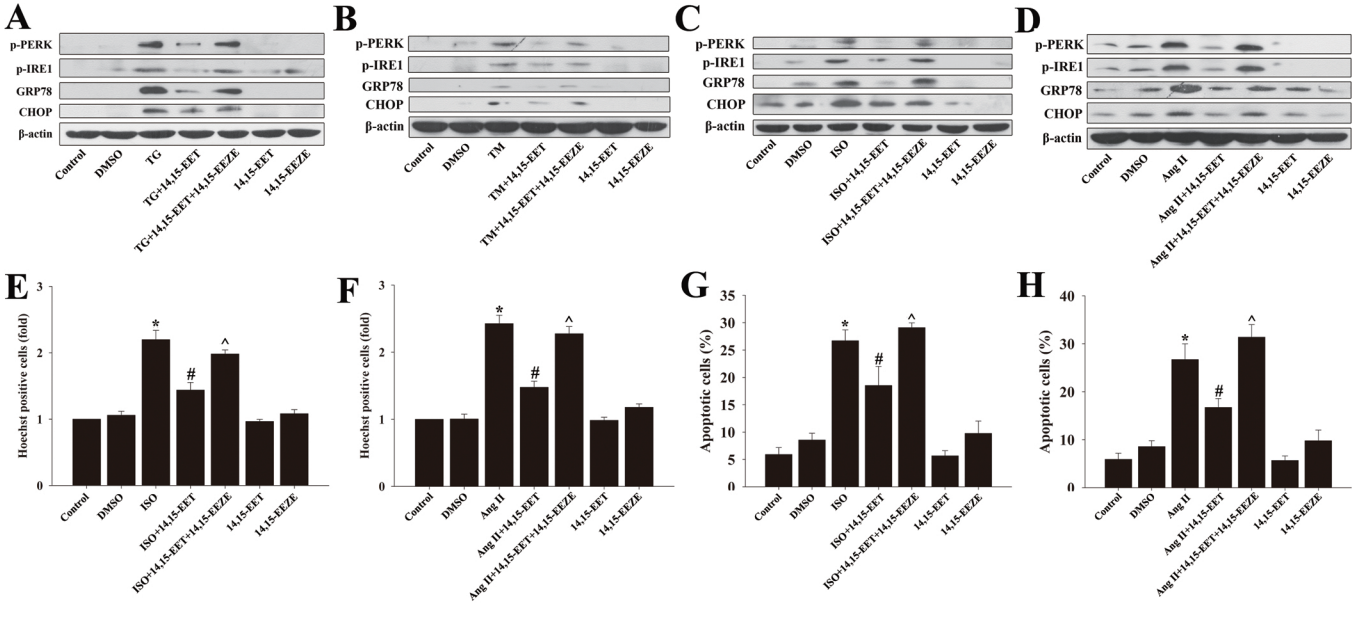

A
p-PERK
p-IRE1
GRP78
CHOP
β-actin
Control
DMSO
TG
TG+14,15-EET
TG+14,15-EET+14,15-EEZE
14,15-EET
14,15-EEZE
B
p-PERK
p-IRE1
GRP78
CHOP
β-actin
Control
DMSO
TM
TM+14,15-EET
TM+14,15-EET+14,15-EEZE
14,15-EET
14,15-EEZE
C
p-PERK
p-IRE1
GRP78
CHOP
β-actin
Control
DMSO
ISO
ISO+14,15-EET
ISO+14,15-EET+14,15-EEZE
14,15-EET
14,15-EEZE
D
p-PERK
p-IRE1
GRP78
CHOP
β-actin
Control
DMSO
Ang II
Ang II+14,15-EET
Ang II+14,15-EET+14,15-EEZE
14,15-EET
14,15-EEZE
E
Hoechst positive cells (fold)
Control
DMSO
ISO
ISO+14,15-EET
ISO+14,15-EET+14,15-EEZE
14,15-EET
14,15-EEZE
F
Hoechst positive cells (fold)
Control
DMSO
Ang II
Ang II+14,15-EET
Ang II+14,15-EET+14,15-EEZE
14,15-EET
14,15-EEZE
G
Apoptotic cells (%)
Control
DMSO
ISO
ISO+14,15-EET
ISO+14,15-EET+14,15-EEZE
14,15-EET
14,15-EEZE
H
Apoptotic cells (%)
Control
DMSO
Ang II
Ang II+14,15-EET
Ang II+14,15-EET+14,15-EEZE
14,15-EET
14,15-EEZE

Figure 6

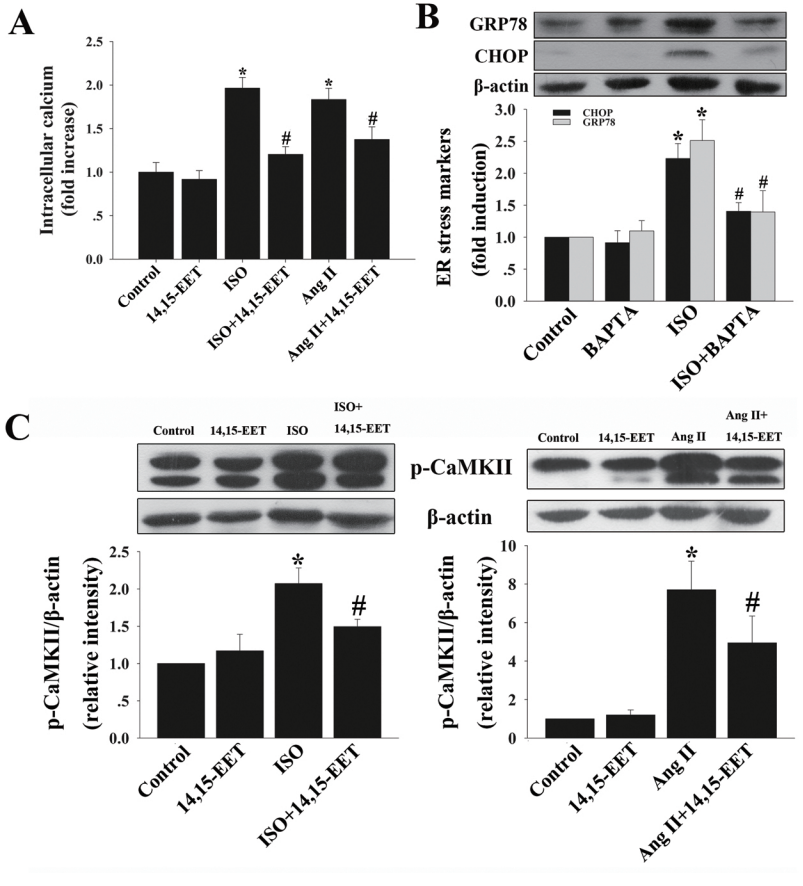
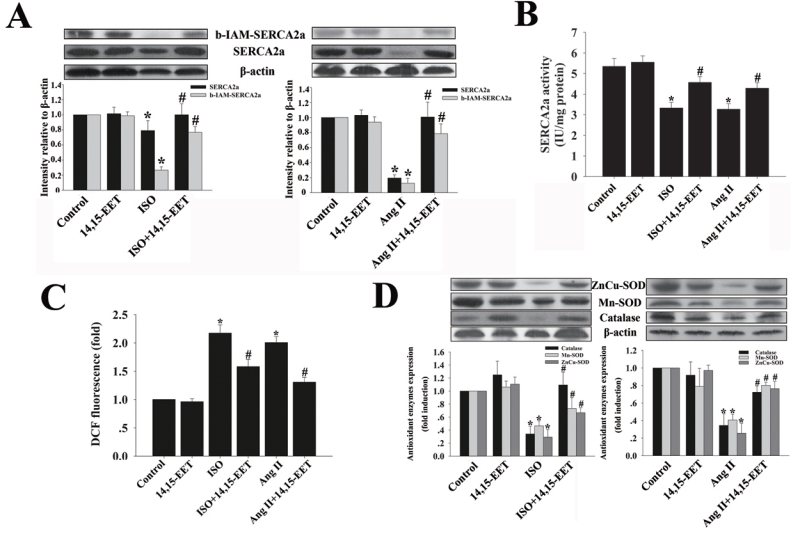

A
Intracellular calcium (fold increase)
2.5
2.0
1.5
1.0
.5
0.0
*
*
#
#
Control
14,15-EET
ISO
ISO+14,15-EET
Ang II
Ang II+14,15-EET
B
GRP78
CHOP
β-actin
CHOP
GRP78
ER stress markers (fold induction)
3.0
2.5
2.0
1.5
1.0
.5
0.0
*
*
#
#
Control
BAPTA
ISO
ISO+BAPTA
C
Control
14,15-EET
ISO
ISO+ 14,15-EET
p-CaMKII
β-actin
p-CaMKII/β-actin (relative intensity)
2.5
2.0
1.5
1.0
.5
0.0
*
#
Control
14,15-EET
ISO
ISO+14,15-EET
Control
14,15-EET
Ang II
Ang II+ 14,15-EET
p-CaMKII/β-actin (relative intensity)
10
8
6
4
2
0
*
#
Control
14,15-EET
Ang II
Ang II+14,15-EET

Figure 7


A
b-IAM-SERCA2a
SERCA2a
β-actin
SERCA2a
b-IAM-SERCA2a
Intensity relative to β-actin
Control
14,15-EET
ISO
ISO+14,15-EET
Ang II
Ang II+14,15-EET
B
SERCA2a activity (IU/mg protein)
C
DCF fluorescence (fold)
D
ZnCu-SOD
Mn-SOD
Catalase
β-actin
Antioxidant enzymes expression (fold induction)