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## Insulin-Like Growth Factor I (IGF-1) Deficiency Ameliorates Sex Difference in Cardiac Contractile Function and Intracellular Ca<sup>2+</sup> Homeostasis

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### Abstract

Sex difference in cardiac contractile function exists which may contribute to the different prevalence in cardiovascular diseases between genders. However, the precise mechanisms of action behind sex difference in cardiac function are still elusive. Given that sex difference exists in insulin-like growth factor I (IGF-1) cascade, this study is designed to evaluate the impact of severe liver IGF-1 deficiency (LID) on sex difference in cardiac function. Echocardiographic, cardiomyocyte contractile and intracellular Ca<sup>2+</sup> properties were evaluated including ventricular geometry, fractional shortening, peak shortening, maximal velocity of shortening/relengthening ( $\pm$  dL/dt), time-to-peak shortening (TPS), time-to-90% relengthening (TR<sub>90</sub>), fura-fluorescence intensity (FFI) and intracellular Ca<sup>2+</sup> clearance. Female C57 mice exhibited significantly higher plasma IGF-1 levels than their male counterpart. LID mice possessed comparably low IGF-1 levels in both sexes. Female C57 and LID mice displayed lower body, heart and liver weights compared to male counterparts. Echocardiographic analysis revealed larger LV mass in female C57 but not LID mice without sex difference in other cardiac geometric indices. Myocytes from female C57 mice exhibited reduced peak shortening,  $\pm$  dL/dt, longer TPS, TR<sub>90</sub> and intracellular Ca<sup>2+</sup> clearance compared with males. Interestingly, this sex difference was greatly attenuated or abolished by IGF-1 deficiency. Female C57 mice displayed significantly decreased mRNA and protein levels of Na<sup>+</sup>-Ca<sup>2+</sup> exchanger, SERCA2a and phosphorylated phospholamban as well as SERCA activity compared with male C57 mice. These sex differences in Ca<sup>2+</sup> regulatory proteins were abolished or overtly attenuated by IGF-1 deficiency. In summary, our data suggested that IGF-1 deficiency may significantly attenuated or mitigate the sex difference in cardiomyocyte contractile function associated with intracellular Ca<sup>2+</sup> regulation.

### Keywords

IGF-1; cardiomyocytes; sex; intracellular Ca<sup>2+</sup>; SERCA

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## INTRODUCTION

Although cardiovascular diseases affect both genders, there are distinct gender-related differences in a broad spectrum of cardiovascular incidence and mortality (Ren, 2006; Shirato and Swan, 2010). Several factors have been speculated to contribute to the gender disparity such as sex hormones, the gender-specific intrinsic organ function, difference in body size and other cardiovascular risk profiles (Ceylan-Isik *et al.*, 2006b; Ren, 2006; Perez-Lopez *et al.*, 2010; Shirato and Swan, 2010). It is well known that premenopausal women possess advantage in the onset and progression of cardiovascular diseases compared with the postmenopausal women and the age-matched men (Ren and Kelley, 2009; Shirato and Swan, 2010). Given that estrogen is a key regulator of maturation, growth, differentiation and function of tissues, including those in the cardiovascular system (Younes and Honma, 2011), the “female advantage” in cardiovascular disease risk may be attributable to the female sex hormone estrogen (Ren and Kelley, 2009; Lombardi *et al.*, 2010; Perez-Lopez *et al.*, 2010). Estrogen exerts its biological actions via estrogen receptor  $\alpha$  found in abundance in endometrium, breast cancer cells, ovarian stroma cells and hypothalamus; and estrogen receptor  $\beta$  found in kidney, brain, bone, heart, lungs, intestinal mucosa, prostate and endothelial cells (Billon-Gales *et al.*, 2009; Arnal *et al.*, 2010). Estrogen has been shown to mediate a wide array of beneficial cardiovascular effects including cardiac excitability, regulation of ion channel function and  $\text{Ca}^{2+}$  regulatory proteins (Ling *et al.*, 2006; Perez-Lopez *et al.*, 2010). Ovariectomy has been linked to both enhanced and depressed cardiac contractility (Ren *et al.*, 2003; Kravtsov *et al.*, 2007) while selective estrogen receptor modulators are capable of inhibiting L-type  $\text{Ca}^{2+}$  current and thus decrease myocyte contraction (Liew *et al.*, 2004). Nonetheless, many of the sex difference in cardiovascular function including cardiac contractile function may be intrinsic to the target organ/tissue and thus be secondary to cardiovascular regulation elicited by sex hormones such as estrogen (Ren and Ceylan-Isik, 2004; Ceylan-Isik *et al.*, 2006a).

Insulin-like growth factor-1 (IGF-1), an important cardiac survival, maintain cardiac function, cardiac growth and energy metabolism in both healthy and failing hearts (Ren *et al.*, 1999). Severe IGF-1 deficiency is associated with the altered body composition, neuroendocrine activation, and cardiac atrophy and compromised cardiac function (Gola *et al.*, 2005; Yakar *et al.*, 2005). Nonetheless, liver IGF-1 deficiency retards cardiac dysfunction in aging (Li *et al.*, 2008) and antagonizes pro-oxidants such as paraquat-induced cardiac contractile dysfunction (Li *et al.*, 2007), indicating a rather unique role of IGF-1 deficiency or depletion in the regulation of cardiovascular function. Given the apparent sex difference in IGF-1 signaling cascade including lower IGF-1 levels in males (Chaler *et al.*, 2009; Gupta *et al.*, 2011), the aim of our present study was to examine the influence of liver IGF-1 deficiency on cardiac contractile function and intracellular  $\text{Ca}^{2+}$  handling between males and females. In an effort to explore the potential mechanism(s) of action behind sex difference in cardiac mechanical function, levels of the key intracellular  $\text{Ca}^{2+}$  regulatory proteins including sarco(endo)plasmic reticulum  $\text{Ca}^{2+}$ -ATPase activity (SERCA),  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchanger and phospholamban as well as estrogen receptor  $\beta$  (ER $\beta$ ) were examined in myocardium from male and female mice.

## MATERIALS and METHODS

### Experimental animals and genotyping

The experimental procedure was approved by the Institutional Animal Use and Care Committee at the University of Wyoming (Laramie, WY). All animal procedures were in accordance with the National Institutes of Health standard. LID mice on a mixed C57BL/6, FVB/N and 129sv background were generated using *Cre/loxP* system (Yakar *et al.*, 1999). To determine the presence of the IGF/loxP and *Cre* transgenes, genomic DNA was isolated

from tail clips using a Quick extraction and amplification kit (BioPioneer Inc. San Diego, CA). Homozygous or heterozygous mice for IGF-1/loxP carrying the albumin-*Cre* transgene were crossed. The homozygous offspring, along with negative controls, were used for our experiment. The mouse genotyping was executed using a double PCR strategy. To identify the genotype IGF-1/loxP, primers of IA6, IA8 and ID3 were used in PCR reaction. Mice that yielded one 0.4 kb band were considered to be negative for IGF-1/loxP, whereas those with one 0.2 kb band were positive. Heterozygous IGF/loxP was identified with the presence of both 0.4 and 0.2 kb bands. To determine the presence of the *Cre* transgene, primers Cre-5 and Cre-3 were used, which yielded a 0.6 kb band. Mice positive for both IGF-1/loxP and *Cre* transgenes were deemed LID mice, whereas IGF-1/loxP-negative mice with or without the *Cre* transgene were used as the LID-negative mice (Li *et al.*, 2007; Li *et al.*, 2008). Male or female LID mice and their negative littermates at 9–10 weeks of age were used. Plasma IGF-1 levels were measured using an ELISA commercial kit from Diagnostic System Laboratory (Webster, TX) as described previously (Li and Ren, 2007).

### Isolation of murine cardiomyocytes

Murine cardiomyocytes were isolated as described (Ceylan-Isik *et al.*, 2011). After ketamine/xylazine sedation, hearts were removed and perfused with Ca<sup>2+</sup>-free Tyrode's solution containing (in mM): NaCl 135, KCl 4.0, MgCl<sub>2</sub> 1.0, HEPES 10, NaH<sub>2</sub>PO<sub>4</sub> 0.33, glucose 10, butanedione monoxime 10, and the solution was gassed with 5% CO<sub>2</sub>/95% O<sub>2</sub>. Hearts were digested with Liberase Blendzyme 4 (Hoffmann-La Roche Inc., Indianapolis, IN) for 20 min. Left ventricles were removed and minced before being filtered. Tissue pieces were gently agitated and pellet of cells was resuspended. Extracellular Ca<sup>2+</sup> was added incrementally back to 1.20 mM over a period of 30 min. Isolated myocytes were used within 8 hrs of isolation. Normally, a yield of 50–60% viable rod-shaped cardiomyocytes with clear sarcomere striations was achieved. Only rod-shaped myocytes with clear edges were selected for mechanical study.

### Cell shortening/relengthening

Mechanical properties of cardiomyocytes were assessed using a SoftEdge MyoCam system (IonOptix Corporation, Milton, MA). In brief, cardiomyocytes were placed in a chamber mounted on the stage of an inverted microscope (Olympus, Model IX-70, Olympus Ltd., Tokyo, Japan) and superfused at 25°C with a buffer containing (in mM): 131 NaCl, 4 KCl, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 Glucose and 10 HEPES, at pH 7.4. Cells were field stimulated with suprathreshold voltage at frequencies of 0.5 Hz using an FHC stimulator (Brunswick, NE). The myocyte being studied was displayed on a computer monitor using an IonOptix MyoCam camera. IonOptix SoftEdge software was used to capture changes in cell shortening and relengthening. Cell shortening and relengthening were assessed using the following indices: peak shortening, indicative of cell contractile capacity; maximal velocities of cell shortening and relengthening ( $\pm$  dL/dt), indicative of maximal rate of ventricular tension development and decline; time-to-PS (TPS) - indicative of systolic duration; time-to-90% relengthening (TR<sub>90</sub>) - indicative of diastolic duration (90% rather than 100% relengthening was used to avoid noisy signal near baseline). In the case of altering stimulus frequency, the steady-state contraction of myocyte was achieved (usually after the first six beats) before PS amplitude was recorded at 0.1, 0.5, 1.0, 3.0 and 5.0 Hz (Li *et al.*, 2005; Ceylan-Isik *et al.*, 2011).

### Intracellular Ca<sup>2+</sup> transients

Separate cohorts of myocytes were loaded with fura-2/AM (0.5  $\mu$ M) for 15 min, and fluorescence intensity was measured with a dual-excitation fluorescence photomultiplier system (IonOptix). Myocytes were placed on an inverted Olympus microscope and imaged through a Fluor 40x-oil objective. Cells were exposed to light emitted by a 75 W mercury

lamp and passed through either a 360 nm or a 380 nm filter. The myocytes were stimulated to contract at 0.5 Hz. Fluorescence emissions were detected between 480 nm and 520 nm by a photomultiplier tube after cells were first illuminated at 360 nm for 0.5 sec and then at 380 nm for the duration of the recording protocol (333 Hz sampling rate). The 360 nm excitation scan was repeated at the end of the protocol, and qualitative changes in intracellular  $\text{Ca}^{2+}$  concentration were inferred from the ratio of the fluorescence intensity at two wavelengths. Intracellular  $\text{Ca}^{2+}$  decay was calculated using curve fitting (Ceylan-Isik *et al.*, 2011).

### Western blot analysis

Protein expression of estrogen receptor  $\beta$  (ER $\beta$ ),  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger, SERCA2a and phospholamban were examined by Western blot analysis. Left ventricular tissues were homogenized and centrifuged at 70,000 g for 20 min at 4°C. The supernatants were used for immunoblotting. The extracted proteins were separated on 10–15% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After being blocked, the membrane was incubated with rabbit monoclonal anti-ER $\beta$ , rabbit polyclonal anti- $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger, mouse polyclonal anti-SERCA2a, mouse monoclonal anti-phospho-phospholamban (Ser16) and  $\beta$ -actin (loading control) antibodies at 4°C overnight. Anti-ER $\beta$  antibody was purchased from Cell Signaling Technology (Beverly, MA). Anti-NCX antibody was purchased from Swant (Bellinzona, Switzerland). Anti-SERCA2a antibody was from Affinity BioReagents (Golden, CO) and anti-phospholamban antibody was obtained from Abcam (Cambridge, MA). After incubation with the primary antibodies, blots were incubated with horseradish peroxidase-linked secondary antibodies (1:5,000) for 60 min at room temperature. Immunoreactive bands were detected using the Super Signal West Dura Extended Duration Substrate (Pierce, Milwaukee, WI). The intensity of bands was measured with a scanning densitometer (Model GS-800; Bio-Rad) coupled with a Bio-Rad personal computer analysis software (Ceylan-Isik *et al.*, 2011).

### SERCA activity measured by $^{45}\text{Ca}^{2+}$ -uptake

Cardiomyocytes were sonicated and solubilized in a tris-sucrose homogenization buffer containing 30 mM Tris-HCl, 8% sucrose, 1 mM PMSF, and 2 mM dithiothreitol (pH 7.1). To determine SERCA-dependent  $\text{Ca}^{2+}$  uptake, samples were treated with and without the SERCA inhibitor thapsigargin (10  $\mu\text{M}$ ) for 30 min. The difference between the two readings was deemed the thapsigargin-sensitive uptake through SERCA. Uptake was initiated by the addition of an aliquot of supernatant to a solution consisting of (in mM) 100 KCl, 5  $\text{NaN}_3$ , 6  $\text{MgCl}_2$ , 0.15 EGTA, 0.12  $\text{CaCl}_2$ , 30 Tris-HCl (pH 7.0), 10 oxalate, 2 ATP, and 1  $\mu\text{Ci}$   $^{45}\text{CaCl}_2$  at 37°C. Aliquots of samples were injected onto glass filters on a suction manifold and washed three times. Filters were removed from the manifold and placed in scintillation fluid. SERCA activity was expressed as counts per million per mg protein (Ceylan-Isik *et al.*, 2011).

### Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Analysis of ER $\beta$ ,  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger, SERCA2a and phospholamban was performed using real time RT-PCR as described previously (Elsherif *et al.*, 2004; Mathiyalagan *et al.*, 2010). In brief, myocardium was homogenized to obtain a clear solution in TriZol and cells were handled in the presence of RNaseIn (Invitrogen, Carlsbad, CA). Total RNA was prepared using RNeasy minikit preparation columns (Qiagen, Valencia, CA). RNA was reverse transcribed with a reverse transcriptase kit (Invitrogen, Carlsbad, CA). SYBR Green (Bio-Rad, USA) real-time quantitative PCR was performed after first strand cDNA synthesis. Designed forward and reverse primers for SERCA,  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger and phospholamban were purchased from Integrated DNA Technologies (Coralville, IA) and the ER $\beta$  primer was obtained from Qiagen (Qiagen, Valencia, CA) (Table 1). GAPDH was used as the internal control for normalization. Relative differences were evaluated using the cycle

time valued and expresses as the fold increase of control. Assuming that the cycle time value is reflective of the initial starting copy and that there is 100% efficacy, a difference of one cycle is calculated from each gene's standard curve.

### Statistical analysis

Data were Mean  $\pm$  SEM. Statistical significance ( $p < 0.05$ ) for each variable was determined by a one-way ANOVA followed by the Tukey's *post hoc* analysis.

## RESULTS

### General features and echocardiographic properties of male and female mice

As shown in Table 2, female C57BL/6 mice exhibited significantly lower body, heart and liver weights compared with the age-matched male C57BL/6 mice. This sex difference prevailed in LID mice with the exception of liver weight. Liver IGF-1 deficiency led to overtly increased liver weight and size (normalized to body weight) in both sexes and nullified the sex difference in liver weight in C57BL/6 mice. Neither female sex nor LID affected the cardiac size although the size of heart was significantly enhanced by LID in female mice. Neither female sex nor LID significantly altered the weight and size (normalized to body weight) of kidney. Plasma IGF-1 levels were significantly higher in female C57BL/6 mice while liver IGF-1 gene deletion resulted in a comparable and dramatic drop in plasma IGF-1 levels in both males and females. Echocardiographic examination revealed comparable heart rate, left ventricular end systolic diameter (LVESD), LV end diastolic diameter (LVEDD), LV wall thickness and fractional shortening in male or female C57BL/6 and LID groups. However, the calculated LV mass was significantly lower in female C57BL/6 mice compared with male C57BL/6 mice, the effect of which was not present in LID mice. LID itself did not significantly affect the LV mass. Most likely due to the lower body weight in female mice, the normalized LV mass was comparable among male or female C57BL/6 and LID mice.

### Mechanical and intracellular Ca<sup>2+</sup> properties of cardiomyocytes

Evaluation of cardiomyocyte mechanical properties revealed similar resting cell length in male or female C57BL/6 and LID mice. Female C57BL/6 mice displayed significantly reduced peak shortening and maximal velocity of shortening/relengthening ( $\pm$  dL/dt) as well as prolonged time-to-PS (TPS) and time-to-90% relengthening (TR<sub>90</sub>) compared with C57BL/6 male mice. Interestingly, while liver IGF-1 deficiency itself did not affect the cardiomyocyte contractile function in male mice, it nullified the sex difference in cardiomyocyte mechanical function (Fig. 1). To explore to intracellular Ca<sup>2+</sup> handling contributes to IGF-1 deficiency-induced effect on sex difference in cardiomyocyte contractile properties, Fura-2 fluorescence microscopy was used to examine the intracellular Ca<sup>2+</sup> handling in cardiomyocytes. Our data shown in Fig. 2A-D revealed that resting, peak and the electrically-stimulated rise in intracellular Ca<sup>2+</sup> ( $\Delta$ FFI) were comparable in cardiomyocytes from male or female C57BL/6 and LID mice. Intracellular Ca<sup>2+</sup> decay rate was significantly prolonged in female C57BL/6 mice compared with male C57BL/6 mice. Although liver IGF-1 deficiency itself did not affect any of the intracellular Ca<sup>2+</sup> handling parameters tested, it significantly attenuated female sex-induced prolongation in intracellular Ca<sup>2+</sup> transient clearance.

### Effect of increasing stimulation frequency on cardiomyocyte shortening

Murine hearts beats at high frequencies (~450 beats/min), whereas our baseline stimulus in this study was only set at 0.5 Hz (30 beats/min). To probe possible derangement of cardiac contractile function at higher frequencies, stimulating frequency was increased stepwise



from 0.1 Hz to 5.0 Hz pe before steady-state peak shortening was recorded. All values were normalized to peak shortening (Duan *et al.*) obtained at 0.1 Hz of the same cardiomyocyte. Fig. 2E displays a comparable negative staircase in PS (change from PS obtained at 0.1 Hz) among all four myocyte groups with increasing stimulus frequencies, suggesting minimal effect of female sex and/or IGF-1 deficiency on intracellular  $\text{Ca}^{2+}$  cycling.

### **Influence of IGF-1 deficiency on expression of ER $\beta$ and intracellular $\text{Ca}^{2+}$ regulatory proteins**

To further determine the potential mechanisms involved in LID-elicited antagonism against sex difference in cardiomyocyte mechanical function, we evaluated the expression and/or activity of the estrogen receptor ER $\beta$ , the key intracellular  $\text{Ca}^{2+}$  regulatory proteins  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchanger, SERCA2a and the SERCA inhibitor phospholamban in myocardium. As depicted in Fig. 3, Western blot analysis demonstrated significantly downregulated levels of expression of  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchanger, SERCA2a and phosphorylated phospholamban with unchanged ER $\beta$  in female C57BL/6 mice compared with male C57BL/6 mice. Although IGF-1 deficiency itself did not affect these intracellular  $\text{Ca}^{2+}$  regulatory proteins, it significantly attenuated or mitigated female sex-induced change in  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchanger, SERCA2a and phosphorylated phospholamban. Consistent with the protein expression, SERCA activity measured by  $^{45}\text{Ca}^{2+}$  uptake was significantly decreased in female C57BL/6 mice, the effect of which was lessened by IGF-1 deficiency (with little effect on SERCA activity itself). Last but not least, although LID failed to elicit any effect on ER $\beta$  expression in male mice, it significantly enhanced the expression of ER $\beta$  in female mice.

### **Influence of IGF-1 deficiency on mRNA of ER $\beta$ and intracellular $\text{Ca}^{2+}$ regulatory proteins**

Fig. 4 illustrates the fold increase in mRNA expression of ER $\beta$ ,  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchanger, SERCA2a and phospholamban using qRT-PCR. Our result indicated that mRNA expression of  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchanger, SERCA2a and phospholamban was significantly lower in female C57BL/6 mice compared with those in male C57BL/6 mice. Although LID itself did not elicit any notable effect on mRNA levels of these intracellular  $\text{Ca}^{2+}$  regulatory proteins, it ablated or partially alleviated the sex difference in mRNA levels of  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchanger and phospholamban but not SERCA2a. Neither sex nor LID significantly affected the mRNA expression of ER $\beta$  although the combination of both significantly elevated ER $\beta$  mRNA expression.

## **DISCUSSION**

The salient finding from our study suggested that liver IGF-1 gene deletion attenuated or mitigated the sex difference in cardiomyocyte contractile function possibly due to an intracellular  $\text{Ca}^{2+}$  regulation-dependent mechanism. Our data revealed higher plasma IGF-1 levels in female C57BL/6 mice compared with those in male C57BL/6 mice, consistent with the previous notion of a sex difference in circulating IGF-1 levels (Chaler *et al.*, 2009; Gupta *et al.*, 2011). Liver IGF-1 gene deletion significantly lowered the plasma IGF-1 levels to comparable levels in both male and female LID mice. More importantly, we found that female C57BL/6 mice exhibited significantly lower levels (both mRNA and protein) of the intracellular  $\text{Ca}^{2+}$  regulator proteins  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchanger, SERCA2a, and phospholamban (phosphorylated) compared to those from male C57 mice. Similarly, SERCA activity measured by  $^{45}\text{Ca}^{2+}$  uptake assay was reduced in female C57BL/6 mice, the effect of which was reversed by IGF-1 deficiency. Measurement of ER $\beta$  levels in both mRNA and protein expression did not reveal any significant sex difference in C57BL/6 mice nor did IGF-1 deficiency overtly alter ER $\beta$  expression. Interestingly, liver IGF-1 gene deletion significantly enhanced ER $\beta$  expression (both mRNA and protein) in female mice.

Epidemiological evidence has demonstrated that premenopausal women possess a lower incidence for cardiovascular diseases compared to the age-match men and postmenopausal women (Ren, 2006; Shirato and Swan, 2010). This female bias is disappeared once menopause starts (Ren, 2006). It has been shown that animal models of ovariectomy exhibited increased vascular reactivity (Ceylan-Isik *et al.*, 2009), heart rate (Marni *et al.*, 2009) and  $\text{Ca}^{2+}$  fluxes (Kravtsov *et al.*, 2007). On the other hand, estrogen or estrogen replacement may protect cardiomyocytes from hypoxia-induced  $\text{Ca}^{2+}$  overload (Liao *et al.*, 1995), decrease the development of atherosclerosis (Mendelsohn and Karas, 1999; Kim *et al.*, 2006) and increase endothelium-dependent relaxation (Ceylan-Isik *et al.*, 2009). Gender difference in intrinsic cardiomyocytes contractile and intracellular  $\text{Ca}^{2+}$  properties have been well documented (Ren and Ceylan-Isik, 2004). Data from our current study revealed that reduced peak shortening, lessened maximal velocity of shortening and relengthening and, prolonged duration of shortening and relengthening in female C57BL/6 mouse cardiomyocytes compared with male C57BL/6 mouse cardiac myocytes. This gender-related myocardial contractile difference is consistent with our previous findings (Zhang *et al.*, 2003; Ceylan-Isik *et al.*, 2006a; Ceylan-Isik *et al.*, 2006b). The mechanisms behind the sex difference in cardiomyocyte contractile function remain unclear but may be associated with in altered intracellular  $\text{Ca}^{2+}$  handling as slowed intracellular  $\text{Ca}^{2+}$  clearing was seen in female C57BL/6 mice, which was cancelled off by IGF-1 deficiency. In our hand, we failed to observe any difference in the baseline, peak and electrically-stimulated intracellular  $\text{Ca}^{2+}$  levels between male and female C57BL/6 mice. The apparent sex difference in cardiomyocyte mechanical and intracellular  $\text{Ca}^{2+}$  properties is still elusive but may be associated with sex hormone-induced changes in myofilament  $\text{Ca}^{2+}$  sensitivity. Ovarian hormones (e.g., estrogen) are well known to alter myocardial contractile function such as myofilament  $\text{Ca}^{2+}$  sensitivity (Bupha-Intr *et al.*, 2007). This is also reflected in the peak shortening-stimulus frequency response where little sex difference was observed. The similar pattern in the negative staircase in peak shortening-stimulus frequency response among male or female C57BL/6 and LID mice may indicate a comparable efficiency in intracellular  $\text{Ca}^{2+}$  replenishing from sarcoplasmic reticulum. The fact that cardiomyocyte contractile indices displayed a sex difference in the absence of changes in baseline, peak intracellular  $\text{Ca}^{2+}$  and  $\Delta\text{FFI}$  as well as intracellular  $\text{Ca}^{2+}$  cycling denotes a possible role of myofilament  $\text{Ca}^{2+}$  responsiveness in the sex difference of cardiac contractile function. It is also noteworthy of the subtle but significant difference between whole hearts and cardiomyocytes. This discrepancy between hearts and heart cells may indicate possible contributions from the non-myocyte components such as fibroblasts and cell matrix.

Our echocardiographic result indicated significantly lower calculated LV mass in female C57BL/6 mice compared with that from male C57BL/6 mice. This is consistent with observation from human in that Japanese women display a smaller LV mass compared with age-matched Japanese men (Katayama *et al.*, 2010). In a smaller-scale study performed in Nigerians, women display a trend but insignificant decrease in LV mass compared with age-matched men (Ogah and Bamgboye, 2010). This subtle difference in the LV mass between men and women may be related to the apparent differences in ethnicity and blood pressure (Nigerian participants were hypertensive). Our study did not reveal any sex difference in fractional shortening, again similar that reported in the human setting (Katayama *et al.*, 2010). Interestingly, the sex difference in LV mass was absent in LID mice. In addition, liver IGF-1 deficiency itself did not significantly affect LV mass. These findings seem to contradict the commonly accepted concept that IGF-1 promotes cardiac growth (Ren *et al.*, 1999) and data from our recent report that IGF-1 overexpression in the heart promotes cardiac hypertrophy (Zhang *et al.*, 2010). Several scenarios may be considered for the paradoxical effect of IGF-1. First, cardiac IGF-1 mRNA expression was found to be normal in these LID mice compared with wild-type controls despite of the severely reduced circulating IGF-1 levels (Yakar *et al.*, 1999). The cardiac growth in LID mice appears to be

normal (Yakar *et al.*, 1999). It is possible that locally released IGF-1 may override the reduced circulating IGF-1 levels in LID mice and thus maintain the normal cardiac growth and geometry in LID mice. However, excessive IGF-1 will trigger cardiac hypertrophy (Ren *et al.*, 1999). Secondly, IGF-1 is known to regulate cardiac function and architecture through an endocrine/paracrine fashion (Ren *et al.*, 1999). Severe loss of circulating IGF-1 may turn on other compensatory pro-growth mechanisms to cope with the reduced circulating levels of IGF-1 although further study is warranted.

Perhaps the most intriguing finding from our study is that liver IGF-1 gene deficiency attenuated or abolished the sex difference in cardiomyocyte contractile function, indicating a role of IGF-1 in sex difference in cardiovascular function and thus risk of cardiovascular diseases. IGF-1 is essential in the regulation of growth and cell proliferation (Juul, 2003). Decreased levels of IGF-1 have been reported in chronic heart failure (Broglia *et al.*, 1999; Jankowska *et al.*, 2006) and IGF-1 treatment increased cardiac contractility and reduced ischemia-induced apoptosis in myocytes (Lee *et al.*, 1999). The beneficial effects of cardiac specific overexpression of IGF-1 has been shown to protect cardiac anomalies in sepsis (Zhao *et al.*, 2009), alcoholism (Zhang *et al.*, 2010) and aging (Li and Ren, 2007). Data from our present study depicted that liver IGF-1 deficiency did not lead to any deterioration in cardiac contractile and intracellular  $\text{Ca}^{2+}$  property in male mice, suggesting that deficiency of IGF-1 may not be innately harmful to myocardial contractile function at least at relatively young age used in our study (9-10 weeks). Several scenarios may be considered behind the IGF-1 deficiency-induced effect in neutralizing sex difference in cardiac mechanical mechanics. Considering IGF-1 deficiency nullified sex difference in intracellular  $\text{Ca}^{2+}$  clearance observed in our current study, regulation through intracellular  $\text{Ca}^{2+}$  regulatory proteins including SERCA2a,  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger and phospholamban seems to play a major role in LID-mitigated sex difference in cardiomyocyte contractile function.  $\text{Ca}^{2+}$  regulatory proteins display different activities in the heart in a sex-dependent manner. Phospholamban is linked to cardiac function and phosphorylation state of phospholamban is reported to be different between sexes, leading to differences in the affinity of the SERCA for  $\text{Ca}^{2+}$  reuptake (Dash *et al.*, 2001). In our hand, the phosphorylation of phospholamban is significantly lessened in female C57BL/6 hearts, favoring a lower SERCA function (lessened removal of the phospholamban inhibition on SERCA pump). This was in line with both expression and activity of SERCA observed in our study. Along the same line, SERCA2a mRNA levels were reported to be more abundant in males than those in females (Tappia *et al.*, 2007). It is quite possible that IGF-1 deficiency nullified the sex difference in phospholamban phosphorylation en route to a better SERCA function and intracellular  $\text{Ca}^{2+}$  extrusion. In addition, our Western blotting and mRNA data also favored a role of the  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger in LID-mediated neutralization of the sex difference in intracellular  $\text{Ca}^{2+}$  clearance and cardiomyocyte contractile function. The disparate findings between mRNA and protein in SERCA2a levels (female LID displayed a reduced mRNA level associated with normal protein expression) suggest possible involvement of translational modification for the combined IGF-1 deficiency and sex hormone.

Sex differences in the cardiovascular function have been attributed to the effects of sex steroids. Both estrogen and androgen receptors are found in human and rodent hearts as well as in vascular endothelial and smooth muscle cells, as well as cardiac fibroblasts (Grohe *et al.*, 1997). Estrogens exert their effects via both  $\text{ER}\alpha$  and  $\text{ER}\beta$ . It has been implicated that induction of  $\text{ER}\beta$  is stronger in female than in male hearts while the increase of  $\text{ER}\alpha$  is similar in both sexes (Fliegner *et al.*, 2010). Cardiac protective role has been associated with  $\text{ER}\beta$  (Skavdahl *et al.*, 2005; Pedram *et al.*, 2008). It has been shown that, deletion of  $\text{ER}\beta$  in mice led to cardiac hypertrophy in pressure overloaded female mice not male mice (Skavdahl *et al.*, 2005). Although our finding revealed higher  $\text{ER}\beta$  mRNA and protein levels in female LID mice, data from our present study are inclusive at this point to suggest a key



role of ER $\beta$  in the regulation of sex difference in cardiac contractile function. Additional work should focus on the precise role of estrogen receptor signaling in sex difference of cardiac contractile function.

Experimental limitation: Our study suffers from a number of experimental limitations. First, the current study is mostly phenomenological and lacks mechanistic depth with regards to how IGF-1 deficiency mitigates or attenuates sex difference in cardiac function and intracellular Ca<sup>2+</sup> handling. Although our data suggest existence of possible interactions among gender, IGF-1 and intracellular Ca<sup>2+</sup> regulatory proteins (SERCA, Na<sup>+</sup>-Ca<sup>2+</sup> exchanger and phospholamban), such interaction, if any, seems to occur at the transcriptional level as suggested by our qRT-PCR data. Further study is needed to better understand the transcriptional or epigenetic regulation of sex hormones and IGF-1 on intracellular Ca<sup>2+</sup> regulatory or other cardiac proteins governing cardiac contractile function. Furthermore, although LID cancelled off the sex difference in plasma IGF-1 levels and cardiac contractile function, it is premature to draw any conclusion that IGF-1 is one of the major contributing factors for sex difference in cardiac physiology. Although a cardiac regulatory role has been well defined for IGF-1 (Ren *et al.*, 1999; Zhang *et al.*, 2010), little information is available on the interaction between IGF-1 and sex hormones. Estrogen and IGF-1 are known to interact in the brain to regulate a variety of developmental and neuroplastic events involving control of hormonal homeostasis and reproduction (Garcia-Segura *et al.*, 2010). Several molecular mechanisms are speculated for the interaction of estrogen and IGF-1 including the cross-regulation of the expression of estrogen and IGF-1 receptors, the regulation of estrogen receptor-mediated transcription by IGF-1 and the regulation of IGF-1 receptor signaling by estrogen (Garcia-Segura *et al.*, 2010). Nonetheless, little is known for the interaction between estrogen and IGF-1 in the heart. Lastly, as evidenced by findings from our laboratory (Li *et al.*, 2007; Li *et al.*, 2008; Ge *et al.*, 2010), IGF-1 deficiency or depletion may have a greater impact on cardiac structure and performance under pathological stress conditions such as aging, alcoholism and oxidative stress.

In conclusion, data from our present study revealed for the first time that IGF-1 deficiency attenuates or abolishes the sex difference in cardiac function possibly through the intracellular Ca<sup>2+</sup> regulatory proteins SERCA2a, Na<sup>+</sup>-Ca<sup>2+</sup> exchanger and phosphorylation state of phospholamban. These findings seem to suggest a role of IGF-1 in the interplay between sex hormones and endocrine factors in the maintenance of heart function. In light of gender bias in cardiac function and risk for cardiac event, it is attempting to speculate that a better understanding of IGF-1 and its interaction with sex hormones is of clinical value in the sex-associated prevalence in cardiovascular diseases.

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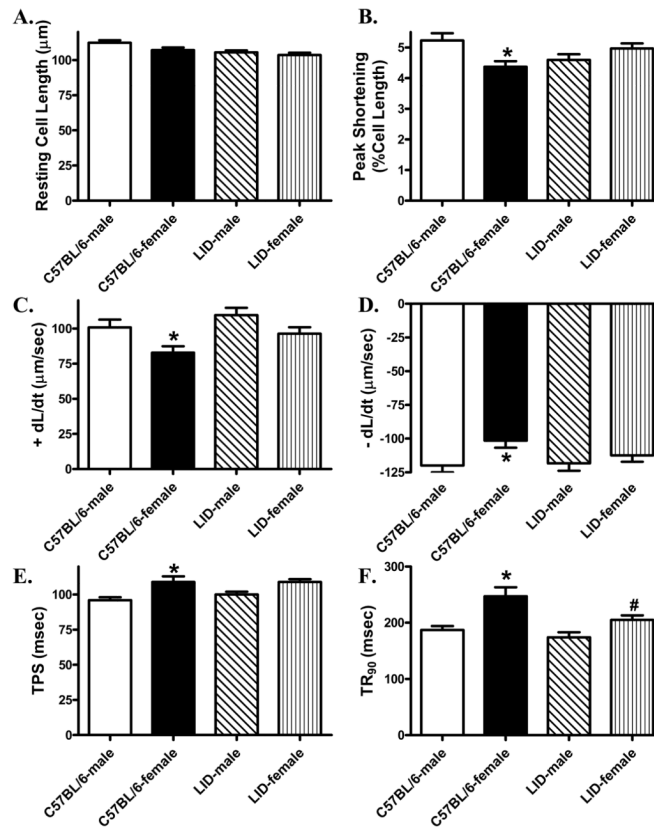
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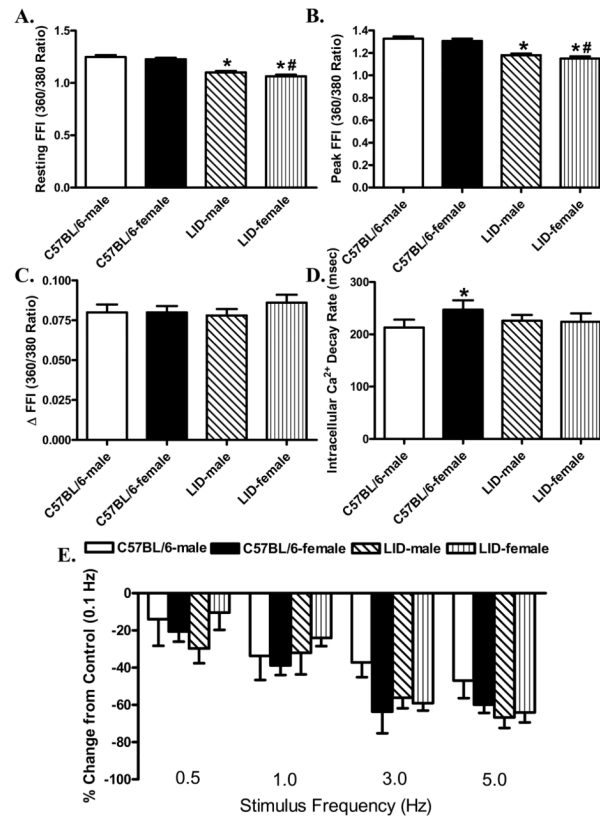
### Highlights

- Liver IGF-1 deficiency attenuates or abolishes the sex difference in cardiac contractile function and intracellular  $\text{Ca}^{2+}$  handling.
- Sex difference in cardiac function is associated with sex difference in intracellular  $\text{Ca}^{2+}$  regulatory proteins SERCA2a,  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger and phospholamban, which may be alleviated by liver IGF-1 deficiency.
- IGF-1 may interact with sex hormones to regulate cardiac contractile function and intracellular  $\text{Ca}^{2+}$  handling.

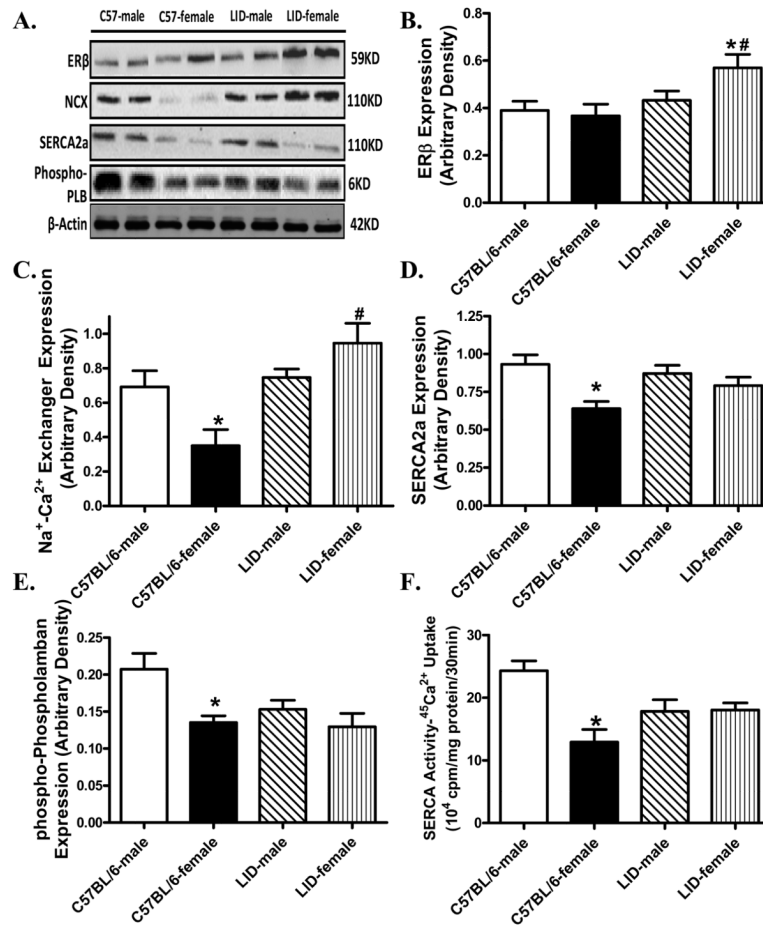




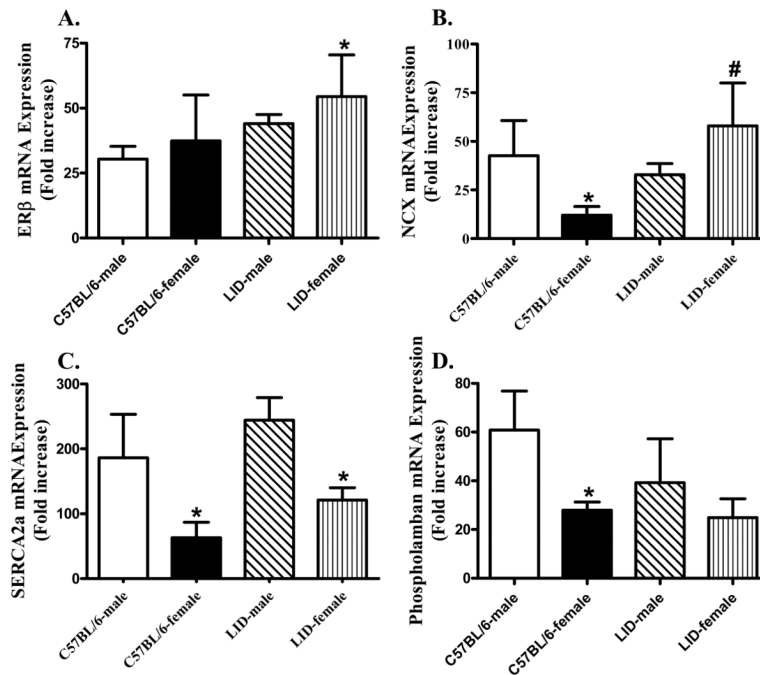
**Fig. 1.** Contractile properties of cardiomyocytes isolated from male or female C57BL/6 and LID mouse hearts. A: Resting cell length; B: Peak shortening (PS, normalized to cell length); C: Maximal velocity of shortening (+ dL/dt); D: Maximal velocity of relengthening (- dL/dt); E: Time-to-PS (TPS); F: Time-to-90% relengthening (TR<sub>90</sub>); Mean  $\pm$  SEM, n = 150–166 cells/group, \*p < 0.05 vs. corresponding male group, #p < 0.05 vs. C57BL/6-female group.



**Fig. 2.** Intracellular Ca<sup>2+</sup> transient properties and stimulus frequency-peak shortening response in cardiomyocytes isolated from male or female C57BL/6 and LID mouse hearts. A: Resting fura-2 fluorescence intensity (FFI); B: Peak FFI; C: Change in FFI ( $\Delta$ FFI) in response to electrical stimuli; D: Intracellular Ca<sup>2+</sup> decay rate; and E: Influence of stimulus frequency (0.1 – 5.0 Hz) on PS amplitude. PS at each stimulus frequency was normalized to that of 0.1 Hz from the same cell. Mean  $\pm$  SEM, n = 91-92 (panel A-D) and 21-25 (panel E) cells per group, \*p < 0.05 vs. corresponding male group, #p < 0.05 vs. C57BL/6-female group.



**Fig. 3.** Protein expression of ER $\beta$ , Na<sup>+</sup>-Ca<sup>2+</sup> exchanger (NCX), sarco(endo)plasmic reticulum Ca<sup>2+</sup>-ATPase 2a (SERCA2a) and phosphorylated phospholamban (PLB) as well as SERCA activity in myocardial tissue from male or female C57BL/6 and LID mice. Panel A: Representative gel blots of ER $\beta$ , NCX, SERCA2a, phosphor-PLB and  $\beta$ -actin (loading control) using specific antibodies; Panel B: ER $\beta$  expression; Panel C: NCX expression; Panel D: SERCA2a expression; Panel E: phosphorylated (Ser16) PLB expression; and Panel F: SERCA activity measured using <sup>45</sup>Ca<sup>2+</sup> uptake technique. Mean  $\pm$  SEM, n = 4–6 hearts per group, \*p < 0.05 vs. corresponding male group, #p < 0.05 vs. C57BL/6-female group.



**Fig. 4.** mRNA expression of ER $\beta$ , Na<sup>+</sup>-Ca<sup>2+</sup> exchanger (NCX), sarco(endo)plasmic reticulum Ca<sup>2+</sup>-ATPase 2a (SERCA2a), and phospholamban in myocardial tissue from male or female C57BL/6 and LID mice. Panel A: ER $\beta$  mRNA expression; Panel B: NCX mRNA expression; Panel C: SERCA2a mRNA expression; and Panel D: Phospholamban mRNA expression. Mean  $\pm$  SEM, n = 4-6 hearts per group, \*p < 0.05 vs. corresponding male group, #p < 0.05 vs. C57BL/6-female group.

**Table 1**

General features of male or female C57BL/6 and LID mice

Genes	Primer
Sarco(endo)plasmic reticulum Ca <sup>2+</sup> ATPase (SERCA)	F: GAGGGTCTGCCTGCTGTCA R: CAGAAGGCAGACTTCGAACGA
Na <sup>+</sup> -Ca <sup>2+</sup> exchanger (NCX)	F: GATTCCGTGACTGCCGTTGT R: CCTGGGTAGCTGCTACTTTGCT
Phospholamban (PLB)	F: CAGACCTGCAACATGCCAACT R: GCAGCGGTGCGTTGCT
Estrogen receptor beta (ER $\beta$ )	Catalog # QT10761879 (lot #97833315) Sequence information not disclosed per policy of Qiagen
GAPDH	F: TGAAGCAGGCATCTGAGGG R: CGAAGGTGGAAGAGTGGGAG



**Table 2**

General features of male or female C57BL/6 and LID mice

	<b>C57-Male</b>	<b>C57-Female</b>	<b>LID-Male</b>	<b>LID-Female</b>
Body Weight (g)	27.5 ± 0.6	22.7 ± 0.4*	27.0 ± 0.3	23.2 ± 0.5*
Heart Weight (Ogah and Bamgboye)	156 ± 8	123 ± 3*	169 ± 8	141 ± 6*
HW/BW (mg/g)	5.69 ± 0.31	5.43 ± 0.13	6.24 ± 0.27	6.11 ± 0.31#
Liver Weight (g)	1.35 ± 0.09	0.93 ± 0.15*	1.56 ± 0.10*	1.45 ± 0.09#
LW/BW (mg/g)	49.2 ± 3.9	40.6 ± 6.6	57.8 ± 3.5*	62.5 ± 3.7*
Kidney Weight (g)	0.35 ± 0.02	0.28 ± 0.01	0.32 ± 0.02	0.26 ± 0.01
KW/BW (mg/g)	12.6 ± 0.7	12.5 ± 0.3	11.7 ± 0.7	11.4 ± 0.5
Plasma IGF-1 (ng/ml)	125.7 ± 8.7	160.5 ± 8.2*	46.4 ± 7.1*	48.2 ± 11.4*,#
End Diastolic Diameter (mm)	2.83 ± 0.12	2.53 ± 0.17	2.76 ± 0.26	2.70 ± 0.10
End Systolic Diameter (mm)	1.59 ± 0.09	1.30 ± 0.09	1.39 ± 0.19	1.55 ± 0.11
Diastolic Wall Thickness (mm)	0.88 ± 0.10	0.89 ± 0.08	0.87 ± 0.05	0.78 ± 0.70
Heart Rate (beat per minute)	459 ± 33	465 ± 31	451 ± 20	462 ± 15
LV Mass (Ogah and Bamgboye)	75.6 ± 7.4	60.1 ± 3.5*	77.4 ± 10.7	66.0 ± 2.8
Normalized LV Mass (mg/g)	2.83 ± 0.25	2.58 ± 0.15	2.89 ± 0.35	2.86 ± 0.16
Fractional Shortening (%)	44.0 ± 1.4	48.5 ± 1.3	49.2 ± 3.1	42.9 ± 2.0

BW= Body weight; HW = Heart weight; LW = Liver weight; KW = Kidney weight; Mean ± SEM,

\* p < 0.05 vs. corresponding male group,

# p < 0.05 vs. C57BL/6-Female group, n = 8–10 mice per group.