# IGF-1 Alleviates High Fat Diet-Induced Myocardial Contractile Dysfunction: Role of Insulin Signaling and Mitochondrial Function
# ONLINE SUPPLEMENT

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## MATERIALS AND METHODS

***Experimental animals, high fat diet feeding, exogenous IGF-1 treatment and intraperitoneal glucose tolerance test (IPGTT):*** The experimental procedure described here was approved by the Institutional Animal Use and Care Committee at the University of Wyoming (Laramie, WY). In brief, 2-3 month-old male FVB and cardiomyocyte-specific IGF-1 overexpression transgenic mice were randomly assigned to low fat (10 and 70% of total calorie from fat and carbohydrate, respectively, catalogue #D12450B) or high fat (45 and 35% of total calorie from fat and carbohydrate, respectively, catalogue #D12451) diets from Research Diets Inc (New Brunswick, NJ) for 5 months. The high fat diet was calorically rich (4.83 kcal/g vs. 3.91 kcal/g in low-fat diet) due to higher fat composition. However, the two diets possessed similar nutrient composition. Generation of cardiomyocyte-specific IGF-1 overexpression transgenic mice using the α-myosin heavy chain (MHC) promoter was previously described in detail.[1] The fur pigmentation was used as a marker for IGF-1 (light brown) or FVB (white) mouse identification. For exogenous IGF-1 treatment, a group of low fat and high fat-fed FVB mice was supplemented with the recombinant IGF-1 (3 mg/kg/d, i.p., National Hormone and Peptide Program, NIDDK) for 8 weeks [2] after being placed on low and high fat diets for 3 months. The IGF-1-treated mice were kept on their respective low or high fat diet for the entire duration (8 weeks) of IGF-1 supplementation. Mice were housed individually in a climate-controlled environment with a 12/12-light/dark cycle and free access to diets and water. After 5 months of feeding, all mice fasted for 12 hrs were given an intraperitoneal injection of glucose (2 g/kg b.w.). Blood samples were drawn from the tail vein immediately before glucose challenge, as well as 15, 60 and 120 min thereafter. Serum glucose levels were determined using an Accu-Chek III glucose analyzer.[3] The area under the curve (AUC) was calculated using trapezoidal analysis for each adjacent time point and serum glucose level. Blood pressure was recorded using a CODA non-invasive blood pressure system (Kent Scientific Co, Torrington, CT) according to the instructions and reported values were averaged from 3 readings each mouse.[4] Blood insulin, leptin, interleukin-6 (IL-6), IGF-1 and triglycerides levels were measured using mouse insulin, leptin, IL-6, IGF-1 and triglyceride ELISA commercial kits from Diagnostic System Laboratory (Webster, TX) and Pierce Biotechnology (Rockford, IL).[4, 5] The homeostasis model assessment of insulin resistance (HOMA-IR) was used to estimate insulin resistance based on the following equation: fasting insulin (μU/ml) x fasting blood glucose (mmol/l)/22.5.[6]

***Measurement of tissue IGF-1 levels:*** Myocardial IGF-1 levels were determined using our previously described procedure.[5] Briefly, lyophilized myocardial tissues were pulverized and duplicate aliquots were extracted with 1 M acetic acid by end-to-end rotation for 4 hrs at 4°C. The extraction mixtures were centrifuged. IGF-1 concentration of the supernatant was determined using a competitive binding enzyme immunoassay kit (Diagnostic Systems) after pre-treatment to remove IGF-1 binding proteins. The recovery of an internal standard of IGF-1 added at the beginning of the extraction procedure was approximately 99%. The inter-assay and intra-assay variability is 4.9-11.9% and 5.3-9.1%, respectively.

***Echocardiographic assessment:*** Cardiac geometry and function were evaluated in anesthetized (Avertin 2.5%, 10 μl/g b.w., i.p.) mice using a 2-D guided M-mode echocardiography (Sonos 5500, Phillips Medical System, Andover, MA) equipped with a 15-6 MHz linear transducer. Left ventricular (LV) wall thickness, diastolic and systolic LV dimensions were recorded from the M-mode images. Fractional shortening was calculated from end-diastolic diameter (EDD) and end-systolic diameter (ESD) using the equation of (EDD-ESD)/EDD. Estimated echocardiographic LV mass was calculated as [(LVEDD + septal wall thickness + posterior wall thickness)$^3$-LVEDD$^3$]*1.055, where 1.055 (mg/mm$^3$) represents the density of myocardium. Heart rates were averaged over 10 cardiac cycles.[7]

***Isolation of murine cardiomyocytes:*** After ketamine/xylazine sedation, hearts were removed and perfused with Krebs-Henseleit bicarbonate (KHB) buffer containing (in mM): 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 $KH_2PO_4$, 25 $NaHCO_3$, 10 HEPES and 11.1 glucose. Hearts were digested with collagenase D (223 U/ml) for 20 min. Left ventricles were removed and minced before being filtered. Myocyte yield was ~ 75% which was not affected by high fat diet or IGF-1 overexpression. Only rod-shaped myocytes with clear edges were selected for mechanical study.[3] To examine the causal relationship in IGF-1-induced actions in mechanical and mitochondrial responses, cardiomyocytes from FVB mice were maintained in a DMEM culture medium while being treated with palmitate (100 μM) at 37$^o$C for 6 hrs in the absence or presence of IGF-1 (10 nM), the mitochondrial uncoupler FCCP (1 μM), or the GSK3β inhibitor SB216763 (10 μM) prior to mechanical or biochemical assessment.[8]

***Cell shortening/relengthening:*** Mechanical properties of cardiomyocytes were assessed using a SoftEdge MyoCam® system (IonOptix Corporation, Milton, MA). In brief, cells were placed in a Warner chamber mounted on the stage of an inverted microscope (Olympus, IX-70) and superfused (~1 ml/min at 25 °C) with a buffer containing (in mM): 131 NaCl, 4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES, at pH 7.4. The cells were field stimulated with supra-threshold voltage at a frequency of 0.5 Hz (unless otherwise stated), 3 msec duration, using a pair of platinum wires placed on opposite sides of the chamber connected to a FHC stimulator (Brunswick, NE). The myocyte being studied was displayed on the computer monitor using an IonOptix MyoCam camera. An IonOptix SoftEdge software was used to capture changes in cell length during shortening and relengthening. Cell shortening and relengthening were assessed using the following indices: peak shortening (PS) - indicative of peak ventricular contractility, time-to-PS (TPS) - indicative of contraction duration, and time-to-90% relengthening ($TR_{90}$) - represents cardiomyocyte relaxation duration, maximal velocities of shortening (+dL/dt) and relengthening (−dL/dt) - indicatives of maximal velocities of ventricular pressure rise/fall. In the

case of altering stimulus frequency from 0.1 to 5.0 Hz, the steady state contraction of myocyte was achieved (usually after the first 5–6 beats) before PS was recorded.[3]

***Intracellular $Ca^{2+}$ transient measurement:*** Myocytes were loaded with fura-2/AM (0.5 μM) for 10 min and fluorescence measurements were recorded with a dual-excitation fluorescence photomultiplier system (IonOptix). Cardiomyocytes were placed on an Olympus IX-70 inverted microscope and imaged through a Fluor × 40 oil objective. Cells were exposed to light emitted by a 75W lamp and passed through either a 360 or a 380 nm filter, while being stimulated to contract at 0.5 Hz. Fluorescence emissions were detected between 480 and 520 nm by a photomultiplier tube after first illuminating the cells at 360 nm for 0.5 sec 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 $Ca^{2+}$ concentration were inferred from the ratio of fura-2 fluorescence intensity (FFI) at two wavelengths (360/380). Fluorescence decay time was measured as an indication of the intracellular $Ca^{2+}$ clearing rate. Single exponential curve fit was applied to calculate the intracellular $Ca^{2+}$ decay constant.[3]

***ROS production:*** Cellular ROS was evaluated by analyzing changes in fluorescence intensity resulting from oxidation of the intracellular fluoroprobe 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (DCF). In brief, cardiomyocytes were loaded with 10 μM DCF at 37°C for 30 min. The myocytes were rinsed and the fluorescence intensity was then measured using a fluorescent micro-plate reader at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. Untreated cells showed no fluorescence and were used to determine background fluorescence.[9]

***Caspase-3 assay:*** Tissue homogenates were centrifuged (10,000 *g* at 4°C, 10 min) and pellets were lysed in 100 μl of ice-cold cell lysis buffer [50 mM HEPES, pH 7.4, 0.1% CHAPS, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.1% NP40]. The assay was carried out in a 96-well plate with each well containing 30 μl cell lysate, 70 μl of assay buffer (50 mM HEPES, 0.1% CHAPS, 100 mM NaCl, 10 mM DTT and 1 mM EDTA) and 20 μl of caspase-3 colorimetric substrate Ac-DEVD-pNA (Sigma). The 96-well plate was incubated at 37°C for 1 hr, during which time the caspase in the sample was allowed to cleave the chromophore p-NA from the substrate molecule. Absorbency was detected at 405 nm with caspase-3 activity being proportional to color reaction. Protein content was determined using the Bradford method. The caspase-3 activity was expressed as picomoles of pNA released per μg of protein per minute.[10]

***MTT assay for cell viability:*** [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay is based on transformation of the tetrazolium salt MTT by active mitochondria to an insoluble formazan salt. Cardiomyocytes were treated with or without palmitate for 6 hrs in a DMEM culture medium in the absence or presence of IGF-1 (10 nM), the mitochondrial uncoupler FCCP (1 μM), or the GSK3β inhibitor SB216763 (10 μM). The cells were then plated in microtiter plate at a density of $3 \times 10^5$ cells/ml. MTT was added to each well with a final concentration of 0.5 mg/ml, and the plates were incubated for 2 hrs at 37°C. The formazan crystals in each well were dissolved in dimethyl sulfoxide (150 μl/well). Formazan was quantified spectroscopically at 560 nm using a SpectraMax® 190 spectrophotometer.[11]

***Aconitase activity:*** Mitochondria prepared from whole heart homogenate were resuspended in 0.2 mM sodium citrate. After determination of protein concentration, aconitase activity assay (Aconitase activity assay kit, Aconitase-340 assay$^{tm}$, Oxisresearch, Portland, OR) was performed according to manufacturer instructions with minor modifications. Briefly, mitochondrial sample (50 µl) was mixed in a 96-well plate with 50 µl trisodium citrate (substrate) in Tris-HCl pH 7.4, 50 µl isocitrate dehydrogenase (enzyme) in Tris-HCl, and 50 µl NADP in Tris-HCl. After incubating for 15 min at 37°c with 50 rpm shaking, the absorbance was dynamically recorded at 340 nm every min for 5 min with a spectramax 190 microplate spectrophotometer. During the assay, citrate is isomerized by aconitase into isocitrate and eventually α-ketoglutarate. The aconitase-340 assay$^{tm}$ measures NADPH formation, a product of the oxidation of isocitrate to α-ketoglutarate. Tris-HCl buffer (pH 7.4) served as a blank. All results were normalized to respective protein content.[12]

***Mitochondrial cytochrome c release:*** The ventricles were minced and homogenized by Polytron in the ice-cold MSE buffer [220 mM mannitol, 70 mM sucrose, 2 mM EGTA, 5 mM 3-(4-morpholino) propane sulfonic acid (MOPS), pH 7.4, 0.2% bovine serum albumin (BSA) and a protease inhibitor cocktail containing 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), E-64, bestatin, leupeptin, aprotinin, and EDTA obtained from Sigma Chemicals (St. Louis, MO)]. The homogenates were centrifuged for 10 min at 600 x g to remove unbroken tissue and nuclei, and the supernatants were centrifuged for 10 min at 3000 x g to pellet mitochondria. The supernatants were further centrifuged for 30 min at 100,000 x g to obtain cytosolic fraction. The mitochondrial pellet was dissolved in a lysis buffer and centrifuged at 10,000 x *g* for 30 min at 4°C to make a soluble protein. Fifty µg of the mitochondrial or cytosolic protein was separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and was analyzed by western blot using the anti-cytochrome c antibody (Upstate, Waltham, MA).[13]

***TUNEL assay:*** TUNEL staining of myonuclei positive for DNA strand breaks were determined in myocardium using a fluorescence detection kit (Roche, Indianapolis, IN) and fluorescence microscopy. Briefly, paraffin-embedded sections (5 µm) were deparaffinized and rehydrated. The sections were then incubated with Proteinase K solution at room temperature for 30 min. TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and fluorescein-dUTP was added to the sections in 50-µl drops and incubated for 60 min at 37°C in a humidified chamber in the dark. The sections were rinsed three times in PBS for 5 min each. Following embedding, sections were visualized with an Olympus BX-51 microscope equipped with an Olympus MaguaFire SP digital camera. DNase I and label solution were used as positive and negative controls. To determine the percentage of apoptotic cells, micrographs of TUNEL-positive and DAPI-stained nuclei were captured using an Olympus fluorescence microscope and counted using the ImageJ software (ImageJ version 1.43r; NIH) from 10 random fields at 400× magnification At least 100 cells were counted in each field .[10]

***Histological examination:*** Following anesthesia, hearts were arrested in diastole with saturated KCl, excised and fixed in 10% neutral-buffered formalin at room temperature for 24 hrs. The specimen was processed through graded alcohols, cleared in xylenes, and embedded in paraffin; serial sections were cut at 5 µm. Deparaffinized slides were briefly rinsed with PBS and incubated in 0.1 mg/ml fluorescein isothiocyanate (FITC)-tagged lectin (SIGMA, L-4895) for 2 hrs in the dark. Thereafter, the slides were washed with PBS 3 times, mounted with the aqueous

mounting media and coverslipped. Cardiomyocyte cross-sectional areas were digitalized using an Olympus BX-51 microscope (Olympus America Inc., Melville, NY) equipped with a fluorescence filter and measured with the Image J (version1.44C) software.[14]

***HPLC assay of ATP content:*** Myocardial tissues were extracted by 6% perchloric acid and the acidic homogenate was kept on ice for 30 min before being centrifuged at 14,000 rpm at 4°C for 10 min. An aliquot of pellets was set aside for protein measurements. The supernatant was neutralized with 1 M $K_2CO_3$ (to pH of 3.5) and was stored at -80°C until HPLC assay. The chromatographic separation of ATP was performed using a Grace Partisil SAX column (250 mm × 4 mm i.d., particle size 10 μm) (Deerfield, IL). The mobile phases were composed of a gradient of 5 mM ammonium dihydrogen phosphate (pH 2.8) and 750 mM ammonium dihydrogen phosphate (pH 3.9). The flow rate was varied from1-2 ml/min over the course of the gradient profile to provide a reasonable assay time of 25 min. The sample injection volume was 50 μl and the components were monitored at 254 nm. ATP concentration was determined by construction of a calibration curve range from 1 to 80 nmoles per 50 μl injected. Standard stock solutions for calibration included 6.4 μmole/ml ATP in 5 mM ammonium dihydrogen phosphate. These solutions were used as references for peaks quantification by adding 5 mM ammonium dihydrogen phosphate to obtain 1, 5, 10, 20, 40 and 80 nmoles per 50 μl injected.[15]

***Lipid peroxidation assay:*** The lipid peroxidation end products malondialdehyde (MDA) and 4-hydrononenal (4-HNE) levels were measured in myocardial homogenates. A colorimetric assay (LPO-586 Kit; Oxis International, Portland, OR) was used to determine levels of lipid peroxidation. Briefly, a 20-30% tissue homogenate was prepared using 20 mM Tris buffer containing 5 mM butylated hydroxytoluene (BHT) to prevent sample oxidation. Protein samples were stored at –70$^{o}$C until analysis. To 200 μl of sample, 10 μl of 0.5 M BHT, and 650 μl of Reagent 1 were added. The tubes were gently vortexed and 150 μl of 15.4 M methanesulfonic acid (Reagent 2) were added. Samples were incubated at 45$^{o}$C for 45 min followed by centrifugation for 10 min at 15,000 x g. The clear supernatant was transferred to a cuvette and the absorbance measured at 586 nm. Sample blanks, reagent blanks and MDA and 4-HNE standards were included each time the assay was performed. The concentration of the analyte in each sample was calculated using the molar extinction coefficient at 586 nm of 110,000.[5]

***Data analysis:*** Data were Mean ± SEM. Statistical comparison was performed by a one-way analysis of variance [1] (two-way ANOVA for IPGTT test) followed by the Tukey's post hoc test. Significance was set as $p < 0.05$.

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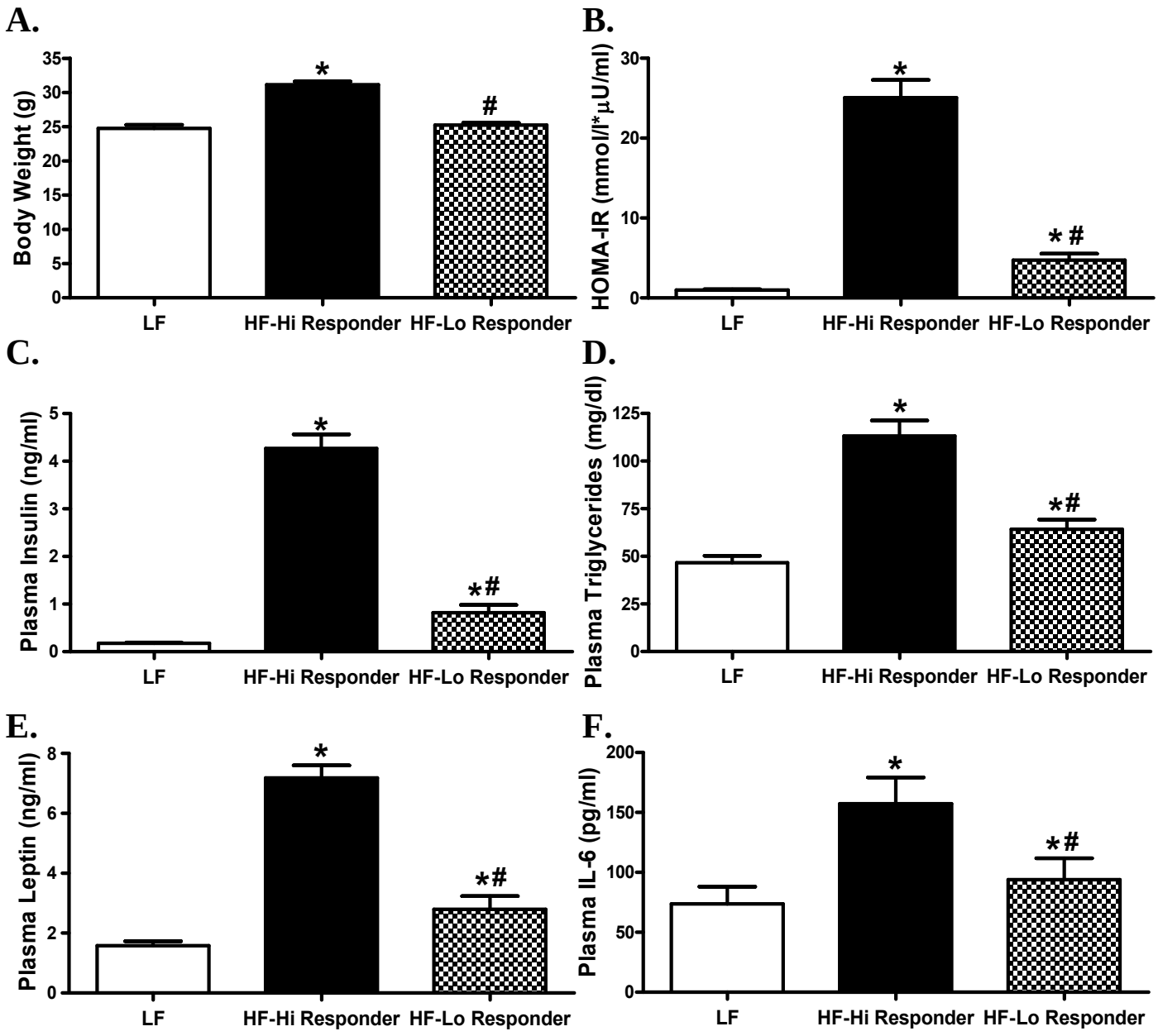


**Fig. S1:** Influence of low fat (LF) and high fat (HF) diet feeding (5 months) on body weight and plasma markers. Following high fat diet feeding, mice were divided into low (Lo) and high (Hi) responder groups. Low responders were those failed to display a final body weight greater than 115% of the average final body weight of low fat group. Other high fat diet-fed mice displayed a final body weight greater than 130% of the low fat group average. A. Body weight; B: HOMA-IR; C: Plasma insulin; D: Plasma triglycerides; E: Plasma leptin; and F: Plasma IL-6. Mean ± SEM, n = 26 for LF and HF-Hi responders or 5 for HF-Lo responders, *$p < 0.05$ *vs.* LF group, # $p < 0.05$ *vs.* HF-Hi Responder group.

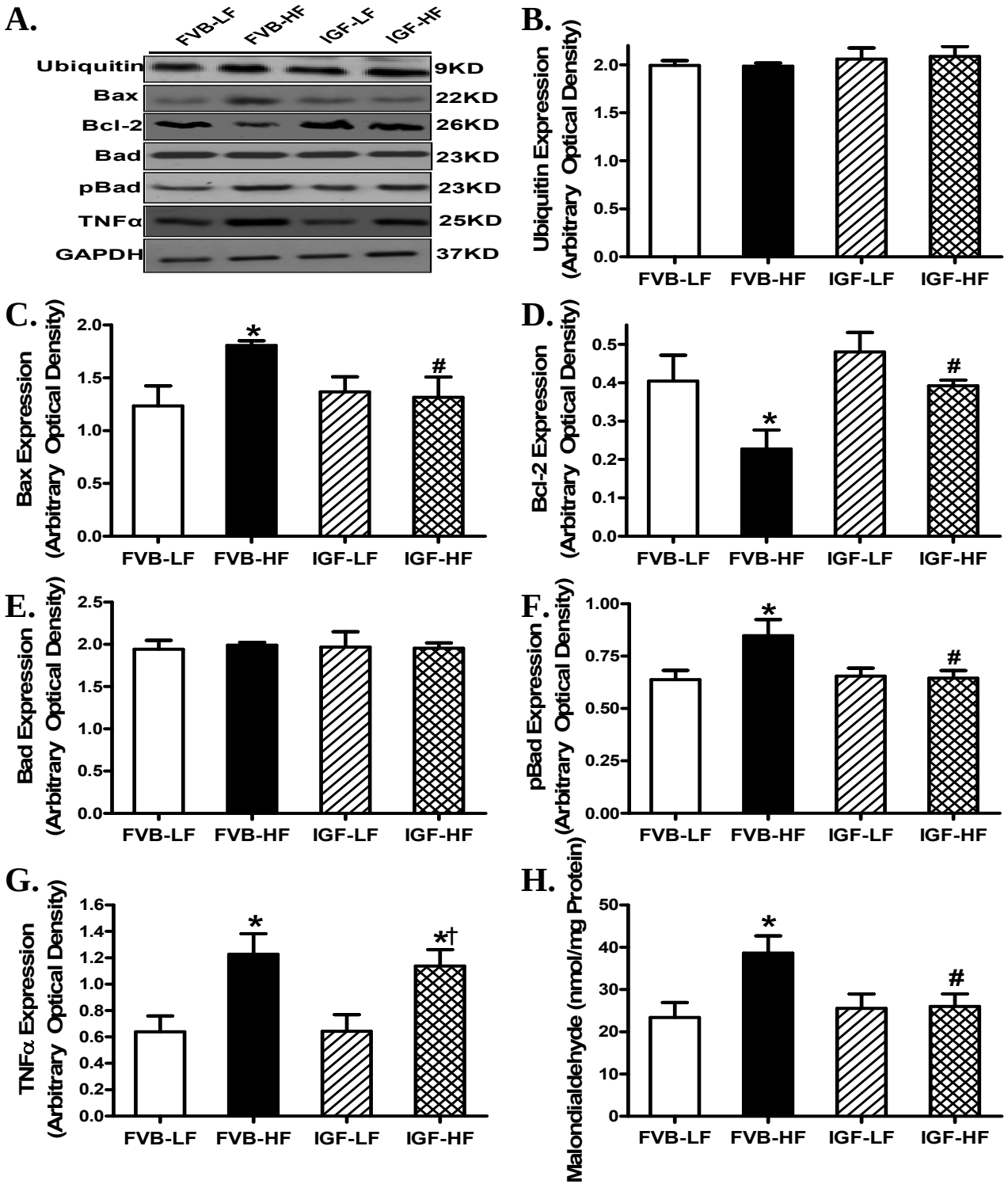


**Fig. S2:** Western blot analysis of the ubiquitin-proteasome system protein marker ubiquitin, the apoptotic proteins Bax, Bcl-2 and Bad (pan and phosphorylated), the inflammation makers TNFα and accumulation of the lipid peroxidation end product malondialdehyde in myocardium from low fat (LF) and high fat (HF)-fed FVB and IGF-1 mice. A: Representative gel blots of ubiquitin, Bax, Bcl-2, Bad, phosphorylated Bad (pBad), TNFα and GAPDH (loading control) using respective specific antibodies; B: Ubiquitin; C: Bax; D: Bcl-2; E: Bad; F: pBad; G: TNFα; and H: Malondialdehyde levels. All expressions are normalized to GAPDH. Mean ± SEM, n = 4-7 per group, *$p < 0.05$ *vs*. FVB-LF group, #$p < 0.05$ *vs*. FVB-HF group, †$p < 0.05$ *vs*. IGF-LF group.

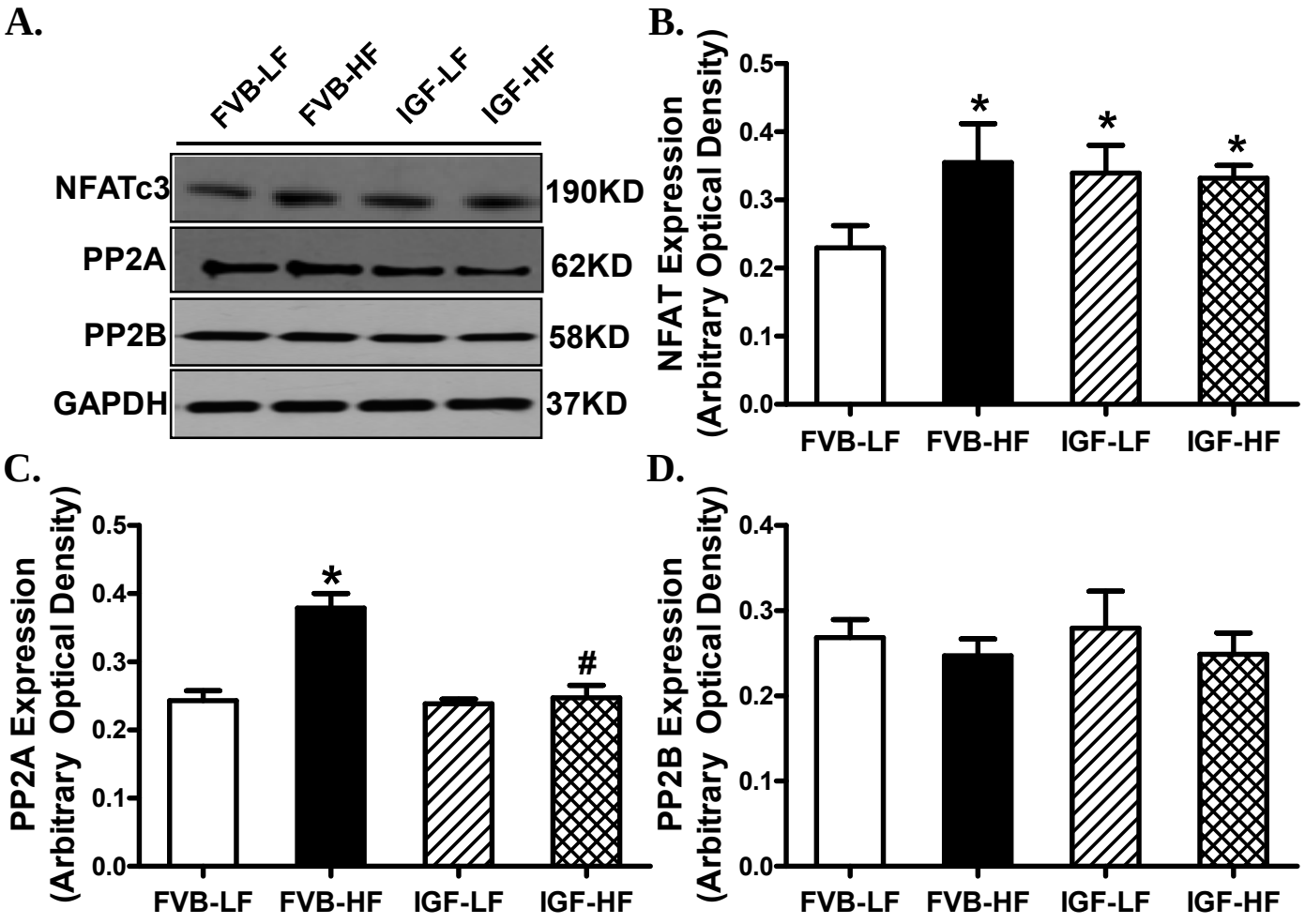


**Fig. S3:** Western blot analysis of the hypertrophic marker NFATc3, protein phosphatase 2A (PP2A) and protein phosphatase 2B (PP2B) in myocardium from low fat (LF) and high fat (HF)-fed FVB and IGF-1 mice. A: Representative gel blots of NFATc3, PP2A, PP2B and GAPDH (loading control) using specific antibodies; B: NFATc3 expression; C: PP2A expression; and D: PP2B expression. All protein expressions were normalized to GAPDH. Mean ± SEM, n = 5-8 mice per group, *$p < 0.05$ *vs*. FVB-LF group, #$p < 0.05$ *vs.* FVB-HF group.

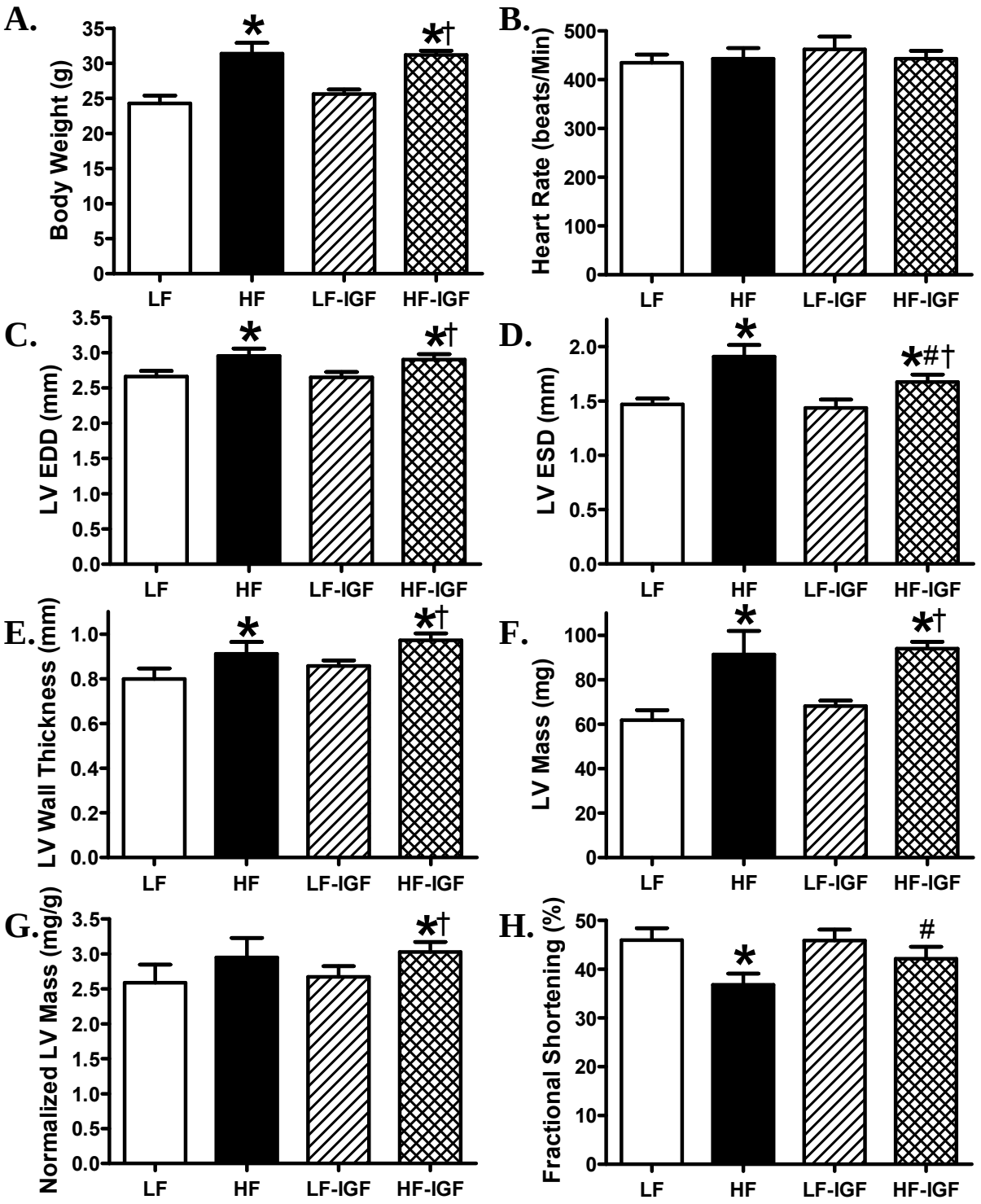


**Fig. S4:** Effect of exogenous IGF-1 treatment on high fat diet feeding-induced echocardiographic changes. Cohorts of low fat (LF) and high fat (HF)-fed FVB mice were supplemented with IGF-1 (3 mg/kg/d, i.p.) for 8 weeks after being placed on LF or HF diet for 3 months. IGF-1-treated mice were maintained on their respective diet while receiving IGF-1. A. Body weight; B: Heart rate; C: LV EDD; D: LV ESD; E: LV wall thickness; F: LV mass; G: Normalized LV mass; and H: Fractional shortening. Mean ± SEM, n = 6 mice per group, *$p < 0.05$ *vs.* LF group, # $p < 0.05$ *vs.* HF group, †$p < 0.05$ *vs.* LF-IGF group.

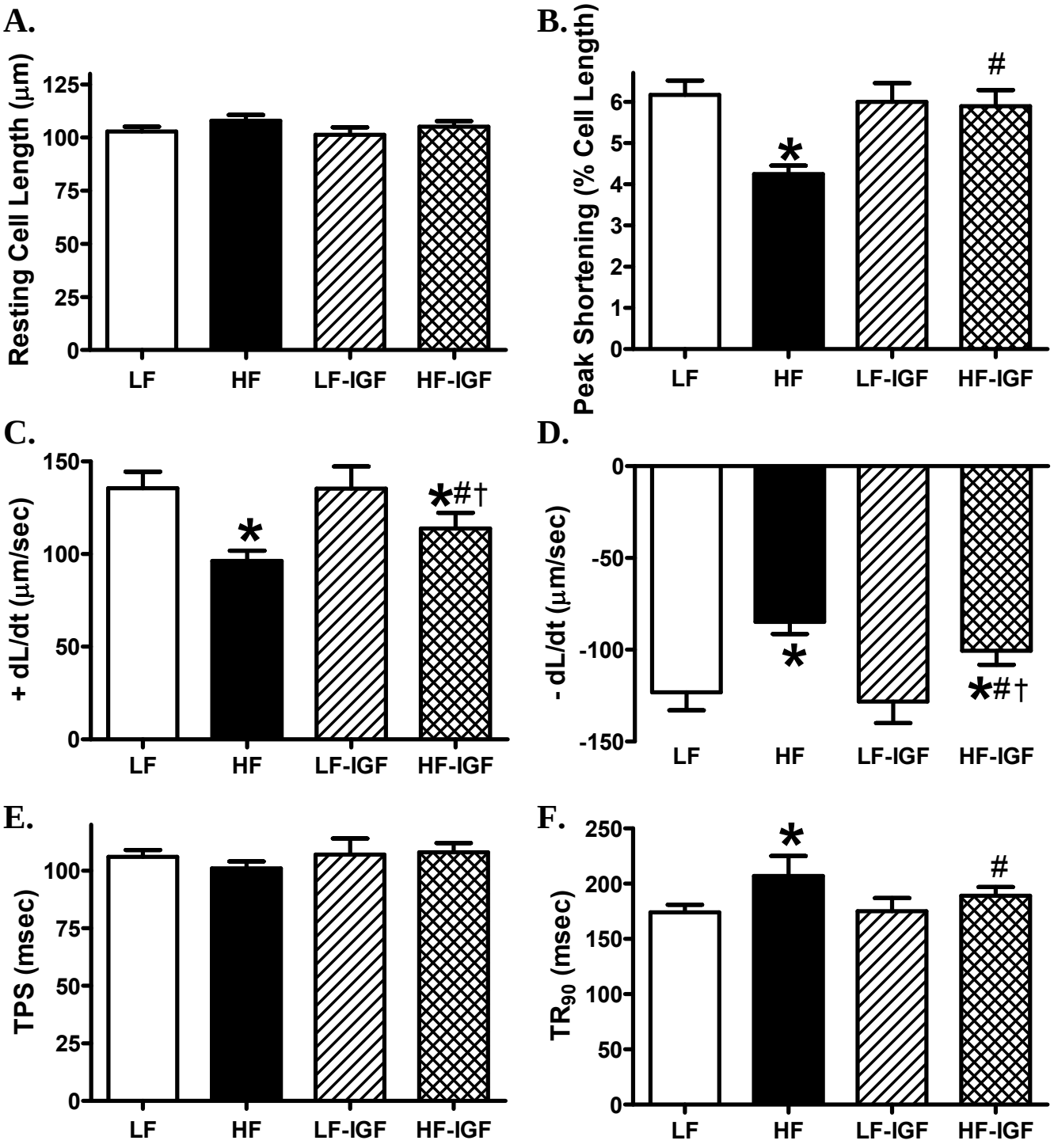


**Fig. S5:** Effect of exogenous IGF-1 treatment on high fat diet feeding-induced cardiomyocyte contractile properties. Cohorts of low fat (LF) and high fat (HF)-fed FVB mice were treated with IGF-1 (3 mg/kg/d, i.p.) for 8 weeks after being placed on LF or HF diet for 3 months. IGF-1-treated mice were maintained on their respective diet while receiving IGF-1. A: Resting cell length; B: Peak shortening (normalized to cell length); C: Maximal velocity of shortening (+ dL/dt); D: Maximal velocity of relengthening (- dL/dt); E: Time-to-PS (TPS); and F: Time-to-90% relengthening ($TR_{90}$). Mean ± SEM, n = 78-80 cells from 3 mice/group, *$p < 0.05$ *vs.* LF group, #$p < 0.05$ *vs.* HF group, †$p < 0.05$ *vs.* LF-IGF group.

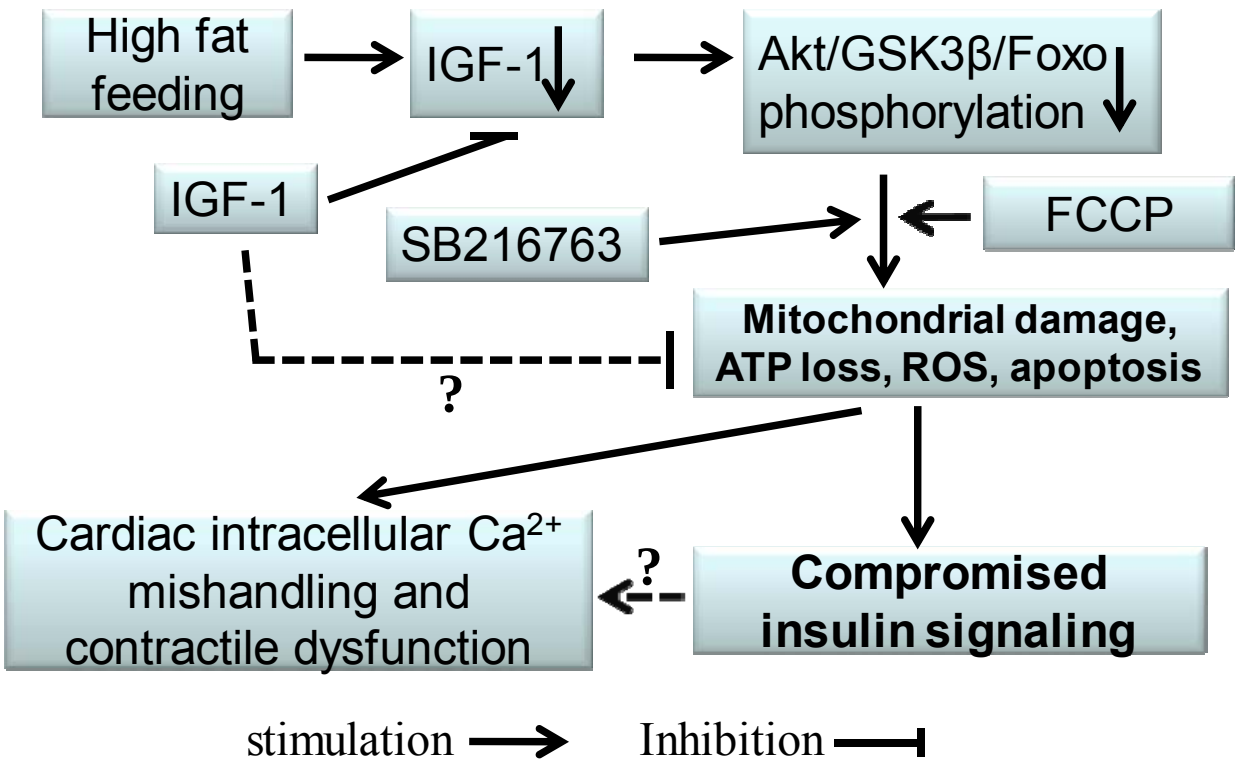


**Fig. S6:** Schematic diagram depicting proposed signaling events in high fat diet/IGF-1-induced cardiac responses and signaling mechanisms involved. Dashed lines and question mark (?) denote effects likely to occur but have not fully validated by the current experimental evidence.