

Original Article

Synergism between arrhythmia and hyperhomocysteinemia in structural heart disease

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Abstract: Elevated levels of homocysteine (Hcy) known as hyperhomocysteinemia (HHcy) is associated with cardiac arrhythmia and sudden cardiac death (SCD). Hcy increases iNOS, activates matrix metalloproteinase (MMP), disrupts connexin-43 and increases collagen/elastin ratio. The disruption of connexin-43 and accumulation of collagen (fibrosis) interrupt cardiac conduction and attenuate NO transport from endothelium to myocyte (E-M) causing E-M uncoupling. We hypothesize that Hcy increases mtNOS, metalloproteinase activity, disrupts connexin-43, exacerbates endothelial-myocyte uncoupling, and induces cardiac failure by activating NMDA-R1 in structural heart disease. Chronic volume overload heart failure was created by aorta-venacava (AV) fistula. HHcy was induced by administering Hcy in drinking water. NMDA-R1 was blocked by dizocilpine (MK-801). EKG and M-mode Echocardiography was performed. The E-M coupling was determined in cardiac rings. LV mitochondria was isolated. Levels of NMDA-R1, peroxiredoxin, NOX4, and mtNOS were measured. The degradation of connexin-43, collagen and elastin was measured by Western blot analysis. Mouse cardiac endothelial cells were cultured with or without Hcy or MK-801. The results suggest systolic and diastolic heart failure in HHcy and AVF mice. The levels of connexin, collagen degradation and MMP-9 were increased. The elastin was decreased in HHcy and AVF hearts. The mitochondrial NOX4 increased and peroxiredoxin was decreased. The mtNOS activity was synergistically increased in HHcy, AVF and HHcy+AVF hearts. The cardiac contraction and endothelial dependent relaxation was attenuated in HHcy and AVF hearts. Interestingly, the treatment with MK-801 mitigated the contractile dysfunction. These studies delineated the mechanism of Hcy-dependent endothelial-myocyte uncoupling in cardiac arrhythmia and failure, and have therapeutic ramifications for sudden cardiac death.

Keywords: A-V fistula, MMP-9, connexin, NO, endothelial myocyte coupling, PVC, mitochondria, calpain, NOX4, peroxiredoxin, heart failure

Introduction

Sudden cardiac death (SCD) is a major cause of mortality [1, 2]. Approximately 65% of SCD cases occur in patients with underlying acute or chronic heart disease. The incidence of SCD increases 2 to 4 fold in the presence of coronary disease and 6 to 10 fold in the presence of structural heart disease. Ventricular tachycardia (VT) leading to ventricular fibrillation (VF) is the primary cause of cardiac arrest and SCD. One of the challenges in preventing SCD lies in identifying individuals at highest risk for SCD within a lower risk population [3]. The identification of conventional risk factors for coronary artery disease and structural heart disease during progression to arrhythmogenesis and SCD can be very daunting. However, increased serum homocysteine (Hcy) has been identified as a risk fac-

tor for SCD resulting from coronary fibrous plaques [4-6]. Hcy prolongs the QRS interval, causes cardiac interstitial fibrosis and structural heart disease [7]. Hcy increases mitochondrial oxidative stress and activates MMPs. The MMPs are activated in VT, VF and SCD [8-10]. The metalloproteinases degrade connexin-43 [11], therefore, it is important to measure metalloproteinases and connexin alterations in Hcy-mediated E-M uncoupling and CHF.

The blockade of NMDA-R1 mitigates SCD [12-15]. Although both ischemia and reperfusion induce arrhythmia, only reperfusion-induced arrhythmia is sensitive to NMDA-R1 blockade [16]. This may suggest that arrhythmia in high cardiac output is influenced by circulating factors and is mitigated by NMDA-R1 blockade. Therefore, we hypothesize that Hcy amplifies

arrhythmogenic heart failure by generating arrhythmogenic substrates in aorta-caval (AV) fistula model [17, 18] of chronic volume overload heart failure and NMDA-R1 antagonist (dizocilpine, MK-801) ameliorates Hcy-mediated cardiac arrhythmia and heart failure.

Increased levels of Hcy causes myocardial conduction abnormalities and are associated with SCD [3, 5, 19, 20]. Though acute ischemic events are associated with arrhythmia, it is unclear whether chronic volume overload heart failure exacerbates arrhythmogenesis. Although Hcy behaves as an agonist to NMDA-R1, and NMDA induces Ca²⁺ and K⁺ currents [21, 22], and NMDA-R1 blocker (MK-801) reduces NMDA-analog-mediated increase in heart rate and SCD, it is unclear whether Hcy creates pro-arrhythmogenic condition by activating NMDA-R1 and E-M uncoupling.

Majority of genetic causes of hyperhomocysteinemia are primarily due to the heterozygosity in cystathionine β synthase (CBS). The CBS heterozygous (-/+) mice demonstrated a 4-fold increase in the levels of Hcy [23], analogous to the hyperhomocysteinemic human. Although the defect in cardiac metabolism of Hcy leads to decrease in oxygen consumption [24], and high levels of Hcy increases iNOS, causes oxidative stress, and SCD [25], it is unclear whether Hcy enhances NMDA-R1 expression and oxyradicals, and causes E-M uncoupling.

Although endothelial-myocyte and neuronal-myocyte coupling plays an important role in regulation of cardiac relaxation and contraction, the endothelial-myocyte coupling mechanisms are the least studied. This is, in part, due to two reasons: 1) since the endothelium is within the muscle, it is difficult to remove; and 2) there is no easy way to measure the contribution of the endothelium to cardiac muscle, unlike in vessels. Measuring cardiac endothelial function using the isolated papillary muscle preparation does not demonstrate what happens in the entire transmural wall. To determine endothelial function in the isolated heart, acetylcholine has been perfused in the Langendorff preparation [26]. This approach does not differentiate the specific contribution due to regional ischemia, hypertrophy, stunning, and/or hibernation in the myocardial wall. Rather, it gives an assessment of the global contractile response to cardiotoxic agents. Furthermore, it does not

separate the effects of the LV from the RV. We have compared the data using our cardiac ring preparations from hypertensive animals, with the isolated heart preparation and found similar results [27]. In addition, the cardiac ring preparation separates the effect of LV from RV. Additionally, the specific regional differences in the contractile function of the heart can be measured by including or excluding the homogenous or inhomogeneous regions of the transmural myocardial wall in preparation of cardiac rings [7, 27].

Connexin-43 -/- promotes cardiac arrhythmia and SCD [28-30]. In end-stage human heart failure, metalloproteinases including ADAM-12, are activated and connexin-43 is disrupted [11]. We showed that most of the MMPs in the heart are latent [31] due to active-site Zn²⁺ coordination with constitutive NO in a tertiary complex (MMP/NO/TIMP) in the basement-membrane-matrix of endothelium. Increased oxidative stress leads to generation of nitrotyrosine residues in TIMP and liberates active MMP [32]. This, in turn, degrades the connective tissue matrix. Since collagen turnover is faster than other ECM components, degraded matrix is replaced by oxidized collagen (fibrosis). Two detrimental consequences of this process are: 1) degradation of ultra-structural matrix which causes disconnection (i.e. degradation of connexin-43) of the endothelium from myocytes; and 2) accumulation of oxidized collagen, which impairs the delivery of metabolites to underlying muscle, causing uncoupling.

Generalized MMP activation is implicated in development of VF, SCD and CHF [8-10]. Furthermore, increased oxidative-modification of collagen by cross-linking is associated with diastolic dysfunction in CHF [33-36]. Although NMDA-R1 antagonist ameliorates MMP activation [37], the mechanism of MMP activation, connexin disruption and collagen accumulation in HHcy and CHF is unclear.

TIMP-1 is induced in fibrotic myocardium [38] and Hcy induces TIMP-1 [39]. TIMP-4 is highly expressed in the heart and is decreased during cardiac failure [40]. MMP-2 degrades interstitial collagen as well as elastin [41, 42], and under pathophysiological conditions MMP-9 at 92 kDa (gelatinase b) is induced. ADAM-12 is increased and connexin-43 is degraded in CHF [11]. Therefore, it is important to measure MMP-9,

connexin-43, collagen-elastin synthesis and degradation.

Although oxidative stress plays significant role in VT, arrhythmia, and SCD during CHF [43, 44], the role of Hcy in increasing oxidative stress in arrhythmia and SCD is unclear. Previous studies from our laboratory showed that Hcy increases oxidative stress by generating ROS, RNS and nitrotyrosine [45, 46]. In tissues NADH oxidase is a primary source of ROS generation. To determine whether the increase in ROS and RNS by Hcy is due, in part, to the increase in NADH oxidase and mtNOS activities, it is essential to measure mitochondrial NADH oxidase and mtNOS activities. Since most of the peroxidase activity depends on the levels of peroxiredoxin III (Prx-III), the tissue level of Prx-III is decreased as a result of increased oxidative stress [47-50]. Therefore, it is important to measure the levels of cardiac mitochondrial peroxiredoxin III.

Methods

Animals

Wild type (WT, C57BL/6J) were obtained from Jackson Laboratories, MA. To induce hyperhomocysteinemia (HHcy), mice were supplemented with 0.67 mg/ml in drinking water as described previously [51]. Volume overload heart failure was created by aorta-venacava fistula (AVF). The AVF was created as described previously [52-54]. To inhibit the NMDA-R1, dizocilpine (MK801, Sigma Chemical Co) was administered through drinking water, 8 µg/ml. Vehicle (V) saline was used as control. The mice were grouped: 1) Sham; 2) AVF; 3) Hcy; 4) AVF+Hcy, treated with or without the MK-801. The concentration of MK was based on the fact that the binding constant between MK and NMDA-R1 is in the micromole range [55]. To determine selectivity of MK in the absence of Hcy, MK was administered to WT mice. To determine whether the MK treatment causes any change in food intake, food intake was measured every 2-days during the treatment period for MK content. Because previous studies demonstrated significant cardiac dysfunction at 8-12 weeks of homocysteinemia [56], we administered MK for 8 weeks. Animal room temperature was maintained between 22°C and 24°C. A 12-hour light-dark cycle was maintained by artificial illumination. The animals were fed standard chow and water ad libitum. Levels of water

and food intake, and changes in body weight were measured every other day. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University Of Louisville School Of Medicine, Louisville, in accordance with the National Institute of Health Guidelines for animal research.

Electrophysiology and echocardiography

At the end of protocol mice were anesthetized with tribromoethanol [57]. 3-lead ECG (Micro-Med Dig-meter) was obtained and P-R, QRS intervals were measured. A SONO-5500 echocardiography system, equipped with a 12-MHz shallow-focus phased-array transducer for mice was used to study left ventricular function. Only M-mode echocardiography with well-defined continuous interfaces of the septum and posterior wall was collected. Ventricle cavity, wall thickness, and axis lengths were measured [58, 59].

The E-M coupling

The hearts were prepared by arresting in diastole by injecting (i. v.) 0.2 ml/100g body weight of a 20% solution of KCl, rapidly excised, and placed in freshly prepared cold physiological salt solution (PSS). The heart was perfused at end-diastolic pressure by a catheter in the aorta (attached to perfusion apparatus). The LV and RV were separated. The "deli" shape LV rings were mounted in a tissue myobath [9, 29]. One of two mounting wires was connected to a force transducer. The rings were stretched and brought to resting tension at which 1 µM ET-1 was added to a bath. At the maximum ET-1 contraction, acetylcholine (endothelial-dependent) was added and dose-response curves were generated. The % relaxation was calculated based on 100% contraction to 1 µM ET-1. The data were fitted to a non-linear least squares equation: % Relaxation = $A/(1+\exp^{B(Dose - C)}) + D$, where A, C and D are constants. The validity of measurements regarding endocardial endothelial function using "deli" shape LV rings has been established by measuring responses to various cardiotoxic agents [7, 27, 52].

To determine the functional uncoupling, the endothelial-myocyte contraction/relaxation was determined by the responses to ET-1 and acetylcholine in our ex vivo innovative LV ring preparation as described [7, 27, 52]. To confirm endo-

thelial-dependent cardiac relaxation, hearts were perfused with L-NAME, and rings were prepared. To ensure homogenous predictable endothelial denudation, 0.001% Triton X-100 was perfused for 20 sec through the entire heart [60] prior to preparing the rings. The percent relaxation was estimated based on 100% contraction to 1 μ M ET-1 as described [52]. The endothelial-dependent cardiac relaxation was induced by acetylcholine (EDRF-dependent).

Preparation of Hcy, acetylcholine, and MK-801 solutions

The concentration of Hcy was determined by spectrophotometric titration with dithio-bis-nitrobenzoate (absorption measured at 412 nm) using $\epsilon_{412\text{ nm}}$ of 13,600 $\text{M}^{-1}\text{cm}^{-1}$ [61]. The concentrations of acetylcholine, L-NAME, and MK-801 were based on weight measurements based on the molecular weight of the drug. All dilutions from stock solutions were made immediately prior to the experiment. Buffer was used as vehicle control.

Levels of connexin-43, elastin, collagen-1 and MMPs

LV tissue homogenates were prepared. The Bio-Rad dye binding assay was done to estimate total protein concentration in the tissue extracts according to the method of Bradford [62]. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) was performed with and without reduction following the method of Laemmli [63].

The levels of connexin-43 and its degradation, elastin, and collagen-1 were estimated by Western blot analysis, using respective antibodies. The MMPs were estimated by gelatin gel zymography [64].

Isolation of mitochondria

Isolation buffer contains 0.3 M sucrose (Sigma), 0.1% BSA (Sigma), 0.2 mM EDTA, 10 mM HEPES, adjust pH 7.4 with KOH, including freshly prepared protease inhibitors leupeptin, aprotinin, pepstatin (10 μ g/ml), PMSF (200 μ M). MVEC (10^7) were trypsinized and washed with cold PBS, resuspended in cold isolation buffer (use 5 times pellet volume). Then, the cells were homogenized on ice with a 2-ml glass homogenizer (Dounce: loose x 5 times then tight x 5 times), and centrifuged at 1000g for 10 min at

4°C. The supernatant was collected and the pellet (whole cells and nuclei) discarded. The supernatant was centrifuged at 14,000g for 15 min at 4°C, and saved as the cytosolic fraction. The resulting pellet represents the mitochondrial fraction, and was washed twice with cold Isolation Buffer. The protein concentration was estimated by the Bio-Rad dye-binding assay. The pellet was diluted in SDS-containing buffer, aliquoted, and stored at -20°C until ready for electrophoresis.

Nitric Oxide Assay

Nitric oxide (NO) generation was recorded by using NO detection probe following manufacturer instructions (Apollo 4000 Free Radical Analyzer, WPI Inc.) with a single dose of LPS in the presence of L-arginine.

Statistical analysis

Dose-response curves for cardioactive agents were compared using multigroup analysis according to the Bonferroni correction [65]. A two-way ANOVA analysis of variance was used to compare the respective values, biochemical and physiological data of sham, AVF, HHcy, HHcy+AVF with and without the MK-801 treatment (NMDA-R1 blocker) groups. The n value and a minimum of 6 mice in each group were used to evaluate the significance of the results. Data will be presented as mean + SEM. Differences were considered statistically significant at $p < 0.05$.

Results

Synergism in diastolic (D) and systolic (S) heart failure

Hcy induced diastolic and AV-fistula induced systolic heart failure. The M-mode echocardiographic data suggested diastolic heart failure in HHcy, and AV-fistula-induced increase in preload creates systolic heart failure. The combination of HHcy and AV-fistula synergizes the diastolic-systolic heart failure (**Table 1** and **Figure 1**).

Hcy amplified the connexin-43 degradation in CHF

LV tissues of sham, Hcy, CHF and Hcy+CHF animals were analyzed for connexin-43, elastin, collagen (Western blots), and MMP-9 activity

Table 1. Gravimetric measurements of control sham, AVF, HHcy, AVF+HHcy mice treated with or without MK-801. Body weight (BW) in grams and heart weight (HW) in mg are reported. Plasma levels of Hcy and mitochondria ONOO- and numbers of premature ventricle contraction (PVC) are presented.

	BW, g	HW, mg	HW/BW, mg/g	Hcy, μ Mole/L	mtONOO- nMole/L	Number PVC/min
Sham	25 $\pm$ 1	161 $\pm$ 10	6.44 $\pm$ 0.9	5 $\pm$ 1	10 $\pm$ 2	0
AVF	24 $\pm$ 1	188 $\pm$ 11	7.83 $\pm$ 0.75*	7 $\pm$ 1	60 $\pm$ 4*	2-5
HHcy	23 $\pm$ 1	174 $\pm$ 9	7.57 $\pm$ 0.8*	28 $\pm$ 2*	40 $\pm$ 3*	1-2
AVF+HHcy	26 $\pm$ 1	192 $\pm$ 12	7.38 $\pm$ 0.7*	32 $\pm$ 3*	90 $\pm$ 8*	10-15*
Sham+MK	25 $\pm$ 1	160 $\pm$ 13	6.40 $\pm$ 0.9	4 $\pm$ 1	10 $\pm$ 1	0
AVF+MK	24 $\pm$ 1	166 $\pm$ 12	6.92 $\pm$ 1.1	7 $\pm$ 1	25 $\pm$ 3	1-2
HHcy+MK	26 $\pm$ 1	165 $\pm$ 14	6.35 $\pm$ 0.6**	25 $\pm$ 2	20 $\pm$ 2**	1-2
AVF+HHcy+MK	25 $\pm$ 1	170 $\pm$ 15	6.80 $\pm$ 0.75	26 $\pm$ 3	60 $\pm$ 5	3-5***

Values are mean $\pm$ SEM of 10 animals. * p <0.05 vs sham; **p<0.05 vs HHcy; ***P<0.05 vs HHcy or AVF.

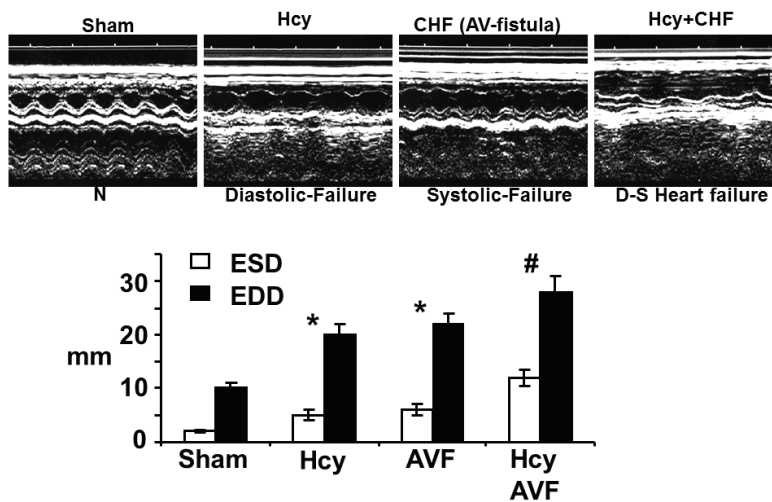


Figure 1. M-mode Echocardiography of Sham, Hcy, AVF and AVF+Hcy mice: End-systolic and diastolic LV diameter (ESD and EDD) in millimeter (mm) was measured. The bar graph represents mean $\pm$ SEM from n=10 in each group. *p<0.05 compared to sham; #p<0.05 compared to Hcy or AVF.

(zymography). The results suggest that although the expression of connexin-43 was increased by Hcy, the degradation of con-43 was outweighed the synthesis. This suggests disconnect between endothelial and myocyte by Hcy in CHF. The levels of elastin decreased and collagen increased (Figure 2), leading to peri-capillary fibrosis. The degradation of con-43, decrease in elastin, increase in collagen, and MMP-9 expression in Hcy and CHF groups were amplified in Hcy+CHF group.

To determine mitochondrial (mt) function, the mitochondria from LV tissue were isolated. SDS-PAGE and Western blot analyses of calpain

(cytosolic marker), prohibitin (a mitochondrial (mt) marker) & mt peroxiredoxin III (Prx-III) and NADH oxidase subunit (Nox-4) were performed. There was robust decrease in mt-peroxiredoxin-III and increase in Nox-4 in Hcy+AV fistula model of CHF as compared to control, AV-fistula or Hcy groups (Figure 3).

To determine whether mtNOS activity (an isoform of iNOS) is increased in Hcy+CHF, we measured NO generation in isolated mitochondria from hearts of sham, Hcy, AV-fistula (CHF) and AV-fistula + Hcy mice. The results suggested that there was robust increase in lipo-polysaccharide (LPS)-induced mtNOS activity in AVF + Hcy mice. The activity was

mitigated by Ca²⁺-chelator (EDTA) [Figure 4A and B]. This may suggests that Ca²⁺ influx activates mtNOS and generates peroxynitrite, leading to oxidative stress and arrhythmia. Thus Ca²⁺-channel blockers may be protective against arrhythmias.

ET-1 response was enhanced and acetylcholine response was attenuated in HHcy mice compared to WT mice. Typical example of cardiac ring contraction to ET-1 and relaxation to acetylcholine (ACH) in WT (N) and HHcy mice is shown in Figure 5. The experiments with cardiac rings suggest that both the contractile and relaxation responses of the heart were impaired in HHcy

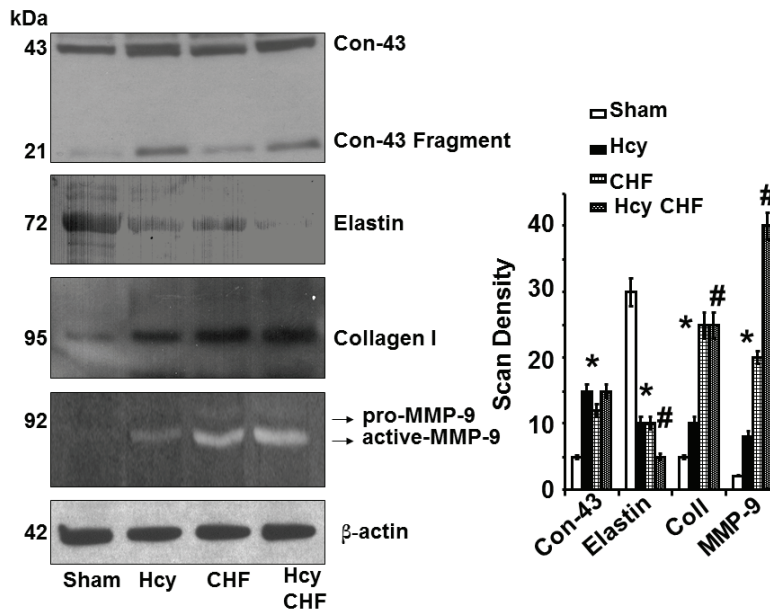


Figure 2. Typical Western blot analyses of collagen I, elastin and connexin-43 degradation; MMP-9 activity by zymography: The LV tissue from Sham, Hcy, AVF and AVF+Hcy mice was analyzed. Beta-actin was used as loading control. The bar graph represents the mean $\pm$ SEM (n=10) of scan density normalized with beta-actin. *p<0.05 compared to sham; #p<0.05 compared to Hcy or AVF.

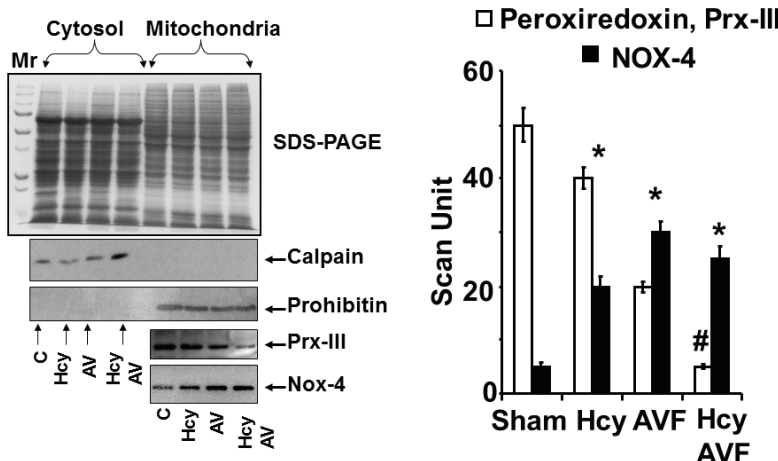


Figure 3. The LV mitochondria and cytosol were separated and characterized using prohibitin and calpain, Western blot analysis, respectively. The total SDS-PAGE is shown. The mitochondria was analyzed for NOX4 (NADPH oxidase subunit) and peroxiredoxin-III (Prx-III). The bar graph represents the mean $\pm$ SEM (n=10) of scan density. *p<0.05 compared to sham; #p<0.05 compared to Hcy or AVF.

mice as compared to WT. The responses were increased in CHF+Hcy. The treatment with MK 801 mitigates the contractile and relaxation dysfunction due to Hcy and CHF (Figure 5).

Hcy induces cardiac arrhythmia

Hcy induced dyssynchronous heart rate (arrhythmogenesis), premature ventricle contraction (PVC), and increased P-R intervals in CHF. The ECG data suggests increase in P-R interval and QRS duration in Hcy-mice as compared to normal (N). The P-R interval was prolonged in Hcy-CHF model, suggesting Hcy exacerbation of arrhythmogenesis in CHF. There was 20-30% higher PVC and SCD in Hcy+CHF group

over 15 wks period than CHF or Hcy groups alone (Figure 6A and B). The chronic treatment with MK-801 (NMDA-R1 blocker) mitigates both the Hcy- and CHF-mediated increase in PVC, P-R duration, arrhythmogenesis and SCD (Figure 6A and B). MK-801 decreases Hcy-induced P-R and QRS durations. These results suggest that Hcy amplifies arrhythmogenic responses in CHF, in part, by behaving as an agonist to NMDA-R1.

Hcy activated NMDA-R1

To determine cellular mechanism of Hcy and NMDA-R1 in relation to connexin, elastin and collagen remodeling, MVEC were treated with hcy, without (C) hcy, and Hcy pretreated MVEC

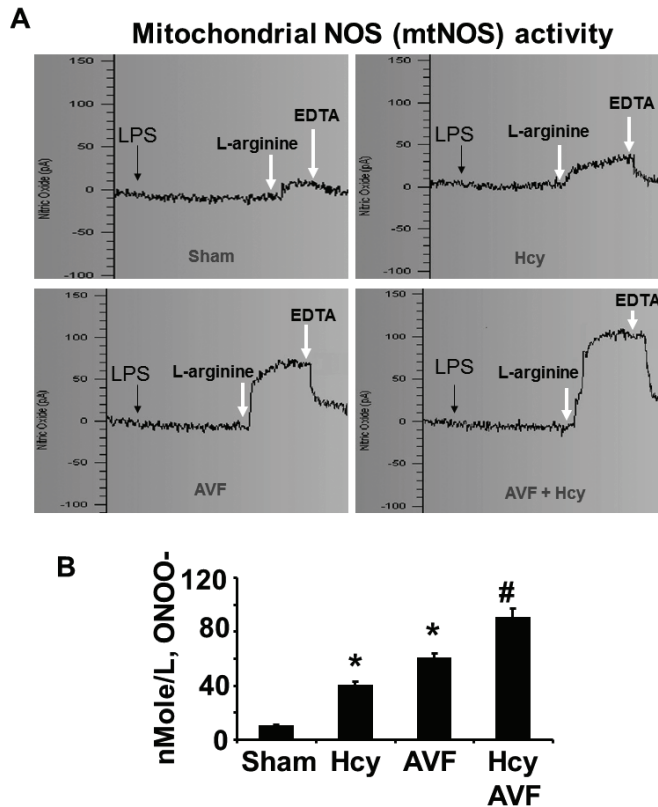


Figure 4. A. Mitochondrial nitric oxide species (ONOO-) generation: The mitochondria was activated with LPS. The L-arginine was added. The ONOO- was detected using NO-sensitive electrode. To block Ca-dependent mtNOS EDTA was added. The mitochondrias from Sham, Hcy, AVF and AVF+Hcy mouse hearts were analyzed. The LPS-induced mtNOS activity measured by NO-electrode (WPI Apollo-4000). L-arginine (1 mM) and EDTA (1 mM) were added. **B.** The bar graph represents the levels of mtONOO- generated. Each bar represents the mean $\pm$ SEM from 10 separate experiments. *p < 0.05 compared to sham; #p < 0.05 compared to Hcy or AVF.

with MK 801. Elastin (E), connexin-43 (Con-43), collagen I (Coll), NMDA-R1 and GAPDH gene expressions were measured by RT-PCR. The connexin-43 protein levels and fragments were measured by Western blot analysis. There was decrease in elastin, increase in collagen I and connexin-43 (con-43) expression. Also, con-43 was degraded in the presence of Hcy and the co-treatment of Hcy with MK ameliorates the con-43 degradation and induction of NMDA-R1 (**Figure 7**).

Discussion

The comparison of HR, P-R and QRS durations between WT, Hcy, AVF and Hcy+AVF treated with and without MK-801 suggests that Hcy causes arrhythmia in structural heart disease. Because Hcy behaves as an agonist to NMDA-R1, and inhibitor of NMDA-R1 decreases HR and SCD, we see reduction in increase in HR in Hcy and AV fistula after MK801 treatment. Attenuation of the acetylcholine response in Hcy mice demonstrates a loss-of-function due to decrease in endothelial NO availability to the adjacent muscle. Although previous reports suggest that the levels of Hcy after MK treatment did not change, a gain-of-endothelial function was attributed to decrease in Hcy-mediated oxidative stress and increase in NO availability. Acetylcholine stimulates NO release in the isolated heart preparation [26], and ROS abrogates NO. Therefore, the magnitude of relaxation and the acetylcholine response was significantly depressed in endocardial rings from Hcy and AVF hearts. Because MK suppresses NMDA-R1, oxidative stress and ROS, the treatment with MK normalizes the relaxation

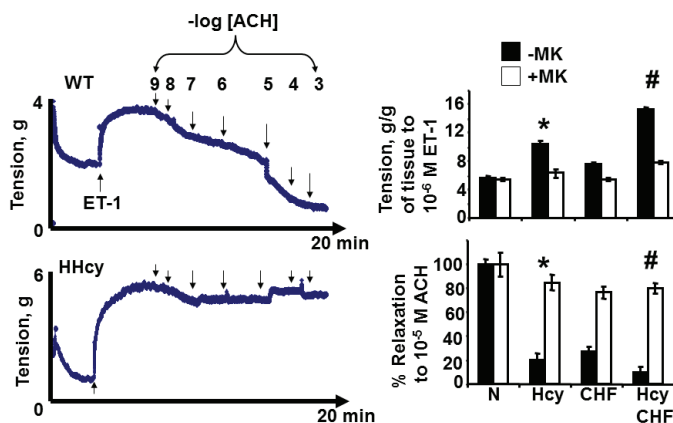


Figure 5. A typical WT mouse LV ring reactivity by endothelin-1 (ET-1) and relaxation by acetylcholine (ACH) different doses. The bar graph represents accumulative data of contraction by ET-1 and relaxation by ACH in cardiac LV rings from Sham, Hcy, AVF and AVF+Hcy mouse treated with or without MK-801 (NMDA-R1 blocker). *p < 0.05 compared to sham; #p < 0.05 compared to Hcy or AVF.

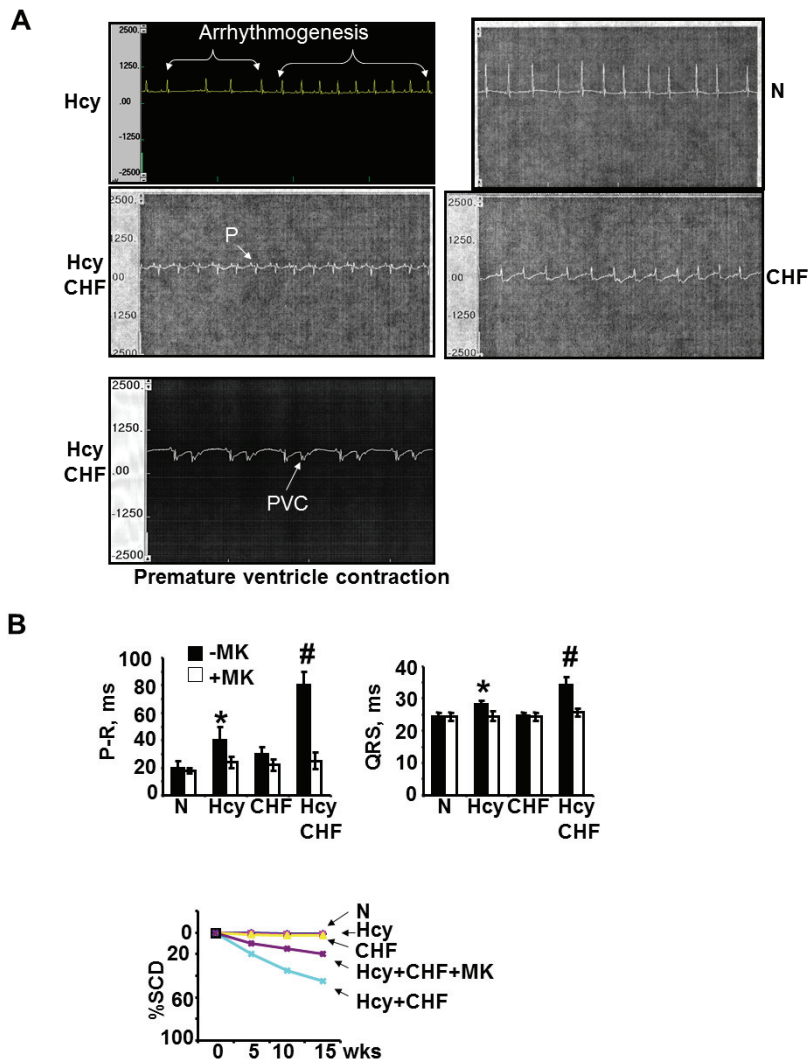


Figure 6. A. The 3-lead ECG recording of Sham, Hcy, AVF and AVF+Hcy mouse treated with or without MK-801 (NMDA-R1 blocker). Irregular heart beat (arrhythmogenesis) in Hcy mice, premature ventricular contraction (PVC) in Hcy+AVF mice. **B.** Bar graph representation of P-R, QRS and % sudden cardiac death in Sham, Hcy, AVF and AVF+Hcy mouse treated with or without MK-801 (NMDA-R1 blocker). * $p < 0.05$ compared to sham; # $p < 0.05$ compared to Hcy or AVF.

to control values. The results reveal that the treatment with MK restores these levels to normal, and ameliorates the oxidative-stress and repairs the endothelial-myocyte coupling in HHcy and AVF+Hcy mice with heart failure and SCD.

Hcy decreases endothelial NO and increases production of nitrotyrosine [46]. Therefore, de-

creased NO and increased redox stress was expected in the Hcy mice. Because NMDA-R1 is induced by Hcy, we see an increase in NMDA-R1 expression in Hcy treatment. These effects were amplified by AV fistula. Interestingly, treatment with MK801 decreases the NMDA-R1 levels in Hcy group as compared to controls. Because blocking of NMDA-R1 has been shown to decrease heart rate [66], it was anticipated that MK 801 also increases constitutive NO and mitigates E-M uncoupling.

MK treatment, does not necessarily suggest that endothelial NO is increased. Therefore, we measured ONOO. This supports that mitigation of NMDA-R1 activation improves Hcy-mediated cardiac remodeling, but does not affect the Hcy metabolism. It is possible that the increase in Hcy, post MK treatment, is due to a decrease in renal clearance. However, this will be counterintuitive given the fact that the NMDA-R1 antagonist reduces cardiac dysfunction by increasing endothelial NO [67].

Because, there is an increased oxidative stress in Hcy and AVF, we anticipated a decrease in peroxiredoxin in Hcy mice. Previous reports suggest that Hcy de-

creases peroxiredoxin, and increases ROS [68], causing arrhythmia that may lead to SCD. However, the inactivation of NMDA-R1 increases redoxins and decreases oxidative stress. These results suggest that suppression of NMDA-R1 decreases NADH oxidase in a murine model of hyperhomocysteinemia, in part, by increasing peroxiredoxin. This will have implication for the treatment of cardiac arrhythmia, SCD and CHF.

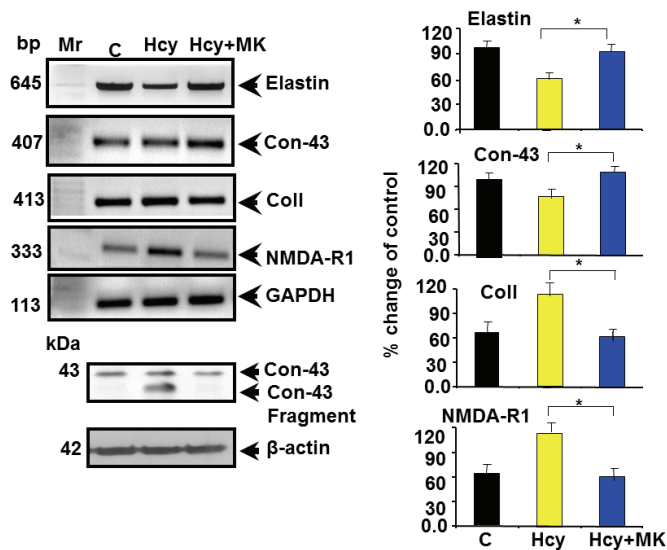


Figure 7. In vitro cardiac endothelial cells cultured with Hcy with and without MK-801 treatment. The mRNA was analyzed by RT-PCR for elastin, connexin-43, collagen and NMDA-R1 expression. The cell homogenate was analyzed for connexin-43 fragment by Western blot analysis. The bar graph represents the densitometric scan unit of PCR products and normalized with GAPDH. * $p < 0.05$, $n = 10$.

Conclusion

Hcy causes systolic hypertension in genetic model of HHcy. The echo data suggests amplification of diastolic and systolic heart failure by Hcy in AV-fistula model of CHF. The ECG data suggests PVC and SCD in Hcy+CHF groups. The treatment with MK-801 (a NMDA-R1 blocker) ameliorates the SCD in Hcy+CHF mice. The cardiac contraction to ET-1 was enhanced in CBSKO mice. This contraction was amplified in Hcy+CHF mice. The treatment with MK-801 mitigates the cardiac systolic contractile and diastolic relaxation dysfunction in Hcy+CHF groups. The mtNOS activity was robustly increased in Hcy-CHF group as compared to controls. In MVEC we show that Hcy increases NMDA-R1 and connexin-43 expression. However, there was robust degradation of connexin-43 in Hcy treated MVEC. The co-treatment with MK-801 ameliorates the Hcy-mediated degradation of connexin-43. Collectively, our study supports the hypothesis that Hcy increases mtNOS activities, superoxide levels, metalloproteinase activity, disrupts connexin-43, exacerbates endothelial-myocyte uncoupling, and induces cardiac failure by activating NMDA-R1 (Figure 8).

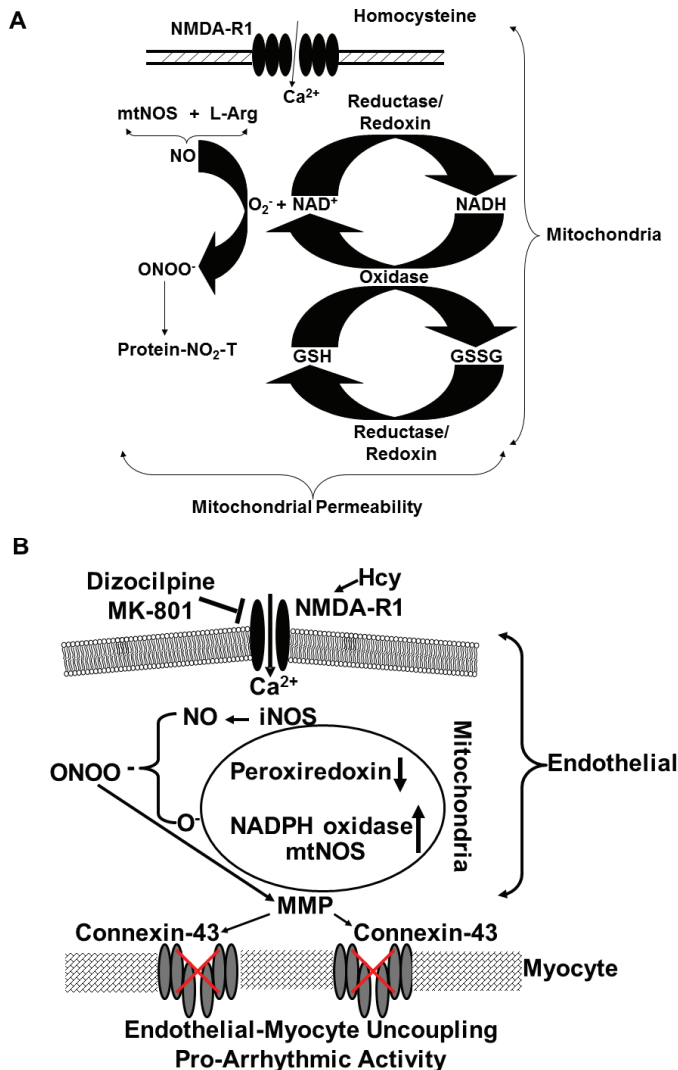


Figure 8. A. Hypothesis of mitochondrial mechanism of ONOO⁻ generation; B. The mechanism of endothelial/mitochondria-myocyte uncoupling and connexin-43 degradation, causing pro-arrhythmic activity (revised from [69]).

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