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Original Article

Exercise mitigates homocysteine - β2-adrenergic receptor interactions to ameliorate contractile dysfunction in diabetes

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Abstract: We tested the hypothesis that exercise ameliorates contractile dysfunction by interfering with homocysteine – β2-adrenergic receptor (AR) interactions, inducing β2-adrenergic response and Gs (stimulatory G adenylyl cyclase dependent protein kinase), and lowering homocysteine level in diabetes. The effect of homocysteine on β2-AR was determined by (a) scoring the β2-AR in the cardiomyocytes treated with high dose of homocysteine using flow cytometry, and (b) co-localizing homocysteine with Gs (an inducer of β2-AR) in the cardiomyocytes obtained from C57BL / 6J (WT) and db/ db mice using confocal microscopy. The effect of exercise on the protein-protein interactions of homocysteine and β2-AR in diabetes was evaluated by co-immunoprecipitation in the four groups of db/db mice: (1) sedentary, (2) treated with salbutamol (a β2-AR agonist), (3) swimming exercise, and (4) swimming + salbutamol treatment. The effect of exercise on β2-AR was determined by RT-PCR and Western blotting while cardiac dysfunction was assessed by echocardiography, and contractility and calcium transient of cardiomyocytes from the above four groups. The results revealed that elevated level of homocysteine decreases the number of β2-AR and inhibits Gs in diabetes. However, exercise mitigates the interactions of homocysteine with β2-AR and induces β2-AR. Exercise also ameliorates cardiac dysfunction by enhancing the calcium transient of cardiomyocytes. To our knowledge, this is the first report showing mechanism of homocysteine mediated attenuation of β2-AR response in diabetes and effect of exercise on homocysteine - β2-AR interactions.

Keywords: Cardiomyopathy, Heart failure, echocardiogram, Gs, HL1, db/db

Introduction

Exercise exerts myriad effects on the heart. In diabetes, exercise improves cardiac function by influencing multiple peripheral and central mechanisms [1-6]. The induction of beta-adrenergic response is one of the central mechanisms by which exercise mitigates cardiac dysfunction [7,8]. β-adrenergic receptors (AR) are transmembrane G protein-coupled receptors (GPCR) that maintain sympathetic tone and modulate cardiac contractility [9]. There are three subtypes of β-AR: β2-AR, β2-AR and β3-AR. These subtypes are highly conserved across the species. Both β1-AR and β2-AR increases whereas β3-AR decreases contractility of the heart [9]. The diabetic heart shows less response to the β1-AR agonist stimulation than that of the β2-agonist stimulation [10]. Also, the effect of Insulin treatment is more on β2-AR response than that on β1-AR response [10]. Earlier, we have reported that β2-AR response is attenuated in the diabetic heart that leads to contractile dysfunction [11]. However, the underlying mechanism was not clear. Therefore, we investigated the mechanism of exercise mediated induction of β2-adrenergic response in diabetes. It is reported that hyperglycemia induces hyperhomocysteinemia - an independent risk factor for cardiovascular diseases [12]. Homocysteine (Hcy- a non coding amino acid) inhibits Gs (stimulatory G adenylyl cyclase dependent protein kinase) signaling. Gs is an inducer of β-AR [13]. Hcy also competes for β2-AR binding [14]. Therefore, it is worthwhile to investigate the effect of homocysteine on β-AR re-

sponse in diabetes.

Type 2 diabetes (T2D) is more prevalent form of diabetes and is often associated with obesity. Clinical studies revealed that diabetes causes cardiomyopathy and the chances of heart failure increases if the patient has diabetes. On the other hand, there are empirical evidences suggesting that exercise training mitigates cardiac dysfunction in diabetes [2,4]. The beneficial effects of exercise training include improvement in sympathetic tone by influencing β-AR [15], and lowering of Hcy level [16]. However, the effect of exercise on the modulation of cross talk between β2-AR and Hcy in diabetes is unknown. To address this issue, we used leptin receptor mutant db/db mice that resemble metabolic syndrome found in the clinical course of human T2D [17]. We determined the effect of exercise on (i) the expression of β2-AR and Hcy, (ii) the cross-talk between β2-AR and Hcy, and (iii) the contractile dysfunction of cardiomyocytes in db/db mice.

Materials and methods

Animal model

Ten week old male leptin receptor deficient db/db and control C57BL/6J (WT) mice were procured from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in the animal care facility of University of Louisville with controlled temperature (22-24 ^{0}C) and 12 hr light-dark cycle. They were allowed free access to the standard chaw and drinking water. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of University of Louisville, School Of Medicine in accordance with the animal care and use program guidelines of National Institute of Health.

Exercise and β2-AR agonist treatment

The ten weeks db/db mice were housed into the animal facility for two weeks for adaptation to the new environment and used for the experiments at the age of twelve weeks. For exercise, mice were allowed to swim in a water tub for 1hr /day. The water temperature was maintained at 31±1^{o}C during the course of exercise. To induce beta2-AR response, salbutamol (a β2-AR agonist) was injected into mice (200μg/Kg body weight/day i.p.). The total duration of experiment was ten days.

Measurement of LV wall function by echocardiography

Mice were anaesthetized by tribromoethanol [18]. The M-Mode echocardiography was performed on the SON05500 instrument equipped with a 12-mHz shallow-focus phased- array transducer for mice. The transducer was placed on the hemi- thorax region of the mice for echocardiography. The fractional shortening (FS) was calculated from the formula, % FS= 100x (LVIDd - LVIDs) / LVIDd, where LVIDd stands for left ventricular internal diameter in diastole, and LVIDs stands for left ventricular internal diameter in systole.

Western Blotting

The total heart protein was extracted by using RIPA buffer, quantified by Bradford assay and expression of different proteins were determined by Western blotting as previously described [19]. The primary antibodies for β2-AR (Santa Cruz Biotechology, cat # sc-569), Hcy (abcam, cat # ab 15154) and GAPDH (Millipore, cat # MAB374) were used at the dilution of 1: 1000 and incubated for overnight at 4^{o}C. The β2-AR and Hcy antibodies were raised in rabbit whereas GAPDH was raised in mouse. The secondary antibodies for rabbit (Santa Cruz Biotechnology, cat # sc-2054) and mouse (Santa Cruz Biotechnology, cat # sc-2005) were diluted in the ratio of 1:2000 and incubated for 2 hr at RT.

Confocal microscopy

Cardiomyocytes were fixed in 4% paraformaldehyde for 20 min and washed with PBS (three times with 5 min intervals). They were permeabilized in 0.02% TritonX100 in PBS for 20 min and washed with PBS. The blocking was done in 1% BSA in PBS for 1 hr. The primary antibodies for Gs (Santa Cruz Biotech, cat # sc-26766) and Hcy (abcam cat # ab 15154) were diluted in the ratio of 1:50 in PBS and cardiomyocytes were incubated in primary antibody for 2 hrs at RT. After washing the primary antibodies with PBS, the cardiomyocytes were incubated with secondary antibody (dilution 1:100) for 1 hr at RT. The secondary antibody for Gs was FITC conjugated rabbit- anti goat IgG (Invitrogen, cat # 61-1611) and for Hcy was Alexa fluor 647, goat anti-rabbit IgG (Invitrogen, cat # A21245). The cardiomyocytes were washed with PBS and incubated with

Dapi for 30 min for staining the nucleus. After washing with PBS, cardiomyocytes were mounted in FluoroGel mounting medium (GTX 28214) and observed under confocal microscope (Olympus). The intensity of fluorescence was measured by Image-Pro software.

In vitro studies on cardiomyocytes

The HL1 cardiomyocytes that possess the characteristics of murine cardiomyocytes [20] were treated with 5mM (normal dose) and 25mM (high dose) of sucrose (Sigma, cat # S-2395) for 24 hr. For control, same dose of medium was used. The expression of β2-AR was measured in the control and treated groups by Western blotting.

Flow cytometry

The HL1 cardiomyocytes were treated with 100 μM of Hcy for 24 hr in a cell culture incubator maintained at 37°C with 5% CO_2 as described earlier [19]. They were washed with PBS, dislodged by trypsin and precipitated by centrifugation at 500 rpm for 5 min at 37°C. The supernatant was removed and cells were resuspended in PBS. They were counted using hemocytometer (Fisher Scientific, Cat # 0267110) and equal number of cells was used for all the experimental groups. The cells were fixed in 3.7% formaldehyde for 10 min, washed with PBS (three times with 5 min interval) and incubated with β2-AR antibody (abcam cat # ab13300) for 30 min. For control, the cells were treated with Rabbit IgG FITC Isotype (abcam cat # ab37406). After washing with PBS, cells were run through accury flow cytometer (10,000 events). The results were analyzed using the software of accury flow cytometer.

Measurement of blood glucose level and gravimetric data

Mice were kept on fast for 10 hrs (overnight) and fasting blood glucose was determined by Glucometer (ONETOUCH Ultra). The heart and body weight of mice was measured at the time of sacrifice.

The isolation of ventricular cardiomyocytes

Cardiomyocytes were isolated from adult mice by the enzymatic dissociation of Liberase Blendzyme 4 (Roche Diagnostics, IN) following the described protocol [21]. Briefly, heart was perfused first in the oxygenated (5% CO_2 and 95% O_2) perfusion buffer maintained at 37°C and then in the digestion buffer containing liberase enzyme to dissociate the cardiomyocytes. Extracellular calcium (1.2 mM) was added in increments to the digestion buffer to maintain the contractility of cardiomyocytes.

Measurement of contractile dysfunction of cardiomyocytes

The contractility and calcium consumption of cardiomyocytes were measured by video-edge based detection system (Ion Optics, Milton, MA) following the described protocol [21]. A minimum of 30 myocytes with good contractile pattern were scored from single animal. To rule out the prolong effect of stimulation, cardiomyocytes were observed in batches.

Co-immunoprecipitation (Co-IP)

The protocol was followed from the "abcam" company with a little modification. In brief, equal amounts (450 μg) of protein were mixed with 5-10 μl of antibody (β2-AR) and the volume was maintained to 100 μl by adding phosphate buffered saline (PBS). The CNBr-activated Sepharose 4B beads (Pharmacia LKB Biotechnology AB Uppsala, Sweden) were mixed with PBS (100 mg/ ml). Both protein samples and beads were incubated overnight at 4°C under agitation, centrifuged (100 rpm for 5 min) and pellet was blocked with PBS-BSA (1% weight / volume) for 1 hr on a shaker at RT. After washing with PBS (twice at 5 min interval), beads were incubated with the protein samples at 4°C for 4 hr under slow agitation that allows protein binding. They were centrifuged at 4°C and supernatant was discarded. To elute the bound protein, 30 μl of loading sample buffer was added to the pellet and boiled at 95 °C for 5 min. It dissociated the protein from the beads, which was collected and loaded onto the SDS-PAGE gel following the protocol of Western blotting. The membrane was probed with β2-AR to detect the immunoprecipitation of β2-AR with the beads. After confirming the binding of β2-AR with the beads, co-immunoprecipitation of beta2-AR with Hcy was performed. All the steps in the protocol were same as mentioned for immunoprecipitation except that the membrane was probed with Hcy antibody (abcam, cat # ab 15154).

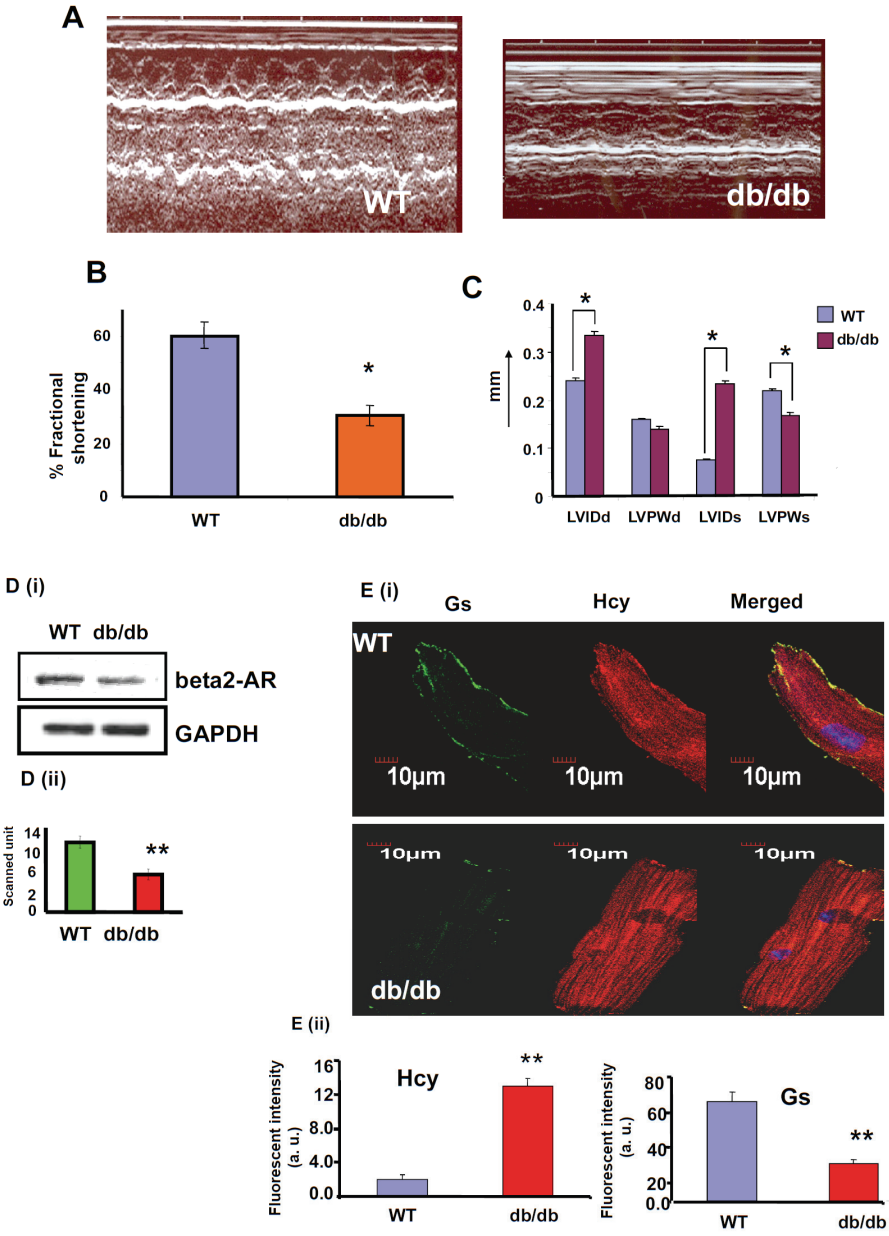


Figure 1. A. The echocardiogram of WT and db/db mice. **B.** The percentage fractional shortening. **C.** Bar graph of the echocardiogram of db/db and WT representative as bar graph in comparing the chamber dilation (LVIDd and LVIDs) and wall thickness (LVPWd and LVPWs). *p<0.05, n=6. **D.** (i): Western blotting of the heart tissue from WT (C57BL/6J) and db/db mice. GAPDH is used as a loading control. (ii). Representative bar graph with densitometry analysis. **p<0.01, n=6. E. (i). Co-localization of homocysteine (red color) and Gs (green color) in db/db and WT cardiomyocytes. (ii). Bar graph for fluorescent intensity. Arbitrary unit (a.u) *p<0.01, n=5.

Statistical analyses

The results were represented as average ± standard error. The two tailed t-test with unequal variance was used to compare the groups. The significance level was denoted with "*" symbol. The number of "*" corresponds to the "p" value (*, p<0.05; **, p<0.01; ***, p<0.001).

Results

Cardiac dysfunction in the db/db mice

The echocardiogram showed significant decrease in the percentage fractional shortening in db/db mice (**Figure 1A-B**). There was significant increase in the left ventricle internal diameter in diastolic (LVIDd) and left ventricle internal diameter in systole (LVIDs). On the contrary, left ventricle posterior wall thickness in systole (LVPWs) was decreased and there was tendency of decrease in the left ventricle posterior wall thickness in diastole (LVPWd) in db/db mice (**Figure 1C**).

β2-AR, homocysteine and Gs in db/db mice

The Western blot show attenuation of β2-AR in

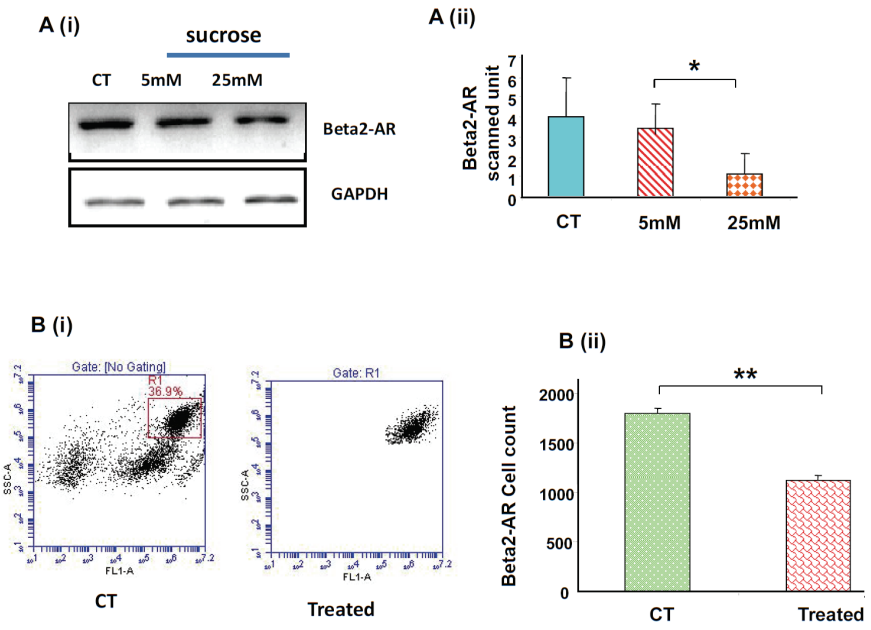


Figure 2. A. (i): Western blotting showing expression of β-AR in HL1 cardiomyocytes treated with high dose of sucrose. GAPDH is a loading control. (ii): The scanned unit of bands is shown in the left panel. *p<0.05, n=4. **B.** (i): Flow cytometry of HL1 cardiomyocytes treated with homocysteine (100μM). (ii): The gated population is analyzed for the β2-AR stained cell counts. *p<0.01, n= 4.

db/db mice (**Figure 1D i-ii**). The co-localization of Hcy with Gs in the cardiomyocytes from WT and db/db mice revealed increase in Hcy (red color) and decrease in Gs (green color) in db/db mice (**Figure E i - ii**).

Hyperglycemia and β2-AR expression

The high dose of sucrose simulates diabetic condition. The HL1 cardiomyocytes treated with high dose of sucrose show down regulation of β2-AR (**Figure 2A i-ii**).

Hyperhomocysteinemia and β2-AR expression

Treatment of HL1 cardiomyocytes with the high dose of Hcy resembles hyperhomocysteinemic condition. The flow cytometry analyses of hyperhomocysteinemic cardiomyocytes show significant decrease in the number of β2-AR (**Figure 2B i-ii**).

Effect of exercise and β2-AR agonist on cardiac dysfunction

Increase in the heart to body weight ratio (HW/BW) indicates cardiac hypertrophy. There was significant decrease in the HW/BW in the treated groups of db/db mice (**Figure 3A**) suggesting mitigation of cardiac hypertrophy due to exercise and treatment with β2-AR agonist. Also, the percentage fractional shortening was increased (**Figure 3B i-ii**) and LVIDs was decreased (**Figure 3C**) in the treated groups indicating improvement in cardiac function. The measurement of contractility of cardiomyocytes revealed shortening of time to 90% peak height in the DBES group (**Figure 3D**). However, calcium transient was increased in all the treated groups (**Figure 3E i-ii**). Interestingly, there was additive effect of salbutamol treatment and exercise on calcium transient (**Figure 3E ii**). The calcium handling enzyme SERCA2 was also up regulated in all the treated groups (**Figure 3 F i-ii**).

Effect of exercise on β2-AR

There was induction in transcription of β2-AR after exercise training (**Figure G i-ii**) and it was translated at protein level (**Figure H i-ii**).

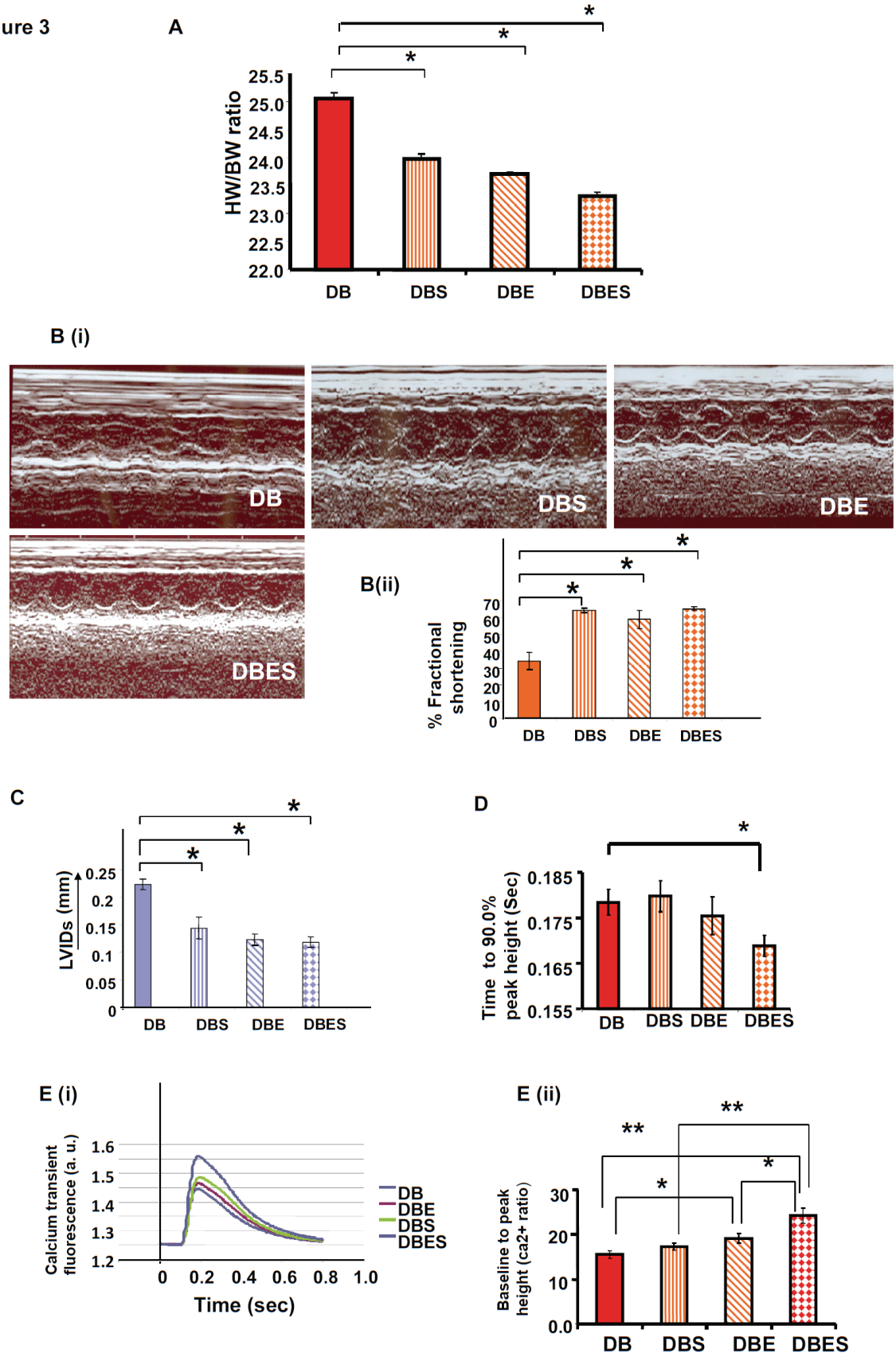
Effect of exercise on interactions of Hcy with β2-AR

The co-immunoprecipitation of β2-AR with Hcy revealed that exercise mitigates the cross talk between β2-AR and Hcy in diabetes (**Figure 3 I i-ii**).

Discussion

The increasing number of patients with obesity and diabetes across the world attracts the attention of scientists for investigating the causes and underlying mechanisms for these diseases.

Figure 3


A
HW/BW ratio
25.5
25.0
24.5
24.0
23.5
23.0
22.5
22.0
DB
DBS
DBE
DBES
B (i)
DB
DBS
DBE
DBES
B(ii)
% Fractional shortening
70
60
50
40
30
20
10
0
DB
DBS
DBE
DBES
C
LVIDs (mm)
0.25
0.2
0.15
0.1
0.05
0
DB
DBS
DBE
DBES
D
Time to 90.0% peak height (Sec)
0.185
0.175
0.165
0.155
DB
DBS
DBE
DBES
E (i)
Calcium transient fluorescence (a. u.)
1.6
1.5
1.4
1.3
1.2
0
0.2
0.4
0.6
0.8
1.0
Time (sec)
DB
DBE
DBS
DBES
E (ii)
Baseline to peak height (ca2+ ratio)
30
20
10
0.0
DB
DBS
DBE
DBES

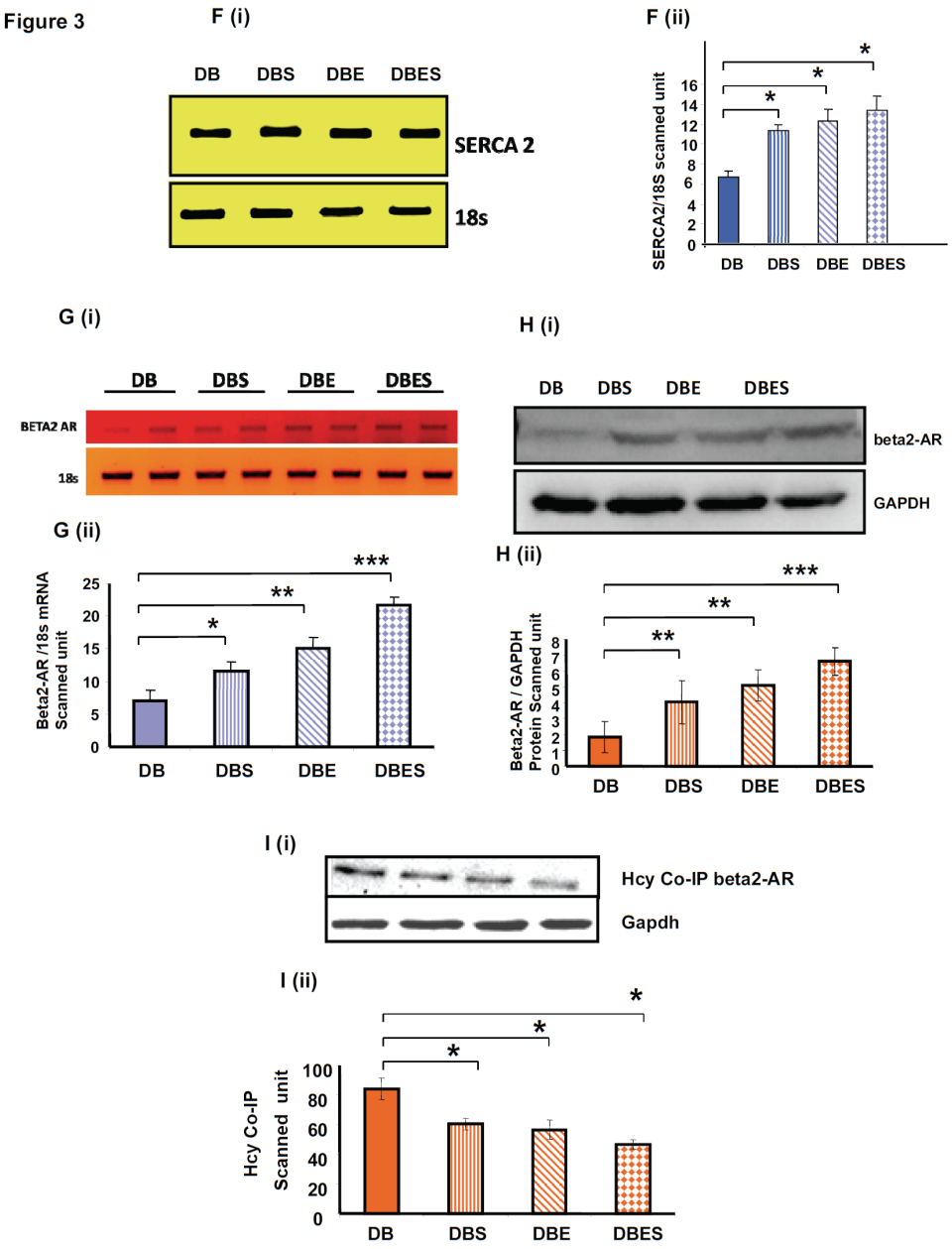


Figure 3. A. The ratio of the heart weight to body weight in the four groups of db/db mice. *p<0.05, n=6. **B.** (i): Representative echocardiograms of the four groups: db/db (DB), DB+ salbutamol (DBS), DB+ exercise (DBE), DB+ salbutamol + exercise (DBES). (ii): The percentage fractional shortening of the above four groups. *p<0.05, n=6. **C.** Comparison of left ventricle internal dimension in systole (LVIDs) among the four groups: DB, DBS. DBE and DBES. *p<0.05, n=6. **D.** The time required to the 90% peak height during systolic contraction of cardiomyocytes among the four groups: *p<0.05, n=6. **E.** (i): A representative graph for the calcium transient of cardiomyocytes from the four groups. (ii): The bar graph in the left panel shows the calcium consumption during baseline to peak height. *p<0.05; **, p< 0.01, n=6. **F.** (i): A representative RT-PCR for SERCA2 mRNA expression among the four groups. The 18s is used for normalization. (ii): The bar graph showing the relative expression of SERCA2 among the four groups. *p<0.05, n=5. **G.** (i): A representative RT-PCR showing mRNA expression of β2-AR among the four groups. (ii): The bar graph showing the relative expression of β2-AR among the four groups. *p<0.05, ** p<0.01, *** p<0.001, n=5. **H.** (i): A representative Western blots showing expression of β2-AR protein among the four groups. (ii): The bar graph showing relative expression of β2-AR protein among the four groups. *p<0.05, ** p<0.01, *** p<0.001, n=5. **I.** (i): Co-immunoprecipitation of Hcy with β2-AR using the heart tissue from the four groups. (ii): The bar graph represents the relative expression of Hcy among the four groups. *p<0.05, ** p< 0.01, n=5.

The translation of obesity first into insulin resistance and then into diabetic cardiomyopathy (DCM) provides a clue that the major changes that resulted into DCM starts in the obese individuals. One of the important caveats is that obese individual show over activity of sympathetic tone that results into spillover of noradrenaline into different organs including skeletal muscles. Excess noradrenaline causes vasoconstriction that impairs glucose uptake in the skeletal muscle resulting into insulin resistance - the hallmark of T2D [22] that ultimately leads to DCM. On the other hand exercise training ameliorates cardiac dysfunction by improving insulin sensitivity and sympathetic tone [8]. Nevertheless, the underlying mechanism is unclear.

Diabetes induces hyperhomocysteinemia [11,23,24] that causes attenuation of sympathetic tone by inhibiting β-AR [14,13]. Contrary to that exercise mitigates hyperhomocysteinemia [16] and ameliorates DCM. These reports indicate the relationships among exercise, β-AR, hyperhomocysteinemia and cardiac dysfunction. To study the cross talk among β2-AR, Gs, Hcy and cardiac dysfunction, we first determined cardiac function of WT and db/db mice by echocardiography. The results show decrease in the percentage fractional shortening and LVPWs, and increase in the LVIDd and LVIDs in db/db mice (**Figure 1A-C**) suggesting cardiac dysfunction. These findings concur with the earlier report that twelve week db/db mice have systolic and diastolic dysfunction [25]. After confirming the cardiac dysfunction, we measured the expression of β2-AR in the heart of WT and db/db mice. As expected, there was attenuation of β2-AR in db/db mice (**Figure 1 D i-ii**). The down regulation of β2-AR response in diabetes was also supported by the in vitro studies, where treatment with high dose of sucrose down regulated β2-AR (**Figure 2 A i-ii**). These data corroborate the previous reports that sympathetic tone is impaired in diabetes [22,26]. To investigate the effect of Hcy on β2-AR, we performed two experiments: 1) confocal microscopy to co-localize Hcy with Gs in the cardiomyocytes extracted from the WT and db/db mice, and 2) flow cytometry analyses of β2-AR number in HL1 cardiomyocytes treated with high dose of Hcy. Interestingly, co-localization of Hcy with Gs revealed antagonistic relationship between Hcy and Gs expression in WT and db/db cardiomyocytes (**Figure 1E i-ii**). The increase in Hcy and decrease in Gs expression in db/db cardiomyocytes indicates that Hcy might be inhibiting β2-AR response by down regulating Gs. The direct effect of Hcy on β2-AR was evaluated by flow cytometry studies. The results revealed that hyperhomocysteinemia depletes β2-AR (**Figure 2 B i-ii**). To our knowledge this is the first report that Hcy modulates β-adrenergic drive in the diabetic heart by either a) directly decreasing the number of β2-AR, or b) indirectly attenuating β2-AR response by inhibiting Gs. The above exciting findings lead us to investigate the effect of exercise on the cross talk among Hcy, β2-AR and cardiac dysfunction in db/db mice. The beneficial effect of exercise on β2-AR, Hcy and their cross talk in diabetes was determined by treating db/db mice with (a) salbutamol, (b) exercise, and (c) exercise + salbutamol and comparing them with (d) sedentary group. We chose ten days exercise period (more than acute and less than long term exercise) to observe the overall effect of exercise. Similarly, swimming was preferred because it was a complete exercise. To rule out the stress generated by swimming, the mice were allowed to rest for one day after treatment and then sacrificed. We first looked at the mitigation of cardiac dysfunction by measuring the HW/BW ratio, fractional shortening and LVIDs in the four groups of mice (DB, DBS, DBE and DBES). There was decrease in the HW/BW ratio and LVIDs, and increase in the fractional shortening in the treated groups (**Figure 3 A, B i-ii and C**) suggesting amelioration of cardiac dysfunction. However, there was no significant change in the rate of contraction and relaxation (± dL/dt) in the treated groups except the significant decrease in the time to 90% peak height in DBES group (**Figure 3D**). These findings indicate that exercise and β2-AR agonist might not have considerable effects on the contractile apparatus of cardiomyocytes. Nevertheless, there was improvement in calcium transient in the treated groups (**Figure E i-ii**) suggesting that exercise and β2-AR agonist have major influence on calcium handling proteins in diabetes. Importantly, the β2-AR agonist and exercise have additive effect on calcium transient (**Figure E ii**). We also measured the expression of SERCA2 (a well established calcium handling protein) in the four groups, which was up regulated in the treated groups (**Figure F i-ii**). These findings revealed that exercise mitigates cardiac dysfunction by improving calcium handling of cardiomyocytes in diabetes.

The direct effect of exercise on β2-AR expression was determined both at mRNA (RT-PCR)

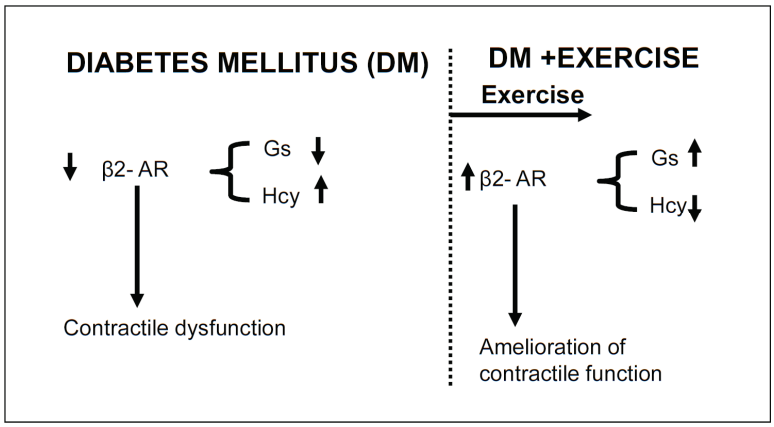


Figure 4. A schematic representation of the effect of exercise on contractile dysfunction in diabetes. The impairment of contractility is due, in part, to the attenuation of β2-AR response caused by inhibition of Gs and up regulation of homocysteine. Exercise lowers homocysteine level, induces Gs, restores β2-AR response and mitigates contractile dysfunction.

and protein (Western blotting) levels. The results show induction of β2-AR by exercise (**Figure G i-ii and H i-ii**). The effect of exercise on cross- talk between Hcy and β2-AR was determined by co-IP. Interestingly, the interactions between Hcy and β2-AR were alleviated by exercise (**Figure I i-ii**). It is an important finding because Hcy influences cardiac dysfunction, in part, by interacting through β2-AR. Exercise interferes with the protein-protein interactions of Hcy and β2-AR and ameliorates β2-adrenergic drive in diabetes. These findings elicit that elevation of Hcy level and inhibition of Gs causes attenuation of β2-AR that leads to contractile dysfunction in diabetes. Exercise lowers the level of Hcy, interferes with Hcy-β2-AR interactions and induces Gs that altogether enhances β2-AR response and mitigates contractile dysfunction in diabetes (**Figure 4**).

Novelty and limitations

The present study revealed for the first time that (1) Hcy attenuates β2- AR by inhibiting Gs in diabetes, (2) exercise mitigates the protein-protein interactions between Hcy and β2-AR in diabetes, and (3) exercise ameliorates cardiac dysfunction mainly by improving the calcium transient of cardiomyocytes in diabetes.

The major limitations are (1) the data required to be confirmed by over expression of β2-AR in the diabetic mice, (2) expression of β2-AR needs to be measured in the hyperhomocysteinemic mice (such as cystathionine β synthase mutant mice- a genetic model for mild hyperhomocysteinemia, or mice treated with high methionine diet) to confirm the interactions of Hcy with β2-AR, and (3) further investigations are required to substantiate the cross- talk among Hcy, β2-AR, Gs in different models of diabetes and obesity.

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