

Impaired Cardiac Efficiency and Increased Fatty Acid Oxidation in Insulin-Resistant *ob/ob* Mouse Hearts

Pradip K. Mazumder, Brian T. O'Neill, Matthew W. Roberts, Jonathan Buchanan, Ui Jeong Yun, Robert C. Cooksey, Sihem Boudina, and E. Dale Abel

Diabetes alters cardiac substrate metabolism. The cardiac phenotype in insulin-resistant states has not been comprehensively characterized. The goal of these studies was to determine whether the hearts of leptin-deficient 8-week-old *ob/ob* mice were able to modulate cardiac substrate utilization in response to insulin or to changes in fatty acid delivery. *Ob/ob* mice were insulin resistant and glucose intolerant. Insulin signal transduction and insulin-stimulated glucose uptake were markedly impaired in *ob/ob* cardiomyocytes. Insulin-stimulated rates of glycolysis and glucose oxidation were 1.5- and 1.8-fold higher in wild-type hearts, respectively, versus *ob/ob*, and glucose metabolism in *ob/ob* hearts was unresponsive to insulin. Increasing concentrations of palmitate from 0.4 mmol/l (low) to 1.2 mmol/l (high) led to a decline in glucose oxidation in wild-type hearts, whereas glucose oxidation remained depressed and did not change in *ob/ob* mouse hearts. In contrast, fatty acid utilization in *ob/ob* hearts was 1.5- to 2-fold greater in the absence or presence of 1 nmol/l insulin and rose with increasing palmitate concentrations. Moreover, the ability of insulin to reduce palmitate oxidation rates was blunted in the hearts of *ob/ob* mice. Under low-palmitate and insulin-free conditions, cardiac performance was significantly greater in wild-type hearts. However, in the presence of high palmitate and 1 nmol/l insulin, cardiac performance in *ob/ob* mouse hearts was relatively preserved, whereas function in wild-type mouse hearts declined substantially. Under all perfusion conditions, myocardial oxygen consumption was higher in *ob/ob* hearts, ranging from 30% higher in low-palmitate conditions to greater than two-fold higher under high-palmitate conditions. These data indicate that although the hearts of glucose-intolerant *ob/ob* mice are capable of maintaining their function under conditions of increased fatty acid supply and hyperinsulinemia, they are insulin-resistant, metabolically inefficient, and unable to modulate substrate utilization in response to changes in insulin and fatty acid supply. *Diabetes* 53:2366–2374, 2004

From the Program in Human Molecular Biology and Genetics, Division of Endocrinology, Metabolism and Diabetes, The University of Utah School of Medicine, Salt Lake City, Utah.

Address correspondence and reprint requests to E. Dale Abel, MD, PhD, Program in Human Molecular Biology and Genetics, Division of Endocrinology, Metabolism and Diabetes, University of Utah, 15 North 2030 East, Building #533, Room 3410B, Salt Lake City, UT 84112. E-mail: dale.abel@hmbg.utah.edu.

Received for publication 9 January 2004 and accepted in revised form 3 June 2004.

CPT-1, carnitine palmitoyl transferase-1; DMEM, Dulbecco's modified Eagle's medium; FFA, free fatty acid; IR, insulin receptor; MCD, malonyl CoA decarboxylase; WHW, wet heart weight.

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Diabetes is associated with a switch in myocardial substrate utilization that results in increased fatty acid utilization and decreased glucose utilization (1,2). Most studies of myocardial energy metabolism in diabetes have been performed in models of insulin deficiency. Fewer studies in insulin-resistant animals with type 2 diabetes have also revealed that glucose and/or lactate oxidation rates are decreased and that palmitate oxidation rates are increased (3–5). Moreover, this metabolic profile is associated with reduced myocardial function. Most studies in insulin-resistant mouse models have been performed at fatty acid concentrations that are similar to those seen in lean controls. Furthermore, some studies have been performed in the presence of added insulin, whereas others have been performed in the absence of insulin. Thus, it is possible that the experimental conditions might not reflect insulin and fatty acid concentrations that exist in these insulin-resistant models in vivo.

It is well established that insulin and fatty acids are important modulators of cardiac substrate utilization (6). Insulin will increase glucose utilization by augmenting glucose uptake and glucose oxidation. This in turn will increase the concentrations of malonyl CoA, which will suppress fatty acid oxidation rates by allosteric inhibition of carnitine palmitoyl transferase-1 (CPT-1). The opposite effect is seen when fatty acid supply to the heart is increased. In this case, fatty acid uptake and oxidation are proportionately increased and glucose utilization is reduced. Therefore, a goal of these studies was to determine whether cardiac metabolism in a model of insulin resistance and glucose intolerance remains responsive to insulin and to changes in fatty acid substrate delivery. Studies were performed in the absence and presence of 1 nmol/l insulin and at palmitate concentrations of 0.4 and 1.2 mmol/l. This study design enabled us to determine whether the metabolic phenotype of the *ob/ob* heart varied in response to changes in substrate availability and allowed us to determine whether insulin is able to modulate substrate metabolism in these hearts.

We elected to perform these studies in hearts that were obtained from 8-week-old *ob/ob* mice. In contrast to *db/db* mice, in which most studies have been performed to date, *ob/ob* mice, although possessing similar underlying pathophysiology (e.g., deficient leptin action), do not develop severe hyperglycemia. Indeed at the age studied, *ob/ob* mice are glucose intolerant but are profoundly hyperinsu-

linemic and insulin resistant. This therefore provided an opportunity to determine whether the previously reported metabolic phenotypes of the *db/db* mouse heart are similar to those of the less hyperglycemic *ob/ob* heart. These studies demonstrate that a profound state of insulin resistance exists in the hearts of *ob/ob* mice and that the ability of these hearts to modulate substrate utilization in response to insulin and changes in fatty acid supply is altered.

RESEARCH DESIGN AND METHODS

The institutional animal care and use committee of the University of Utah approved all studies. Mice were cared for according to the "Guiding Principles for Research Involving Animals and Human Beings." Homozygous male C57BL/6J-*lep^{ob}* (*ob/ob*) and their respective wild-type control (C57BL/6J) mice were obtained at 6 weeks of age from The Jackson Laboratories (Bar Harbor, ME) and were studied when they were 8 weeks of age. The animals were fed a standard laboratory diet and housed in temperature-controlled facilities with a 12-h light and 12-h dark cycle (lights on at 6:00 A.M.). All *ex vivo* cardiac studies were performed on hearts that were obtained from mice that were killed between 8:00 A.M. and 4:00 P.M.

Glucose tolerance tests and determination of serum concentrations of insulin and free fatty acids and of serum and tissue concentrations of triglycerides. Glucose tolerance tests were performed after a 6-h fast (food removed at 6:00 A.M.). A glucose bolus was injected intraperitoneally (1 mg/g body wt), and blood samples were obtained from the tail vein at 30, 60, 90, and 120 min after glucose administration. Blood glucose was determined using a glucose oxidase method with OneTouch test strips (Lifescan; Johnson & Johnson, Milpitas, CA). An additional blood sample was collected at time 0 for serum insulin determination, which was measured by radioimmunoassay using the sensitive rat insulin radioimmunoassay kit (Linco Research, St. Charles, MO). Insulin assays were performed in 25- μ l samples. In a second cohort of mice, blood samples were obtained during peak feeding (5:00 A.M.) and 6 h after food removal (postabsorptive state) and assayed for free fatty acids (FFAs), insulin, and triglycerides. FFA concentrations were determined in 50- μ l serum samples using the 1/2-micro fatty acid test kit (Roche Diagnostics, Mannheim, Germany). Triglyceride concentrations were determined in 10- μ l serum samples using the L-type TG H kit (Wako, Richmond, VA). Myocardial triglyceride content of mouse hearts (removed between 10:00 A.M. and 12:00 P.M.) was measured as described (7).

Substrate metabolism in isolated working mouse hearts. Cardiac metabolism was measured in hearts that were isolated from 8-week-old male *ob/ob* mice and age-matched C57BL/6J controls. All hearts were prepared and perfused in the working mode, using protocols that have been previously described (7,8). In brief, the working heart buffer was Krebs Henseleit buffer that contained (in mmol/l) 118.5 NaCl, 25 NaHCO₃, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 2.5 CaCl₂, 0.5 EDTA, and 5 glucose, gassed with 95% O₂ and 5% CO₂ and supplemented with either 0.4 or 1.2 mmol/l palmitate bound to 3% BSA in the presence or absence of 1 nmol/l insulin. Glycolytic flux was determined by measuring the amount of ³H₂O released from the metabolism of exogenous [⁵-³H]glucose (specific activity, 177 Gbq/mol). Glucose oxidation was determined by trapping and measuring ¹⁴CO₂ released by the metabolism of [U-¹⁴C]glucose (specific activity, 296 Mbq/mol). Palmitate oxidation was determined in separate perfused hearts by measuring the amount of ³H₂O released from [9,10-³H]palmitate (specific activity, 42 Gbq/mol).

Determination of myocardial oxygen consumption. Myocardial oxygen consumption (MVO₂) was measured at 20-min intervals in hearts that were used for the determination of palmitate oxidation, as the glucose oxidation protocols require that the perfusion apparatus be totally sealed to prevent loss of CO₂. Oxygen concentration was measured in pulmonary artery effluent that was collected using a capillary tube (venous partial pressure of oxygen [PvO₂]). The oxygen content of freshly oxygenated buffer was also determined (arterial partial pressure of oxygen [PaO₂]). Oxygen concentration in the sample was measured using a fiber-optic oxygen sensor (Ocean Optics, Orlando, FL). The sensor consists of a probe that is coated with the fluorescent dye, ruthenium red, which emits fluorescence at 600 nm when excited with a light source that is also integrated with a spectrophotometer. In the presence of oxygen, the fluorescence is quenched in proportion to the oxygen content of the sample, and the data are analyzed using accompanying software (Ocean Optics). The following formulas were used to determine MVO₂, cardiac hydraulic work, and cardiac efficiency:

$MVO_2 \text{ (ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1} \text{ wet heart weight [WHW])} = [(PaO_2 - PvO_2)/100] \times (\text{coronary flow/WHW}) \times (725/760) \times (1,000 \times C)$; where C is Bunsen

coefficient for plasma, i.e., 0.0212, and 725 and 760 mmHg are atmospheric pressures at the University of Utah and at sea level, respectively.

Cardiac hydraulic work ($J \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ WHW) = CO (ml/min) \times DevP (mmHg) \times 1.33 \times 10⁻⁴/g WHW, where CO is cardiac output and DevP = developed pressure.

Cardiac efficiency (%) = hydraulic work/MVO₂ \times 100. MVO₂ (ml/min) was converted to μ mol/min by multiplying by the conversion factor 0.0393 and then to Joules (J/min) using the conversion of 1 μ mol O₂ = 0.4478 J as described by Suga (9).

Determination of insulin signaling. The ability of insulin to increase the tyrosine phosphorylation of the insulin receptor (IR) and the p85 subunit of phosphatidylinositol-3 kinase and to increase serine phosphorylation of Akt was determined in extracts that were obtained from isolated working hearts that were perfused with working heart buffer that contained 5 mmol/l glucose and 0.4 mmol/l palmitate \pm 1 nmol/l insulin for 15 min. Hearts were freeze-clamped and homogenized as previously described (7). Samples were immunoprecipitated with antiphosphotyrosine antibodies as described (7), and the immunoprecipitates were blotted with anti-IR β and anti-p85 antibodies. Proteins were separated on SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Fisher Scientific, Pittsburgh, PA). For Akt and p85, membranes were blocked for 1 h at room temperature with PBST (PBS that contained 0.1% Tween 20) that contained 5% nonfat milk and then incubated for 15–20 h at 4°C in PBST that contained 5% nonfat milk with anti-phospho Akt (ser473), anti-Akt, or anti-p85 antibodies. For IR immunoblots, membranes were blocked for 15–20 h at 4°C in PBST-5% milk and then incubated for 1 h at room temperature with the anti-IR antibody in PBST-5% milk. GLUT4 immunoblots were performed as described (7). SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, IL) was used for detection. Antibodies against IR β and Akt were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against phospho Akt (phosphorylation of serine at position 473), p85, and phospho-tyrosine were obtained from Cell Signaling Technology (Beverly, MA); and anti-GLUT4 antibodies were obtained from Chemicon (Temecula, CA).

Isolation of cardiac myocytes and determination of glucose uptake.

Cardiomyocytes were isolated using collagenase digestion of Langendorff-perfused mouse hearts as previously described by our group (7). This isolation procedure initially yields between 80 and 90% viable rod-shaped myocytes, the majority of which attach to laminin-coated wells and maintain their morphology for the duration of the protocol. Basal and insulin-stimulated glucose uptake was measured using 2-deoxyglucose as previously described (7). In brief, cardiomyocytes were prepared from mouse hearts that were obtained from heparinized mice and retrograde perfused with perfusion buffer (pH 7.3; in mmol/l): 126 NaCl, 4.4 KCl, 1.0 MgCl₂, 4.0 NaHCO₃, 10.0 HEPES, 30.0 2,3-butanedione monoxime, 5.5 glucose, 1.8 pyruvate, and 0.025 CaCl₂ supplemented with 16 U/ml type I collagenase for 10 min. Myocytes were dispersed for 10 min at 37°C in perfusion buffer that contained 2% BSA and 0.2 mmol/l CaCl₂ and cultured in modified Dulbecco's modified Eagle's medium (DMEM) that contained 5% FBS, 5.0 mmol/l glucose, and 1.0 mmol/l CaCl₂ on laminin-coated plates at 37°C in humidified 95% O₂-5% CO₂. Cells were allowed to attach to the wells for 60 min and were subsequently cultured for 30 min in DMEM that contained 0.1% BSA, 5.0 mmol/l glucose, and 1.0 mmol/l CaCl₂ before measuring glucose uptake. Cells were then incubated for 40 min in the presence or absence of 0.1–10 nmol/l insulin in glucose-free DMEM supplemented with 1 mg/ml BSA and 1 mmol/l pyruvate. Glucose uptake was performed by adding 0.1 mmol/l 2-deoxy-D-glucose and 3.33 nCi/ml 2-[1,2-³H]-deoxy-D-glucose for 30 min. Glucose transport experiments were terminated after 30 min by aspiration of the buffer followed by two washes with ice-cold PBS and then cells were lysed in 1 N NaOH for 20 min at 37°C. Nonspecific uptake was assessed in the presence of 10 μ mol/l cytochalasin B and subtracted from all of the measured values. The radioactivity was counted by liquid scintillation spectroscopy using a Beckman LS 5000 TD instrument (Beckman Coulter, Fullerton, CA) and normalized to protein amount measured with a Micro BCA Protein Assay Kit (Pierce Chemical, Rockford, IL).

Statistical analysis. Data are expressed as mean \pm SE. Differences in the serum concentrations of glucose and insulin and myocardial triglyceride content were assessed using the unpaired Student's *t* test. All other differences were analyzed by ANOVA, and significance was assessed by Fisher's protected least significant difference test. For all analyses, *P* < 0.05 was accepted as indicating a significant difference. Statistical calculations were performed using the Statview 5.0.1 software package (SAS Institute, Cary, NC).

RESULTS

Systemic metabolic parameters in *ob/ob* mice. As expected, *ob/ob* mice were significantly more obese than controls (48.2 \pm 0.9 vs. 24.8 \pm 0.4 g; *P* < 0.0001). Glucose

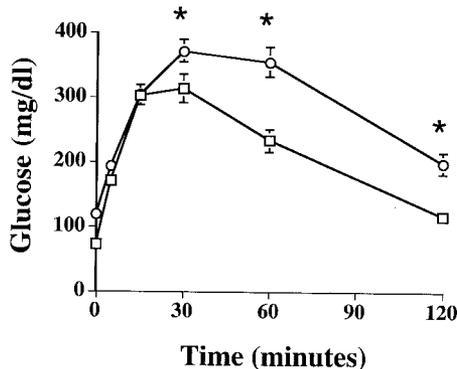
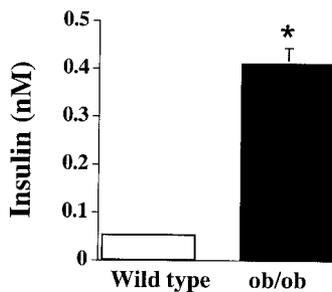
A Glucose Tolerance Test**B**

FIG. 1. Glucose tolerance tests (GTTs) and insulin concentrations. **A:** Glucose tolerance tests in 8-week-old *ob/ob* (○) and age-matched C57BL/6J controls (wild type; □). * $P < 0.05$ vs. wild type; $n = 9$ *ob/ob*, $n = 8$ wild type. **B:** Fasting insulin concentrations were measured in serum obtained at time 0 of the GTT from *ob/ob* and wild-type mice. * $P < 0.04$ vs. wild type.

concentrations after a 6-h fast were 120 ± 6 in *ob/ob* mice vs. 74 ± 3 mg/dl in controls ($P < 0.001$). As shown in Fig. 1, glucose tolerance was impaired in *ob/ob* mice and insulin concentrations were increased by eightfold. Insulin, triglycerides, and FFA concentrations were also measured at peak feeding and 6 h after food removal (postabsorptive; Table 1). In the postabsorptive state, FFA concentrations were 0.6 ± 0.08 and 1.3 ± 0.1 mmol/l and insulin concentrations were 0.03 ± 0.009 and 0.57 ± 0.15 nmol/l in control and *ob/ob* mice, respectively. Levels of insulin and FFAs increased in both groups of animals during peak feeding but remained significantly higher in *ob/ob* mice relative to controls. Serum triglycerides were similar in both groups of mice in the postabsorptive state but were twofold higher in *ob/ob* mice during peak feeding. Intramyocardial triglyceride content was 10.8 ± 1.1 μ mol/g WHW in controls and 16.5 ± 1.4 μ mol/g WHW in *ob/ob* mice ($P < 0.002$).

Substrate metabolism in isolated working hearts. Substrate metabolism and cardiac performance were determined in hearts that were perfused in the presence or absence of 1 nmol/l insulin and at palmitate concentrations of 0.4 (low palmitate) and 1.2 mmol/l (high palmitate). Functional parameters of the working heart preparations are summarized in Table 2. Under low-palmitate conditions, rates of glycolysis in the absence of insulin were 22% lower in *ob/ob* hearts ($P < 0.04$ vs. controls; Fig. 2A). Insulin failed to increase glycolytic rates in *ob/ob* mouse hearts but increased glycolytic rates in wild-type hearts by 19% ($P < 0.06$), thereby magnifying the differences in glycolytic flux between *ob/ob* and controls. Increasing palmitate concentrations to 1.2 mmol/l resulted in a significant decline in glycolytic rates in all hearts. Under high-palmitate conditions in the presence or absence of insulin, rates of glycolysis were similar in *ob/ob* and control hearts. The 58% reduction in glycolytic rates in

control hearts from low- to high-palmitate conditions was greater than the 36–45% reduction in *ob/ob* hearts. Moreover, the ability of insulin to augment glycolysis in wild-type hearts was completely abrogated under high-palmitate conditions.

Differences in the rates of glucose oxidation between *ob/ob* and control hearts were even more magnified (Fig. 2B). Under low-palmitate conditions, glucose oxidation rates were 1.4-fold greater in control hearts relative to *ob/ob* hearts, and insulin increased glucose oxidation rates in wild-type hearts by an additional 27% ($P < 0.002$). Increasing palmitate concentrations led to a substantial reduction in glucose oxidation rates in control hearts. In addition, the ability of insulin to increase glucose oxidation was blunted in control hearts, perfused under high-palmitate conditions (411 ± 28 nmol \cdot min $^{-1}$ \cdot g dry wt $^{-1}$ in the presence of insulin vs. 322 ± 23 nmol \cdot min $^{-1}$ \cdot g dry wt $^{-1}$ in the absence of insulin; $P = 0.07$). In contrast, insulin failed to augment glucose oxidation rates in *ob/ob* hearts, which were 31–46% lower in the absence and presence of insulin, respectively, relative to low-palmitate controls and did not change any further under high-palmitate conditions. Thus, *ob/ob* hearts exhibit metabolic inflexibility in terms of modulating glucose utilization in response to insulin or to changes in fatty acid supply.

Rates of palmitate oxidation were higher in *ob/ob* than controls under all conditions (Fig. 2C). In the absence of insulin, the difference in palmitate oxidation rates between *ob/ob* and wild-type hearts was 142 nmol \cdot min $^{-1}$ \cdot g dry heart wt $^{-1}$ in low-palmitate-perfused hearts. When hearts were perfused at 1.2 mmol/l palmitate (in the absence of insulin), wild-type and *ob/ob* hearts displayed a similar fold increase in fatty acid oxidation rates, so the difference in palmitate oxidation rates at high palmitate (148 nmol \cdot min $^{-1}$ \cdot g dry heart wt $^{-1}$) between *ob/ob* mice and their controls was similar to the difference observed under

TABLE 1

Serum metabolites in wild-type and *ob/ob* mice obtained at 5:00 A.M. (peak fed) and 6 h after food removal (postabsorptive)

	Wild-type peak fed	<i>Ob/ob</i> peak fed	Wild-type postabsorptive	<i>Ob/ob</i> postabsorptive
Serum insulin (nmol/l)	0.071 ± 0.016 (9)‡	1.586 ± 0.317 (9)*‡	0.03 ± 0.009 (9)	0.569 ± 0.149 (9)†
Serum triglycerides (mmol/l)	3.998 ± 0.353 (9)‡	8.051 ± 1.413 (9)*‡	0.875 ± 0.105 (9)	0.966 ± 0.245 (9)
Serum FFAs (mmol/l)	1.387 ± 0.154 (9)‡	2.618 ± 0.171 (10)*‡	0.601 ± 0.079 (9)	1.273 ± 0.105 (10)†

Data are means \pm SE (no. of samples). * $P < 0.0005$ vs. wild-type peak fed; † $P < 0.04$ vs. wild-type postabsorptive; ‡ $P < 0.04$ vs. postabsorptive of the same genotype.

low-palmitate conditions. Insulin suppressed fatty acid oxidation rates in control mice by 30% under low-palmitate conditions and by 66% under high-palmitate conditions. In contrast, insulin had a negligible effect on rates of palmitate oxidation in *ob/ob* hearts under low-palmitate conditions and modestly inhibited palmitate oxidation rates by 23% under high-palmitate conditions. Thus, *ob/ob* hearts maintain the ability to enhance fatty acid oxidation rates in the face of increased supply, but the ability of insulin to modulate fatty acid utilization is impaired.

MVO₂ and cardiac performance. Under low-palmitate conditions, insulin significantly increased cardiac power by 43% in wild-type mice ($P < 0.0001$) and increased cardiac power in *ob/ob* hearts by a more modest 23% ($P < 0.05$). Under low-palmitate conditions, cardiac power in *ob/ob* hearts was significantly reduced relative to controls that were perfused with equivalent insulin concentrations. Increasing fatty acid delivery resulted in a dramatic reduction in cardiac performance in control hearts, particularly in the presence of insulin. In contrast, the decline in cardiac power in *ob/ob* hearts from low- to high-palmitate conditions was more gradual. Thus, under high-palmitate conditions, cardiac power was actually higher in *ob/ob* hearts, particularly in the presence of insulin (Fig. 3A). Taken together, these data suggest that adaptations have occurred in the hearts of *ob/ob* mice that enable them to maintain cardiac performance in the presence of hyperinsulinemic conditions and in the face of increased fatty acid supply.

MVO₂ was increased in *ob/ob* hearts relative to controls in the presence or absence of insulin and under high- and low-palmitate conditions (Fig. 3B). Moreover, increased concentrations of palmitate were associated in *ob/ob* mice with progressively increased oxygen consumption that paralleled the increased fatty acid utilization. In contrast, increasing palmitate concentrations had a small effect on MVO₂ in control hearts that were perfused in the absence of insulin. In the presence of insulin, MVO₂ fell in control hearts in concert with the decreased myocardial performance. When cardiac efficiency (which reflects work performed per unit of oxygen consumed) was calculated, it became evident that cardiac efficiency was greatest in wild-type hearts that were perfused under low-palmitate conditions (Fig. 3C). Indeed, in control hearts, insulin significantly enhanced cardiac efficiency under low-palmitate conditions. In contrast, cardiac efficiency was significantly reduced in *ob/ob* mouse hearts under low-palmitate conditions and was not altered by insulin treatment. Increasing palmitate concentrations led to a dramatic decline in cardiac efficiency in control hearts, whereas cardiac efficiency was not affected by changing palmitate concentrations in *ob/ob* hearts. Thus, under high-palmitate and insulin-free conditions, *ob/ob* and control hearts exhibited equivalent impairment in cardiac efficiency. Cardiac efficiency was further impaired in control hearts under high-palmitate conditions in the presence of insulin. The basis for the reduced cardiac efficiency under high-palmitate conditions in *ob/ob* and wild-type hearts was distinct, however. In wild-type hearts, the reduction in myocardial efficiency reflects in large part the dramatic reduction in cardiac performance, whereas in *ob/ob* hearts,

TABLE 2
Characteristics of wild-type and *ob/ob* working hearts perfused at 0.4 and 1.2 mmol/l palmitate

	0.4 mmol/l palmitate wild type (insulin 0 mmol/l)	0.4 mmol/l palmitate <i>ob/ob</i> (insulin 0 mmol/l)	0.4 mmol/l palmitate wild type (insulin 1 mmol/l)	0.4 mmol/l palmitate <i>ob/ob</i> (insulin 1 mmol/l)	1.2 mmol/l palmitate wild type (insulin 0 mmol/l)	1.2 mmol/l palmitate <i>ob/ob</i> (insulin 0 mmol/l)	1.2 mmol/l palmitate wild type (insulin 1 mmol/l)	1.2 mmol/l palmitate <i>ob/ob</i> (insulin 1 mmol/l)
Body weight (g)	25.16 ± 0.71 (8)	50 ± 2.88 (8)*	24.21 ± 0.79 (8)	48.73 ± 1.59 (9)*	23.65 ± 0.95 (6)	42.94 ± 1.31 (6)*	26.34 ± 0.85 (8)	53.17 ± 1.04 (6)*
WHW (mg)	149.63 ± 5.29 (8)	195.75 ± 5.55 (8)*	131 ± 2.52 (8)	200.22 ± 3.65 (9)*	292.57 ± 6.26 (6)	206.08 ± 5.21 (6)	257.04 ± 11.68 (8)	224.70 ± 9.81 (6)*
Dry heart weight (mg)	29 ± 0.54 (8)	34.63 ± 1.90 (8)*	28.38 ± 1.09 (8)	35.89 ± 1.43 (9)*	26.62 ± 2.48 (6)	30.08 ± 1.12 (6)	35.04 ± 1.76 (8)	31.85 ± 1.99 (6)
Heart rate (beats/min)	287.03 ± 9.19 (8)	293.75 ± 12.25 (8)	322.19 ± 7.17 (8)	307.2 ± 7.51 (9)	336.57 ± 13.03 (6)	276.02 ± 20.91 (6)*	268.45 ± 13.22 (8)	278.55 ± 10.10 (6)
LVSP (mmHg)	87.85 ± 1.54 (8)	82.88 ± 1.67 (8)	89.75 ± 2.46 (8)	80 ± 1.73 (9)*	85.83 ± 3.81 (6)	85.42 ± 3.60 (6)	89.69 ± 4.64 (8)	92.08 ± 4.59 (6)
LVEDP (mmHg)	48.23 ± 0.64 (8)	46.13 ± 2.21 (8)	34.13 ± 0.73 (8)	33.92 ± 0.28 (9)	60.88 ± 3.54 (6)	59.46 ± 3.56 (6)	67.59 ± 4.11 (8)	65.21 ± 4.15 (6)
LV DevP (mmHg)	39.63 ± 1.28 (8)	36.75 ± 1.69 (8)	55.63 ± 1.92 (8)	46.08 ± 1.68 (9)*	24.96 ± 0.85 (6)	25.96 ± 1.22 (6)	22.09 ± 0.83 (8)	26.88 ± 0.95 (6)*
Coronary flow (ml/min)	3.05 ± 0.04 (8)	3.07 ± 0.04 (8)	2.87 ± 0.06 (8)	3.02 ± 0.04 (9)	2.76 ± 0.14 (6)	2.98 ± 0.12 (6)	2.61 ± 0.07 (8)	3.30 ± 0.12 (6)*
Aortic flow (ml/min)	8.42 ± 0.40 (8)	7.67 ± 0.41 (8)	9.05 ± 0.50 (8)	7.24 ± 0.34 (9)*	6.53 ± 0.38 (6)	6.17 ± 0.54 (6)	4.37 ± 0.28 (8)	8.08 ± 0.51 (6)*
Cardiac output (ml/min)	11.47 ± 0.41 (8)	10.73 ± 0.40 (8)	11.91 ± 0.53 (8)	10.25 ± 0.34 (9)*	9.30 ± 0.43 (6)	9.15 ± 0.61 (6)	6.98 ± 0.30 (8)	11.38 ± 0.49 (6)*
Cardiac power (mW/g)	36.89 ± 2.14 (8)	26.31 ± 1.62 (8)*	53.49 ± 3.67 (8)	31.92 ± 2.25 (9)*	15.99 ± 1.90 (6)	17.41 ± 1.34 (6)	10.33 ± 0.73 (8)	23.62 ± 1.62 (6)*

Data are means ± SE (no. of heart perfusions). LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; LV DevP, left ventricular developed pressure. * $P < 0.05$ vs. wild type perfused under similar conditions.

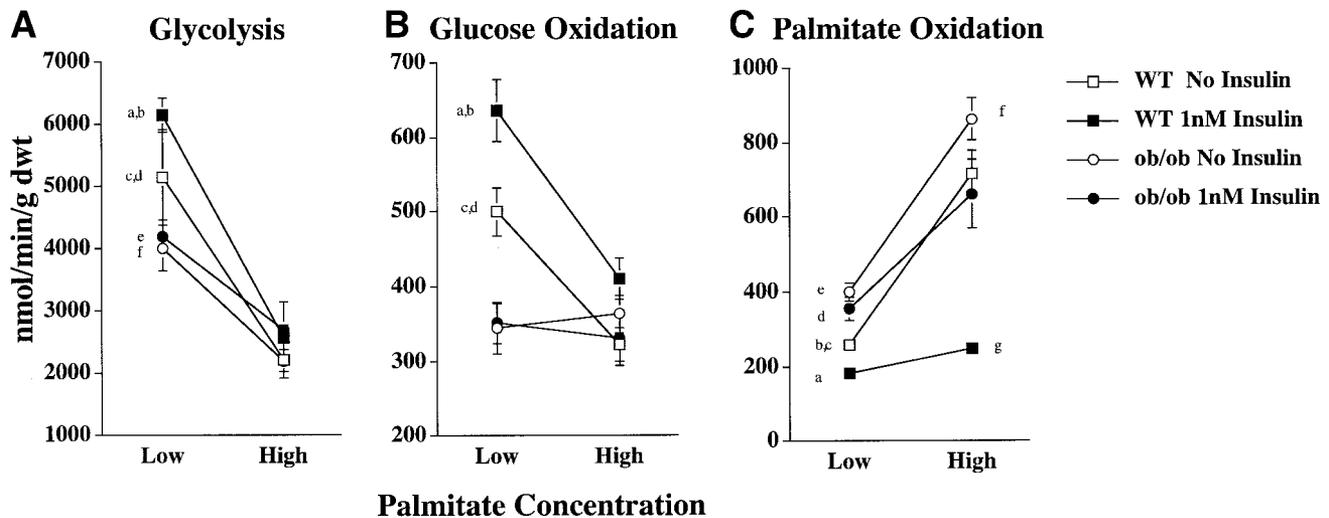


FIG. 2. Substrate metabolism in isolated working hearts. Rates of glycolysis, glucose oxidation, and palmitate oxidation were determined in isolated working hearts obtained from 8-week-old *ob/ob* and C57BL/6J (wild-type [WT]) mice. Data from *ob/ob* mice are represented by circles, and data from WT mice are represented by squares. Open and closed symbols represent perfusions performed in the absence or presence of 1 nmol/l insulin, respectively. Perfusions were performed at 0.4 mmol/l palmitate (low) or 1.2 mmol/l palmitate (high). Data were obtained from three to five hearts per genotype. **A:** Glycolysis. ^a $P < 0.06$ vs. WT basal and $P < 0.0001$ vs. both low-palmitate *ob/ob* results; ^b $P < 0.0001$ vs. high-palmitate wild-type hearts perfused with insulin; ^c $P < 0.04$ vs. low palmitate *ob/ob* hearts perfused without insulin; ^d $P < 0.0001$ vs. high-palmitate wild-type hearts perfused without insulin; ^e $P < 0.01$ vs. high-palmitate *ob/ob* hearts perfused with insulin; ^f $P < 0.003$ vs. high-palmitate *ob/ob* hearts perfused without insulin. **B:** Glucose oxidation. ^a $P < 0.002$ vs. all other low-palmitate results; ^b $P < 0.0001$ vs. high-palmitate wild-type hearts perfused with insulin; ^c $P < 0.001$ vs. low-palmitate *ob/ob* hearts perfused with and without insulin; ^d $P < 0.0005$ vs. high-palmitate wild-type hearts perfused without insulin. **C:** Palmitate oxidation. ^a $P < 0.004$ vs. low-palmitate *ob/ob* hearts perfused with and without insulin; ^b $P < 0.01$ vs. low-palmitate *ob/ob* hearts perfused without insulin; ^c $P < 0.0001$ vs. high-palmitate wild-type hearts perfused without insulin; ^d $P < 0.0001$ vs. high-palmitate *ob/ob* hearts perfused with insulin; ^e $P < 0.0001$ vs. high-palmitate *ob/ob* hearts perfused without insulin; ^f $P < 0.03$ vs. all other high-palmitate results; ^g $P < 0.0001$ vs. high-palmitate wild-type hearts perfused without insulin and high-palmitate *ob/ob* hearts perfused with insulin.

reduced efficiency is largely accounted for by the high rates of oxygen consumption.

Insulin signaling in hearts and glucose transport in cardiomyocytes. We speculated that impaired insulin signal transduction was responsible in part for the reduced ability of insulin to modulate substrate metabolism in *ob/ob* mouse hearts and that impaired, insulin-stimulated glucose transport contributed to the impaired ability of insulin to increase glycolysis. We therefore assayed various components of the insulin signaling pathway in working hearts and measured insulin-stimulated glucose uptake in isolated cardiomyocytes. Figure 4A shows that basal levels of IR tyrosine phosphorylation were increased in *ob/ob* hearts, but in contrast to wild-type hearts that exhibited minimal IR tyrosine phosphorylation under basal conditions and a robust increase with insulin stimulation, the fold increase in IR-tyrosine phosphorylation upon insulin stimulation of *ob/ob* hearts was blunted. Despite the increased basal phosphorylation state of the IR, there was no increase in the basal phosphorylation of p85 or Akt (Fig. 4A and B). Moreover, the levels of phosphorylated p85 and Akt were reduced in *ob/ob* hearts relative to controls after insulin stimulation (Fig. 4A and B). We also observed a 30% reduction in abundance of phosphorylated glycogen synthase kinase β ($P < 0.05$) in insulin-stimulated *ob/ob* hearts relative to controls (data not shown). We then measured the dose response of insulin-stimulated glucose uptake in *ob/ob* and control cardiomyocytes (Fig. 4C). Basal rates of glucose uptake were similar in *ob/ob* and control cardiomyocytes. However, the ability of insulin to stimulate glucose uptake was completely blunted. The decline in insulin-stimulated glucose uptake was not associated with decreased total

GLUT4 expression (Fig. 4C) but likely represents a defect in GLUT4 translocation on the basis of impaired insulin signaling. These data are consistent with multiple defects in insulin signaling in *ob/ob* mouse hearts.

DISCUSSION

Impaired insulin signal transduction in adipose tissue, skeletal muscle, and hepatocytes have long been recognized as features of the insulin resistance syndrome (10). Although a small number of studies have described impaired insulin signaling in the hearts of obese Zucker rats (11,12), little is known about the relationship between myocardial insulin resistance and substrate preference in the heart. In this study, we characterized myocardial substrate utilization and insulin signaling in the hearts of obese, insulin-resistant *ob/ob* mice. We demonstrated that cardiomyocytes from *ob/ob* mice are profoundly insulin resistant and show that impaired insulin action in the heart is associated with a metabolic profile in which glucose utilization (glycolysis and glucose oxidation) is reduced and fatty acid utilization is increased. *Ob/ob* mouse hearts exhibit a fixed defect in glucose oxidation and fail to modulate glucose oxidation in response to insulin or to changes in the delivery of fatty acids. In contrast, fatty acid oxidation rates and MVO₂ are increased in the hearts of *ob/ob* mice, but despite this, cardiac performance is decreased, indicating that the efficiency of coupling of respiration to myocardial work is impaired. In control hearts, the combination of hyperinsulinemia and increased fatty acid supply was associated with a marked reduction in MVO₂ and cardiac performance. This contrasts with *ob/ob*

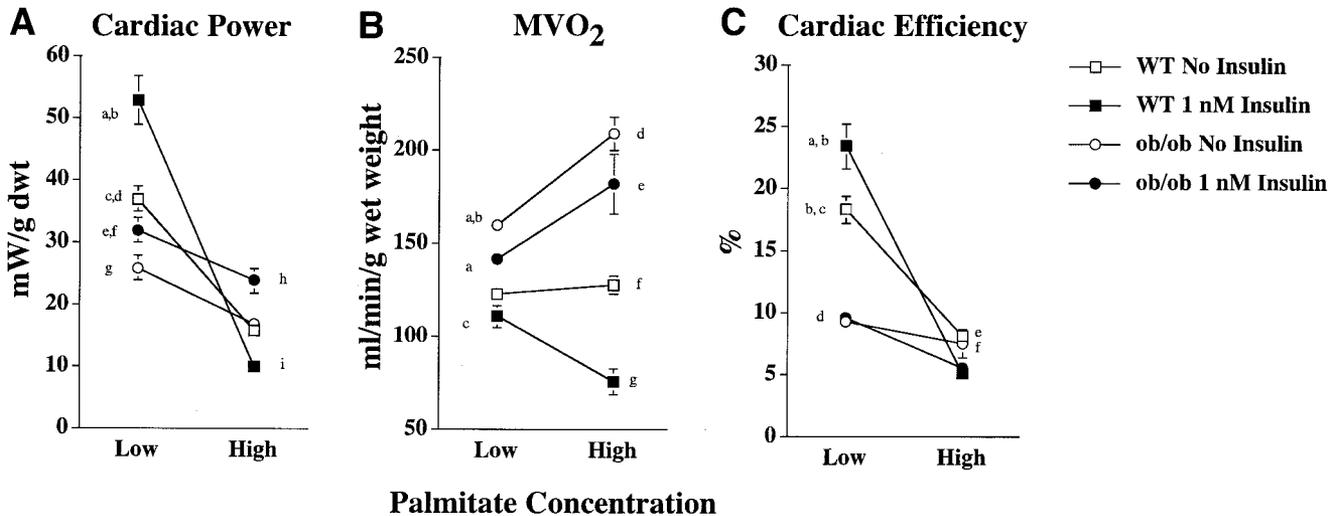


FIG. 3. Cardiac performance and oxygen consumption in isolated working hearts. Cardiac power, MVO_2 , and cardiac efficiency were determined in the same experiments depicted in Fig. 2. Symbols remain the same. The hearts used for MVO_2 and cardiac efficiency calculations are those used for palmitate metabolism measurements. For each perfusion condition, cardiac power data from glucose oxidation and palmitate oxidation experiments were combined. **A:** Cardiac power. ^a $P < 0.0001$ vs. all other low-palmitate results; ^b $P < 0.0001$ vs. high-palmitate wild-type hearts perfused with insulin; ^c $P < 0.0003$ vs. low-palmitate *ob/ob* hearts perfused without insulin; ^d $P < 0.0001$ vs. high-palmitate wild-type hearts perfused without insulin; ^e $P < 0.05$ vs. low-palmitate *ob/ob* hearts perfused without insulin; ^f $P < 0.007$ vs. high-palmitate *ob/ob* hearts perfused with insulin; ^g $P < 0.005$ vs. high-palmitate *ob/ob* hearts perfused without insulin; ^h $P < 0.06$ vs. *ob/ob* hearts perfused without insulin and $P < 0.02$ vs. both groups of high-palmitate wild-type hearts; ⁱ $P < 0.02$ vs. high palmitate *ob/ob* hearts perfused without insulin. **B:** MVO_2 . ^a $P < 0.03$ vs. low-palmitate wild-type hearts perfused with and without insulin; ^b $P < 0.04$ vs. low-palmitate *ob/ob* hearts perfused with insulin; ^c $P < 0.0001$ vs. high-palmitate wild-type hearts perfused with insulin; ^d $P < 0.0001$ vs. low-palmitate *ob/ob* hearts perfused without insulin; ^e $P < 0.0001$ vs. low-palmitate *ob/ob* hearts perfused with insulin; ^f $P < 0.0001$ vs. high-palmitate *ob/ob* hearts perfused with and without insulin; ^g $P < 0.0001$ vs. all other high-palmitate results. **C:** Cardiac efficiency. ^a $P < 0.0001$ vs. all other low-palmitate results; ^b $P < 0.0001$ vs. all high-palmitate results; ^c $P < 0.0001$ vs. low-palmitate *ob/ob* hearts perfused with and without insulin; ^d $P < 0.0007$ vs. high-palmitate *ob/ob* hearts perfused with insulin; ^e $P < 0.0001$ vs. low-palmitate *ob/ob* hearts perfused with and without insulin; ^f $P < 0.0001$ vs. all other low-palmitate results; ^g $P < 0.0001$ vs. all high-palmitate results; ^h $P < 0.0001$ vs. low-palmitate *ob/ob* hearts perfused with and without insulin; ⁱ $P < 0.0007$ vs. high-palmitate *ob/ob* hearts perfused with insulin; ^j $P < 0.06$ vs. high-palmitate *ob/ob* and $P < 0.02$ vs. high-palmitate wild-type hearts both perfused with insulin; ^k $P = 0.05$ vs. high-palmitate wild-type hearts perfused with insulin.

mouse hearts that maintain their function, albeit with increased rates of MVO_2 under these conditions.

The greatest differences in glycolytic rates between wild-type and *ob/ob* hearts were observed under low-palmitate conditions. Under these conditions, the normal heart will utilize relatively more glucose, and the ability of insulin to augment glycolysis further is readily apparent. Insulin increases glycolysis, in part by translocating GLUT4 glucose transporters to the plasma membrane (13). In studies performed in isolated cardiomyocytes, we observed a complete failure of insulin to increase glucose uptake in cardiomyocytes from *ob/ob* mice. This occurred without any changes in GLUT4 protein content, which leads us to conclude that insulin-stimulated GLUT4 translocation is impaired in these hearts. Normal GLUT4 levels in *ob/ob* hearts contrast with findings reported in 12-month-old Zucker rats (14). As our study was performed in relatively young animals (8 weeks of age), the possibility remains that GLUT4 levels could decline further with age. Although the failure of insulin to increase glycolytic flux in *ob/ob* mouse hearts may be due in part to the profound insulin resistance in these hearts, glycolytic rates were also reduced in *ob/ob* hearts that were perfused in the absence of insulin, indicating that additional mechanisms might be responsible for decreasing glycolysis in the *ob/ob* mouse hearts. Increasing fatty acid concentrations impaired glycolytic rates to a greater extent in wild-type hearts than in *ob/ob* hearts. Thus, increased fatty acid flux independently impairs myocardial glycolysis. The mechanisms by which increased fatty acid utilization impairs myocardial glucose uptake in the absence of insulin are partially understood. Exposure of cardiomyocytes to long-

chain fatty acids was shown to lead to increased intracellular retention of GLUT4 and to reduced abundance of GLUT4 and GLUT1 on sarcolemmal membranes (15). Moreover, inhibition of fatty acid oxidation in cardiomyocytes increased glucose transport, and the increase was due in part to enhanced intrinsic activity of GLUT4 (16,17). Thus, it is probable that the increased fatty acid utilization in the hearts of *ob/ob* mice even under low-palmitate conditions contributes to the impaired glycolysis in these hearts. Myocardial contraction is associated with translocation of GLUT4 transporters to the plasma membrane (18). The possibility therefore exists that a generalized defect in GLUT4 translocation might be present in the hearts of *ob/ob* mice. Additional mechanisms that could also impair glycolytic flux in these hearts include independent effects on other rate-limiting steps involved in glycolysis, such as hexokinase and phosphofructokinase, and will be the subject of future investigations.

Ob/ob mouse hearts exhibited a fixed defect in glucose oxidation rates. Rates of glucose oxidation were not modulated by insulin and were already maximally impaired, even under low-palmitate perfusion conditions. Perfusion of control hearts with insulin results in a reduction in fatty acid utilization and an increase in glucose utilization. It is not clear whether the stimulatory effect of insulin on glucose oxidation in the heart is secondary to the ability of insulin to reduce fatty acid utilization or vice versa. It is clear, however, that the ability of insulin to alter both fatty acid and glucose utilization was completely absent in *ob/ob* hearts that were perfused under low-palmitate conditions. The integrated and coordinated regulation of fatty acid and glucose metabolism in the heart is

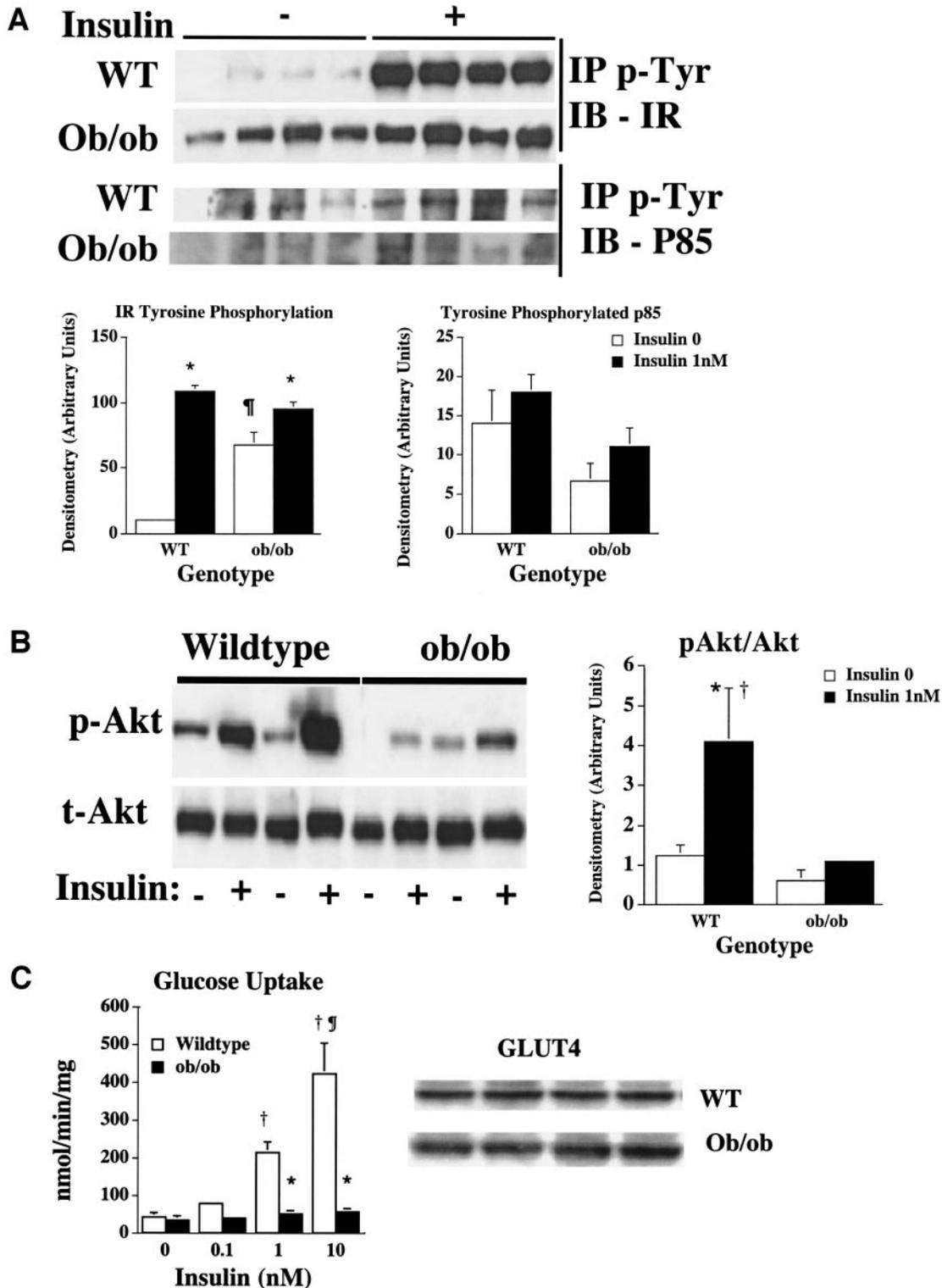


FIG. 4. Insulin signaling and glucose uptake. *A*: Top panel shows immunoblots of phosphotyrosine immunoprecipitates (IP *p*-Tyr) obtained from working hearts perfused for 15 min with (+) or without (-) 1 nmol/l insulin and blotted with antibodies to the IR and the p85 subunit of phosphatidylinositol-3 kinase. Bottom panel shows densitometric analyses of these blots. **P* < 0.01 vs. 0 insulin of the same genotype, †*P* < 0.001 vs. wild type (WT) 0 insulin and WT 1 nmol/l insulin. Wild type, *n* = 3; *ob/ob*, *n* = 4. *B*: Representative Western blots showing protein levels of phosphorylated Akt (pAkt) and total levels of Akt (t-Akt) in working hearts perfused for 15 min with (+) or without (-) 1 nmol/l insulin (*left*). Densitometric analysis of Akt blots showing the ratio of pAkt to Akt in immunoblots obtained from four hearts per genotype and treatment, respectively (*right*); **P* < 0.01 vs. WT 0 insulin, and †*P* < 0.01 vs. *ob/ob* ± insulin. *C*: Glucose uptake was measured in isolated cardiomyocytes obtained from 9-week-old *ob/ob* mice and C57BL/6J controls (wild type), incubated with insulin at the concentrations shown (*left*). **P* < 0.0004 vs. wild type incubated at the same insulin concentration; †*P* < 0.003 vs. wild type at 0 and 0.1 nmol/l insulin; ‡*P* < 0.0001 vs. wild type at 1.0 nmol/l. Data are from cardiomyocytes isolated from one heart per genotype in three separate experiments (six mice total), and transport measurements at each insulin dose were performed in triplicate. GLUT4 immunoblots of whole-heart homogenates from four wild-type and four *ob/ob* mice, respectively, demonstrate no difference in GLUT4 protein content (*right*).

believed to be mediated via the allosteric regulation of CPT-1 activity by malonyl CoA (19). Malonyl CoA is generated by acetyl CoA carboxylase and degraded by malonyl CoA decarboxylase (MCD). The increased fatty acid utilization in *ob/ob* mouse hearts is consistent with activation of the peroxisome proliferator-activated receptor- α regulatory pathway (20), and MCD is a known peroxisome proliferator-activated receptor- α target (19). Thus, the possibility exists that increased activity of MCD in *ob/ob* mouse hearts could chronically reduce malonyl CoA levels, leading to constant activation of CPT-1.

A second mechanism for the reduction in glucose oxidation rates could be reduced flux through pyruvate dehydrogenase. This possibility is supported by observations that were made in the hearts of Zucker diabetic fatty rats shortly after the onset of diabetes. In those studies, Chatham and Seymour (5) reported that the reduction in glucose oxidation rates in Zucker diabetic fatty rats was associated with reduced flux through pyruvate dehydrogenase. The possibility that impaired insulin signaling in the cardiomyocytes of *ob/ob* mice could directly impair the oxidative capacity of cardiac mitochondria for glucose should also be entertained. The 30% reduction (relative to controls) in rates of glucose oxidation in *ob/ob* hearts that were perfused in the absence of insulin are similar to findings that we obtained in the hearts of mice with cardiomyocyte-restricted deletion of insulin receptors (CIRKO) (7). In CIRKO hearts, the reduction in glucose oxidation was not associated with reduced rates of glycolysis. Thus, the defect seemed to be primarily in pathways that mediate the oxidative metabolism of glucose. Thus, impaired insulin signaling per se could also contribute to defective glucose oxidation in *ob/ob* mouse hearts.

Fatty acid oxidation rates were increased in the hearts of *ob/ob* mice, even at low-palmitate concentrations. The increase in fatty acid metabolism was associated with a striking increase in MVO_2 . Despite the increase in MVO_2 , cardiac performance was reduced in *ob/ob* mouse hearts relative to controls under low-palmitate conditions. These observations suggest that the mitochondria of *ob/ob* mice are uncoupled. The mechanism for uncoupling remains to be elucidated as we did not observe any change in the protein content of uncoupling protein-3 in *ob/ob* hearts relative to controls (data not shown), and we do not detect significant amounts of uncoupling protein-2 in the mouse heart. However, chronically elevated delivery of fatty acids (from extrinsic sources as well as from intrinsic myocardial triglyceride stores) could lead to increased uncoupling of the mitochondria in *ob/ob* mouse hearts. The implication of increased mitochondrial uncoupling would be reduced myocardial ATP production, which would be consistent with the reduction in cardiac performance. We did not measure the content of high-energy phosphates in the hearts of *ob/ob* mice in this study. However, a recent study performed in individuals with type 2 diabetes revealed that patients with diabetes had significantly lower myocardial phosphocreatine/ATP ratios than the healthy volunteers, and the phosphocreatine/ATP ratio was inversely correlated with serum concentrations of FFAs (21). Thus, one mechanism for myocardial dysfunction, despite increased rates of fatty acid metabolism that develop in the hearts of *ob/ob* mice and indeed in all of the

other models of diabetes in which this has been examined, could be reduced myocardial energetics on the basis of mitochondrial uncoupling.

MVO_2 and fatty acid oxidation rates increased further in the *ob/ob* hearts under high-palmitate conditions, yet cardiac performance declined modestly, and this was true regardless of whether insulin was present. In contrast, wild-type hearts exhibited a precipitous decline in performance under conditions of increased fatty acid supply. We believe that this is due in part to the significant negative effect of increased palmitate oxidation on glucose utilization in these hearts. Increased mitochondrial uncoupling as evidenced by the modest increase in MVO_2 , and reduced myocardial efficiency in wild-type hearts (high palmitate, no insulin) could also play a role. Myocardial performance declined further in high-palmitate-perfused wild-type hearts in the presence of 1 nmol/l insulin. We believe that this reflects the combined effect of reduction in glucose utilization, coupled with the additional effect of insulin to suppress fatty acid oxidation rates. The net effect was a reduction in substrate availability, as evidenced by the reduction in MVO_2 . This contrasts with the low palmitate plus insulin perfusion conditions in which glucose utilization and myocardial efficiency are optimal in wild-type hearts. Thus, the reduction in myocardial efficiency in insulin-perfused high-palmitate wild-type hearts is due to the relatively greater decrease in myocardial work, whereas in *ob/ob* hearts that are perfused under similar conditions, the reduction in cardiac efficiency is a reflection of the increased MVO_2 . The divergent responses of *ob/ob* and wild-type hearts to increased fatty acid delivery and hyperinsulinemia indicate that chronic adaptations have developed to maintain cardiac performance under such conditions in the hearts of *ob/ob* mice. These adaptations are absent in wild-type hearts when they are presented with an acute increase in fatty acid delivery coupled with hyperinsulinemia.

Some limitations of our study need to be acknowledged. Our analysis was limited to two substrates. Furthermore, there is evidence that a major source of myocardial fatty acids in vivo derives from the hydrolysis of lipoproteins by lipoprotein lipase (22,23). Our experiments were performed at two FFA concentrations that encompass the dynamic range of FFAs observed in control animals. Perfusions were not performed at the highest levels of FFAs (2.6 mmol/l), which were observed in *ob/ob* mice. The reduction in cardiac performance in the presence of increased palmitate, observed in control hearts, could reflect direct cardiotoxic effects of palmitate on isolated hearts ex vivo, and it is possible that such dramatic reductions in cardiac function might not occur in control animals under peak fed conditions, when FFA concentrations might be as high as 1.4 mmol/l. Second, the insulin concentrations used in the perfusions and signaling studies more closely mimic levels in *ob/ob* mice, as opposed to those that occur in control mice in vivo. Nevertheless, our overall conclusions are bolstered by recent in vivo observations in humans with morbid obesity that provided evidence for increased myocardial fatty acid utilization and oxygen consumption and decreased cardiac efficiency (24).

In conclusion, we have presented the first comprehen-

sive metabolic characterization of the hearts of *ob/ob* mice. In terms of metabolic substrate utilization, *ob/ob* hearts are characterized by decreased glucose utilization and increased fatty acid utilization. Whereas wild-type hearts are unable to adapt acutely to increased fatty acid delivery and hyperinsulinemia, our study reveals that hearts from *ob/ob* mice exhibit numerous adaptations that maintain high oxygen consumption rates and fatty acid oxidation rates in the milieu of increased fatty acid supply and hyperinsulinemia. Thus, myocardial function (although reduced) is maintained, albeit with decreased efficiency.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (HL 73167, U01-70525), the American Diabetes Association (ADA), and the Ben and Iris Margolis Foundation. E.D.A. is an Established Investigator of the American Heart Association. B.T.O. is supported by a physician scientist-training award from the ADA. M.W.R. was supported by a summer undergraduate fellowship from the American Heart Association, Western Affiliates. S.B. is supported by a postdoctoral fellowship from the Juvenile Diabetes Foundation.

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