Transition From Obesity to Metabolic Syndrome Is Associated With Altered Myocardial Autophagy and Apoptosis

Zi-Lun Li, John R. Woollard, Behzad Ebrahimi, John A. Crane, Kyra L. Jordan, Amir Lerman, Shen-Ming Wang, Lilach O. Lerman

Objective—Transition from obesity to metabolic-syndrome (MetS) promotes cardiovascular diseases, but the underlying cardiac pathophysiological mechanisms are incompletely understood. We tested the hypothesis that development of insulin resistance and MetS is associated with impaired myocardial cellular turnover.

Methods and Results—MetS-prone Ossabaw pigs were randomized to 10 weeks of standard chow (lean) or to 10 (obese) or 14 (MetS) weeks of atherogenic diet (n=6 each). Cardiac structure, function, and myocardial oxygenation were assessed by multidetector computed-tomography and Blood Oxygen Level-Dependent–MRI, the microcirculation with microcomputed-tomography, and injury mechanisms by immunoblotting and histology. Both obese and MetS showed obesity and dyslipidemia, whereas only MetS showed insulin resistance. Cardiac output and myocardial perfusion increased only in MetS, yet Blood Oxygen Level-Dependent–MRI showed hypoxia. Inflammation, oxidative stress, mitochondrial dysfunction, and fibrosis also increased in both obese and MetS, but more pronouncedly in MetS. Furthermore, autophagy in MetS was decreased and accompanied by marked apoptosis.

Conclusion—Development of insulin resistance characterizing a transition from obesity to MetS is associated with progressive changes of myocardial autophagy, apoptosis, inflammation, mitochondrial dysfunction, and fibrosis. Restoring myocardial cellular turnover may represent a novel therapeutic target for preserving myocardial structure and function in obesity and MetS. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: obesity ■ autophagy ■ cardiac function ■ inflammation ■ metabolic-syndrome

Obesity has reached epidemic proportions and risen to 34% among adults in the United States.1 Obese is related to increased prevalence of type 2 diabetes, with excess adiposity being a critical contributor to the development of insulin resistance (IR).2 The incidence of metabolic syndrome (MetS), a cluster of cardiovascular risk factors with obesity and IR as major components, increases with the severity of obesity and reaches 50% in severely obese youngsters.3 MetS is associated with increased cardiovascular morbidity and mortality,4 partially related to left ventricular (LV) diastolic dysfunction.5

One of the mechanisms by which development of IR in obese subjects might exacerbate cardiac injury is inflammation, which macrophages that infiltrate adipose tissue in obesity play a pivotal role in obesity and IR.6 Furthermore, myocardial inflammation, evidenced by increases in cardiac macrophages and cytokines in obesity, suppresses myocardial glucose metabolism7 and increases fibrosis in obesity-induced MetS.8

Normal myocardial cellular turnover depends on adequate disposal of damaged proteins and organelles (autophagy) or cells (apoptosis). Autophagy, a critical catabolic process through which damaged cytoplasmic components are degraded and recycled, controls cellular contents’ quality and homeostasis.9 Increasing evidence indicates that cardiovascular diseases might be associated with maladaptation of autophagy.10,11 Although autophagic activation is a cellular response to starvation, autophagy is also impaired in hypercholesterolemia12 and obesity,13 which may lead to accumulation of damaged mitochondrial14 in muscle and defective insulin signaling in the liver.15 Conversely, myocardial autophagy is activated in nonobese mice with fructose-induced IR.16 Yet, whether myocardial autophagic activity is altered by the microenvironment characterizing obesity and IR remains largely unknown.

Therefore, this study aimed to test the hypothesis that transition from obesity to MetS, as reflected by development of IR, is characterized by impaired myocardial autophagy, in association
with inflammation and fibrosis. We studied both in vivo and ex vivo hearts of Ossabaw pigs, a unique large animal model that progressively gains components\textsuperscript{17} mimicking human MetS.

Methods

The study was approved by the Institutional Animal Care and Use Committee. Littermate 3-month-old Ossabaw pigs (Swine Resource, Indiana University) started a 10-week standard chow (lean), or 10 (obese) or 14 (MetS) weeks of atherogenic diet (SB4L; Purina Test Diet, Richmond, Indiana)\textsuperscript{18} (n = 6 each group). Blood and urine samples under fasting conditions were subsequently collected, and the pigs studied with MRI (for myocardial oxygenation) followed by multidetector computed-tomography (for cardiac structure, function, and myocardial perfusion) 2 days later. Three days following the completion of in vivo studies, pigs were euthanized with pentobarbital-sodium (100 mg/kg IV, Sleepaway\textsuperscript{8}, Pr Dodge Laboratories, Fort Dodge, Iowa), hearts were removed, immediately shock-frozen in liquid nitrogen, and stored at −80°C, preserved in formalin or prepared for micro-CT studies. Myocardial autophagy, apoptosis, oxidative stress, inflammation, lipid accumulation, microvascular (MV) architecture, and fibrosis were then determined.

Systemic Measurements

Blood pressure and heart rate were recorded with an intraarterial catheter during the multidetector computed-tomography study. Rate-pressure product, an index of myocardial oxygen consumption, was calculated by heart rate x systolic blood pressure x 10\textsuperscript{-7}. Levels of plasma renin activity, endothelin-1, tumor necrosis factor-\alpha (TNF-\alpha), and monocyte-chemoattractant-protein (MCP)-1 were tested as previously described,\textsuperscript{19,20} and total cholesterol, triglycerides (TG), LDL, and HDL by standard procedures. Intravenous glucose-tolerance test was performed before CT scanning,\textsuperscript{17} and the homeostasis model assessment insulin resistance index (fasting plasma glucose x fasting plasma insulin/22.5) was used as an index of IR.\textsuperscript{19} Systemic oxidative stress was evaluated by plasma levels of oxidized LDL (Ox-LDL, Alpco Diagnostics, Windham, NH) and 8-isoprostanes (EIA, Cayman Chemical, Ann Arbor, MI).\textsuperscript{18}

In Vivo Studies

For each in vivo study animals were weighed, induced with IM Telazol (5 mg/kg) and xylazine (2 mg/kg), intubated, and ventilated with room air.

Imaging

Blood Oxygen Level-Dependent-MRI was used to acquire myocardial oxygenation data (Blood Oxygen Level-Dependent index, R\textsubscript{0}), subsequently analyzed using MATLAB 7.10 (MathWorks, Natick, MA), as we have shown.\textsuperscript{21} Multidetector computed-tomography studies performed 2 days after MRI evaluated cardiac structure and function, as shown previously.\textsuperscript{22} LV muscle mass, systolic function, early and late LV filling rates were calculated, as well as myocardial perfusion and MV permeability index before and during adenosine infusion, as we described.\textsuperscript{20,22,23} Intra-abdominal adipose tissue measured using multidetector computed-tomography was expressed as volume and fraction.\textsuperscript{18,19} For detail, see the online-only Data Supplement.

In Vitro Studies

Myocardial hypoxia was evaluated by Western blotting of hypoxia-inducible factor-1\alpha and angiogenic activity by vascular endothelial growth factor (VEGF) and its receptor Fk-1. Myocardial redox status was evaluated by in situ production of superoxide anion, detected by dihydroethidium (Sigma, 20 \textmu M/MLL), and by protein expression of the P47, P67, and GP91 subunits of NAD(P)H oxidase, and superoxide dismutase-1. Inflammation was investigated by staining for macrophage markers CD163 and CD8, and by protein expression of the activated T-lymphocyte marker CD134, interleukin-6, MCP-1, its receptor C-C motif receptor-2, and TNF-\alpha. Mitochondrial biogenesis and energy metabolism were assessed by protein expression of sirtuin-1, peroxisome proliferator-activated receptor-\gamma-coactivator 1-\alpha (PGC-1\alpha), ATP synthase mitochondrial-F1–complex-assembly–factor-1, uncoupling protein-2, and creatine transporter-1. Fibrosis was studied by Sirius-red and trichrome staining, as well as protein expression of matrix-metalloproteinase (MMP)-2, tissue inhibitor of metalloproteinase-1, plasminogen-activator inhibitor-1 and transforming growth-factor-B1. Autophagy was assessed by protein expression of unc-51-like kinase-1, Beclin-1, autophagy-related gene (Atg)-12, microtubule-associated protein-1 light-chain (LC)-3, phospho-AMP-activated protein kinase (p-AMPK), and mammalian target of rapamycin (mTOR). Apoptosis was evaluated by Terminal deoxynucleotidyl transferase dUTP nick end labeling (green) and cleaved (active) caspase-3 (red) double-staining, as well as expression of CCAAT/enhancer binding protein homologous protein and phosphorylated signal-transducer and activator of transcription (STAT)-3. For Western blotting, GAPDH was used as loading control, except for pro-MMP–2 and total STAT-3 for corresponding proteins. LC3 conversion was evaluated by LC3 II-to-LC3 I ratio. LV triglyceride levels were quantified as recently described.\textsuperscript{18} For detail, see the online-only Data Supplement.

Micro-CT

The left-anterior–descending coronary artery was perfused with a radio-opaque polymer, and a transmural portion of the LV myocardium then scanned as previously described.\textsuperscript{22,24,25} Microvessels were classified using Analyse\textsuperscript{TM} as small (diameters 20–200 \textmu m), medium (201–300 \textmu m), or large (301–500 \textmu m). For detail, see the online-only Data Supplement.

Histology

Staining performed on 5-\textmu m-thick myocardial cross-sections was semiautomatically quantified by a computer-aided image analysis program (MetaMorph\textsuperscript{\textregistered}, Molecular Devices, Sunnyvale, CA).\textsuperscript{22} Staining in 10 random fields was quantified and expressed as fraction of surface area or of total cells stained with DAPI.

Effects of Obesity and IR on Myocardial Tissue Pathomechanisms in Domestic Pigs

To further confirm that development of IR rather than duration of obesity altered myocardial autophagy and apoptosis, 3 additional groups of domestic farm pigs were studied: normal (standard chow, n = 6), obese domestic (high-fat diet, n = 6),\textsuperscript{19} and IR domestic (IR-D) pigs (atherogenic diet, n = 2), all for 12 weeks.\textsuperscript{18} These models (on a different background than the MetS-prone Ossabaw model) were achieved using different diets that we have found to induce obesity\textsuperscript{19} in domestic pigs, and the same atherogenic diet used for the Ossabaw model, which led to mild but significant IR in the domestic swine. After 12 weeks of diet, representative in vivo and in vitro studies were performed.

Statistical Analysis

Continuous data are expressed as mean±SEM. Comparisons within groups were performed using the paired Student t test and among groups using ANOVA and unpaired t test with Bonferroni correction. P ≤ 0.05 was considered statistically significant.

Results

Systemic Characteristics

Body weight and visceral fat accumulation were greater in obese Ossabaw pigs than lean, and greater yet in MetS (Table). Compared with lean pigs, total cholesterol, LDL, HDL, and LDL/HDL ratio were similarly elevated in both obese and MetS pigs, whereas serum triglycerides tended to be higher (P = 0.09) in MetS than lean pigs. Blood pressure, heart rate, rate-pressure product (Table), plasma renin activity, endothelin-1, TNF-\alpha, and MCP-1 (data not shown) remained unchanged. Basal insulin levels tended to increase in obese compared with lean pigs (P = 0.06) but were signif-
Cardiac Hemodynamics and Function

The relatively low heart rate observed might have been attributable to the effect of xylazine anesthesia. LV muscle mass increased in obese pigs and tended to increase compared with lean pigs (P<0.07), and their homeostasis model assessment insulin resistance were elevated compared with both groups (Figure 1A). Compared with lean, 8-epi-isoprostane increased in both obese and MetS pigs, whereas ox-LDL increased only in MetS.

<table>
<thead>
<tr>
<th>Cardiac characteristic</th>
<th>Lean (n=6)</th>
<th>Obese (n=6)</th>
<th>MetS (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (bpm)</td>
<td>63.6±8.3</td>
<td>73.3±10.2</td>
<td>74±9.2</td>
</tr>
<tr>
<td>Rate-pressure product (mm Hg·bpm)</td>
<td>99.0±10.8</td>
<td>102.8±13.5</td>
<td>100.2±13.3</td>
</tr>
<tr>
<td>Stroke volume (ml)</td>
<td>261.2±2.0</td>
<td>289±1.5</td>
<td>33.7±3.3</td>
</tr>
<tr>
<td>Cardiac output (L/min)</td>
<td>1.7±0.3</td>
<td>2.1±0.3</td>
<td>2.7±0.3</td>
</tr>
<tr>
<td>Cardiac index (L/min/m²)</td>
<td>4.1±0.6</td>
<td>3.8±0.6</td>
<td>3.8±0.3</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>57.1±4.6</td>
<td>60.0±3.4</td>
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</tbody>
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Cardiac Adiposity

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Table. Characteristics (Mean±SEM) of Lean, Obese, and Metabolic Syndrome (MetS) Pigs

<table>
<thead>
<tr>
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<th>MetS (n=6)</th>
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<tbody>
<tr>
<td>Body weight (kg)</td>
<td>16.6±2.1</td>
<td>24.1±2.2*</td>
<td>33.6±1.1†</td>
</tr>
<tr>
<td>Intra-abdominal fat (%)</td>
<td>9.6±2.0</td>
<td>20.0±1.7*</td>
<td>32.3±3.4†</td>
</tr>
<tr>
<td>Cholesterol (mg/dl): Total</td>
<td>79.8±6.5</td>
<td>364.8±90.6*</td>
<td>300.2±61.8*</td>
</tr>
<tr>
<td>Low-density lipoprotein</td>
<td>36.0±3.0</td>
<td>258.0±75.1*</td>
<td>193.7±34.7*</td>
</tr>
<tr>
<td>High-density lipoprotein</td>
<td>39.4±4.6</td>
<td>100.8±25.2*</td>
<td>97.8±29.6*</td>
</tr>
<tr>
<td>LDL/HDL</td>
<td>1.0±0.1</td>
<td>2.5±0.6*</td>
<td>2.5±0.5*</td>
</tr>
<tr>
<td>Plasma triglycerides (mg/dl)</td>
<td>22.0±5.7</td>
<td>29.8±8.2</td>
<td>43.4±13.7</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>0.1±0.05</td>
<td>0.4±0.1</td>
<td>0.9±0.2†</td>
</tr>
<tr>
<td>Oxidized low-density lipoprotein (ng/ml)</td>
<td>319.5±41.2</td>
<td>620.7±237.8</td>
<td>709.3±199.4*</td>
</tr>
<tr>
<td>8-epi-Isoprostane (pg/ml)</td>
<td>175.8±27.0</td>
<td>406.4±33.2*</td>
<td>551.0±124.2*</td>
</tr>
<tr>
<td>E/A</td>
<td>1.2±0.09</td>
<td>2.0±0.2*</td>
<td>2.3±0.3*</td>
</tr>
<tr>
<td>Mean arterial pressure (mm Hg)</td>
<td>115.7±10.1</td>
<td>108.0±11.1</td>
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Myocardial Oxygenation

Basal myocardial R₂* values were higher in MetS compared with lean and obese pigs (Figure 1B–1D), suggesting myocardial hypoxia. R₂* values strongly tended to decrease (possibly due to substantial variation) in lean pigs in response to adenosine (P=0.06) compared with lean pigs and was significantly blunted in MetS (Table). Contrarily, the permeability index in MetS significantly increased in response to adenosine, suggesting impaired myocardial microvascular integrity.

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increased (Figure 1K–1M). Myocardial tissue triglyceride levels were also higher in obese and MetS (Figure 1S).

**Microcirculation**

The density of subendocardial microvessels (20–200 μm) tended to increase \( (P=0.08) \) in obese hearts and increased significantly in MetS (Figure 2A-D), whereas subepicardial MV density was unchanged (data not shown). MV tortuosity, an index of angiogenesis and vascular immaturity, increased in both obese and MetS pigs (Figure 2E). Interestingly, protein expression of VEGF increased in obese but declined to normal levels in MetS pigs. Conversely, protein expression of FLK-1 was upregulated only in MetS pigs (Figure 2F–2G), possibly as a compensatory mechanism.

**Inflammation and Oxidative Stress**

Infiltration of CD163+ macrophage and CD8+ T-cells increased only in MetS compared to lean and obese, as was the expression of CD134 (Figure 3). Myocardial expression of interleukin-6 increased in obese and further enhanced in MetS, whereas receptor C-C motif receptor-2 and TNF-α increased only in MetS and MCP-1 remained unaltered. Superoxide anion production and superoxide dismutase-1 expression increased in both the obese and MetS groups (Figure 3), whereas myocardial expression of the NAD(P)H oxidase subunits was magnified in MetS compared to obese pigs (Figure 3).

**Mitochondrial Function and Energy Metabolism**

Myocardial expression of SIRT1 and ATP synthase mitochondrial-F1–complex-assembly–factor-1 decreased in MetS compared with both lean and obese pigs. PGC-1α similarly decreased in obese and MetS pigs, whereas uncoupling protein decreased in obese and further decreased in MetS. Creatine transporter-1 expression increased in MetS compared with lean and obese pigs (Figure 4). Collectively,
these suggest impaired mitochondrial function and energy metabolism.

Myocardial Fibrosis
The level of total collagen (Sirius red staining) was elevated in obese and further increased in MetS pigs (Figure 4). Trichrome staining showed that fibrosis tended to increase \( (P=0.06) \) in obesity, and increased markedly in MetS compared with lean pigs. Protein expression of plasminogen-activator inhibitor-1 and MMP-2 was also upregulated in MetS compared with obese pigs, whereas transforming growth-factor \(-\beta\) and tissue inhibitor of metalloproteinase-1 were unchanged (Figure 4).

Autophagy and Apoptosis
Protein expression of unc-51–like kinase-1 was lower in MetS compared with obese pigs. Conjugated Atg12-Atg5 was elevated in both obese and MetS, whereas decreases in Beclin-1 and LC3 conversion did not reach statistical significance in obese pigs \( (P=0.06 \text{ and } 0.08, \text{ respectively}) \) but were pronounced in MetS. Expression of mTOR increased markedly in MetS compared with lean pigs (Figure 5), whereas p-AMPK remained unchanged. Apoptotic activity assessed by deoxyribonucleotidyl transferase dUTP nick end labeling and caspase-3 staining has not reached statistical significance levels in obese pigs \( (P=0.06 \text{ and } 0.09, \text{ respectively}) \) but was pronounced in MetS. This was accompanied by elevated protein expression of CCAAT/enhancer binding protein homologous protein in MetS and decreased phospho-STAT–3 in both groups (Figure 5).

Obesity and IR in Domestic Swine
Obese-D and IR-D pigs had similarly elevated body weight, whereas IR only developed in IR-D pigs (Table II in the online-only Data Supplement). IR-D pigs showed greater hyperlipidemia compared with obese pigs, but their intra-abdominal fat was not increased compared with normal pigs and was lower than in obese pigs. Plasma triglycerides in IR-D pigs were lower than normal and tended to be lower than in obese domestic pigs \( (P=0.07) \). In vitro studies showed that obese domestic and IR-D pigs had similar inflammation and oxidative stress (receptor C-C motif receptor-2 and P47, respectively). IR-D pigs showed increased fibrotic activity reflected by increased expression of active MMP2 and presented mitochondrial dysfunction (decreased PGC-1α and uncoupling protein-2 expression). Obese-D pigs showed depressed autophagy (increased mTOR), which was exacerbated in IR pigs, as demonstrated by an additional tendency \( (P=0.088) \) of Atg12-Atg5 to decline. Further, Obese-D pigs showed slightly increased apoptosis, which was magnified in IR-D, as shown by deoxyribonucleotidyl transferase dUTP nick end labeling and cleaved caspase-3 staining (Figure I in the online-only Data Supplement). Taken together, development of IR exacerbated myocardial autophagy and apoptosis in domestic pigs with IR.

Discussion
This study shows that the early phase of visceral obesity is characterized by myocardial adiposity, accompanied by diastolic dysfunction, mild inflammation, oxidative stress, and mitochondrial dysfunction. Subsequent development of IR magnifies MV proliferation and dysfunction, and aggravates myocardial inflammation, oxidative stress, mitochondrial dysfunction, and fibrosis. Furthermore, we demonstrated in several different swine models that IR might suppress autophagy and trigger apoptosis, suggesting attenuated defense mechanisms leading to cell death.

Obesity has become pandemic that causes devastating health problems, with excess fat accumulation being a major contributor to the development of IR and a transition to MetS. However, the structural and functional myocardial alterations that characterize this transition are poorly understood. In this study we took advantage of a unique MetS-prone swine model and state-of-the-art imaging technologies to reveal underlying pathomechanisms of myocardial injury in obesity and its transition to MetS. After 10 weeks of atherogenic diet, the Ossabaw pigs developed central obesity and pericardial fat. Extending the atherogenic diet for another 4 weeks invoked marked IR, expanding the cluster of risk factors typical for MetS, including obesity, dyslipidemia, and IR.
Importantly, IR is considered a critical factor linking visceral adiposity to cardiovascular risk. Although TNF-α and MCP-1 have been implicated in obesity-induced IR, the unchanged levels that we observed argue against a major role of these inflammatory factors in our swine model. Conversely, an increase in systemic oxidative stress in obese pigs that was exacerbated in MetS paralleled the development of IR, consistent with accumulating evidence implicating oxidative stress in contributing to development of IR. In some models, appearance of metabolic and cardiovascular components tracks together during development of MetS, whereas in our model obesity preceded IR, and MetS developed as obesity worsened.

The risk imposed by obesity for increased cardiovascular morbidity may involve several mechanisms. Excess adipose tissue with high metabolic activity increases total blood volume and cardiac output, which may eventually lead to LV dilation, increased wall stress, compensatory hypertrophy, and diastolic dysfunction. Thus, augmented systolic function in MetS pigs might be attributed to their higher body weights. An E/A ratio ≥2 in obese and MetS pigs may imply LV diastolic dysfunction of restrictive filling pattern, as observed in obese rats. Accumulation of pericardial fat pads may increase ventricular stiffness and restrict filling, and excessive cardiomyocytes lipid deposits (cardiac steatosis) may cause contractile dysfunction. The similar cardiac diastolic dysfunction in obesity and MetS was accompanied by comparable myocardial fat accumulation, although the increased end-diastolic volume in MetS may suggest greater cardiac dilation. The association between lipids and myocardial dysfunction is likely mediated by toxic fatty-acid intermediates, which increase oxidative stress and apoptosis. Pertinently, development of IR instigates a vicious cycle of increased cardiomyocytes fatty-acid uptake, oxidative-stress, and triglycerides deposit.

Impaired myocardial metabolism is associated with diastolic dysfunction in diabetes. Indeed, we observed in obese pigs impaired mitochondrial function and energy metabolism, which was exacerbated in MetS. Accordingly, SIRT1 is downregulated in MetS, particularly in IR compared with insulin-sensitive subjects. We found lower level of SIRT1 in MetS but not in obese pigs, indicating a link between IR and depressed SIRT1. Importantly, SIRT1 activates PGC-1α, a master regulator of mitochondrial biogenesis and energy metabolism. Downregulation of SIRT1 and PGC-1α suggests compromised mitochondrial biogenesis in MetS pigs. Of note, PGC-1α was also inhibited in obese pigs. Reduction of PGC-1α is associated with a conversion from fatty acid oxidation to glycolytic metabolism under stress, possibly constituting a compensatory mechanism in obesity, which IR impairs in MetS. Furthermore, PGC-1α-deficient hearts show decreased levels of ATP and phosphocreatine. Indeed, decreased activity of ATP synthase (downregulated ATP synthase mitochondrial-F1–complex-assembly–factor-1) was only found in MetS pigs, likely implying decreased formation of ATP, whereas creatine transporter-1 upregulation in MetS was possibly compensatory to insufficient phosphocreatine, given a feedback-loop of creatine transporter-1. Thus, decreased myocardial oxygenation detected by Blood Oxygen Level-Dependent–MRI after development of IR might be secondary to impaired glucose utilization, as oxygen consumption remained unchanged. Nonetheless, decreased hemoglobin saturation may contribute to lower resting R*, as does elevated capillary volume (increasing deoxygenated blood availability). Furthermore, macrophages and cytokines like interleukin-6 detected in MetS may blunt myocardial glucose metabolism. This is in agreement with evidence from Framingham Offspring Study that MetS requires IR to increase risk for cardiovascular disease.
Figure 4. Mitochondrial biogenesis, energy metabolism, and fibrosis. Myocardial protein expression of sirtuin 1 (SIRT1), peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α), ATP synthase mitochondrial F1 complex assembly factor 1 (ATPAF1), uncoupling protein-2 (UCP2), and creatine transporter 1 (CT1) and their quantification (A, B). Sirius red (C–E) and Trichrome (F–H) staining and quantifications (J, K); myocardial protein expression of the fibrogenic factors (I) plasminogen-activator-inhibitor (PAI)-1, tissue-inhibitor of metalloproteinase (TIMP)-1, transforming growth-factor (TGF)-β1, and matrix metalloproteinase (MMP)-2, and their quantifications (L). P<0.05 vs lean, †P<0.05 vs obese. Black arrows indicate collagen in Sirius red and fiber in Trichrome staining, respectively. N=6 for staining, N=5 for Western blot.
Increased myocardial fibrosis and stiffness observed in obese and MetS pigs may also contribute to LV diastolic dysfunction, as collagen and fibrosis determine tissue compliance, which is inversely associated with LV diastolic function. Although cardiac TGF-β expression was not different among the groups, plasminogen-activator inhibitor-1 was upregulated in MetS, likely secondary to IR. The increased expression of MMP-2 also implicates abnormal matrix turnover in the fibrogenesis.

We found enhanced myocardial angiogenesis in obese and MetS pigs, as observed in kidneys of obese pigs and rats. Indeed, hypercholesterolemia per se induces myocardial neovascularization in domestic pigs, associated with myocardial ischemia, inflammation, and oxidative stress. Notably, MV proliferation was elevated in MetS, likely because of their increased systolic function. However, newly formed vessels may not function properly because of their impaired integrity, suggested by enhanced permeability but blunted perfusion response to adenosine in MetS. Furthermore, permeable vessels permit extravasation of inflammatory cells releasing inflammatory mediators, which were all increased in MetS. Notably, myocardial expression of VEGF initially increased in obese pigs but subsequently fell in MetS, yet microvascular density showed no difference between them, likely due to increased expression in MetS of the FLK-1 receptor, which mediates the angiogenic effects of VEGF.

Our study shows for the first time that in Ossabaw swine excessive nutrition progressively inhibits cardiac autophagic activity, which might be at least partially responsible for myocardial injury. The transition from obesity to MetS in our study prominently involved attenuated autophagy, which may contribute to development of IR. Although cardiac conjugated Atg12-Atg5 expression increased in obese and MetS, as also found in defective hepatic autophagy in obesity, unc-51–like kinase-1, Beclin-1, and LC3 conversion all decreased in MetS, suggesting inhibited formation of nascent and mature autophagosomes. Furthermore, upregulation of the autophagy inhibitor mTOR in MetS underscores impairment in autophagy as IR develops. Indeed, SIRT1 can activate autophagy by inhibition of mTOR, and its down-regulation, linked to IR, may contribute to inhibition of autophagy in MetS. Chronic lipid load and cytokines may blunt autophagy, in line with the myocardial adiposity in obese and MetS pigs and exacerbated inflammation in MetS.

Importantly, inhibition of autophagy may lead to accumulation of damaged cellular components that eventuates in cell death. Impaired mitochondrial biogenesis evidenced by decreased PGC-1α was observed in both obese and MetS. Further, decreased autophagy in MetS compromises autophagic clearance of damaged mitochondria, resulting in their accumulation and thereby apoptosis. Indeed, we found marked apoptosis in the MetS myocardium, accompanied by increased CCAAT/enhancer binding protein homologous protein and decreased p-STAT3 expression. In turn, apoptosis...
of hepatocytes and adipocytes has been linked to development of IR. Notably, increased apoptosis was observed in early diabetic cardiomyopathy, in which diastolic dysfunction preceded systolic dysfunction. As apoptosis leads to systolic dysfunction, altered survival signaling may be partially responsible for the transition from diastolic to systolic dysfunction in diabetic cardiomyopathy. Indeed, marked myocardial apoptosis was observed after 12 weeks of established diabetes with coexistent impaired LV diastolic and systolic function. Conceivably, apoptosis may represent a mechanism underlying the progression from diastolic dysfunction at early MetS, as suggested in our study, to systolic dysfunction at later stage. Importantly, our observations made in several different pig models underscore the important role of IR underlying this pathophysiology. Thus, suppressed autophagy and increased apoptosis may be critical elements in the cascade of cardiac injury in MetS.

Limitations
The present study design did not afford establishing a causal relationship among the functional and structural variables in obesity and MetS, which are rather complex biological systems. Indeed, the clinically relevant swine models of diet-induced obesity and MetS successfully mimicked the complexity of obesity-induced metabolic disorders, and thus allowed us to correlate the dynamic pathophysiological changes in the heart in vivo and ex vivo. In addition, autophagy flux is difficult to measure in vivo, especially in large swine models. We also cannot rule out the possibility that some of the exacerbated cardiac injury in Ossabaw MetS is secondary to more severe or prolonged obesity rather than IR. However, the observation that similar mechanisms were activated in domestic pig models with selectively-induced IR and comparable duration of obesity supports the link between IR, attenuated autophagy, and magnified apoptosis. Hence, our study enabled demonstration of progression of myocardial insult due to obesity and development of IR and exploring their incremental effects on heart.

In summary, our study demonstrated pathophysiological changes in the heart of several different swine models of experimental obesity and MetS both in vivo and in vitro. Transition from obesity to MetS and development of IR are associated with impairment of myocardial autophagy and progressive increase in inflammation, mitochondrial dysfunction, apoptosis, and fibrosis. Maintaining the balance of myocardial organellar and cellular turnover and reducing inflammation may represent novel therapeutic targets for preserving myocardial structure and function in obesity and MetS.

Acknowledgments
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Disclosures
None.

References


Transition From Obesity to Metabolic Syndrome Is Associated With Altered Myocardial Autophagy and Apoptosis
Zi-Lun Li, John R. Woollard, Behzad Ebrahimi, John A. Crane, Kyra L. Jordan, Amir Lerman, Shen-Ming Wang and Lilach O. Lerman

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Supplemental Material

Supplemental Methods, Figures, Figure Legends and Tables

Supplemental Methods

Magnetic resonance imaging (MRI)

Under 1-2% isoflurane anesthesia, pigs were positioned in the MRI scanner (Signa EXCITE 3T system, GE, Waukesha, WI) and Blood Oxygen Level Dependent (BOLD) images acquired before and after intravenous injection of adenosine (400 mg/kg/min) through the ear vein catheter.

BOLD images (4-5 axial-oblique) were acquired along the cardiac short axis during suspended respiration. Gated Fast Gradient Echo (FGRE) sequence was used with TR/TE/number of echoes/Matrix size/FOV/Slice thickness/Flip angle= 6.8 ms/1.6-4.8 ms/8/128×128/35/0.5 cm/30°.

The BOLD index, R₂*, was estimated in each voxel by fitting the MR signal intensity vs. echo times to a single exponential function and calculating the MR intensity decay rate. A region of interest in the septum, which is least influenced by air-induced artifacts, was traced in each slice on T₂*-weighted images obtained.

MDCT

64-slice MDCT (Somatom Definition-64, Siemens Medical Solution, Forchheim, Germany) was performed 2 days after MRI to evaluate cardiac structure and function, as shown previously.¹ Briefly, two parallel 6-mm-thick mid-left-ventricle (LV) levels were selected for evaluation of microvascular perfusion and function. A bolus injection of nonionic, low osmolar contrast medium (Isovue-370, 0.33 ml/kg over 2 sec) into the right atrium was followed by a 50-s flow study during respiratory suspension. Fifteen minutes later, this was repeated during a 5-min intravenous infusion of adenosine (400 μg/kg/min). Subsequently, the entire LV was
scanned 20 times throughout the cardiac cycle to obtain cardiac systolic and diastolic functions and LV muscle mass.

Images were analyzed with Analyze™ (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN), as reported previously. For LVMM, the end-diastolic LV endocardial and epicardial borders were traced at each tomographic level to sum the products of myocardial volume and specific gravity. LV cavity was also traced at end-diastole and systole to calculate stroke volume, cardiac output, and LV ejection fraction. Cardiac index was calculated by cardiac output normalized for swine body-surface-area. Early (E) and late (A) LV filling rates were calculated from the positive slopes of the curve, describing the change in LV cavity volume during the cardiac cycle. Myocardial perfusion and microvascular permeability index before and during adenosine infusion were calculated from time-attenuation curves obtained from the anterior cardiac wall ROI, as we previously described.

**Fat measurement**

For pericardial fat volume, regions of interests were traced around the heart on the MDCT-derived cross-sections, and expanded proportionally in 3-dimensions within the chest wall, bordered by the descending thoracic aorta and thoracic musculature. The pericardial fat was then measured based on the attenuation range for fat and expressed as ratio to the entire cardiac volume.

**Companies and dilutions for antibodies**

Santa Cruz (1:200 for all): vascular endothelial growth factor (VEGF) and its receptor Flk-1, P47, P67, superoxide dismutase (SOD) -1, TNF-α, matrix metalloproteinase (MMP)-2, tissue inhibitor of metalloproteinase (TIMP)-1, and transforming growth-factor (TGF)-β1; Abcam: CD134 (1:500), interleukin (IL)-6 (1:1000), sirtuin 1 (SIRT1, 1:5000), peroxisome proliferator-activated receptor gamma coactivator 1- α (PGC-1α, 1:1000), ATP synthase mitochondrial F1
complex assembly factor 1 (ATPAF1, 1:1000), uncoupling protein-2 (UCP2, 1:500), unc-51-like kinase-1 (ULK1, 1:500), microtubule-associated protein-1 light chain (LC3) (LC3B, 1:500), and phospho-AMP-activated protein kinase (p-AMPK, 1:1000); Cell Signaling Technology: Beclin-1 (1:1000), autophagy-related gene (Atg) 12 (1:1000), mammalian target of rapamycin (mTOR, 1:1000), and phosphorylated signal-transducer and activator of transcription (STAT)-3 (1:1000); Thermo: C-C motif receptor (CCR)-2 (1:1000), CCAAT/enhancer binding protein (C/EBP) and homologous protein (CHOP, 1:100); Millipore: GP91(1:500); Serotec: CD163(1:20); Novocastra: CD8 (1:20); MyBioSource: monocyte chemoattractant protein (MCP)-1 (1:7500); Alpha Diagnostic International: creatine transporter 1 (CT1, 1:400); BD Biosciences: plasminogen-activator inhibitor (PAI)-1 (1:2500).

Micro-CT

The subepicardial and subendocardial spatial density, average diameter, and tortuosity of microvessels were classified using Analyze™ as small (diameters 20-200 µm), medium (201-300 µm), or large (301-500 µm) microvessels. Analysis was performed by an experienced imaging analyst who was blinded to the study groups. In order to minimize the influence of potential variability, the operator combined 2-D and 3-D images to determine the threshold for each sample. Furthermore, similar incremental intervals (about 20 slices) were used to sample 30 representative slices for vessels density measurement.
Supplemental Figures

Figure I.

A

<table>
<thead>
<tr>
<th>Normal</th>
<th>Obese-D</th>
<th>IR-D</th>
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<tr>
<td>CCR-2 42KD</td>
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<tr>
<td>P47 47KD</td>
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<td>TGF-β 12.5KD</td>
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<td>GAPDH 37KD</td>
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<td>pro-MMP2 72KD</td>
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<tr>
<td>Active-MMP2 63KD</td>
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B

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<tr>
<th>Normal</th>
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<tr>
<td>PGC-1α 91KD</td>
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<td>UCP2 30KD</td>
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<td>Beclin-1 60KD</td>
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<tr>
<td>Atg12-Atg5 53KD</td>
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<td>LC3-I 19KD</td>
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<td>LC3-II 17KD</td>
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<td>mTOR 289KD</td>
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<td>CHOP 28KD</td>
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<td>GAPDH 37KD</td>
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<td>p-STAT-3 88KD</td>
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<td>STAT-3 79KD</td>
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C

**Protein expression (relative to GAPDH)**

- **Normal**
- **Obese-D**
- **IR-D**

D

**Protein expression (relative to GAPDH)**

- **PGC-1α**
- **UCP2**
- **Beclin-1**
- **Atg12-Atg5**
- **LC3-I/II**
- **mTOR**
- **CHOP**

E

**Protein expression (relative to STAT-3)**

- **Normal**
- **Obese-D**
- **IR-D**

F

**Positive cells (%)**

- **TUNEL**
- **Caspase-3**

G

**TUNEL staining**

- **Normal**
- **Obese-D**
- **IR-D**

H

**Caspase-3 staining**

- **Normal**
- **Obese-D**
- **IR-D**
Figure legends

Figure I. Myocardial inflammation, oxidative stress, fibrosis, mitochondrial function, autophagy, and apoptosis in domestic normal, obese, and insulin resistance (IR) pigs. Myocardial protein expression of C-C motif receptor (CCR)-2, P47, transforming growth-factor (TGF)-β1, and matrix metalloproteinase (MMP)-2 (A), and their quantification (C). Myocardial protein expression of peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α), uncoupling protein-2 (UCP2), Beclin-1, conjugated autophagy-related gene (Atg)12-Atg5, microtubule-associated protein-1 light chain 3 (LC3), and mammalian target of rapamycin (mTOR), and apoptosis-related proteins including CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) and phosphorylated signal-transducer and activator of transcription (STAT)-3 (B), and their quantifications (D-E). Representative images of TUNEL (G-I), and Caspase-3 (J-L) staining, and their quantifications (F). IR, insulin resistance. (L); p≤0.05 vs. normal, †p≤0.05 vs. obese-D. White arrows indicate positive cells. N=6, 6, and 2 for staining and Western blot. Taken together, these suggested exacerbated inhibition of autophagy and increase in apoptosis in IR-D pigs compared to obese-D pigs.
**Supplemental Tables**

Table I. Cardiac size (mean±SEM) in lean, obese, and MetS Ossabaw pigs.

<table>
<thead>
<tr>
<th>Cardiac size</th>
<th>Lean (n=6)</th>
<th>Obese (n=6)</th>
<th>MetS (n=6)</th>
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<tbody>
<tr>
<td><strong>LV Volume (ml)</strong></td>
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<td></td>
</tr>
<tr>
<td>EDV</td>
<td>37.9±4.3</td>
<td>43.0±3.3</td>
<td>50.2±3.7*</td>
</tr>
<tr>
<td>ESV</td>
<td>13.4±1.9</td>
<td>18.3±2.3</td>
<td>17.7±2.9</td>
</tr>
<tr>
<td><strong>LA Volume (ml)</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>EDV</td>
<td>2.5±0.6</td>
<td>4.0±0.9</td>
<td>2.8±0.3</td>
</tr>
<tr>
<td>ESV</td>
<td>7.4±0.7</td>
<td>9.4±0.9</td>
<td>8.7±1.1</td>
</tr>
<tr>
<td><strong>LVMM (g)</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>EDV</td>
<td>30.6±2.0</td>
<td>36.4±2.2*</td>
<td>42.2±3.3*</td>
</tr>
<tr>
<td><strong>LV wall area (cm²)</strong></td>
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<td></td>
</tr>
<tr>
<td>EDV</td>
<td>44.8±4.9</td>
<td>44.9±2.7</td>
<td>49.3±2.6</td>
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<tr>
<td>ESV</td>
<td>58.2±7.4</td>
<td>58.6±2.2</td>
<td>59.2±3.2</td>
</tr>
</tbody>
</table>

For LV wall area (representing its thickness), comparable LV levels (defined by landmarks) were selected in all pigs during each cardiac phase, yet systole and diastole are not directly compared, as it is difficult to track and ascertain precisely the same level for both cardiac phases. *p≤0.05 vs. lean, †p≤0.05 vs. obese. MetS, metabolic syndrome; LV, left ventricle; EDV, end diastolic volume; ESV, end systolic volume; LVMM, left ventricle muscle mass.
Table II. Characteristics (mean±SEM) of normal, obese-D, and insulin resistance (IR)-D pigs.

<table>
<thead>
<tr>
<th></th>
<th>Normal (n=6)</th>
<th>Obese-D (n=6)</th>
<th>IR-D (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (kg)</strong></td>
<td>61.9±1.3</td>
<td>69.3±1.5*</td>
<td>68.0±5.0*</td>
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<tr>
<td><strong>Intra-abdominal fat (%)</strong></td>
<td>6.9±0.4</td>
<td>17.1±1.1*</td>
<td>8.2±1.5†</td>
</tr>
<tr>
<td><strong>Total cholesterol (mg/dl)</strong></td>
<td>75.8±8.0</td>
<td>126.0±25.1*</td>
<td>598.0±127*†</td>
</tr>
<tr>
<td><strong>LDL cholesterol (mg/dl)</strong></td>
<td>35.0±3.0</td>
<td>74.9±75.1</td>
<td>400.4±128.2†</td>
</tr>
<tr>
<td><strong>HDL cholesterol (mg/dl)</strong></td>
<td>39.4±4.6</td>
<td>100.8±22.0*</td>
<td>196.5±1.5*</td>
</tr>
<tr>
<td><strong>LDL/HDL</strong></td>
<td>1.2±0.6</td>
<td>1.6±0.3</td>
<td>2.0±0.7</td>
</tr>
<tr>
<td><strong>Plasma triglycerides (mg/dl)</strong></td>
<td>24.8±2.1</td>
<td>23.7±5.9</td>
<td>5.5±1.5*</td>
</tr>
<tr>
<td><strong>HOMA-IR (µU/ml×mg/dl)</strong></td>
<td>0.8±0.3</td>
<td>1.0±0.3</td>
<td>1.8±0.04*</td>
</tr>
</tbody>
</table>

*p≤0.05 vs. normal, †p≤0.05 vs. obese-D. LDL: low-density lipoprotein; HDL: high-density lipoprotein; HOMA-IR: homeostasis model assessment insulin resistance.
References


