Multiple abnormalities of myocardial insulin signaling in a porcine model of diet-induced obesity

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Lee J, Xu Y, Lu L, Bergman B, Leitner JW, Greyson C, Draznin B, Schwartz GG. Multiple abnormalities of myocardial insulin signaling in a porcine model of diet-induced obesity. Am J Physiol Heart Circ Physiol 298: H310–H319, 2010. First published November 6, 2009; doi:10.1152/ajpheart.00359.2009.—Heightened cardiovascular risk among patients with systemic insulin resistance is not fully explained by the extent of atherosclerosis. It is unknown whether myocardial insulin resistance accompanies systemic insulin resistance and contributes to increased cardiovascular risk. This study utilized a porcine model of diet-induced obesity to determine if myocardial insulin resistance develops in parallel with systemic insulin resistance and investigated potential mechanisms for such changes. Micropigs (n = 16) were assigned to control (low fat, no added sugars) or intervention (25% wt/wt coconut oil and 20% high-fructose corn syrup) diet for 7 mo. Intervention diet resulted in obesity, hypertension, and dyslipidemia. Systemic insulin resistance was manifest by elevated fasting glucose and insulin, abnormal response to intravenous glucose tolerance testing, and blunted skeletal muscle phosphatidylinositol-3-kinase (PI 3-kinase) activation and protein kinase B (Akt) phosphorylation in response to insulin. In myocardium, insulin-stimulated glucose uptake, PI 3-kinase activation, and Akt phosphorylation were also blunted in the intervention diet group. These findings were explained by increased myocardial content of p85α (regulatory subunit of PI 3-kinase), diminished association of PI 3-kinase with insulin receptor substrate (IRS)-1 in response to insulin, and increased serine-307 phosphorylation of IRS-1. Thus, in a porcine model of diet-induced obesity that recapitulates many characteristics of insulin-resistant patients, myocardial insulin resistance develops along with systemic insulin resistance and is associated with multiple abnormalities of insulin signaling.

INSULIN RESISTANCE IS AN INCREASINGLY common condition in modern societies and usually underlies the clinical “metabolic syndrome” (10). Data from the most recent National Health and Nutrition Examination Survey identified hyperinsulinemia suggestive of insulin resistance among 35% of nondiabetic U.S. adults during the period 1999–2002 compared with 25% during the period 1988–1994 (31). These statistics are sobering because insulin resistance and metabolic syndrome are associated with increased cardiovascular risk, even in the absence of diabetes. Among nondiabetic patients with established coronary heart disease, a majority of studies indicate that insulin resistance or metabolic syndrome is associated with poor prognosis that is not fully explained by angiographic severity of disease (43, 54, 56). While it seems plausible that adverse cardiac outcomes are mediated in part through alterations in myocardial insulin signaling, there is a paucity of data to support this hypothesis. Therefore, a critical and as yet unanswered question is whether patients with systemic insulin resistance also develop myocardial insulin resistance.

The metabolic actions of insulin are transduced primarily through a pathway that involves binding of insulin to its cell surface receptor, tyrosine phosphorylation of insulin receptor substrate (IRS)-1, activation of phosphatidylinositol-3-kinase (PI 3-kinase), and phosphorylation of protein kinase B/Akt and its downstream targets. Insulin signaling through this pathway mediates not only glucose uptake in insulin-sensitive tissues but also adaptive and protective responses to physiological stresses, including ischemia-reperfusion injury, thermal injury, and sepsis, thereby mitigating tissue injury and dysfunction (2, 13, 28, 46, 51).

In skeletal muscle of insulin-resistant humans (8, 50), and in cardiac muscle of insulin-resistant rodents (24, 36, 52), insulin-stimulated PI 3-kinase activation and Akt phosphorylation are blunted. Among the mechanisms underlying these abnormalities is increased serine phosphorylation of IRS-1, which attenuates tyrosine phosphorylation of IRS-1 in response to insulin and thereby impedes the interaction of IRS-1 with PI 3-kinase (3, 29). Another mechanism of impaired PI 3-kinase/Akt signaling identified in insulin-resistant skeletal muscle is increased expression of the p85 (regulatory) subunit of PI 3-kinase (3, 15); a surfeit of p85 leads to competition between p85 monomer and p85-p110 dimer (the entire PI 3-kinase) for the IRS-1 binding sites, thereby reducing the capacity for insulin-stimulated association of PI 3-kinase dimers with IRS-1 and blunting insulin-stimulated activation of this enzyme (27).

To date, it remains unknown whether similar abnormalities of insulin signaling occur in the myocardium of humans or large animals with diet-induced systemic insulin resistance. A well-characterized large animal model of insulin resistance could provide an important translational step between rodent models and clinical cardiac pathophysiology.

In this study, we describe a new porcine model of diet-induced obesity and systemic insulin resistance without overt diabetes that recapitulates many of the features of the clinical metabolic syndrome. Using this model, we tested the hypo-
esis that myocardial insulin resistance develops along with systemic insulin resistance and is associated with defects in PI 3-kinase/Akt signaling.

MATERIALS AND METHODS

Animal model and dietary intervention. All experiments were approved by the Institutional Animal Care and Use Committee. Sixteen Yucatan micropigs (S&S Farms, Ramona, CA) were obtained at 3 mo of age and baseline weight 18 ± 1 kg. After a 2-wk period of acclimatization to the laboratory environment, pigs were assigned to either a control diet or an intervention diet (4 castrated males and 4 females in each diet group). Diet compositions and energy provision are indicated in Table 1. Control diet was a high-fiber commercial minipig chow (LabDiet 5L80). Intervention diet used a base of standard minipig chow (LabDiet 5L80) to which coconut oil (Cargill Ultimate 76, 25% wt/wt) and high-fructose corn syrup (Cargill Isoclear 55, 20% wt/wt) were added. The intervention diet provided excess fat, simple sugars, and calories, but both diet regimens provided nearly equal quantities of protein, essential fatty acids, and minerals. No cholesterol was added to either diet.

Study procedures are schematized in Fig. 1. Body weight, fasting blood glucose, plasma insulin, and free fatty acids (FFA) were measured under sedation with ketamine (25 mg/kg im) at baseline and monthly during 6 mo of assigned diet. Glucose was measured with an autoanalyzer (YSI 2300, Yellow Springs, OH), insulin was measured using an automated sandwich enzyme-linked immunosorbent assay method (Beckman Coulter Access Ultrasensitive Insulin, Fullerton, CA), and FFA were measured by colorimetric assay (Wako Diagnostics, Richmond, VA). Serum adiponectin was measured at baseline and at 6 mo by semiquantitative Western blotting using antibodies to the porcine protein (25).

Intravenous glucose tolerance testing. Intravenous glucose tolerance testing (IVGTT) was performed in each pig at baseline and after 3 and 6 mo of assigned diet to assess serial changes in systemic insulin sensitivity. After initial sedation with ketamine (25 mg/kg im), moderate sedation was maintained by continuous infusion of ketamine (15 mg kg⁻¹ h⁻¹ iv) and midazolam (0.35 mg kg⁻¹ h⁻¹ iv). An internal jugular vein was cannulated percutaneously to withdraw blood samples. Blood glucose was monitored at 5-min intervals until concentration remained constant (±0.2 mmol/l) for 30 min. A bolus of glucose (0.3 g/kg iv) was then administered over 5 min, and blood samples were collected 2, 3, 4, 6, 8, 10, 12, 14, 16, 19, 22, 25, 30, 40, 50, 70, and 100 min from the beginning of glucose infusion for measurement of glucose and insulin concentrations. Areas under the curve (AUC) for the transient glucose and insulin responses were computed.

Instrumentation of the heart and hemodynamic measurements. At 7 mo of assigned diet, pigs were sedated with ketamine (25 mg/kg im), anesthetized with α-chloralose (100 mg/kg iv induction, 20–30 mg kg⁻¹ h⁻¹ iv maintenance), and wrapped in a recirculating warm water blanket to maintain body temperature. After endotracheal intubation, pigs were mechanically ventilated with oxygen-enriched air; ventilation was adjusted based on measurements of arterial blood gases (Radiometer ABL-30, Copenhagen, Denmark). Fluid-filled catheters were inserted in the jugular veins and a carotid artery for administration of fluids and drugs, blood sampling, and measurement of aortic pressure. The chest was opened by median sternotomy, and the heart was suspended in a pericardial cradle. Bipolar pacing electrodes were attached to the left atrial appendage to maintain a constant heart rate slightly higher than the intrinsic rate. A solid-state catheter was inserted in the left ventricle for pressure measurement. An array of four subendocardial sonomicrometry crystals was implanted in the anterior free wall of the left ventricle to measure fractional systolic area reduction in the area subtended by the crystals (i.e., the 2-dimensional equivalent to fractional segment shortening in one dimension). Hemodynamic data were digitized at 200 Hz and analyzed using custom software.

Myocardial glucose and FFA uptake. Fluid-filled catheters were inserted in the left atrium for injection of microspheres and in the anterior interventricular vein for coronary venous blood sampling. In pigs, 90% of the blood flow of the anterior interventricular vein is derived from the perfusion territory of the left anterior descending coronary artery (7). Arterial blood glucose was measured every 5 min, and an iterative computer algorithm (14) was used to adjust the rate of infusion of 20% glucose solution through a jugular vein to clamp arterial blood glucose at 4.5 mmol/l. When glucose infusion rate was stable (defined as a 30-min period with <10% maximum fluctuation), an arterial blood sample was obtained for measurement of plasma insulin, and paired arterial and anterior interventricular vein blood samples were withdrawn for measurement of blood glucose and plasma FFA concentrations (FFA). Hematocrit was used to convert plasma to blood [FFA]. Simultaneously, 3–4 × 10⁵ 15-μm polystyrene microspheres labeled with gold, samarium, lutetium, lanthanum, ytterbium, or europium (Biopal, Worcester, MA) were injected in the left atrium while a reference arterial blood sample was withdrawn from a carotid artery with a calibrated pump. An insulin infusion was begun at a rate of 1 mU kg⁻¹ min⁻¹ iv while the glucose infusion rate was adjusted to maintain the arterial blood glucose concentration at 4.5 mmol/l. After at least 45 min of insulin infusion at this rate and at least 30 min at a steady-state glucose infusion rate, a second set of paired arterial and coronary venous blood samples was obtained, and a second injection of microspheres was performed. The insulin infusion rate was then increased to 2 mU kg⁻¹ min⁻¹ and after 30 min at steady-state glucose infusion rate a third set of measurements was performed. Myocardial tissue blood flow (ml g⁻¹ min⁻¹) was calculated from neutron excitation of microspheres in tissue and

Table 1. Diet composition

<table>
<thead>
<tr>
<th></th>
<th>Control Diet</th>
<th>Intervention Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Content, % by wt</td>
<td></td>
<td></td>
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<tr>
<td>Total fat</td>
<td>4.5</td>
<td>26.9</td>
</tr>
<tr>
<td>Saturated fat</td>
<td>0.5</td>
<td>24.3</td>
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<tr>
<td>Simple sugars</td>
<td>2.2</td>
<td>16.5</td>
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<tr>
<td>Fructose</td>
<td>0.7</td>
<td>11.0</td>
</tr>
<tr>
<td>Protein</td>
<td>14.0</td>
<td>8.5</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>15.0</td>
<td>2.9</td>
</tr>
<tr>
<td>%Energy from:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td>Fat</td>
<td>9</td>
<td>45</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>71</td>
<td>47</td>
</tr>
<tr>
<td>Physiological fuel value</td>
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</tr>
<tr>
<td>Energy content of chow, kcal/g</td>
<td>2.9</td>
<td>4.4</td>
</tr>
<tr>
<td>Quantity fed, g chow·kg body wt⁻¹·day⁻¹</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td>Energy provided, kcal·kg body wt⁻¹·day⁻¹</td>
<td>87</td>
<td>220</td>
</tr>
</tbody>
</table>

Fig. 1. Schema of experimental design. BP, blood pressure; IVGTT, iv glucose tolerance test; TG, triglyceride.
reference blood samples (41). The product of tissue blood flow and the coronary arteriovenous concentration difference for glucose or FFA (μmol/ml) yielded a measurement of myocardial glucose uptake (μmol·g⁻¹·min⁻¹). The relationship between arterial plasma insulin concentration and myocardial glucose uptake at each insulin infusion rate was examined.

In vivo cardiac and skeletal muscle biopsies before and after insulin stimulation. After the completion of a hyperinsulinemic-euglycemic clamp, at least 2 h were allowed before the following procedures to assess insulin signaling in skeletal and cardiac muscle. A 3-mm transmural drill biopsy of the left ventricular free wall was rapidly frozen in liquid nitrogen (n = 7 each group). Hemostasis was achieved with a purse string suture around the biopsy site. The extensor muscles of the proximal forelimb were exposed, and an excisional biopsy was obtained and frozen in liquid nitrogen. Insulin (10 U/kg iv) was then administered. Later (10 min), a blood sample was obtained for plasma insulin concentration, and a second set of myocardial and skeletal muscle biopsies was taken. After in vivo tissue sampling, pigs were killed, and additional postmortem samples of myocardium and skeletal muscle were obtained and frozen at −80°F.

Assessment of PI 3-kinase/Akt signaling pathway in response to insulin. IRS-1-associated PI 3-kinase activity and phospho-Akt were measured in paired myocardial and skeletal muscle biopsy samples obtained in vivo before and after insulin stimulation. IRS-1-associated PI 3-kinase activity was determined after IRS-1 immunoprecipitation with rabbit anti-IRS-1 antibody (06–248; Upstate, Lake Placid, NY) and incubation with protein A-sepharose. Lipids were resolved by thin-layer chromatography in CHCl₃-CH₃OH-H₂O-NH₄OH (60:47:11:3), dried, visualized by autoradiography, and quantified using a phosphorimaging device (4). Phospho-Akt was determined in tissue homogenates by Western blotting with 7% SDS-PAGE using anti-phospho-Akt (Ser473) antibody (4058; Cell Signaling Technology, Danvers, MA) at 1:1,000 dilution. Bands were visualized with secondary horseradish peroxidase-conjugated antibody and chemiluminescence and quantified by densitometry (44).

Serine-307 phosphorylation of IRS-1 and total IRS-1 was measured by Western blotting in myocardium obtained after insulin stimulation in vivo. IRS-1 immunoprecipitates were probed with rabbit anti-phosphoserine-IRS-1 (Ser307) antibody (07–247; Cell Signaling Technology, Danvers, MA) at 1:1,000 dilution. The blot was reprobed with a monoclonal mouse anti-IRS-1 antibody (sc-8038; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1,000 dilution to detect levels of total IRS-1.

Total myocardial p85α was measured in homogenates of myocardium obtained after insulin stimulation in vivo by Western blotting using monoclonal p85α antibody at 1:1,000 dilution (05–212; Upstate). IRS-1-associated p85α was measured by Western blotting using the same antibody in IRS-1 immunoprecipitates prepared from paired myocardial samples obtained before and after insulin stimulation in vivo. IRS-1-associated p110 was measured by Western blotting using monoclonal p110α antibody at 1:1,000 dilution (611398; BD Transduction Laboratories, San Jose, CA) in IRS-1 immunoprecipitates prepared from myocardium obtained after insulin stimulation in vivo.

Measurement of myocardial lipid content. Myocardial triglyceride and diacylglyceride content and fatty acyl composition were measured as previously described (5). Under a dissecting microscope with samples on ice, extramyocardial adipose tissue was removed. Samples remained on ice for dissection for 2 min or less. With the use of 20 mg dry dissected myocardium, tripentadecanoic and dipentadecanoic acids were added as internal standards. Lipids were extracted in chloroform, isolated using solid phase extraction, and converted to fatty acid methyl esters (FAME) using sodium methoxide. FAME identities were determined by gas chromatography and mass spectrometry by comparing peak elution times and fragment species with known standards (Supelco FAME mix RM-6) and quantified by comparing abundance with internal standards.

**RESULTS**

**Weight gain.** Pigs fed intervention diet gained weight more rapidly than pigs fed control diet and became obese (Fig. 2). At 7 mo, the weight of the intervention diet group was 82% higher than the control group (73 ± 4 vs. 40 ± 4 kg, respectively, P < 0.001). Heart weight was also significantly greater in the intervention diet group (231 ± 11 g) than in the control diet group (159 ± 8 g).

**Noninvasive blood pressure, lipid, and adiponectin.** Pigs in the intervention diet group developed several characteristics commonly found in humans with “metabolic syndrome.” At baseline, noninvasive blood pressure measured under conditions of moderate sedation was equal in both groups (Table 2). However, over 6 mo of assigned diet, systolic blood pressure (P = 0.04) and pulse pressure (P = 0.01) rose to higher levels in the intervention diet group.

Effects of diet on fasting blood lipids and adiponectin are also shown in Table 2. At baseline, serum lipids were similar in both groups. However, over 6 mo of assigned diet, pigs in the intervention diet group developed higher cholesterol (P < 0.01), triglycerides (P < 0.05), and nonesterified fatty acids (P = 0.01). Serum adiponectin levels were similar at baseline but tended to be lower at 6 mo in pigs in the intervention diet group (diet-time interaction P = 0.14).

**Fasting glucose and insulin and responses during IVGTT.** Fasting glucose and insulin measurements are shown in Fig. 3, A and B. Fasting blood glucose rose in the intervention diet group at 1 mo and remained higher than in the control group throughout the observation period (P < 0.001, Fig. 3A). Similarly, fasting plasma insulin rose at 1 mo in the intervention diet group and remained slightly but significantly elevated compared with the control diet group (P < 0.05; Fig. 3B).

Data from IVGTT are shown in Fig. 3, C and D. The AUC of the transient blood glucose response to intravenous glucose load did not differ between groups or change significantly over time (Fig. 3C). The AUC of the transient insulin response to glucose load (AUCins) was similar in both groups at baseline,
but increased progressively at 3 and 6 mo in the intervention diet group. At 6 mo, AUC\textsubscript{insulin} was approximately two times as great in the intervention diet group as in the control group (Fig. 3D; \(P = 0.02\)). Thus, to dispose of a similar amount of glucose, two times the insulin response was required in the intervention group as in the control group. These findings indicate development of systemic insulin resistance in the intervention diet group.

**Hemodynamics under open-chest, anesthetized conditions.** Table 3 lists hemodynamic measurements performed at 7 mo of assigned diet during terminal experiments under general anesthesia. There were no significant differences between groups in heart rate, left ventricular pressure or peak rate of change of pressure, or fractional systolic area reduction.

**Myocardial glucose uptake, arterial [FFA], and myocardial FFA uptake as functions of plasma insulin concentration.** Figure 4A plots myocardial glucose uptake as a function of plasma insulin concentration during graded hyperinsulinemic-euglycemic clamp. Insulin infusion at the 1 m\text{U} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} dose significantly increased myocardial glucose uptake in both groups. Although further increase of insulin infusion rate to 2 m\text{U} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} caused further, significant increase in myocardial glucose uptake in the control diet group, there was no further increase in the intervention diet group, despite a higher achieved plasma insulin concentration in the latter group. These observations suggest that maximal insulin-stimulated myocardial glucose uptake was blunted in the intervention diet group.

Figure 4B plots arterial [FFA] as a function of plasma insulin concentration. As expected, hyperinsulinemia decreased arterial [FFA] (main effect of insulin concentration \(P < 0.001\) in 2-way repeated-measures ANOVA). Moreover, at any given insulin concentration, arterial [FFA] was higher in the intervention diet group than in the control diet group (main effect of diet, \(P = 0.01\)). The latter finding most likely reflects insulin resistance of adipose tissue in the intervention diet group. Similarly, Fig. 4C shows myocardial FFA uptake as a function of plasma insulin concentration. At a given plasma insulin concentration, myocardial FFA uptake was higher in the intervention diet group than the control diet group (main effect of diet, \(P = 0.05\)). Thus the data of Fig. 4, B and C, indicate greater FFA availability and myocardial uptake in the intervention diet group compared with the control diet group,

### Table 2. Blood pressure, fasting serum lipids, and adiponectin

<table>
<thead>
<tr>
<th>Diet</th>
<th>Control ((n = 8))</th>
<th>Intervention ((n = 8))</th>
<th>Interaction Diet (\times) Time</th>
<th>(P) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Months of assigned diet</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Blood pressure, mmHg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>96±9</td>
<td>102±10</td>
<td>97±9</td>
<td>123±8</td>
</tr>
<tr>
<td>Diastolic</td>
<td>58±6</td>
<td>56±5</td>
<td>58±5</td>
<td>55±6</td>
</tr>
<tr>
<td>Pulse pressure</td>
<td>38±4</td>
<td>47±6</td>
<td>39±5</td>
<td>68±7</td>
</tr>
<tr>
<td>Serum lipids, mmol/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1.8±0.1</td>
<td>1.8±0.1</td>
<td>1.7±0.1</td>
<td>2.4±0.2</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.37±0.07</td>
<td>0.27±0.05</td>
<td>0.28±0.05</td>
<td>0.43±0.06</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>0.6±0.1</td>
<td>0.4±0.1</td>
<td>0.5±0.1</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>Adiponectin, OD units</td>
<td>0.32±0.13</td>
<td>0.32±0.14</td>
<td>0.35±0.05</td>
<td>0.19±0.06</td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n\), no. of pigs. OD, optical density. Interaction \(P\) values computed by 2-way ANOVA with repeated measures. NS, not significant.

Fig. 3. Fasting blood glucose and insulin and responses during intravenous glucose tolerance testing. A and B: fasting blood glucose and insulin levels were similar at baseline in both groups but increased significantly during 6 mo assignment to intervention diet compared with control diet. C and D: area under the curve (AUC) of blood glucose and plasma insulin in response to iv glucose tolerance testing at 0, 3, and 6 mo of assigned diet. The AUC of the transient blood glucose response to intravenous glucose load (AUC\textsubscript{glucose}) did not differ between groups or change over time. However, the AUC of the transient insulin response to glucose load (AUC\textsubscript{insulin}) increased significantly over time in the intervention diet group compared with the control diet group. The greater transient insulin response required to dispose of similar transient glucose load indicates the development of systemic insulin resistance in the intervention diet group. All data are means ± SE; \(n = 8\) in each group.
which may have contributed to blunted myocardial glucose uptake in the former.

**PI 3-kinase activity and Akt phosphorylation in response to insulin in skeletal muscle.** Plasma insulin concentrations corresponding to the preinsulin biopsy specimens were $5 \pm 1 \mu U/ml$ in the control diet group and $16 \pm 3 \mu U/ml$ in the intervention diet group. After the administration of $10 \mu g$ iv insulin, plasma insulin concentrations corresponding to the postinsulin biopsy specimens exceeded $300 \mu U/ml$ in each pig in both groups. Thus the postinsulin specimens reflect maximal insulin stimulation. In the control diet group, insulin stimulation resulted in expected, significant increases in skeletal muscle PI 3-kinase activity (2.5-fold) and phospho-Akt (1.5-fold), both $P < 0.01$ (Fig. 5, A and B). In contrast, in the intervention diet group, insulin stimulation produced no significant change in either PI 3-kinase activity or phospho-Akt. The responses of skeletal muscle PI 3-kinase activity and Akt phosphorylation to insulin were both significantly influenced by diet assignment (interaction $P < 0.01$). These findings confirm that intervention diet leads to blunted insulin-mediated PI 3-kinase/Akt signaling in skeletal muscle.

**PI 3-kinase activity and akt phosphorylation in response to insulin in cardiac muscle.** Findings in cardiac muscle mirrored those in skeletal muscle (Fig. 5, C and D). Insulin produced significant increases in PI 3-kinase activity and Akt phosphorylation in the control diet group (both $P < 0.001$) but no increases in the intervention diet group. The interaction between insulin stimulation and diet was significant for both PI 3-kinase activity and phospho-Akt ($P < 0.01$). These findings confirm that intervention diet leads to blunted insulin-mediated PI 3-kinase/Akt signaling in myocardium.

**Serine-307 phosphorylation of IRS-1.** Total IRS-1 did not differ between immunoprecipitates prepared from the two groups. However, serine-307 phosphorylated IRS-1 was significantly greater in the intervention diet group compared with the control diet group ($P < 0.01$; Fig. 6), supporting this mechanism as a contributor to myocardial insulin resistance.

**Total p85α and IRS-1-associated p85α and p110.** Total p85α content in myocardial homogenates was greater in pigs fed intervention diet than control diet ($P < 0.01$; Fig. 7A). Moreover, the intervention diet group demonstrated an abnormal pattern of insulin-stimulated association of PI 3-kinase subunits with IRS-1. Before insulin stimulation, the intervention diet group demonstrated greater association of p85α with IRS-1 than the control group ($P = 0.04$; Fig. 7B). In response
to insulin stimulation, the association of p85α with IRS-1 increased as expected in the control diet group ($P = 0.03$) but did not increase in the intervention diet group (Fig. 7B). The association of p85α with IRS-1 was influenced by significant interaction between the diet group and insulin stimulation ($P = 0.03$). In addition, postinsulin samples demonstrated less association of p110 with IRS-1 in the intervention diet group than in the control diet group ($P = 0.05$; Fig. 7C).

**Myocardial lipid content.** Total myocardial triglyceride content was not significantly different in hearts of the intervention diet group ($2.6 \pm 0.6 \mu g/mg$ dry wt) compared with the control diet group ($2.1 \pm 0.4 \mu g/mg$ dry wt, $P = 0.19$). However, myocardial triglyceride composition was markedly different in the two groups. In the intervention diet group, there was greater content of saturated fatty acyl residues (12:0, 14:0, and 16:0) and lower content of polyunsaturated residues (18:2) (Fig. 8A). Similarly, the total content of diacylglycerols was not different between groups, but the diacylglycerol pool in the intervention diet group was significantly enriched in saturated (12:0, 14:0, and 16:0) residues and diminished in 18:2 residues (Fig. 8B).

**DISCUSSION**

Much of our knowledge about the effect of insulin resistance on the heart has been derived from small animal models. To date, a large animal model of insulin resistance that recapitu-
lates human pathophysiology has been unavailable. In this study, we describe a novel porcine model of diet-induced insulin resistance with many of the clinical features of metabolic syndrome, including obesity, dyslipidemia, elevated blood pressure, depressed adiponectin levels, and systemic insulin resistance as manifest by elevated fasting blood glucose and insulin and abnormal responses to IVGTT. Despite relatively mild elevation of fasting blood glucose and insulin and relatively short duration of abnormal metabolic conditions, these pigs developed skeletal muscle and myocardial insulin resistance as manifest by blunted insulin-stimulated activation of PI 3-kinase and phosphorylation of Akt.

**Defects in myocardial insulin signaling.** Increased serine phosphorylation of IRS-1 and increased expression of p85α, the regulatory subunit of PI 3-kinase, are likely responsible for the downstream abnormalities in insulin signaling and for blunted maximal insulin-stimulated myocardial glucose uptake. Serine phosphorylation of IRS-1 most likely reflects increased activity of serine kinases, including several isoforms of protein kinase C, mammalian target of rapamycin, c-Jun NH2-terminal kinase, and others (18). These serine kinases may be activated during accumulation of intramyocellular lipids (17), particularly those with saturated fatty acyl residues as observed in the present model. Sustained increases in serine phosphorylation of IRS-1 impair its ability to attract and activate PI 3-kinase, thus weakening insulin signal transduction.

Brief periods of overfeeding in humans increase the expression of p85α, the principal regulatory subunit of PI 3-kinase (11). Increased expression of p85α has also been observed in insulin-resistant rodents (20) and humans (21). Conversely,

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**Fig. 7.** Total and IRS-1-associated p85α and IRS-1-associated p110. Data are means ± SE; n = 7 in each group. A: total myocardial p85α (regulatory subunit of PI 3-kinase) was greater in the intervention diet group than in the control diet group (P = 0.01). B: before maximal insulin stimulation, myocardial IRS-1-associated p85α was greater in the intervention diet group (I) than in the control diet group (C), P = 0.04. Insulin stimulation significantly increased association of p85α with IRS-1 in the control diet group (P = 0.03) but not the intervention diet group. The interaction of diet assignment and insulin stimulation was significant (P = 0.03). C: in myocardial samples obtained after insulin stimulation, IRS-1-associated p110 (catalytic subunit of PI 3-kinase) was lower in the intervention diet group than in the control diet group.

**Fig. 8.** Composition of myocardial lipid pools. Data are means ± SE; n = 8 in each group. In the intervention diet group, compared with the control diet group, the myocardial triglyceride pool (A) and diglyceride pool (B) were enriched in saturated fatty acyl residues (12:0, 14:0, and 16:0) and depleted in linoleic acid residues (18:2).
experimental silencing (1) or targeted disruption of p85α (34) restores insulin sensitivity. Increased expression of p85α provides an explanation for the increased basal and diminished insulin-stimulated association of p85α with IRS-1 observed in the intervention diet group of the present study. Increased IRS-1-associated p85α in the basal state (i.e., in the absence of insulin stimulation) most likely reflects a sequestration of inhibitory p85 monomers bound to IRS-1. This limits the potential for further association of catalytic p85-p110 dimers in response to insulin. Accordingly, the intervention diet group also demonstrated reduced insulin-stimulated association of p110 with IRS-1. These effects are schematized in Fig. 9.

Composition of intervention diet. We employed a diet enriched in both saturated fat and fructose to produce insulin resistance. Although this diet precludes isolation of individual components responsible for the pathological features of the model, it has the advantage of reflecting diet characteristics common among patients who develop obesity and insulin resistance in Western societies. Unlike other models of diet-induced insulin resistance in pigs (19, 55), we avoided animal fat or supplemental cholesterol because our intent was to induce insulin resistance, rather than atherosclerosis. High-fat diet increases intramyocellular lipid content and may thereby play a primary role in the development of insulin resistance in muscle (32, 35). Coconut oil was chosen as the source of fat because it is one of the most highly saturated nutritive vegetable oils, containing substantial amounts of 12:0, 14:0, and 16:0 residues. Development of insulin resistance is particularly associated with dietary content of saturated fatty acids (33, 42). Fatty acids such as palmitic acid promote intramyocellular accumulation of ceramide, which in turn impairs activation of Akt (39) and alters the activation of protein kinase C isoforms to promote pathological serine phosphorylation of IRS-1 (17), as observed in the present study. Accumulation of saturated fatty acyl residues also promotes oxidative stress, inflammation, and apoptosis and impairs endothelial function (9, 47), each of which may contribute to poor tolerance to physiological stressors such as ischemia.

Both high-sucrose and high-fructose diets have been shown to decrease insulin sensitivity in rodents (12); a high-fructose diet has been employed to induce insulin resistance in dogs (37). Although fructose produces smaller postprandial insulin excursions than glucose, it is preferentially metabolized to lipid in the liver and thus may contribute to the development of insulin resistance through the mechanisms indicated above (48).

Factors affecting myocardial glucose uptake. In the intervention diet group, myocardial glucose uptake increased in response to insulin infusion (Fig. 4A), even though PI 3-kinase activation and Akt phosphorylation did not increase under conditions of maximal insulin stimulation (Fig. 5, C and D). There are two potential explanations for these observations. First, insulin may have stimulated myocardial glucose uptake through pathways other than PI 3-kinase/Akt, such as the TC10 pathway (22). Second, the decrease in arterial [FFA] and myocardial FFA uptake with infusion of insulin (Fig. 4, B and C) would be expected to permit greater myocardial glucose uptake through the Randle cycle (40), independent of insulin signaling in the myocardium. In fact, three-way ANOVA with dependent variable myocardial glucose uptake and factors diet, plasma insulin concentration, and arterial [FFA] showed a significant main effect of arterial FFA on myocardial glucose uptake (P = 0.04), i.e., indicating the influence of the Randle cycle in this model.

After in vivo insulin bolus injection, resulting in plasma insulin concentration >300 μU/ml, activation of PI 3-kinase/Akt by insulin was confirmed in myocardial samples from the control diet group, but not from the intervention diet group. During euglycemic-hyperinsulinemic clamp, plasma insulin concentrations were lower (averaging 70–150 μU/ml in the control group). We assume, but cannot prove, that PI 3-kinase and Akt were activated by insulin in the control group at these lower concentrations, contributing to the robust increase in myocardial glucose uptake and systemic glucose disposal rate in response to insulin.

Effects of anesthesia. Under moderate sedation, higher blood pressure was observed in the intervention diet group compared with the control diet group (Table 2). However, there were no hemodynamic differences between groups under general anesthesia (Table 3). The latter observations might reflect the effect of anesthesia per se or the fact that anesthetic was dosed according to total body weight, rather than lean body mass, possibly resulting in higher plasma concentrations of anesthetic in the intervention diet group.

Clinical relevance. The present model of obesity and insulin resistance in juvenile pigs may have relevance to the growing problem of obesity, insulin resistance, and consequent future cardiovascular risk among youth and adolescents in modern times.
societies (49). The model may also be useful to determine whether abnormalities in myocardial insulin signaling contribute to the poor cardiac prognosis of adult patients with insulin resistance. Although cardiac contractile function was normal under baseline conditions in the present model, as it is in most patients with insulin resistance who have not yet suffered an ischemic heart disease event, insulin resistance may set the stage for abnormal myocardial responses to physiological stress. Numerous studies have shown that intact insulin signaling through PI 3-kinase/Akt promotes, whereas impaired insulin signaling through PI 3-kinase/Akt compromises cardiac contractile recovery following stresses such as ischemia-reperfusion injury, heat shock, or sepsis (2, 13, 28, 46, 51).

The beneficial effects of insulin signaling through PI 3-kinase/Akt are related in part to effects on substrate utilization. Glucose utilization is beneficial to the heart, particularly in ischemia-reperfusion (16). Conversely, diminished Akt activation by insulin may be deleterious in myocardial ischemia and reperfusion if glucose transport and utilization are decreased and dependence upon FFAs as an energy source is increased (6). However, the importance of insulin-mediated PI 3-kinase/Akt signaling in myocardial ischemia appears to extend beyond effects on substrate metabolism. Zaha et al. (57) demonstrated that insulin-induced improvement in postischemic recovery of contractile function is dependent on PI 3-kinase activation, rather than glucose uptake. Activation of the PI 3-kinase/Akt pathway is also necessary for myocardial protection conferred by both ischemic preconditioning and postconditioning (58), whereas inhibition of the pathway abolishes such protection. Rodents with insulin resistance are resistant to preconditioning (26, 30), in association with impaired activation of Akt (53).

Other adverse effects of reduced PI 3-kinase/Akt signaling in insulin resistance may include diminished expression of angiogenic factors such as vascular endothelial growth factor, resulting in increased apoptosis and decreased angiogenic response to chronic ischemia (23). Impaired activation of PI 3-kinase by insulin in adipose tissue may also lead to decreased secretion of adiponectin (38), an adipokine that is protective in myocardial ischemic injury (45). Conversely, reduced circulating adiponectin, as demonstrated in the present model, might exacerbate ischemic injury. A goal of further studies may be to determine whether the abnormalities of insulin signaling and substrate utilization demonstrated in the present model lead to impaired responses to physiological stress such as ischemia-reperfusion.

In summary, pigs fed a diet high in saturated fat, simple sugars, and energy content for 7 mo develop features recapitulating the clinical metabolic syndrome, including systemic insulin resistance. In conjunction with these abnormalities, the heart demonstrates blunted maximal insulin-stimulated glucose uptake in vivo and defective insulin-mediated PI 3-kinase/Akt signaling. The basis for defective myocardial insulin signaling may be increased serine phosphorylation of IRS-1 and increased expression of the p85α subunit of PI 3-kinase. The model thus described may prove to be a useful tool to investigate why insulin resistance confers an adverse clinical cardiovascular prognosis.

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