Obesity Alters Molecular and Functional Cardiac Responses to Ischemia-Reperfusion and Glucagon-Like Peptide-1 Receptor Agonism

Daniel J Sassoon[1], Adam G Goodwill[1], Jillian N Noblet[1], Abass M Conteh[1], B. Paul Herring[1], Jeanette N McClintick[2], Johnathan D Tune[1], Kieren J Mather[3]

[1]Department of Cellular & Integrative Physiology, Indiana University School of Medicine; [2]Department of Biochemistry and Molecular Biology, Indiana University School of Medicine; [3]Department of Medicine, Indiana University School of Medicine

Running Heading: Functional and molecular responses in obese myocardium

Correspondence:

Kieren Mather, M.D.

Professor of Medicine

Indiana University School of Medicine

1120 W. Michigan St., Suite CL365

Indianapolis, Indiana 46202

Phone: 317-278-7826

Email: kmather@iu.edu

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Abstract

This study tested the hypothesis that obesity alters the cardiac response to ischemia/reperfusion and/or glucagon like peptide-1 (GLP-1) receptor activation, and that these differences are associated with alterations in the obese cardiac proteome and microRNA (miR) transcriptome. Ossabaw swine were fed normal chow or obesogenic diet for 6 months. Cardiac function was assessed at baseline, during a 30-min coronary occlusion, and during 2 hours of reperfusion in anesthetized swine treated with saline or exendin-4 for 24 hours. Cardiac biopsies were obtained from normal and ischemia/reperfusion territories. Fat-fed animals were heavier, and exhibited hyperinsulinemia, hyperglycemia, and hypertriglyceridemia. Plasma troponin-I concentration (index of myocardial injury) was increased following ischemia/reperfusion and decreased by exendin-4 treatment in both groups. Ischemia/reperfusion produced reductions in systolic pressure and stroke volume in lean swine. These indices were higher in obese hearts at baseline and relatively maintained throughout ischemia/reperfusion. Exendin-4 administration increased systolic pressure in lean swine but did not affect blood pressure in obese swine. End-diastolic volume was reduced by exendin-4 following ischemia/reperfusion in obese swine. These divergent physiologic responses were associated with obesity-related differences in proteins related to myocardial structure/function (e.g. titin) and calcium handling (e.g. SERCA2a, histidine-rich Ca^{2+} binding protein). Alterations in expression of cardiac miRs in obese hearts included miR-15, miR-27, miR-130, miR-181, and let-7. Taken together, these observations validate this discovery approach and reveal novel associations that suggest previously undiscovered mechanisms contributing to the effects of obesity on the heart and contributing to the actions of GLP-1 following ischemia/reperfusion.

Keywords: Obesity, Ischemia, GLP-1, Exendin-4, Cardiovascular, microRNA, Proteomics

New and Noteworthy

Diet-induced obesity/metabolic syndrome in swine was associated with altered cardiovascular functional, miR transcriptome, and proteomic response to cardiac ischemia/reperfusion. The GLP-1 mimetic exendin-4 reduced myocardial damage, and altered these functional, miR and protein responses to ischemia/reperfusion differently in obese versus lean swine. These observations highlight known and novel mechanisms contributing to obesity-related differences in the responses to cardiac ischemia/reperfusion, and in the responses to GLP-1 agonism.

Introduction

Obesity is a complex disease state that is accompanied by a number of cardiac disease risk factors including hypertension and metabolic dysregulation (glucose intolerance, insulin resistance, dyslipidemia)[54] that are associated with pathologic changes in the heart (ventricular hypertrophy, heart failure)[6, 16, 34, 65] and in the vasculature (atherosclerosis, microvascular dysfunction)[8, 31, 60]. In prospective clinical studies, overweight or obese individuals have increased rates of cardiovascular diseases [1, 3, 41], contributing to a 2-3 fold increase in overall mortality relative to normal weight individuals[1, 7, 15, 32, 49, 51]. In addition to a direct link with occlusive vascular disease, obesity is strongly associated with impairment of systolic and diastolic function and the development of cardiac fibrosis (i.e. obesity cardiomyopathy) [3, 41]. Together these changes contribute to an obesity-related increased incidence of heart failure and associated morbidity and mortality [36, 41].

To date studies have investigated a broad set of potential contributors to this impaired function, such as alterations in β-adrenoceptor signaling, oxidative stress, lipotoxicity, epigenetic changes, and autophagy responses[11], but the adverse effects of obesity on cardiac function remain incompletely understood. The recent discovery of the important regulatory effects of small non-coding ribonucleic acid sequences referred to as microRNAs (miRs)[62] presents novel opportunities for discovery in this area. Limited prior investigations suggest that obesity induces alterations in specific cardiac miRs (miR-34b, miR-34c, miR-199b, miR-210, miR-650, and miR-223) in association with altered metabolism, hypertrophy and heart failure[23]. Improvements or exacerbations of various cardiovascular disease states have been described following experimental alterations of miRs using gene therapy or pharmacologic inhibitors of miRs (antimiR)[24], demonstrating the functional relevance of these molecules. Objectively, very little is known about the nature and contributions of miRs to cardiovascular disease in the obese heart.

Regulatory changes ultimately exert effects by changes in the amount and/or activation of cellular proteins. In parallel with advances in techniques for miR profiling, tools for the evaluation

of whole-proteome have been developed. Such techniques have been used to demonstrate obesity-specific changes in abundance and phosphorylation of proteins related to ion transport, mitochondrial metabolism, antioxidant function and cardiac contractile function [13, 14, 17, 63]. Missing are studies exploring obesity-specific proteomic changes in response to cardiac ischemia and with reperfusion following relief of ischemia, and no published work has concurrently evaluated the changes in miRs and the proteome. Evaluating these changes in parallel under an experimental paradigm designed to produce an informative physiological context can produce novel observations of previously unknown factors and pathways associated with obesity-specific alterations in cardiac function and ischemic responses.

Recent work from our laboratory and others indicates that obesity impairs the effects of glucagon like peptide-1 (GLP-1) to modify the cardio-metabolic response to exercise and to regional myocardial ischemia [20, 21, 44]. The underlying mechanisms remain largely unexplained. Here we have evaluated effects of obesity/metabolic syndrome on the cardiac functional response to ischemia/reperfusion injury with and without prior exposure to GLP-1 receptor activation. We tested the hypothesis that these responses differ in obese animals, and that these differences are associated with obesity-specific alterations in the cardiac proteome and microRNA (miR) transcriptome. We observed distinct alterations of functional responses (*in vivo*) accompanied by obesity-related differences in myocardial protein and miR expression profiles, including changes in previously unknown factors that could contribute to the development of obesity cardiomyopathy.

Methods

Animal Models and Surgical Preparation. This protocol and use of animals were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee and were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996). Male Ossabaw swine were placed on a normal (lean; n = 10) or modified obesogenic diet (obese; n = 9) for 6 months beginning at 6 months of age as previously described [9]. These group sizes are historically sufficient to demonstrate important physiologic differences between animals. Briefly, lean control swine were fed ~2,200 kcal/day of standard chow (5L80, Purina Test Diet, Richmond, IN, USA) containing 18% kcal from protein, 71% kcal from complex carbohydrates, and 11% kcal from fat. Obese swine were fed an excess ~8,000 kcal/day high fat/fructose, obesogenic diet containing 17 % kcal from protein, 20% kcal from complex carbohydrates, 20% kcal from fructose, and 43% kcal from fat (mixture of lard, hydrogenated soybean oil, and hydrogenated coconut oil), and supplemented with 2.0% cholesterol and 0.7% sodium cholate by weight.

Following 6 months of their respective diets, animals were anesthetized with isofluorane for placement of a jugular venous catheter. The degradation-resistant GLP-1 analogue Exendin-4 (30 fmol/kg/min; lean; n= 5, obese; n= 5) or an equivalent volume of saline (lean; n= 5, obese; n= 4) was then infused systemically through the jugular venous catheter for 24 hours via an elastomeric balloon (MILA International Inc, Erlanger, Kentucky; 2 mL/hr). Immediately following this 24 hour infusion, swine were anesthetized with telazol (5 mg/kg, sc), ketamine (3 mg/kg, sc), and xylazine (2.2mg/kg, sc) cocktail IM and anesthesia maintained with morphine (3 mg/kg) and α-chloralose (100 mg/kg, i.v). The exendin-4 or saline infusion continued throughout the remaining period of the experimental protocol. Depth of anesthesia was monitored by observing continuous measurements of arterial blood pressure and heart rate as well as regular (15 minute intervals) reflex tests (corneal, jaw, limb withdrawal), beginning after induction of anesthesia and continuing throughout the experimental protocol. Prophylactic supplementation with chloralose

was performed every 1.5 hour to maintain a level, stage 3 plane of anesthesia. The right femoral artery and vein were isolated and cannulated to allow measurement of systemic arterial pressure and venous access, respectively. Next, the heart was exposed by a left lateral thoracotomy. The left circumflex artery was isolated for placement of a perivascular flow probe and a snare occluder. A catheter was then placed in the great cardiac vein to enable sampling of coronary venous blood from the area supplied by the left circumflex artery. A Sci-Sense pressure/volume admittance catheter (Transonic Technologies, London Ontario, Canada) was then placed directly into the left ventricle via an apical transmural stab and secured with a purse-string suture. The pressure-volume system requires input of an estimated stroke volume. This estimate was based on manufacturer's recommendation of 0.9μL/g of body weight and produced baseline cardiac performance values consistent with those established in swine using other measurement modalities. Heparin was administered intravenously at a bolus dose of 500 U/kg to prevent clotting. All data were recorded using IOX acquisition software (EMKA Technologies, Falls Church VA. USA).

Experimental Protocol. Following surgical instrumentation, swine were allowed to recover for ~20 min. Hemodynamic parameters (blood pressure, heart rate, coronary blood flow, left ventricular volume and pressure) were continuously monitored throughout the experimental protocol. Baseline arterial and coronary venous blood samples were obtained at the end of the stabilization period. Next the left circumflex artery was transiently occluded for 30 min with a reversible snare occluder. The snare was then released to allow reperfusion of the circumflex perfusion territory. Hemodynamic parameters were recorded and blood samples were obtained at the end of 30 min of coronary occlusion, and then at 60 min intervals following release of the occlusion (i.e. following coronary reperfusion). At the end of the experimental protocol, while still anesthetized, animals were euthanized by fibrillation (by application of a 9 volt battery placed near the apex in the heart) with subsequent rapid excision of the heart. The heart was reverse perfused

with cold calcium free Krebs solution and transmural left ventricular biopsies were flash frozen in liquid nitrogen for protein mass spectrometry. The biopsies were taken from both the left anterior descending coronary artery perfused region (i.e. normal, non-ischemic territory) and left circumflex coronary artery perfused region (i.e. ischemia-reperfusion territory) taking care to avoid visible vasculature.

Metabolic analysis and troponin measurements. An Instrumentation Laboratories automatic blood gas analyzer (GEM Premier 3000) and CO-oximeter (682) system was used to measure arterial and venous pH, PCO_2, PO_2, hematocrit, hemoglobin, oxygen saturation, and oxygen content. Baseline blood samples were also analyzed commercially (Antech Diagnostics; Fishers, Indiana) for measurements of insulin, triglycerides and cholesterol. Mass of left circumflex perfusion territory was estimated to be 20% of total heart weight, which approximates the "area at risk" in this study. Troponin measures were made using a i-STAT1, model 300-G (Abbott, Illinois, USA), using systemic blood samples taken at baseline prior to coronary occlusion, and 120 min following release of the occlusion.

RNA isolation, quantification and micro-RNA array. Total RNA from myocardial core biopsies was isolated using PureLink RNA Micro Kit (Life Technologies) according to the manufacturer's instructions. Total RNA was eluted from the column in RNase-free water and stored at -80°C. The Agilent 2100 Bioanalyzer Small RNA kit was used by the Center for Genetics in Indiana University School of Medicine to assess quantity and quality of miRNA.

To perform the arrays, total RNA samples were labeled using the Genisphere FlashTag HSR kit. The labeled samples were individually hybridized to Affymetrix GeneChip miRNA 3.0 arrays. They were stained and washed using the standard miRNA protocol. Affymetrix GeneChip Command Console Software (AGCC) was used to scan the arrays and generate CEL files. CEL

files were imported into Partek Genomics Suite (Partek, Inc., St. Louis, Mo). RMA (robust multi-array average) signals were generated for all probe sets using the RMA background correction, quantile normalization and summarization by Median Polish[30]. Summarized signals for each probe set were $\log_2$ transformed. These log-transformed signals were used for Principal Components Analysis, hierarchical clustering and signal histograms to determine if there were any outlier arrays. No outliers were detected in this analysis. Untransformed RMA signals were used for fold change calculations. Contrasts were calculated as required. Fold changes were calculated using the untransformed RMA signals. Probe sets with $\log_2$ expression levels < 1.0 were considered very close to background. Probe sets with average expression levels < 1.0 were removed before the False Discovery Rate (FDR) was calculated using the Storey method[57]. Fold change of miRs whose expression was significantly different ($P < 0.05$) from lean saline-treated normally perfused controls were used to generate heat maps using the NCI CIMminer tool (http://discover.nci.nih.gov/cimminer). Euclidean distance and average linkage were used to create heat maps. To produce similar scales for both heat maps minimum (min) and maximum (max) fold change was added to each heat map.

Proteome Analysis. Myocardial biopsies were transported to the Ohio State University Proteomics Core. Protein was extracted from transmural myocardial biopsies from perfused and ischemia-reperfusion regions, prepared for capillary-LC-nanospray-MS/MS and subsequent label free quantitation. Proteomic quantifications were performed in accordance with previous studies [42, 53] In brief mass spectrometry methodology that is detailed in the online supplement. In brief, protein quantification and identification was accomplished using the NCBInr Other Mammalia Database (version 20150104, 1,412,788 sequences). A decoy database was also searched to determine the false discovery rate (FDR) and peptides were filtered according to the FDR. The significance threshold was set at $P < 0.05$. Percolator score was used to further validate the search results and the actual FDR was less than 1% after using percolator scores. Label-free

quantitation was performed using the spectral count approach, in which the relative protein quantitation is measured by comparing the number of MS/MS spectra identified from the same protein in each of the multiple LC/MSMS datasets. Scaffold was used for quantitation analysis. The protein filter was set at 99% to ensure the false discovery rate is less than 1% and the peptide filter was set at 95%.

Proteome and miR Analysis via Ingenuity Pathway Analysis. Ingenuity Pathway Analysis (IPA) (a Qiagen® product) was used to assist with interpretation and target discovery of proteome and microarray data. As IPA does not query porcine databases, all array and proteomics results were converted to the nearest bovine (protein) or human or mouse (miR) homologs. The proteomics data set was used in conjunction with the miR data set for the miR Target Finder Feature. Proteins and miR thresholds for inclusion in IPA analyses were set for $P<0.05$.

Microarray and Proteomic Database Locations. MiR microarray results were uploaded to GEO (accession GSE77378). Complete microarray and proteomics results are also provided as online supplements.

Statistical Analyses. Data were analyzed using the SigmaPlot statistical package (version 11 Systat Software Inc, San Jose, CA) and SPSS (version 22 IBM, Chicago, IL). Data are presented as mean ± standard error. Comparisons were assessed by two-way repeated measures ANOVA; (Factor A = treatment (saline/exendin-4); Factor B = condition (baseline/occlusion/reperfusion 60 min/ reperfusion 120 min)). When significance was established with ANOVA, Student-Newman-Keuls posthoc testing was performed to identify pairwise differences between groups and conditions. For **Table 1** and **Figure 3A** comparisons were assessed by one way ANOVA. The troponin data (**Table 2**) were significantly right-skewed, particularly for the reperfusion values;

therefore the effect of reperfusion was assessed using the Wilcoxon signed rank test, and to evaluate effects of obesity and treatment on this response generalized linear mixed modeling was applied in parallel to the ANOVA as above, specifying a gamma distribution without assumptions of equal error distributions between groups in order to correctly model this data distribution. Statistical significance was declared when $p<0.05$. Statistical comparisons of proteomic results were performed on proteins which met Scaffold false discovery rate (FDR) criterion by Student's t-test. In accordance with our discovery-based approach, proteins and miRs with $P < 0.05$ after passing this FDR threshold were considered significantly different between conditions. Proteins with average spectral counts of zero for a group were not statistically analyzed.

Results

Phenotypic characteristics and hemodynamics. Phenotypic characteristics of swine at baseline on the day of the physiologic studies following diet ± exendin-4 treatment, are presented in **Table 1.** The excess calorie high fat/fructose obesogenic diet significantly increased body weight (~39%; P = 0.006) and cholesterol (525%; P = 0.005) relative to the lean-control diet. Obese animals were significantly hyperglycemic and hyperinsulinemic, fulfilling criteria for the metabolic syndrome. Exendin-4 for 24 hours did not produce any statistically significant changes in the metabolic characteristics of lean or obese swine at baseline, including no detectable reduction in glycemia.

The ischemia/reperfusion protocol produced marked elevations in troponin I (**Table 2**). The reperfusion data were markedly skewed necessitating nonparametric analytic approaches as described in the Methods. The effect of reperfusion to increase troponin (P = 0.0002) did not differ between lean and obese animals (P = 0.09), though troponin concentrations were skewed to higher values in obese vs lean swine (i.e. larger injuries in obese animals, not reaching statistical significance). Exendin-4 reduced troponin values overall (P = 0.004) but the effect to reduce reperfusion-induced increase in troponin did not achieve significance (P = 0.09), and this effect did not differ between lean and obese animals (P = 0.85). Nevertheless it is noteworthy that the difference in median troponin level in exendin-4 vs. saline following reperfusion was larger in obese than in lean swine, owing to the higher values in the saline-treated obese animals (T**able 2**). Ischemia/reperfusion produced sequential reductions in mean blood pressure in lean (P < 0.001) and obese (P = 0.005) swine (**Tables 3 and 4**). Administration of exendin-4 increased mean blood pressure ~30% in lean swine at baseline and during ischemia/reperfusion (P = 0.06; **Table 3**), while exendin-4 had no effect on blood pressure in obese swine (P = 0.61; **Table 4**). Heart rate was elevated at baseline in exendin-4 treated lean swine relative to lean controls (~30%), and decreased in both treatment groups during ischemia/reperfusion (P = 0.02; **Table 3**). Exendin-4 had no effect on heart rate in obese swine. These data are also presented in a

format that allows direct comparison of hemodynamics and cardiovascular function in lean versus obese animals in **Supplement Table 1** (saline-control) and **Supplement Table 2** (exendin-4).

Cardiac function. In lean swine, ischemia/reperfusion caused progressive reductions in end-diastolic volume (P = 0.002), stroke volume (P < 0.001), cardiac output (P = 0.003) and ejection fraction (P < 0.001) (**Table 3**). Under baseline conditions, exendin-4 administration reduced ejection fraction ~35% (P = 0.03) in lean swine, likely related to the ~30% increase in resting heart rate in exendin-4 vs. control-saline treated swine (**Table 3**). While ejection fraction declined with ischemia/reperfusion in obese control and exendin-4 treated swine, end-diastolic volume, stroke volume, and cardiac output were not significantly altered by ischemia/reperfusion and/or exendin-4 in obese swine (**Table 4**). Of note, however, ejection fraction and cardiac output (untreated only) were higher during ischemia/reperfusion in obese vs. lean swine (**Supplement Tables 1** and **2**).

Left ventricular pressure volume loops representative of average group responses for lean and obese swine are presented in **Figure 1**. In response to ischemia/reperfusion, lean swine exhibited reductions in end-diastolic volume, systolic pressure development, and stroke volume, evident by comparing pressure-volume relationships for saline-treated lean swine at baseline (**Figure 1A**) and following reperfusion (**Figure 1B**). In contrast, obese swine maintained pressure development and stroke volume during ischemia/reperfusion via a relative right-ward shift (increase in end-diastolic volume) of the pressure-volume relationship, observed by comparing saline-treated obese swine at baseline (**Figure 1C)** and following reperfusion (**Figure 1D)**. In lean swine under baseline conditions, exendin-4 treatment increased systolic pressure development and decreased stroke volume (~35%) with essentially no change in diastolic filling (**Figure 1A**). However, the ~30 mmHg increase in systolic pressure in exendin-4 treated lean swine following ischemia/reperfusion was associated with an ~25% increase in end-diastolic volume relative to control (**Figure 1B**). In obese swine, exendin-4 had relatively little effect on the pressure-volume

relationship at baseline (**Figure 1C**), but produced a marked left-ward shift of the loop (decrease in end-diastolic volume with similar pressure development) following ischemia/reperfusion (**Figure 1D**). Overall the effect of exendin-4 was to mitigate the response to ischemia/reperfusion, although these were directionally opposite in lean versus obese swine.

The relationship between stroke volume and end-diastolic volume (i.e. Frank-Starling relationship as an index of cardiac contractility) revealed a markedly lower slope in obese compared to lean swine under saline-treated control conditions (**Figure 2**; $P < 0.001$). Exendin-4 treatment significantly reduced stroke volume at a given end-diastolic volume in lean animals (**Figure 2A**), changing the intercept ($P < 0.001$) but not the slope ($P = 0.21$) of the relationship. Exendin-4 shifted the slope ($P < 0.001$) of this relationship in obese animals (**Figure 2B**); however, the final slopes were not different between lean and obese exendin-4 treated swine.

Protein and miR Expression Patterns. We performed capillary-lc ms/ms proteomic and miR microarray analyses on myocardial biopsies from the ischemic and non-ischemic zones from the same lean and obese swine ± exendin-4 treatment utilized for the *in vivo* studies described above. A complete list of all 678 quantified proteins, and their differences by physiologic and treatment states, is provided in the ***online supplement***. Among all the conditions we observed significant changes in expression of 218 proteins. These proteins were overall related to cell structure, contractile apparatus and calcium handling proteins, cellular metabolism and mitochondrial function.

Figure 3 illustrates the changes in proteins related to cardiac function and calcium handling. An important novel observation is that obesity was associated with increases in multiple isoforms of the myocardial “molecular-spring” and signaling integrator protein, titin (**Figure 2A**). The abundance of titin was further elevated following ischemia/reperfusion in hearts from obese but not lean swine. Exendin-4 decreased the abundance of titin in lean and obese swine, in both normally perfused ($P = 0.02$ for lean, and $P < 0.001$ for obese) and ischemic ($P = 0.01$ for lean, P

< 0.001 for obese) tissues. In addition, the abundance of the sarcoplasmic reticulum ATPase SERCA2A was augmented in hearts from obese but not lean swine following ischemia/reperfusion (**Figure 3C**). This increase in SERCA2A abundance was mitigated by exendin-4. Obesity also altered expression of other Ca^{2+} binding proteins including calsequestrin-2 (**Figure 3B**), S100A1 (**Figure 3D**), and histidine rich calcium binding protein (**Figure 3E**). Differences in expression of Protein Kinase A, a key signaling protein in cardiomyocyte contractility, were also noted across diet and treatment conditions (**Figure 3F**).

Bioinformatics analysis of changes in protein expression using Ingenuity Pathway Analysis (IPA) software identified a number of effects of obesity and exendin-4 treatment on proteins related to cell death and survival. Selected cell death and apoptosis related proteins are presented in **Figure 4** (pro-apoptotic A-D, anti-apoptotic E,F). Individual proteins were identified to exhibit altered amounts related to ischemia (**Figures 4A** and **4E**) or exendin-4 treatment (**Figures 4B, 4C, 4D and 4F**). However, no differences in overall expression patterns of these proteins were attributable to obesity and no systematic effect of exendin-4 was evident.

A listing of all miRs detected by microarray, and their differences in expression in each sample group, are provided in the ***online supplement***. The heat maps shown in **Figures 5** and **6** illustrate that several groups of miRs are concordantly altered by obesity, ischemia/reperfusion and exendin-4 treatment (regions marked A-D on **Figure 5** and A-I on **Figure 6**). For example, miRs 378,423-3p, 133a-5p, 361-3p, and 423-5p (Group A in **Figure 5**) were all down-regulated by ischemia/reperfusion in lean control swine whereas a different group of miRs (miRs 652, 143-5p, 17-3p, 26a, 24, 214, 140-star, 143-3p, 199a, and 152) were down-regulated by ischemia/reperfusion in obese swine (Group D, **Figure 6**). Interestingly, exendin-4 treatment prevented the downregulation of Group A miRs in lean swine during ischemia/reperfusion and resulted in altered expression of a distinct group of miRs (Group D, **Figure 5**). Obesity alone resulted in decreased expression of miRs 378, 130b, 1307, 331-5p, 2320, 129a, 30b-3p, 30e-3p (Group B, **Figure 6**) and upregulation of miRs 497, 494, 30c, 105-1, 331-3p. Interestingly some

of the miRs downregulated in obese hearts (Group B, **Figure 6**) were also downregulated by ischemia/reperfusion in the lean swine (e.g. Group A **Figure 5**, miR 378, and 133a-59). Exendin-4 prevented the alterations observed in obese swine (Groups A and B, **Figure 6**), and resulted in upregulation of miRs 491, and 146a (Group E, **Figure 6**) which were unchanged in all other comparisons. Obese exendin-4 treated hearts during ischemia/reperfusion had the greatest number of miR changes, with 26 of the 36 total unique to the comparison. In particular, miRs decreased in group H (**Figure 6**) (miRs 140, 122,424-star, 345-3p, 15a, 324, let 7 a/f/g/i, 362, 151-5p/3p, 16, 126, 106a, 425-5p, 365-3p, 181-5p, 221, 28-3p, and 199a-3p). The functional or regulatory significance of these differences is not currently known, but can be inferred from bioinformatics analyses that apply known associations between miR species and specific categories of biological function. Therefore the differentially expressed groups of miRs were evaluated using IPA software for analysis of predicted molecular and cellular function (**Table 5** and **Supplemental Table 4**). The most discernable finding of this analysis was that significantly more miRs were altered in obese relative to lean swine and that IPA identified numerous potential links of these miRs to known pathways of injury and disease (**Table 5 and Supplemental Table 4**).

To determine if any of the observed changes in miR expression could account for alterations in protein expression that we identified from our proteomic analysis we used the Target Finder feature within IPA software. A summary of these results and other is presented in **Supplemental Table 4**. 11 miRs were identified for which the change in their expression inversely correlated with expression of their predicted target proteins. These proteins were not readily categorized into a single functional group although they are important metabolic, structural and signaling molecules.

Discussion

Experiments in this study were designed to explore obesity-associated differences in myocardial function in response to ischemia/reperfusion, and how these responses are modified by pharmacologic treatment (i.e. with the GLP-1 agonist exendin-4). Admittance pressure volume catheter technology, the gold standard measure for cardiac function [12, 58], was used in *in-vivo* experiments to provide highly-sensitive, real time measures of cardiac pressures and volumes. We found that obese and lean swine produced divergent physiologic responses to ischemia/reperfusion, and that the effects of exendin-4 treatment on ischemia/reperfusion were dramatically different between lean and obese swine. We performed unbiased analyses of changes in protein and miR expression associated with these differing responses as a platform for discovery of novel underlying molecular changes that could contribute to the observed physiologic effects and differences between groups. The distinctive *in vivo* responses to ischemia/reperfusion and exendin-4 were associated with informative and interestingly different expression profiles of proteins and miRs, including in factors known to be associated with regulation of cardiac function (validating the approach) but also including factors not previously known to play a regulatory or functional role in relation to obesity.

Obesity and cardiac function in response to ischemia/reperfusion. We found that obesity is associated with a number of important functional differences in the regulation of cardiac contractile function and systemic hemodynamics. In particular, under normal baseline conditions obesity tended to produce a leftward shift of the left ventricular pressure-volume relationship relative to lean swine (**Figure 1,** panels A and C solid lines). Such reductions in end-diastolic filling volumes with similar systolic pressure generation have been previously observed in animal models[47] and in obese humans[48]. These changes are likely attributable in part to augmented sympathetic tone, which is well documented in the setting of obesity [28, 31, 66]. While the difference in the pressure-volume relationship between the lean and obese swine was relatively

modest at baseline, more distinct effects were noted following ischemia/reperfusion (**Figure 1** Panels B and D solid lines). These differences were not likely attributable to differing infarct size; on strict statistical criteria the ischemia/reperfusion-induced increase in troponin did not differ between lean and obese animals, but moreover the trend in troponin values suggested if anything greater myocardial injury among the obese saline-treated animals (**Table 2**) whereas the observed responses suggest reduced function with left-shifted pressure-volume loops in lean animals but preserved or augmented function in obese animals with right-shifted pressure-volume loops (**Figure 1**, comparing solid lines within lean or obese animals). Our data demonstrate that regional ischemia/reperfusion produced marked reductions in indices of global contractile function in lean swine, namely significant reductions in arterial pressure, ejection fraction (**Table 3**), and end-diastolic volume (**Figures 1** and **2**). Thus, lean swine exhibited post-ischemic contractile dysfunction consistent with myocardial stunning [55]. In contrast, indices of cardiac function (stroke volume, cardiac output, ejection fraction) were relatively maintained throughout ischemia/reperfusion in obese swine and thus, were significantly higher in obese vs. lean swine (**Table 3**). Again, this preservation of ventricular stroke volume and pressure generation was observed despite the nonsignificantly greater infarct size as measured by troponin I (**Table 2**) as well as the presumed presence of an obesity cardiomyopathy phenotype which one might predict to observe in these animals *a priori* [3, 54]. Overall these data suggest that the relative preservation of ventricular function under ischemia/reperfusion in our model of early obesity was achieved via a Frank-Starling mechanism (increase end-diastolic filling volume) to maintain cardiac output in response to an ischemia/reperfusion insult (**Figures 1 and 2**). While the lack of post-ischemic contractile dysfunction in obese hearts seems unexpected, particularly with troponin data suggesting material myocardial injury, we do see evidence of underlying myocardial dysfunction in the baseline pressure-volume loops (**Figure 1**) and in that the slope of the relationship between stroke volume and end-diastolic volume was significantly lower in obese relative to lean swine (**Figure 2**); furthermore, the observed increases in blood pressure, stroke

volume, and ejection fraction would act to augment myocardial energy demand, increase ventricular wall stretch, and exacerbate underlying ischemic injury (and may have contributed to the greater injury suggested by higher troponin-I values under saline-reperfusion conditions) [55]. Prior echocardiographic studies in humans are in accordance with these findings and suggest that the cardiac effects of obesity occur over a continuum and are directly dependent on the degree and duration of obesity and the degree of underlying metabolic risk factors [8, 31, 40].

Effects of exendin-4 on cardiac responses to ischemia/reperfusion. The current study is the first to describe the functional consequences of altered GLP-1 responses relative to cardiovascular effects of regional ischemia/reperfusion in a large animal model of obesity. There is considerable experimental evidence supporting beneficial cardiovascular effects of treatment with GLP-1 based therapies. In animal models, GLP-1 therapeutics have been associated with increased cardioprotective capacity related to myocardial substrate selection, heart rate effects, blood pressure effects, reduction in myocardial infarct size, and improved cardiac function[21]. In contrast, large clinical trials of GLP-1 based therapies in humans with obesity and type 2 diabetes have failed to show the expected cardiovascular benefits to date [64, 70]. Recent work from our laboratory suggests that cardiometabolic effects of GLP-1 are impaired in the setting of obesity, raising the possibility of "cardiovascular GLP-1 resistance" in the population being treated with these agents [20, 22, 44]. The troponin measures (**Table 2**) support that exendin-4 may diminish infarct size in lean and obese swine, with reductions in the median values in both groups although overall variability in this injury marker produced non-significant p values for this comparison (p=0.09). This protective effect of exendin-4 did not differ significantly between lean and obese animals. Despite these parallel GLP-1 therapeutic responses to ischemia/reperfusion, and contrary to what might have been predicted based on greater mass of injured myocardium, obese exendin-4 treated swine maintained pressure like their untreated counterparts, and did so despite marked reductions in end-diastolic volume (i.e. increases in cardiac contractility). Several

questions arise relating to whether differing initial state and/r differing responses in autonomic, metabolic, or circulating factors contribute to these different responses in obese hearts. Lean treated swine responded to ischemia/reperfusion with increases in pressure generation and end-diastolic volume (**Figure 1**), despite overall reductions in stroke volume at a given ventricular filling volume (**Figure 2**).These observations contribute to the growing body of experimental and clinical literature supporting marked differences in cardiovascular responses to GLP-1 therapeutics in the setting of obesity [20, 44, 46, 64].

Proteomic and miR expression profile in obese hearts. To explore potential molecular changes that could contribute to the differential cardiac phenotype of obese hearts, we performed proteomics and miR microarray analyses on myocardial biopsies from the lean and obese swine following completion of the *in vivo* ischemia/reperfusion protocols, sampling tissue from ischemic and non-ischemic zones. In parallel with our physiological analyses, protein and miR expression analyses revealed obesity-related differences, and differences in changes with exendin-4 treatment, in protein and miR expression. Of particular note we observed increased abundance of titin in the myocardium of obese swine under control conditions, which was mitigated with GLP-1 therapy. The giant protein titin plays multiple roles within the cardiomyocyte including providing structure in the sarcomere (binding myosin to the z-disk); contributing significantly to the passive and restoring force of the cardiac sarcomere; fine-tuning myocardial stiffness (via differential splicing of titin transcripts as well as titin phosphorylation); and mediating hypertrophic signaling [33, 69]. It is known that modulating titin (via altering the predominant isoform or the phosphorylation status) is associated with cardiac pathophysiology [59], but little prior work has evaluated the role of titin in obesity-associated cardiomyopathy. Hamdani et al. found altered phosphorylation status of titin of in the setting of metabolic risk [25, 26]. Also, therapeutics that increase GLP-1 signaling have been associated with alterations in titin phosphorylation status[26]. Our observations suggest a need for further studies to elucidate isoform switching and

phosphorylation status of titin in the setting of obesity, exendin-4 treatment, and ischemia/reperfusion response.

The proteomic data confirm obesity-associated differences in calcium handling molecules predicted by prior work (e.g. SERCA2A [5, 19, 67, 68], calsequestrin [43]). Importantly these data also highlight the involvement of calcium handling proteins not previously associated with obesity-related cardiac dysfunction (e.g. histidine-rich calcium binding protein). GLP-1 mimetics have been found to differently modulate calcium handling in vascular smooth muscle of swine depending on obesity [18], but, until now, have not been associated with direct changes in the level of calcium handling proteins in myocardium. Modulation of calcium handling and titin protein abundance has profound implications for signaling (whether by calcium as a second messenger, or titin hypertrophic signaling e.g. via NFAT [33]), cardiac stiffness, and cardiac contractility. Taken together, it appears that significant insights into the cardiac effects of obesity can be gained from more in depth investigation of these proteins, including for example splice variants of titin (e.g. N2B, N2Ba, FCT)[37], phosphorylation of specific titin residues[2, 25], down-stream NFAT signaling[10, 27, 45] and the interplay with altered calcium handling[38, 59]. [26]. These discoveries underscore the value of the proteomic approach as we have applied it, in the context of a specific set of studies that produce physiologically relevant states.

MicroRNAs are recognized for their widespread regulatory roles (which are predominantly inhibitory). These ancient systems influence multiple pathways concurrently, allowing for coordinated alterations in multiple components of cellular physiology in response to stimuli. Microarray results demonstrate that global miR regulation is different in obese hearts in response to ischemia/reperfusion and drug intervention with exendin-4. Studies investigating miR expression changes in pathology or therapeutic treatment in the setting of underlying metabolic abnormalities are lacking. In this study, we observed different patterns of miR expression between lean and obese animals, and with ischemia/reperfusion injury with and without exendin-4 treatment. Further, the expression patterns associated with combinations of these conditions were

themselves different from the effects of the individual factors (**Figure 5 and 6**), and therefore not predictable based on knowledge of the effects of individual factors. We see important contributions in particular from a relatively small set of miRs (let 7 family, 10a, 15, 30 family,199 family, and 214), some of which have been previously associated with heart disease. In mice, ischemia was found to upregulate miR 15 family members[29, 39, 50], miR 30 family members[56], and miR 214[4]. In contrast to our observations, in that study ischemia/reperfusion did not alter expression of any of these miRs in lean hearts. In our data in obese swine, these miRs were significantly downregulated during ischemia/reperfusion (while miR-30c was upregulated in obese normally perfused myocardium compared to lean). A prior observation of diminished expression of miR-199a in early ischemia in isolated porcine cardiomyocytes[52] is also consistent with our observed tissue level changes, perhaps acting via increases in cardiac Hif-1α expression. Previous studies have demonstrated that GLP-1 mimetics induce miR expression changes in various non-cardiac tissues [35, 61]. However, our study is the first to demonstrate GLP-1-induced miR expression changes in myocardium, and that those effects differ considerably depending on obesity status. Furthermore, miR expression response to ischemia/reperfusion is both GLP-1 and obesity dependent. These changes in miRs thus mimic the different physiological changes and responses to ischemia/reperfusion that occur in response to exendin-4 in lean and obese swine, suggesting that they may be contributing to the pathophysiological changes. Growing evidence suggests that GLP-1 receptor activation alters DNA transcription in addition to the expected direct signaling effects [35, 61], suggesting the potential for a relatively unexplored mechanism through which GLP-1 may impart direct and/or indirect effects. Further investigation into the mechanisms of GLP-1 mimetic-induced transcriptome changes are needed. IPA software was used to generate literature associated predictions of processes effected by changed proteins and miRs (**Table 5, Supplemental tables 3 and 4**) and provide some context in which experimental validation of the effects of these miRs, or miR groups, might be further explored. Again, the value of these observations is primarily

related to the experimental paradigm under which the tissues were collected, which provides a physiological context to allow interpretation of the differences observed.

***Limitations*.** Owing to the resource-intensive nature of these studies and some animal loss during the ischemia/reperfusion event, sample sizes were relatively low in this study. Nevertheless, we noted statistically significant group- and treatment-related differences in key physiologic end points which were consistent with recent data from our laboratory [21, 22, 44] and directly associated with marked differences in the cardiac proteome and miR transcriptome between lean and obese swine both at baseline and in response to ischemic injury. These observations therefore are not compromised on statistical power overall.

Unusually for this model, the group of animals fed the obesogenic diet were also hyperglycemic, hyperinsulinemic, and hypertriglyceridemic, and furthermore they failed to lower glucose values with the 24 hour exposure to exendin-4. Our current data do not allow post-hoc analyses that separate obesity from obesity plus dysglycemia/metabolic syndrome. These factors may have contributed to the observed differences between groups. However, this does not detract from the observations of significant group differences associated with a high-fat, obesogenic diet. Although our experimental protocol included progressive reductions in venous return using a central venous balloon for gold-standard assessments of contractility (i.e. end-systolic pressure volume relationship (ESPVR)), we were unable to acquire adequate reductions in end-diastolic volume in a sufficient number of animals for adequate statistical analysis of cardiac contractility per se. Statistical analysis of troponin I measures were associated with highly variable values in the separate groups, contributing to insufficient power to definitively identify differences in response due to obesity that are suggested by the median values. Nevertheless, the observed trends suggested increased infarct in obese swine, in association with observed increases in cardiac function in these animals. This apparently greater magnitude of infarct in obese animals may not in itself explain the observed differences in function. Moreover, it is unclear whether the

increased injury is possibly a consequence of the obesity-specific paradoxical response. Further work will be needed to understand the directionality of causality in these responses.

The molecular associations presented do not represent direct experimental manipulations of these systems to evaluate causality. This does not diminish the overall power of our hypothesis-free approach as a discovery platform, evident in our novel and unexpected observations. In particular the approach we applied allows us to make observations about the highly integrated and multi-factorial changes in molecular and integrated physiology in response to clearly described experimentally induced physiologic circumstances (in the same animals). These observations provide novel and valuable insights into the functioning of these systems in ischemia, and provide the first ever description of these changes in response to GLP-1 agonist treatment.

Conclusions and Implications. Taken together, these observations validate this discovery approach and reveal novel associations that suggest previously undiscovered mechanisms contributing to the effects of obesity on the heart and contributing to the actions of GLP-1 following ischemia/reperfusion. Further experimental studies will be needed to understand the causal relationships between transcriptome and proteome changes such as those we have observed and the associated changes in myocardial function.

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Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Tables and Figures

Table 1. Phenotypic characteristics of lean and obese swine

	Lean Saline n=5	Lean Exendin-4 n=5	Obese Saline n=4	Obese Exendin-4 n=5
Body Weight (kg)	75 ± 6	67 ± 8	104 ± 6*	106 ± 4*
Heart Weight (g)	212 ± 16	194 ± 18	247 ± 9	224 ± 11
Glucose (mmol/L)	8.6 ± 2.3	6.8 ± 1.8	13.3 ± 1.1	14.1 ± 1.9*
Insulin (pmol/L)	73 ± 13	52 ± 11	111 ± 57	129 ± 35
Triglycerides (mmol/L)	0.6 ± 0.1	0.5 ± 0.1	1.0 ± 0.4	0.9 ± 0.1
Total cholesterol (mmol/L)	1.6 ± 0.4	1.4 ± 0.4	10.0 ± 3.3*	10.1 ± 2.0*

Table 1 Phenotypic characteristics of lean and obese swine. Measurements were performed before at baseline after 24 hours Exendin-4 or saline infusion. Values are mean ± SE. * $P \leq 0.05$ vs. lean same treatment. Measurements were performed after 24 hours Exendin-4 or saline infusion.

Table 2. Troponin-I measurements.

	Lean		Obese	
	Saline	Exendin-4	Saline	Exendin-4
Baseline	0.30 [0.18 – 0.67]	0.37 [0.15 – 0.57]	0.35 [0.03 – 0.66]	0.10 [0.04 – 0.33]
Reperfusion	0.88 [0.39 – 17.53]	0.55 [0.28 – 0.93]	5.05 [0.27 – 15.22]	0.84 [0.41 – 4.30]

Table 2. Systemic troponin-I concentrations (ng/mL) expressed as median [25^{th} – 75^{th} percentile]. Troponin measures were acquired at the end of each experimental time point. Due to the skewed distributions, values are expressed as median [range] and non-parametric analyses were performed (see Methods). Reperfusion values were significantly increased compared to baseline ($P = 0.0002$). Exendin-4 significantly reduced the troponin values overall ($P = 0.004$), although the effect of exendin-4 to reduce the reperfusion-induced increase in troponin did not achieve significance ($P = 0.09$). Obese animals were not statistically different in these effects ($P = 0.85$ overall; $P = 0.09$ comparing reperfusion-induced increase between groups).

Table 3. Hemodynamics and cardiovascular measurements of lean swine

	Saline	Exendin-4	Condition	Treatment	Interaction
Mean Blood Pressure (mmHg)					
Baseline	101 ± 21	131 ± 5			
Occlusion	89 ± 23	127 ± 6†	*P* < 0.001	P = 0.06	*P* = 0.89
Reperfusion 60 min	75 ± 11	108 ± 8			
Reperfusion 120 min	63 ± 11*	91 ± 6*			
Heart Rate (beats/min)					
Baseline	87 ± 20	113 ± 4			
Occlusion	72 ± 8	68 ± 12	*P* = 0.02	*P* = 0.96	*P* = 0.67
Reperfusion 60 min	83 ± 13	84 ± 8			
Reperfusion 120 min	85 ± 14	80 ± 7			
End Diastolic Volume (ml)					
Baseline	83 ± 15	82 ± 7			
Occlusion	60 ± 8*	77 ± 7	*P* = 0.002	*P* = 0.34	*P* = 0.42
Reperfusion 60 min	58 ± 7*	65 ± 6			
Reperfusion 120 min	49 ± 8*	64 ± 6			
Stroke Volume (ml)					
Baseline	41 ± 10	26 ± 5			
Occlusion	18 ± 7*	21 ± 4	*P* < 0.001	P = 0.727	*P* = 0.13
Reperfusion 60 min	12 ± 4*	14 ± 3			
Reperfusion 120 min	11 ± 3*	13 ± 4			
Cardiac Output (L/min)					
Baseline	2.2± 0.5	1.7 ± 0.5			
Occlusion	1.2± 0.4*	1.3 ± 0.2	*P* = 0.003	*P* = 0.97	*P* = 0.52
Reperfusion 60 min	0.9± 0.2*	1.1 ± 0.2			
Reperfusion 120 min	0.9± 0.2*	1.0 ± 0.3			
Ejection Fraction (%)					
Baseline	47 ± 6	31 ± 4†			
Occlusion	27 ± 8*	26 ± 3	*P* < 0.001	*P* = 0.40	*P* = 0.04
Reperfusion 60 min	20 ± 5*	20 ± 3*			
Reperfusion 120 min	21 ± 4*	20 ± 4*			

Values are mean ± SE for lean (n = 4) and lean + exendin-4 (n = 5) swine. By two way repeated measures ANOVA: * = *P* < 0.05 vs. Baseline, same treatment; † = *P* < 0.05 vs. lean control, same condition. Condition denotes effect of sequential ischemia/reperfusion (baseline/occlusion/reperfusion periods), evaluated as a repeated measure comparison of the 4 experimental stages; Interaction tests whether this ischemia/reperfusion effect differs by treatment.

Table 4. Hemodynamics and cardiovascular measurements of obese swine

	Saline	Exendin-4	Condition	Treatment	Interaction
Mean Blood Pressure (mmHg)					
Baseline	99 ± 10	89 ± 5			
Occlusion	91 ± 8	86 ± 4	*P* = 0.005	*P* = 0.61	*P* = 0.45
Reperfusion 60 min	85 ± 11*	86 ± 4			
Reperfusion 120 min	83 ± 8*	77 ± 6			
Heart Rate (beats/min)					
Baseline	83 ± 8	74 ± 12			
Occlusion	90 ± 11	68 ± 8	*P* = 0.80	*P* = 0.35	*P* = 0.28
Reperfusion 60 min	87 ± 11	75 ± 11			
Reperfusion 120 min	86 ± 7	77 ± 10			
End Diastolic Volume (ml)					
Baseline	66 ± 8	59 ± 5			
Occlusion	76 ± 10	65 ± 3	*P* = 0.68	*P* = 0.25	*P* = 0.25
Reperfusion 60 min	97 ± 32	55 ± 7			
Reperfusion 120 min	89 ± 30	48 ± 8			
Stroke Volume (ml)					
Baseline	26 ± 5	28 ± 4			
Occlusion	32 ± 3	31 ± 4	*P* = 0.30	*P* = 0.33	*P* = 0.43
Reperfusion 60 min	23 ± 8	23 ± 3			
Reperfusion 120 min	28 ± 7	17 ± 4			
Cardiac Output (L/min)					
Baseline	2.1 ± 0.4	1.9 ± 0.1			
Occlusion	2.9 ± 0.5	2.0 ± 0.2	*P* = 0.54	*P* = 0.18	*P* = 0.72
Reperfusion 60 min	2.9 ± 0.9	1.8 ± 0.5			
Reperfusion 120 min	2.4 ± 0.6	1.4 ± 0.5			
Ejection Fraction (%)					
Baseline	39 ± 5	46 ± 6			
Occlusion	42 ± 3	47 ± 6	*P* = 0.04	*P* = 0.32	*P* = 0.80
Reperfusion 60 min	36 ± 4	41 ± 1			
Reperfusion 120 min	35 ± 3	35 ± 2			

Values are mean ± SE for obese (n = 4) and obese + exendin-4 (n = 4) swine. * = $P < 0.05$ vs. Baseline, same treatment. The analytic approach is identical to that presented for Table 3.

Table 5. IPA predictions of molecular and cellular functions associated with miR changes

Heat Map	Effect of	Group	IPA Associations: Molecular and Cellular Function
4	I/R in Lean	A	cell death and survival
	EX-4 in Lean	B	cellular movement development growth and proliferation
	EX-4 and I/R in Lean	C	cell-to-cell signaling and interaction cellular development cellular growth and proliferation cell death and survival cell morphology
		D	N/A
5	Obesity	A	cell morphology cellular function and maintenance DNA replication/ recombination/repair cellular assembly and organization
		A + B	cell death and survival
		B	cellular compromise cell cycle cellular development cell-to-cell signaling and interaction
	I/R and Obesity	C	cell cycle

		C + D	cellular development cellular growth and proliferation
		D	cell death and survival cell-to-cell signaling and interaction small molecule biochemistry
	Ex-4 and Obesity	E	cell-to-cell signaling and interaction cellular assembly and organization cellular compromise cellular movement cell death and survival
	EX-4, I/R and Obesity	F	cell-to-cell signaling and interaction cellular assembly and organization cellular function and maintenance
		F + H	cell death and survival
		H	cell cycle cellular movement
		H + I	cellular growth and proliferation
		F + H + I	cellular development
		G	N/A

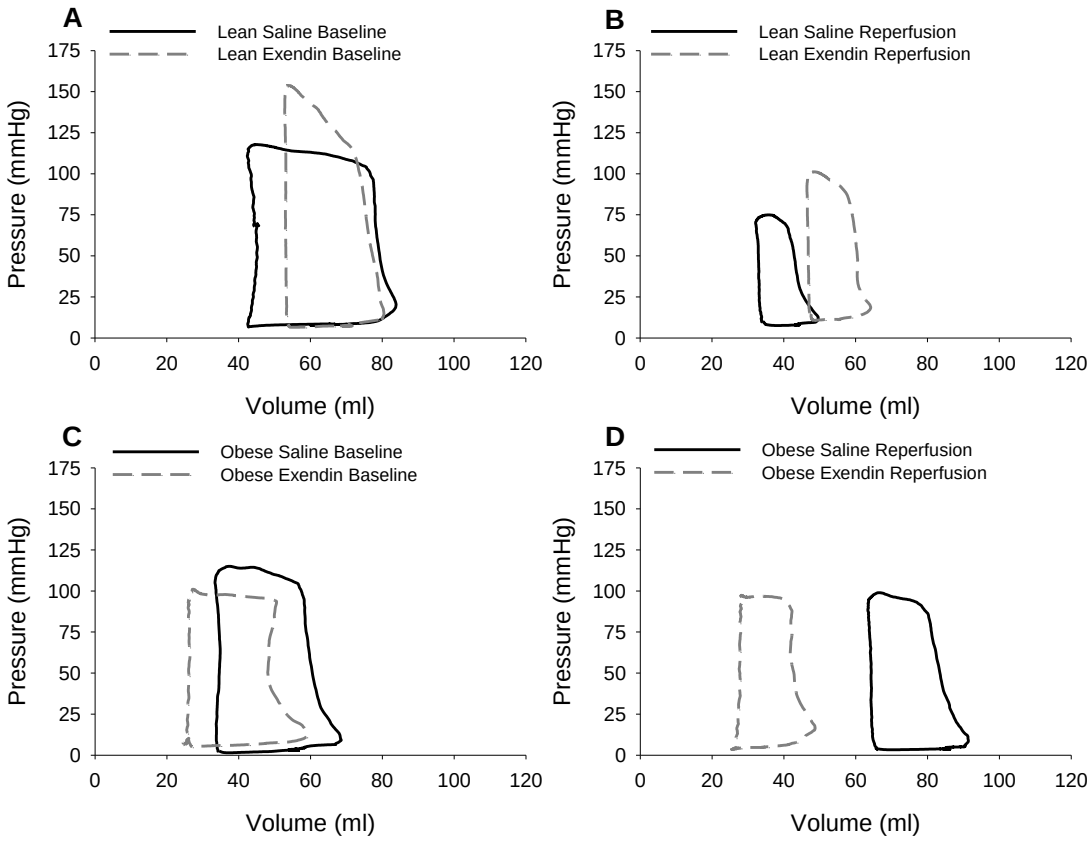

Figure 1. Representative pressure-volume loops for lean and obese swine at baseline, following ischemia-reperfusion injury, in the absence and presence of the GLP-1 receptor agonist exendin-4.

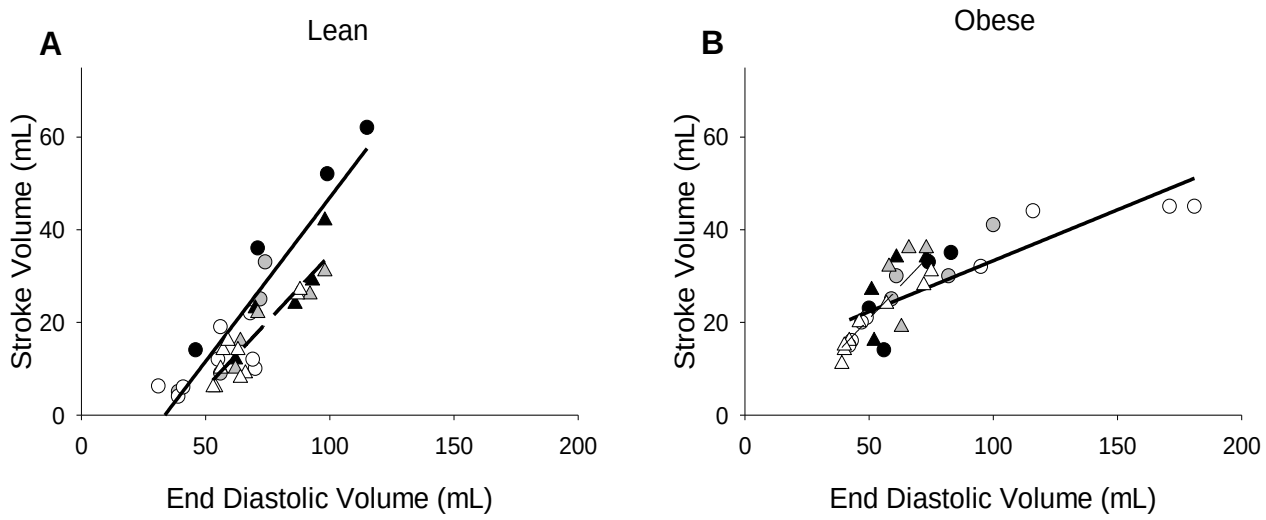

Figure 2. Effects of obesity and exendin-4 on the relationship between stroke volume and end-diastolic volume (i.e. Frank-Starling relationship). Solid lines represent best fit lines for saline (circles); dashed lines are the fit lines for exendin-4 (triangles). Filled symbols are measurements from the baseline pre-occlusion state; gray symbols are measurements during occlusion; and open symbols are measurements at 120 minutes of reperfusion. The slope of this relationship was significantly diminished in obese-control vs. lean-control swine ($P < 0.0001$). Exendin-4 treatment significantly reduced stroke volume at a given end-diastolic volume in lean animals (Panel A), changing the intercept ($P < 0.001$) but not the slope ($P = 0.21$) of the relationship. Exendin-4 shifted the slope ($P = 0.003$) of this relationship in obese animals (Panel B); however, the final slopes were not different between lean and obese exendin-4 treated swine ($P = 0.92$).

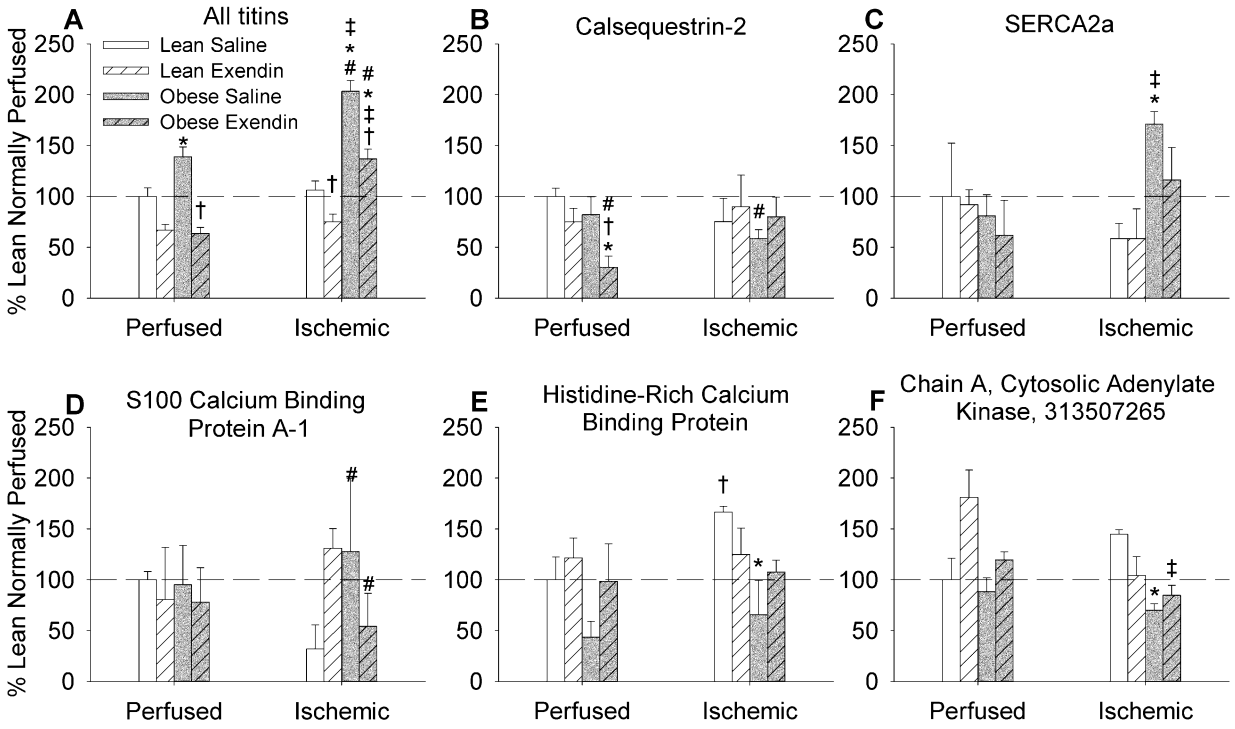

Figure 3. Effects of obesity and exendin-4 on proteins underlying myocardial function and calcium handling in normally perfused hearts and in myocardium following ischemia/reperfusion injury. Bars represent average percent relative to lean normally perfused value and error bars represent standard error. * = *P*<0.05 for comparison of diet within the same treatment and condition; † = *P*<0.05 for comparison of treatment within the same condition and diet; ‡ = *P*<0.05 for condition within the same diet and treatment. # = P > 0.05 for comparison relative to Lean Perfused Untreated myocardium. Panel A shows an aggregate of all titin proteins identified in mass spectrometry. Comparison for panel A by ANOVA All other comparisons by t-test. n=3 for lean perfused saline, n=4 lean perfused exendin-4, n=4 obese perfused saline, n= 4 obese perfused exendin-4, n=4 lean ischemic saline, n= 4 lean ischemic exendin-4, n=3 obese ischemic saline, n=4 obese ischemic exendin-4.

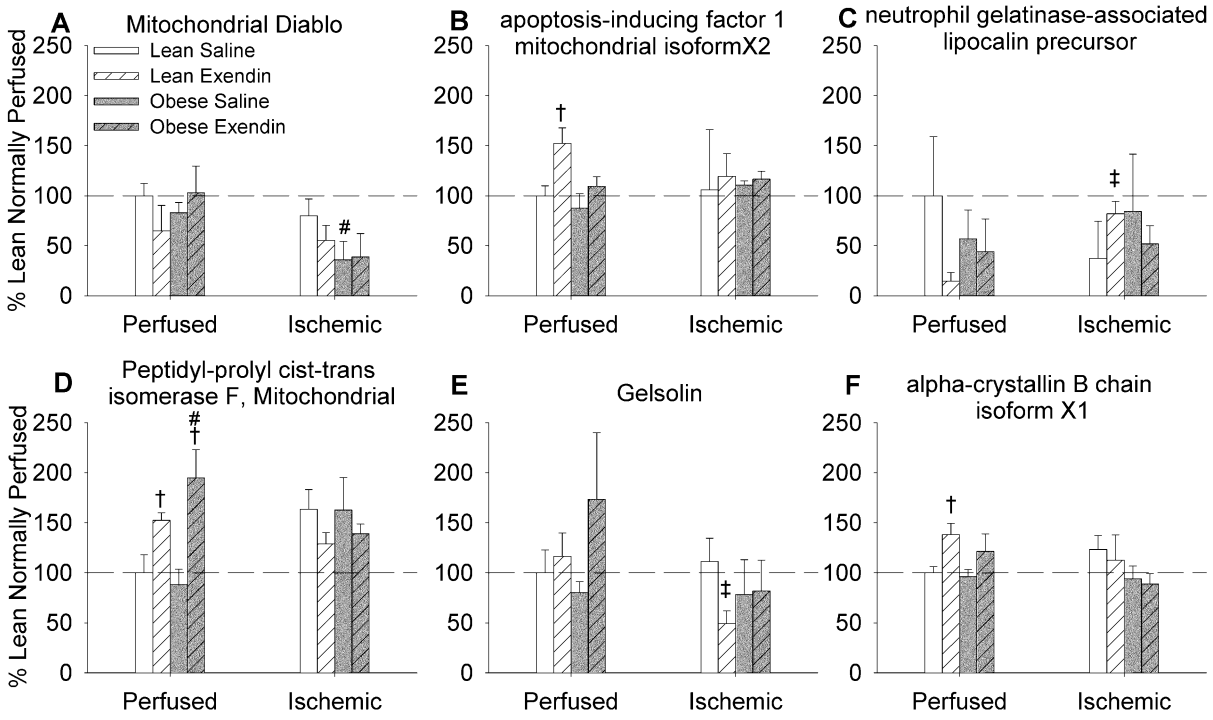

Figure 4. Effects of obesity and exendin-4 on proteins underlying cell death in normally perfused hearts and in myocardium following ischemia/reperfusion injury. Bars represent average percent relative to lean normally perfused value and error bars represent standard error. * = $P<0.05$ for comparison of diet within the same treatment and condition; † = $P<0.05$ for comparison of treatment within the same condition and diet; ‡ = $P<0.05$ for condition within the same diet and treatment. # = P > 0.05 for comparison relative to Lean Perfused Untreated myocardium All comparisons by unpaired T-test. n= 3 for lean perfused saline, n=4 lean perfused exendin-4, n=4 obese perfused saline, n= 4 obese perfused exendin-4, n=4 lean ischemic saline, n= 4 lean ischemic exendin-4, n=3 obese ischemic saline, n=4 obese ischemic exendin-4.

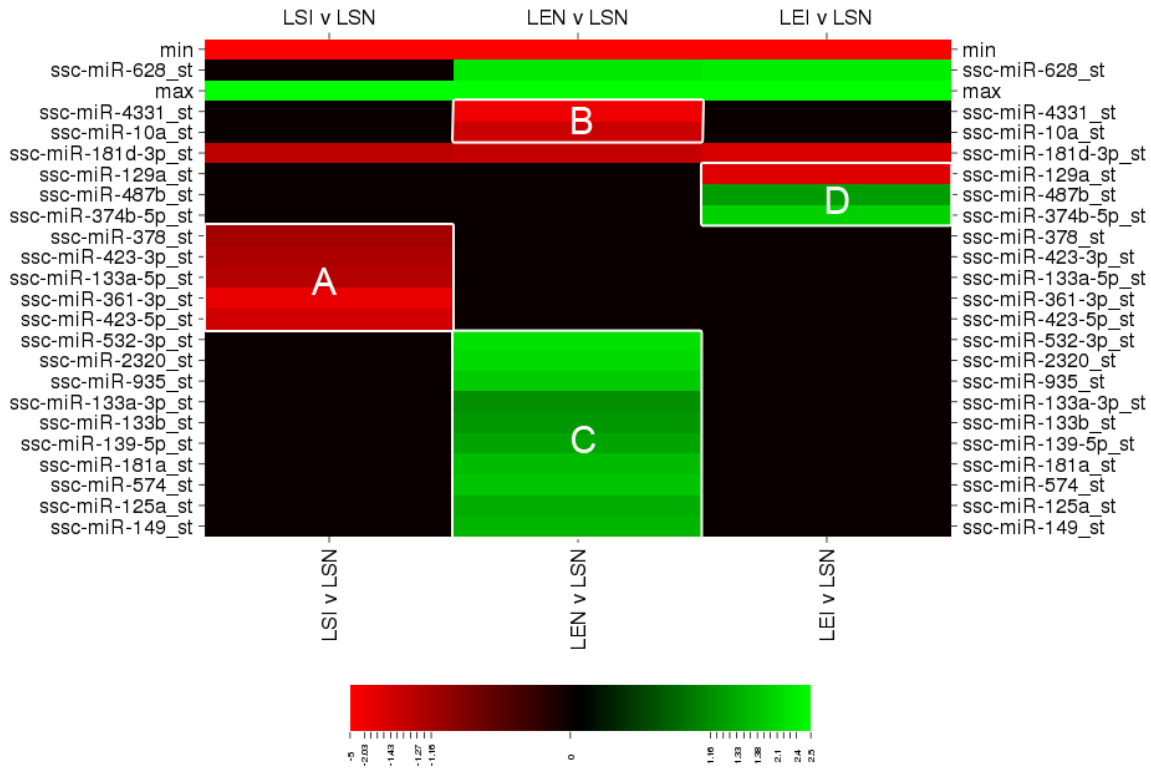

Figure 5. Heat map of miRs changed in lean left ventricular myocardium. n = 5 for all groups within comparisons. Differently regulated clusters of miRs in each comparison were ascribed to a group (indicated by letters A-D), based on common differences within comparisons. For each group the fold change and p-values were submitted to IPA for analysis of each cluster. Results of IPA analysis are presented in **Table 5 and Supplement Table 4**. Rows titled “min” and “max” were added to create similar scales for Figures 5 and 6. LSI = Lean Saline Ischemic. LEN = Lean EX-4 Normally Perfused. LEI = Lean EX-4 Ischemic. LSN = Lean Saline Normally Perfused

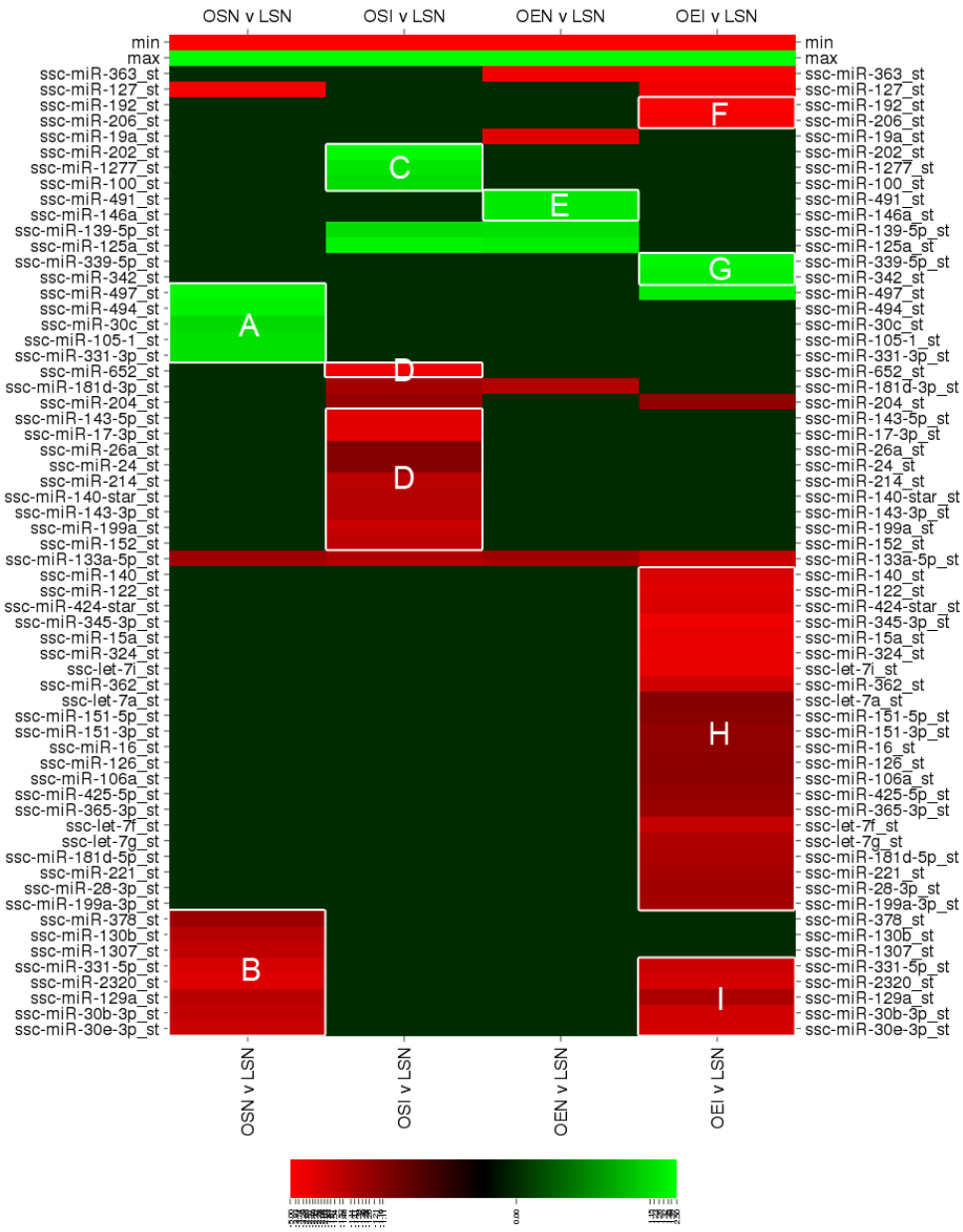

Figure 6. Heat map of miRs changed in obese left ventricular myocardium. For lean saline perfused (n = 5) . n = 4 obese saline, n = 5 obese exendin-4 ischemic and normally perfused. Differently regulated clusters of miRs in each comparison were ascribed to a group (indicated by letters A-I). For each group the fold change and p-values were submitted to IPA for analysis of each cluster. Results of IPA analysis are presented in **Table 5 and Supplement Table 4**. Rows titled “min” and “max” were added to create similar scales for Figures 4 and 5. OSN = Obese Saline Normally Perfused. OSI = Obese Saline Ischemic. OEN = Obese EX-4 Normally Perfused. OEI = Obese exendin-4 ischemic. LSN = Lean Saline Normally Perfused