Effects of Diet-Induced Obesity on Inflammation and Remodeling after Myocardial Infarction

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Short Title: Myocardial infarction in diet-induced obese mice
Abstract:
Epidemiological studies indicate that obesity, insulin resistance and diabetes are important comorbidities of patients with ischemic heart disease and increase mortality and development of congestive heart failure after myocardial infarction. Although \textit{ob/ob} and \textit{db/db} mice are commonly used to study obesity with insulin resistance or diabetes, mutations in the leptin gene or its receptor are rarely the cause of obesity in humans, which is instead primarily a consequence of dietary and lifestyle factors. Therefore, we used a murine model of diet-induced obesity to examine physiological effects of obesity and the inflammatory and healing response of diet-induced obese (DIO) mice after ischemia–reperfusion injury. DIO mice developed hyperinsulinemia and insulin resistance and hepatic steatosis, with significant ectopic lipid deposition in the heart and cardiac hypertrophy in the absence of significant changes in blood pressure. The mRNA levels of chemokines at 24 hours and cytokines at 24 and 72 hours of reperfusion were higher in DIO mice than lean mice. In the granulation tissue at 72 hours of reperfusion, macrophage density was significantly increased, while neutrophil density was reduced in DIO mice compared with lean mice. At 7 days of reperfusion, infarcted DIO mice had significantly reduced collagen deposition in the scar and increased left ventricular (LV) dilation and cardiac hypertrophy, indicative of adverse LV remodeling. The characterization of a murine diet-induced model of obesity and insulin resistance that satisfies many aspects commonly observed in human obesity allows detailed examination at the molecular level of the adverse cardiovascular effects of diet-induced obesity.

\textbf{Key words}: diet-induced obesity, insulin resistance, cardiac lipotoxicity, ischemia–reperfusion injury, left-ventricular remodeling.
Introduction:

The incidence of overweight and obesity in the western world has risen dramatically; two thirds of the US adult population is overweight, and one third is obese (27). Extreme obesity is known to impact the structure and function of the heart in terms of hemodynamic load, altered left ventricular (LV) remodeling and impaired ventricular function leading to overt heart failure (30, 31). In at least four community-based, prospective studies, body mass index (BMI) incrementally predicted heart failure risk above and beyond known risk factors for heart failure. Obesity is known to independently increase the risk for hypertension, diabetes and dyslipidemia, all of which increase the risk for myocardial infarction and subsequent heart failure. On the other hand, when associated with fewer comorbidities, obesity paradoxically has been found to be protective in the short term after a myocardial infarction (39). Nonetheless, the risks for recurrent myocardial infarction and adverse outcome in the long term are increased in patients with BMI >25 kg/m² (40).

In recent years, with the advances made in gene targeting and trapping technologies, the mouse model is increasingly being used to model human diseases for studies at the mechanistic level. To take advantage of the above technologies, we have developed a murine model of myocardial infarction (41) and used it extensively to examine the role of specific genes in the pathobiology of ischemic myocardial injury (5, 14, 23). The process of cardiac repair following myocardial infarction is initiated by an acute inflammatory response largely dictated by temporally and spatially coordinated expression of chemokines and cytokines, cell adhesion molecules, growth factors and extracellular matrix proteins. In the early phase, the recruited leukocytes contribute to the clearing of necrotic cardiomyocytes. Subsequently, granulation tissue is formed consisting of macrophages, myofibroblasts and neovessels. In the final
maturation phase, infarct myofibroblasts deposit a network of collagen-based extracellular matrix, resulting in formation of a scar. Repair of the infarct is accompanied by extensive geometric and structural changes of the ventricle, termed LV remodeling. Adverse remodeling of the infarcted ventricle ultimately leads to dysfunction and is an important predictor of mortality.

The mouse model offers an opportunity to study in detail the pathophysiology of myocardial infarction. The genetic mouse models of obesity and insulin resistance (ob/ob) or diabetes (db/db) are commonly used for obesity-related studies, including myocardial infarction (20, 26, 29). Although ob/ob and db/db are excellent mouse models for studies of some aspects of obesity-related physiology, mutations in the leptin gene (ob/ob) or its receptor (db/db) leading to overt obesity are rarely described in humans. In fact, much of the obesity epidemic is a result of energy imbalance caused by consumption of a high-calorie and high-fat diet combined with a sedentary lifestyle. Furthermore, the pathology in genetic models of obesity develops in combination with several other abnormalities (18). Leptin has profound effects on the immune system which is underscored by the early observation of thymus atrophy in db/db mice (17). Evidence from several studies supports a proinflammatory role for leptin (16). The inherent immune-modulating abnormalities presented by mouse models of leptin or leptin receptor deficiency make these models unattractive for studies that examine inflammatory changes related to cardiac repair after myocardial infarction in the context of obesity. Furthermore, severe hyperglycemia present in mouse models of diabetes may also adversely affect the cardiovascular system. The present study describes a murine dietary model of obesity with an intermediary phenotype in contrast to the morbid obesity displayed by ob/ob and db/db mice and examines the impact of diet-induced obesity on inflammation and cardiac repair following myocardial infarction. We hypothesized that the systemic and cardiac metabolic abnormalities associated
with obesity induced by a "western" diet would adversely influence the ability of the heart to heal effectively.

**Materials and Methods:**

All animals received humane care in compliance with the “Guide for the Care and Use of Laboratory Animals” (NIH publication 85-23, revised 1985). C57BL/6J from The Jackson Laboratory (Bar Harbour, Maine, USA) were bred inhouse. Mice (3–5 per cage) were given ad libitum water and a standard chow diet containing ~5% kcal from fat (Picolab Rodent Diet 5010, Purina Mills, Inc, St. Louis, MO) after weaning until 6–8 weeks of age. Obesity was induced by giving ad libitum access to a high-fat diet containing ~42% kcal from fat (Dyet #112734, Research Diets, Inc, New Brunswick, NJ) starting at age 6–8 weeks for 24 weeks. The lean controls continued to feed on chow diet until the time of experiment.

**Blood Chemistry:** At 32 weeks of age, a retro-orbital bleed was obtained at 10:00 am for nonfasting plasma analysis of glucose, insulin, triglycerides and nonesterified free fatty acids (NEFAs). Food was withdrawn from the cages at 5:00 pm until 10:00 am the next morning to obtain a fasting bleed. Plasma analytes were determined at The Mouse Metabolic Phenotyping Center, University of Cincinnati Medical Center. The following formula was used to calculate homeostasis model assessment for insulin resistance (HOMA-IR): [fasting insulin (pM) × fasting glucose (mM)]/135 (37).

**Lipid Extraction and Triglyceride Determination:** Lipids were extracted with chloroform, methanol and 0.9% sodium chloride as described by Folch (21). One hundred milligrams of liver
or skeletal muscle was used for lipid extraction. Triglyceride content was assayed as described previously (8). For myocardial triglycerides, the hearts were excised, rinsed in cardioplegic solution and blotted onto paper towels. The chambers were flushed with cardioplegic solution to remove any blood by cannulating the aorta with a blunt-ended 22-gauge needle and inserting a PE-50 catheter into the atrium. The entire heart was homogenized and the lipids were extracted in the chloroform layer, which was dried under a nitrogen stream and the residue dissolved in 2-propanol. Triglycerides were measured with a commercially available kit from Sigma Diagnostics Inc. For thin-layer chromatography (TLC), 15 µl of the sample was spotted on silica gel Whatman TLC plates. A two-step TLC method was used to resolve polar and nonpolar lipids by developing the plate first in chloroform:methanol:water (65:25:4) and later in hexane:diethylether:acetic acid (75:35:1) (11). Lipids were visualized by spraying the plate with the fluorescent dye primuline and imaging on a Phosphoimager (Storm 860, Molecular Dynamics, CA) (54).

**Noninvasive Cardiac Indices:** Noninvasive cardiac Doppler measurements were made on mice anesthetized with isoflurane (1% in 100% oxygen) administered at a continuous flow rate (20 ml/min). Doppler signals were obtained from the aortic root and the mitral inflow track using a 10-MHz probe (51). Doppler signals from each mouse were acquired and stored using a Doppler signal processing workstation (Indus Instruments, Houston, TX). Noninvasive cardiac indices of systolic and diastolic function were obtained offline from stored signals.

**Noninvasive Blood Pressure Measurement:** The noninvasive method of measuring blood pressure in mice using tail pressure cuff (or tail-cuff) and Doppler sphygmomanometry is
described elsewhere (44). Briefly, the mouse was anesthetized with 1.5% isoflurane and placed supine on an ECG/heater board. The board temperature was adjusted to maintain the body temperature of the mouse at 37±1°C. A tail-cuff was placed close to the base of the tail and a 20-MHz pulsed Doppler cuff probe was placed immediately distal to the tail-cuff. The tail-cuff was pressurized to suprasystolic levels to occlude the tail artery completely and then released gradually. The cuff pressure at which the tail blood flow reappeared was recorded as systolic pressure and the pressure at which the tail blood flow became continuous was recorded as diastolic pressure. The tail blood flow velocity signal sensed by the Doppler probe and the tail-cuff pressure signal sensed by pressure sensor (Meritrans MER100, Merit Medical Systems, Inc., South Jordan, UT) were acquired by a Doppler signal processing workstation (Indus Instruments, Houston, TX) and stored for offline analysis.

**Murine Myocardial Infarction Protocol:** Male and female lean and diet-induced obese (DIO) mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (10 µg/gm). A closed-chest mouse model of reperfused myocardial infarction was used as previously described (13, 41), to avoid the confounding effects of surgical trauma and inflammation, which may influence the baseline levels of chemokines. The left anterior descending (LAD) coronary artery was occluded for 1 hour then reperfused for 1, 3 and 7 days (n=8 for each group). At the end of the experiment, mice were sacrificed with an overdose of sodium pentobarbital and the hearts were either frozen at –80°C for RNA extraction or fixed in Z-fix and embedded in paraffin for histological studies. Sham-operated animals were implanted with a suture around the LAD but did not undergo coronary artery occlusion and reperfusion.
Perfusion Fixation and Quantitative Morphometry: For assessment of cardiac remodeling postinfarction, sham-operated and 7 day–reperfused hearts were perfusion fixed as described by Michael et al. (36). Briefly, a cardioplegic solution was perfused through the jugular vein to promote relaxation, the heart was excised and rinsed in cold cardioplegic solution, the aorta was cannulated and a PE-50 catheter was pushed through the mitral valve into the LV and secured in place. Hearts were fixed for 10 min by aortic perfusion of 10% zinc-buffered formalin at a constant pressure of ~16 cm H$_2$O. The entire heart was cross-sectioned from base to apex at 250-µm intervals. Ten serial sections of 5 µm were obtained for each interval. LV dimensions were assessed in terms of LV mass and end diastolic volume by quantitative morphometry with ImagePro software by methods described in detail by Michael et al. (36). Scar size was expressed as percentage of LV volume.

Quantitative Histology and Immunohistochemistry: After identifying the area below the suture, serial sections were stained with picrosirius red to identify collagen fibers (22). The picrosirius-stained slides were scanned using a Zeiss Axioskop microscope and quantitative evaluation was performed using ImagePro software (23). Percent collagen staining was assessed in infarcts as well as the peri-infarct area after 7 days of perfusion.

Immunohistochemistry with specific anti-mouse antibody was performed to identify the following cell types: neutrophils (rat anti-mouse neutrophil antibody, Serotec, Oxford, UK), macrophages (rat anti-mouse Mac-2 antibody, Cederlane Laboratories, Ltd., Burlington, NC) and myofibroblasts (monoclonal α–smooth muscle actin antibody, Sigma, St. Louis, MO). Staining was performed using a peroxidase-based technique with the Vectastain Elite rat kit (Vector laboratories, Burlingame, CA). Sections were counterstained with eosin and quantitative
analysis was performed by counting the density of labeled cells in the infarcted area after 3 days of reperfusion. Macrophage, neutrophil and myofibroblast density was expressed as cells/mm².

RNA Extraction, RNase Protection Assay and Quantitative Real-Time PCR: RNA was isolated from hearts using Trizol reagent according to the manufacturer’s instructions. mRNA expression levels of chemokines macrophage inflammatory protein–1α (MIP-1α), MIP-1β, MIP-2, monocyte chemoattractant protein–1 (MCP-1) and interferon-γ–inducible protein–10 (IP-10); and the cytokines tumor necrosis factor–α (TNF-α), interleukin-1β (IL-1β), IL-6, IL-10, macrophage colony–stimulating factor (M-CSF), transforming growth factor–β1 (TGF-β1), TGF-β2, TGF-β3 and osteopontin were determined by using RNase protection assay (RPA; RiboQuant, Pharmingen) according to the manufacturer’s protocol. Phosphorimaging was performed on a Storm 860 (Molecular Dynamics) and the signals were quantified using Image QuaNT software. Expression was normalized to the ribosomal protein L32 mRNA levels.

For real-time PCR studies, total RNA was treated with DNase to remove any genomic contamination as described by the manufacturer (DNA-free; Ambion, Inc.). First-strand cDNA was synthesized using SuperScript II reverse transcriptase and random hexamer primers as described in the manufacturer’s protocol (Invitrogen Corp.). Relative standard curve method was used to measure the expression levels of hypoxia-inducible factor–1α (HIF-1α) using murine TaqMan primers and probes from Applied Biosystems (Foster City, California) on an ABI Prism 7000 Sequence Detection System. Target gene expression was normalized to an internal control, cyclophilin B, which was measured using SYBR Green chemistry and published primer sequences (53).
Statistical Analysis: Statistical differences between groups were analyzed by the unpaired Student’s *t*-test and differences between multiple groups were assessed by ANOVA with Bonferroni’s correction. Data are expressed as mean±SEM. Kaplan–Meier survival statistics were performed with SPSS software. A value of *p*≤0.05 was considered significant.

Results:

Physical and Metabolic Features of DIO Mice: Table 1 is a summary of the physical and metabolic characteristics of lean and DIO male and female mice. Both male and female DIO mice were significantly heavier than their lean counterparts after being fed a high-fat diet for 24 weeks, with an average weight gain of about 1.6- to 1.7-fold. Diet-induced obesity also induced hepatic steatosis in both male and female mice; obese male mice (4.22±0.80 gm) had markedly larger fatty livers than obese females (1.75±1.0 gm, *p*<0.05). This was largely due to the accumulation of neutral lipids in the liver (data not shown). On the other hand, the average fatpad mass in obese female mice (4.41±0.46 gm) was significantly greater than that of obese male mice (2.46±0.09 gm, *p*<0.01). We noted that the heart weights of obese male and female mice were modestly but significantly higher than respective lean controls (p<0.01). The fasting triglyceride levels in the plasma of obese male and female mice were significantly elevated compared with respective controls. In the nonfasted state, both male and female DIO mice had significantly (p<0.05) higher circulating NEFAs than respective lean controls; however, the fasting NEFA levels were not significantly different between the groups.

To monitor hemodynamic changes resulting from obesity, we measured cardiac indices and arterial blood pressure noninvasively in these mice and found no significant differences between lean and obese mice in early peak flow velocity (80.4±3.4 vs. 74.6±0.9 cm/s), atrial
peak flow velocity (52.6±2.5 vs. 55.5±7.0 cm/s), and peak aortic flow velocity (98.7±0.9 vs. 100.0±5.5 cm/s). Also, no significant differences were observed in either the systolic blood pressure (males: 93.0±4.8 vs. 99.0±4.0 mm Hg; females: 89.0±1.8 vs. 85±5.9 mm Hg) or the diastolic blood pressure (males: 75.0±4.8 vs. 78.0±5.8 mm Hg; females: 72.0±5.8 vs. 72.0±4.3 mm Hg) measured by tail-cuff/Doppler sphygmomanometry.

To determine whether diet-induced obesity resulted in development of insulin resistance, we measured plasma insulin and glucose levels after an overnight fast. Both male and female DIO mice exhibited hyperinsulinemia compared with their respective lean controls; the severity of hyperinsulinemia was more pronounced in obese male mice (Fig 1a). In addition, fasting glucose levels were significantly elevated in obese male mice (168.4±10.3 mg/dl) compared with lean controls (126.2±13.3 mg/dl) (Fig 1b). Determination of HOMA-IR indicated that male DIO mice were severely insulin resistant (p=0.001) (Fig 1c).

**Cardiac Triglyceride Levels in Lean and DIO Mice:** Insulin resistance is frequently associated with lipid deposition in nonadipose tissue. As noted earlier, obese mice had significantly greater fatty livers compared with lean mice; we also found lipid accumulation in the skeletal muscle of obese mice (data not shown). We extracted total lipids from the hearts of male lean and obese mice to determine changes in the composition of cardiac lipids. As shown by TLC in Fig 2a, compared with lean mice, obese mice had increased cardiac triglyceride deposition. Biochemical analysis indicated cardiac triglyceride levels in obese male mice were 34% higher than lean controls (p=0.02) (Fig 2b).
**Myocardial Infarction in Lean and DIO Mice:** An established myocardial infarction model of closed-chest ischemia–reperfusion injury was employed to study the myocardial inflammatory and healing response in DIO mice. Fifty-nine DIO animals underwent either occlusion–reperfusion procedure or a sham surgery and 40 survived until sacrifice, representing a survival rate of 67.8%. In the lean group, 63 animals were subjected to either occlusion–reperfusion procedure or a sham surgery and 50 animals survived the procedure, representing a 79.3% survival rate; the difference in survival rate between the two groups was not significant. Mice were subjected to 1 hour of coronary artery occlusion and 24 hours, 72 hours and 7 days of reperfusion for studies on inflammatory and healing response. The mean scar size determined by planimetry (percentage of LV wall mass) in the lean infarcted group (n=11) was 4.2±1.4%, while in the obese group (n=9) it was 9.5±3.6% (p=0.133).

**Chemokine and Cytokine Expression following Myocardial Infarction:** We examined the chemokine mRNA expression profile in sham-operated and infarcted animals at 24 hours of reperfusion by RPA. In sham-operated lean and obese animals, the baseline expression of MIP-1α, MIP-1β, MIP-2 and MCP-1 was minimal and similar. All the chemokines were induced significantly higher in the infarcted animals than in the sham-operated animals; however, the magnitude of induction of MIP-1α, MIP-1β, MIP-2, MCP-1 and IP-10 was significantly higher in the infarcted hearts of obese compared with lean mice (Fig 3).

We examined the expression of IL-1β, IL-6, TNF-α, IL-10, osteopontin, and TGF-β isoforms at 24 and 72 hours of reperfusion. In sham-operated lean and obese animals, we did not detect any difference in the baseline expression levels of the cytokines investigated. The mRNA levels of some but not all cytokines were altered in the infarcted hearts of obese compared with lean...
mice. Increased expression of IL-6, IL-10, osteopontin, TGF-β1 and TGF-β3 was noted in the infarcted hearts of obese mice at 24 and 72 hours of reperfusion (Fig 4). Although relative expression of TGF-β2 was very low in the heart, it was also significantly increased in the infarcted hearts of obese compared with lean mice at both the time points investigated (p<0.05, data not shown). Expression of IL-1β and TNF-α was similar in the infarcted hearts of lean and obese mice (data not shown).

Furthermore, we determined the mRNA expression of HIF-1α, a transcription factor regulating angiogenesis, in the infarcted hearts of lean and obese mice at 24 hours of reperfusion by real-time PCR. The baseline expression of HIF-1α was not significantly different between lean and obese sham-operated animals. At 24 hours of reperfusion, HIF-1α was significantly upregulated in lean infarcted animals (p<0.05) compared with lean sham-operated controls, while obese infarcted animals exhibited a trend toward increased HIF-1α expression compared with obese sham-operated controls (p=0.16). The levels were not significantly different between lean and obese infarcted animals (data not shown).

**Inflammatory Infiltrate in Granulation Tissue:** Immunohistochemistry was used to monitor macrophages and neutrophils in the infarcts of lean and obese mice at 72 hours of reperfusion (Figs 5a and b). Macrophage density was significantly increased in obese compared with lean mice (p=0.036), while neutrophil density in the obese mice was reduced in comparison with lean mice (p=0.056). The density of α–smooth muscle actin–positive fibroblasts, myofibroblasts, was similar in the infarcts of lean and obese mice (205±32 vs 229±29 myofibroblasts/mm²).
**Cardiac Hypertrophy:** The data for LV wall mass and interventricular septal mass are presented in Figs 6a and b. Sham-operated obese animals (59.03±1.8 mg) had significantly higher LV wall mass compared with lean sham-operated controls (46.09±0.75 mg, p=0.001). Also, the interventricular septal mass was increased in obese sham animals (18.85±0.54 mg) compared with lean sham-operated controls (14.98±0.8 mg, p<0.002). At 7 days of reperfusion, the LV wall mass in the obese infarcted group was significantly increased (73.4±4.6) compared with obese sham-operated controls (59.03±1.8 mg, p=0.015). In the lean infarcted group, the change in wall mass was marginal and not significant (51.43±2.35 vs 47.4±2.05 mg in lean sham-operated animals). In addition, there was a similar trend toward increase in the interventricular septal mass in the obese infarcted group compared with obese sham-operated controls (21.7±1.3 vs 18.9±0.54, p=0.067), while no such trend was observed in the lean infarcted versus lean sham group.

**Collagen Accumulation within the Scar:** We assessed collagen deposition in the infarct as well as the peri-infarct area in lean and obese mice at 7 days of reperfusion. The mean collagen content of the remote septum in lean and obese mice was not significantly different (4.88% vs 5.09%, p=NS). Also, the amount of fibrosis in the peri-infarct area in the infarcted hearts of lean (24.28%) and obese (24.47%) mice was similar; however, we noted that the scar in lean mice was densely packed with replacement collagen fibers whereas obese mice had significantly reduced replacement collagen in the scar (Fig 7). The mean percent collagen in the scar of lean and obese mice was 46.6% and 26.7%, respectively (p<0.05).
Left-Ventricular End-Diastolic Volume: For quantitative assessment of ventricular dimensions, the hearts were fixed in end-diastole. **Fig 8** is a graphic representation of the data. We did not observe any significant difference in the end-diastolic volume of the LV in lean and DIO mice in the absence of infarction. After 7 days of reperfusion, hearts of obese infarcted mice (43.22±4.1 mm, n=8) had a significantly larger end-diastolic volume compared with sham-operated obese animals (31.20±1.0, n=7, p=0.023). In the lean group, the increase was not significant.

Discussion:
A relationship has been established for BMI and the risk for development of heart failure in the Framingham Heart Study (30). Obesity-related disorders are linked to the undesirable outcomes of ischemic heart diseases, though the mechanisms responsible are poorly defined. A major limitation of the genetic models of obesity, *ob/ob* and *db/db* mice, for studying inflammation and subsequent remodeling postinfarction is the altered immune system of these animals that may potentially influence the remodeling process in the setting of myocardial ischemia–reperfusion injury. In light of the increased clinical importance of obesity for the development of cardiovascular diseases in humans and the critical need to study the cardiac effects of obesity in an animal with an intact immune system, we report here the characterization of a murine model of diet-induced obesity that we have used to evaluate the effects of diet-induced obesity on inflammation and healing of the myocardium postinfarction. We show that DIO mice developed hyperinsulinemia and insulin resistance. Importantly, we show here for the first time that diet-induced obesity in mice was associated with ectopic cardiac triglyceride deposition along with concentric hypertrophy. Following a myocardial infarction, DIO mice exhibited an enhanced and
prolonged inflammatory reaction that was associated with adverse remodeling as evidenced by reduced collagen deposition in the scar and greater increases in both LV mass and LV end-diastolic volume.

C57BL/6J mice fed a high-fat (21% milk fat), high-sucrose diet for 6 months had increased body weight and fatpad mass; the average weight gain of DIO mice in our study was ~68%. In contrast, at 8 months of age the average body weight of ob/ob mice is ~2.3 times that of wild-type controls (data not shown), making it an extreme model of morbid obesity. The diet-induced model in the mouse thus presents a closer approximation of the severity of obesity commonly observed in humans with myocardial infarction. In atherothrombotic patients in an international registry, 26.6% were obese (BMI ≥30 kg/m²) and 3.6% were morbidly obese (BMI ≥40 kg/m²); respective prevalences in North America were 36.5% and 5.8%, which were statistically significantly higher than other regions (4). As in the genetic models of more severe obesity, DIO mice had increased liver mass indicative of hepatic steatosis. Diet-induced obesity was accompanied with hyperinsulinemia in both male and female mice. Moreover, HOMA-IR indicated gender differences, with male obese mice developing severe insulin resistance compared with female obese mice. A recent report by Park et al. examined the temporal patterns of insulin action and glucose metabolism after feeding a high fat-diet containing 55% fat by kcal and found that insulin-stimulated cardiac glucose metabolism was reduced by as early as 1.5 weeks of high-fat feeding along with blunted AKT-mediated insulin signaling and GLUT4 levels. Long-term (20 weeks) feeding was associated with LV systolic dysfunction and mild hyperglycemia, hyperleptinemia and reduced circulating adiponectin levels (43). The diet used in our study had 42% kcal from fat and is sometimes referred to as a "western" diet, as these values are closer to a high-fat diet in humans in North America and Europe.
Insulin resistance and diabetes are frequently associated with lipid deposition in tissues such as liver and skeletal muscle (34, 47, 52). Lipotoxicity, defined as tissue dysfunction induced due to lipid deposition in nonadipose tissues, is widely believed to be one of the causal factors in the development of insulin resistance. Furthermore, in animal models of diabetes and obesity, perturbations in cardiac fatty acid metabolism lead to ectopic cardiac lipid accumulation. We hypothesized that insulin resistance and elevated circulating NEFAs in diet-induced obesity would also result in lipid accumulation in the heart. Indeed, we show here for the first time that in male DIO mice that are severely insulin resistant, cardiac triglyceride content is significantly elevated. Because the heart relies on fatty acid metabolism to a large extent for its much-needed energy requirements, the balance between uptake and utilization of fatty acids is tightly regulated. Our findings have important implications in diet-induced obese hearts, given that lipid species are potentially capable of generating toxic metabolites that may be harmful to cardiomyocytes compromising cardiac function, especially after an injury. It was demonstrated by Zhou et al. (56) in obese Zucker diabetic fatty (ZDF) rats that cardiac lipid accumulation was associated with contractile dysfunction. To delineate the contributions of global metabolic defects from those of cardiac lipid accumulation in the development of cardiomyopathies, several investigators in recent years have created transgenic mice in which fatty acid uptake is driven in excess of cardiac fatty acid utilization. Cardiac-specific overexpression of acyl-CoA synthetase–1 (10), peroxisome proliferator-activated receptor–α (PPAR-α) or glycosylphosphatidylinositol-anchored lipoprotein lipase each leads to lipid accumulation in the myocardium that is associated with systolic ventricular dysfunction. Transgenic mice with cardiac-specific overexpression of fatty acid transport protein–1 (FATP-1) also import free fatty acids in excess of their capacity to use them and exhibit diastolic ventricular dysfunction (9, 10,
19, 55). Whether intramyocardial lipid deposition is a feature of human heart failure is not entirely clear. Estimates of myocardial lipid by magnetic resonance spectroscopy suggest that obese individuals (BMI >30 kg/m²) with evidence of impaired contractile function have abnormally high levels of triglycerides in the heart (50). Lipid deposition as determined by Oil Red O staining was detected in sections of the LV free wall in a group of obese (BMI >30 kg/m²) diabetic patients with nonischemic heart disease (46).

Long-standing obesity in humans is associated with eccentric hypertrophy and diastolic dysfunctions (2, 3). However, a few studies also report concentric remodeling of the heart in obese subjects in the absence of comorbid conditions such as hypertension. Accordingly, we noted cardiac hypertrophy in DIO mice. Increased wall and septal mass were indicative of concentric hypertrophy in DIO mice. The wet heart weight of both male and female mice was marginally but significantly higher than that of lean controls. To assess whether the mice were hypertensive, an identified factor in inducing cardiac hypertrophy, we measured the tail-cuff blood pressure in these mice and found no significant difference between the groups. Furthermore, the cardiac output was not altered.

In the Framingham study, it was found that the risk for heart failure increased by 5% in men and 7% in women for each 1-kg/m² increment in BMI (30). Loss of functional myocardium subsequent to an acute myocardial infarction is a frequent cause of cardiac remodeling and chronic heart failure. We used a model of reperfused myocardial infarction to examine the inflammatory reaction and LV remodeling in DIO mice. Myocardial repair after a myocardial infarction is a dynamic process, involving an inflammatory process, clearing of necrotic tissue and replacement collagen formation ultimately leading to a healed scar.
Myocardial remodeling is dependent on the initial acute inflammatory reaction that ensues upon reperfusion of the infarcted myocardium and is associated with upregulation of chemokines, cytokines, adhesion molecules and growth factors (24). In a time course study of reperfused infarcted myocardium in wild-type C57BL/6J mice, we have shown that the chemokine response peaks at 3–6 hours after reperfusion and is rapidly downregulated after 24–72 hours of reperfusion (13). At 24 hours of reperfusion, we found that the expression of chemokines in the infarcts of lean mice was almost down to that of sham levels, whereas obese mice displayed an inappropriate response in terms of elevated and prolonged expression of chemokines. The profile of cytokine expression in infarcted hearts of obese mice was also altered; we noted sustained and prolonged expression of IL-6, an inflammatory cytokine, and IL-10, an anti-inflammatory cytokine. Many of the inflammatory cytokines contain nuclear factor-κB (NF-κB) response elements. Ischemia–reperfusion strongly induces NF-κB, thereby upregulating κB-response genes such as IL-6, IL-1β and TNF-α (6, 7, 24, 35). HIP-1α, a transcription factor, is acutely activated during ischemia–reperfusion in animal models and is a regulator of angiogenesis. There is evidence now that HIF-1α also regulates expression of chemokines indirectly via heme oxygenase, which is expressed under hypoxic condition (42). In addition, it has also been reported that cytokines such as TNF-α activate HIF-1α under normoxic conditions (45). The alteration in the expression of chemokines in lean and obese infarcted mice in our study is not entirely explicable by HIF-1α expression, since the mRNA expression of HIF-1α was not significantly different between lean and obese animals at 24 hours of reperfusion. Osteopontin, a multifactorial protein associated with remodeling and matrix organization and a marker of monocyte-to-macrophage differentiation was significantly upregulated in the infarcts of obese mice (25, 32, 33). Recruited inflammatory cells such as macrophages and mast cells are
documented to elaborate TGF-β isoforms, a factor crucial for containing the inflammation as well as an important regulator of collagen deposition (15, 38). We observed increased expression of all three TGF-β isoforms in the infarcts of obese mice. We expected the sustained and prolonged chemokine and cytokine expression in obese mice to influence the leukocyte infiltrate. Indeed, at 72 hours of reperfusion, macrophage density was significantly higher in the infarcts of obese compared with those of lean mice. And although neutrophil chemoattractants MIP-1α and MIP-2 were elevated in the infarcts of obese mice, to our surprise, at 72 hours of reperfusion, obese mice exhibited a trend toward reduced neutrophil density. In contrast to our observation, in a model of obesity and diabetes (db/db), Jones et al. observed increased leukocyte infiltration at 2 hours of reperfusion following a 30-min coronary artery occlusion compared with wild-type animals, leading to greater myocardial necrosis (29). Enhanced phagocytosis of neutrophils by macrophages may possibly be a reason for the reduced neutrophil density we have noted in our study.

Obesity is associated with altered production of proinflammatory cytokines by adipose tissue; these cytokines have been implicated in the metabolic complications of obesity. In addition to triglyceride deposition in the heart, DIO mice in our study had increased levels of free fatty acids, specifically saturated fatty acids and arachidonic acid (data not shown), which are proapoptotic and proinflammatory. Elevated levels of fatty acids are not only causally related to development of insulin resistance but also closely linked to inflammatory pathways by way of activation κB-responsive genes (1, 28).

Macrophages constitute an important component of the inflammatory infiltrate. They are not only responsible for clearing the neutrophils to limit the expansion of the infarct, but also secrete growth factors such as TGF-β1 that regulate the activity of fibroblasts. Phenotypically modified
fibroblasts, myofibroblasts play a central role in progression of fibrosis based on their ability to produce procollagens (12, 49). Collagen deposition within the scar is critical for maintaining the tensile strength of the myocardium and preventing rupture (48). The density of myofibroblasts was not significantly different in the infarcts of lean and obese mice at 72 hours of reperfusion. However, at 7 days of reperfusion, obese mice had significantly reduced replacement collagen in the scar, which may contribute to adverse LV remodeling. Indeed, we noted that the obese mice exhibited adverse remodeling at 7 days of reperfusion. Although the scar size tended to be larger in the obese group, the difference was not statistically significant (p=0.133). We found that obese mice responded with enhanced hypertrophic remodeling to myocardial infarction with reperfusion. At the same time, the LV chamber size was significantly increased in infarcted hearts of obese compared with those of lean mice. In a recent study, Greer et al. report increased short-term mortality following coronary artery occlusion and reperfusion in obese diabetic \( db/db \) mice compared with wild-type controls (26). In addition, they observed significant adverse remodeling in terms of LV dilation, cardiac hypertrophy and contractile dysfunction in these genetically obese and diabetic mice. The authors did not examine infarct size in their study.

In summary, we show that diet-induced obesity induced insulin resistance with significant cardiac lipid deposition and concentric cardiac hypertrophy. Following a myocardial injury, DIO mice exhibited an altered inflammatory response and healing process. The adverse remodeling observed in obese mice in response to myocardial infarction and reperfusion may be detrimental to the heart, leading to heart failure.
Acknowledgements:

This work was supported by National Institutes of Health (NIH) grants P01 HL-42550 (CMB, MLE and NGF), R01 HL-76246 (NGF) and American Diabetes Association grant 1-04-RA-03 (CMB). GDT was supported by NIH training grant T32 HL-007812. We are thankful to Jennifer Pocius, Roy Hendley, Ugochi Uzoka and Christa Unsinn for performing the animal surgeries. We are thankful to Dr. Henry Pownall for useful discussions. Editorial assistance from Kerrie Jara is acknowledged.
References:


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40. Nigam A, Wright RS, Allison TG, Williams BA, Kopecky SL, Reeder GS, Murphy JG, and Jaffe AS. Excess weight at time of presentation of myocardial infarction is associated with lower initial mortality risks but higher long-term risks including recurrent re-infarction and cardiac death. *Int J Cardiol*, 2005.


**Figure Legends:**

**Figure 1:** Diet-induced obese mice were insulin resistant. (A) Overnight fasting plasma insulin and (B) glucose levels in male and female lean and obese mice. Obese male mice had significantly elevated glucose and insulin levels compared with lean controls, and insulin levels in obese female mice were significantly elevated compared with lean controls. (C) HOMA-IR, calculated by a formula described in material and methods, indicated that male obese mice were severely insulin resistant. $\approx P<0.01$ for obese vs lean males, *$p<0.05$ for obese vs respective lean controls.

**Figure 2:** Cardiac triglyceride accumulation in diet-induced obese mice. (A) Thin-layer chromatography of lipids extracted from the hearts of lean and diet-induced obese mice. The following mix of standard lipids was used to identify the individual lipid species in the extracts: CE, cholesteryl ester; TG, triglycerides; FA, fatty acids; Chol, cholesterol; PE, phosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; SM, sphingomyelin; Lyso-PC, lysophosphatidylcholine. (B) Triglycerides in the cardiac extracts were measured using a kit from Sigma Chemical Co. The values were normalized to heart weight.

**Figure 3:** Diet-induced obese mice had increased expression of chemokines after ischemia–reperfusion. RPA demonstrated significantly elevated mRNA expression of the CC chemokines MIP-1\(\alpha\), MIP-1\(\beta\) and MCP-1 and the CXC chemokines MIP-2 and IP-10 in the infarcted hearts of obese compared with lean mice at 24 hours of reperfusion. Expression values were normalized to the housekeeping gene L32. $\approx P<0.01$ for obese vs lean, *$p<0.05$ for obese vs lean (n=8 in each infarcted group, n=3 in each sham group).
**Figure 4:** DIO mice had increased and prolonged cytokine expression after myocardial infarction at 24 and 72 hours of reperfusion. IL-6 was significantly elevated in the infarcted hearts of obese mice at 24 hours of reperfusion, while IL-10, osteopontin and TGF-β3 were markedly elevated at 72 hours of reperfusion compared with lean mice. TGF-β1 was upregulated substantially at both 24 and 72 hours of reperfusion. ∞P<0.05 for obese vs lean at 24 hours, *p<0.05 for obese vs lean at 72 hours (n=8 in each infarcted group, n=3 in each sham group).

**Figure 5:** Leukocyte infiltration in the infarcts of lean and obese mice at 72 hours following myocardial infarction. (A) Macrophages and (B) neutrophils were evaluated by immunohistochemistry in the infarcted region of the left ventricle. Infarcts of obese mice (n=6) had significantly increased number of macrophages compared with lean mice (n=6, p=0.036), while the neutrophil density tended to be lower in the infarcts of obese compared with lean mice (p=0.56).

**Figure 6:** Obese mice exhibited concentric hypertrophy and responded with enhanced hypertrophic remodeling to myocardial infarction with reperfusion. (A) Left ventricular and (B) interventricular septal mass was determined by quantitative morphometry on H&E-stained sections of perfusion fixed hearts. Left ventricular myocardial mass was derived from the muscle tissue density of 1.065 mg/µl. The left ventricular wall mass and septal mass were increased in obese sham animals (n=8) compared with lean sham animals (n=6). Following infarction, at 7 days a significant increase in wall mass (A) was noted in obese (n=9) compared with sham.
controls, but not in the lean group (n=11). *p<0.05 for obese sham vs lean sham, \( \propto P<0.001 \) for obese infarcted vs obese sham.

**Figure 7:** Obese mice had reduced collagen deposition in the scar after a myocardial infarction. (A) Collagen was detected by picrosirius staining and quantitated in the scar of lean (n=13) and obese (n=9) mice after 7 days of reperfusion. Obese mice had significantly reduced collagen in the scar compared with lean mice (p=0.035). (B) A representative image of the scar in lean and obese mice depicting collagen deposition.

**Figure 8:** Obese mice exhibited increased remodeling following myocardial infarction with reperfusion. Hearts of sham-operated and infarcted lean and obese mice were perfusion fixed after 7 days of reperfusion in diastole to measure the left ventricular chamber size. The left ventricular chamber size was significantly increased (p=0.02) in the infarcted hearts of obese mice (n=8) compared with hearts of obese sham animals (n=7), while the increase was not significant in the lean group.
### Table 1. Physical and Metabolic Variables of Lean and Obese Mice.

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lean</td>
<td>Obese</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>27.45±1.26</td>
<td>46.41±2.42*</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>1.35±0.06</td>
<td>4.22±0.80*</td>
</tr>
<tr>
<td>Heart weight, g</td>
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<td>0.15±0.006*</td>
</tr>
<tr>
<td>Fatpad weight, g</td>
<td>1.79±0.14</td>
<td>2.46±0.09*</td>
</tr>
<tr>
<td>TG, mg/dL (nonfasting)</td>
<td>73.01±4.8</td>
<td>61.99±2.1</td>
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<tr>
<td>TG, mg/dL (fasting)</td>
<td>51.13±3.05</td>
<td>67.19±2.52*</td>
</tr>
<tr>
<td>NEFA, mM (nonfasting)</td>
<td>0.75±0.01</td>
<td>0.92±0.04†</td>
</tr>
<tr>
<td>NEFA, mM (fasting)</td>
<td>0.41±0.07</td>
<td>0.50±0.04</td>
</tr>
</tbody>
</table>

*p<0.01, †p<0.05; n>5 per group for body, liver, heart and fatpad weights and fasting triglycerides (TG) and nonesterified fatty acids (NEFA); n=3 per group for nonfasting TG and NEFA.
Figure 2:

A

B

CE

TG

FA

Chol

PE

PA

PC

SM

Lyso-PC

Normal Diet

High Fat Diet

mg/gm wet weight

p=0.02

Lean

Obese

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Figure 3
Figure 4

IL-6

Sham
24h
72h

IL-10

Sham
24h
72h

Osteopontin

Sham
24h
72h

TGF-β1

Sham
24h
72h

TGF-β3

Sham
24h
72h
Figure 5

- For macrophages/mm² (left graph), there is a significant difference between lean and obese groups with a p-value of 0.036.
- For neutrophils/mm² (right graph), while there is a trend, the difference is not statistically significant with a p-value of 0.056.
Figure 6

A

LV wall mass, mg

B

septal mass, mg

Lean sham  Lean I/R  Obese sham  Obese I/R

Lean sham  Lean I/R  Obese sham  Obese I/R

* denotes statistical significance.
Figure 7

A

% collagen

Lean

Obese

\[ p = 0.035 \]

B

Lean scar

Obese scar

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Figure 8.