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Reduced Cardiac Efficiency and Altered Substrate Metabolism Precedes the Onset of Hyperglycemia and Contractile Dysfunction in Two Mouse Models of Insulin Resistance and Obesity

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Hyperglycemia is associated with altered myocardial substrate use, a condition that has been hypothesized to contribute to impaired cardiac performance. The goals of this study were to determine whether changes in cardiac metabolism, gene expression, and function precede or follow the onset of hyperglycemia in two mouse models of obesity, insulin resistance, and diabetes (*ob/ob* and *db/db* mice). *Ob/ob* and *db/db* mice were studied at 4, 8, and 15 wk of age. Four-week-old mice of both strains were normoglycemic but hyperinsulinemic. Hyperglycemia develops in *db/db* mice between 4 and 8 wk of age and in *ob/ob* mice between 8 and 15 wk. In isolated working hearts, rates of glucose oxidation were reduced by 28–37% at 4 wk and declined no further at 15 wk in both strains. Fatty acid oxidation rates and myocardial oxygen consumption were increased in 4-wk-old mice of both strains. Fatty acid oxidation rates progressively increased in *db/db* mice in parallel with the earlier onset and greater duration of hyperglycemia. *In vivo*, cardiac catheterization revealed significantly increased left ventricular contractility and relaxation (positive and negative dP/dt) in both strains at 4 wk of age. dP/dt declined over time in *db/db* mice but remained elevated in *ob/ob* mice at 15 wk of age. Increased β-myosin heavy chain isoform expression was present in 4-wk-old mice and persisted in 15-wk-old mice. Increased expression of peroxisomal proliferator-activated receptor-α regulated genes was observed only at 15 wk in both strains. These data indicate that altered myocardial substrate use and reduced myocardial efficiency are early abnormalities in the hearts of obese mice and precede the onset of hyperglycemia. Obesity *per se* does not cause contractile dysfunction *in vivo*, but loss of the hypercontractile phenotype of obesity and up-regulation of peroxisomal proliferator-activated receptor-α regulated genes occur later and are most pronounced in the presence of long-standing hyperglycemia. (*Endocrinology* 146: 5341–5349, 2005)

CARDIOVASCULAR DISEASE REMAINS the leading cause of mortality and morbidity in individuals with type 2 diabetes and the metabolic syndrome. As the global incidence of obesity and diabetes increases, it is anticipated that the burden of cardiovascular disease will continue to rise (1). The pathophysiology of cardiac disease in insulin-resistant states such as diabetes and obesity is complex and involves an increased incidence of coronary atherosclerosis, hypertension, and hypercoagulability (1, 2). Nevertheless, there is also an independent increase in the risk of congestive heart failure independent of the presence of underlying macroscopic coronary disease (3). Many hemodynamic abnormalities have been described in obesity, including elevated cardiac output, altered vascular reactivity, hypertension, and diastolic dysfunction (4). However, many of the basic mechanisms for cardiac dysfunction in insulin-resistant states remain partially understood.

A large body of work has suggested that altered myocardial substrate metabolism may contribute to contractile dysfunction in the hearts of diabetics (5, 6). Most studies have been performed in models of insulin-deficient (type 1) diabetes (7), and fewer studies have been performed in models of type 2 diabetes. For example, altered substrate use, namely reduced rates of glucose use (glycolysis and glucose oxidation) with a concomitant increase in rates of fatty acid (FA) oxidation were reported in isolated perfused hearts obtained from 12-wk-old *db/db* mice and 8-wk-old *ob/ob* mice (8, 9). Fewer studies have examined the temporal relationship between changes in cardiac metabolism and the onset of hyperglycemia. Aasum *et al.* (10) reported that 6-wk-old *db/db* mice did not exhibit elevated levels of glucose in random-fed animals. In hearts from these mice that were perfused in the absence of insulin and under relatively low FA concentrations, FA oxidation rates were elevated, but glucose oxidation rates were normal, whereas cardiac function was relatively preserved. The transition to hyperglycemia occurs at approximately 6 wk of age in *db/db* mice; thus, it is possible that these animals were already glucose intolerant. Contrac-

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Abbreviations: acyl CoA, Acyl-coenzyme A; FAT/CD36, fatty acid translocase; FA, fatty acid; FFA, free fatty acid; LCAD, long chain acyl CoA dehydrogenase; LV, left ventricular; MCAD, medium chain acyl CoA dehydrogenase; MHC, myosin heavy chain; MVO_2, myocardial oxygen consumption; PDK, pyruvate dehydrogenase kinase; PGC, PPARγ coactivator; PPAR, peroxisome proliferator activated receptor; UCP, uncoupling protein; VLCAD, very long chain acyl CoA dehydrogenase.

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tile dysfunction and/or reduced rates of carbohydrate oxidation have been described *in vivo* and *in vitro* in the hearts of Zucker fatty rats, a model of obesity and type 2 diabetes (11–13). In these studies the changes in carbohydrate metabolism were observed shortly after the onset of diabetes, and the degree of hyperglycemia in this model is less severe than is the case in the studies of *db/db* mice. Thus, analysis of the cardiac phenotypes of obese and insulin-resistant mice, well before the onset of hyperglycemia, remains to be performed. Given the fact that hyperinsulinemia precedes the development of hyperglycemia in all of these models, it is possible that generalized insulin resistance *per se* may play a role in their cardiac phenotypes.

Acute changes in the expression of genes involved in the regulation of contractile function and substrate metabolism have been described in the hearts of animals with insulin-deficient diabetes (14, 15) and may precede the development of overt contractile dysfunction. Consistent changes include increased expression of the fetal myosin isoform β-myosin heavy chain (MHC), increased expression of uncoupling protein (UCP) 3, and increased expression of pyruvate dehydrogenase kinase (PDK) 4. Increased expression levels of genes involved in the mitochondrial β-oxidation of fatty acids have been observed in some but not other studies (14, 16). In models of type 2 diabetes, there may be an imbalance in the expression of genes involved in fatty acid import and mitochondrial fatty acid β-oxidation such that FA entry into the myocyte might exceed mitochondrial oxidative capacity. This has been postulated to contribute to the increase in triglyceride accumulation in the hearts of these models (13). We have shown that selective disruption of insulin signaling in cardiomyocytes results in increased expression of βMHC and reduced expression of genes involved in mitochondrial β-oxidation (17). Thus, it is likely that impaired insulin signaling and hyperglycemia might have divergent or distinct effects on the regulation of gene expression in the myocardium.

The goal of these studies was to identify the early changes that occur in the hearts of obese and insulin-resistant rodent models at a time point that clearly precedes the onset of hyperglycemia. We also chose to study two independent models of leptin deficiency or resistance that differ in the timing of the transition from normoglycemia to glucose intolerance to overt hyperglycemia. Here we show that as early as 4 wk of age, the hearts of *ob/ob* and *db/db* mice exhibit decreased glucose oxidation rates, increased rates of FA oxidation and myocardial oxygen consumption (MVO_2), and decreased cardiac efficiency. These changes are associated with myosin isoform switching but with normal expression levels of peroxisomal proliferator-activated receptor (PPAR)-α regulated genes. Of interest, *in vivo* analysis of cardiac function reveal hypercontractile left ventricular (LV) function, which declines only after hyperglycemia develops, and has been present for more than 6 wk. Thus, altered cardiac metabolism is an early characteristic of leptin-deficient mouse models, precedes the development of hyperglycemia, and appears to develop independently of changes in the expression of PPARα regulated genes.

Materials and Methods

Animals

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 C57BL6/KsJ-*lepr*^{db} *(db/db)*, and their respective wild-type controls (C57BL6/J and C57BL/KsJ), were obtained at ages 4 and 6 wk of age from Jackson Laboratories (Bar Harbor, ME). Four-week-old mice were studied immediately, and 6-wk-old mice were housed until they attained the age of 8 or 15 wk at which time they were studied. The animals were fed standard laboratory chow and housed in temperature-controlled facilities with a 12-h light, 12-h dark cycle. All *ex vivo* cardiac studies were performed on hearts obtained from random-fed mice.

Glucose tolerance tests and the determination of serum concentrations of insulin, free fatty acids (FFAs), and triglycerides

Glucose tolerance tests were performed after a 6-h fast. A glucose bolus was injected ip (1 mg/g body weight) and blood samples 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 one-touch test strips (Lifescan; Johnson & Johnson Co., Milpitas, CA). Concentrations of insulin, FFAs, and triglycerides were measured at 0500 h (peak feeding) and after a 6-h fast. Serum insulin concentrations were measured by RIA using the sensitive rat insulin RIA kit (Linco Research Inc., St. Charles, MO). Insulin assays were performed in duplicate 25-μl samples. FFA concentrations were determined in duplicate 50-μl serum samples that were obtained from all animals at 48 h or greater before performing the glucose tolerance tests. FFA concentrations were measured using the 1/2-micro-fatty acid test kit (Roche Diagnostics, Mannheim, Germany). Triglyceride concentrations were determined in 10-μl serum samples using the L-type TG H kit from Wako (Richmond, VA).

Determination of in vivo cardiac contractility

Mice were anesthetized with 1–1.5% isoflurane. LV pressure was then measured with a temperature-calibrated 1.4 Fr micromanometer-tipped catheter (Millar Instruments, Houston, TX) inserted through the right carotid artery and analyzed as previously described (18).

Substrate metabolism in isolated working mouse hearts

Cardiac metabolism was measured in hearts isolated from 4- and 15-wk-old male *ob/ob, db/db* mice and their age-matched controls. All hearts were prepared and perfused in the working mode, using protocols that have been previously described by our group (9). In brief, the working heart buffer was Krebs Henseleit buffer containing (in millimoles) 118.5 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 0.5 EDTA, and 11 glucose, gassed with 95% O_2-5% CO_2 and supplemented with 1.0 mM palmitate bound to 3% BSA in the presence of 1 nM insulin to mirror the plasma concentrations of FFAs and insulin in *db/db* and *ob/ob* mice. Glycolytic flux was determined by measuring the amount of ^3H_2O released from the metabolism of exogenous [5-^{3}H]glucose (specific activity 400 MBq/mol). Glucose oxidation was determined by trapping and measuring $^{14}CO_2$ released by the metabolism of [U-^{14}C]glucose (specific activity 400 MBq/mol). Palmitate oxidation was determined in separate perfused hearts by measuring the amount of ^3H_2O released from [9,10-^{3}H]palmitate (specific activity 18.5 GBq/mol). MVO_2 and cardiac efficiency were determined as previously described (9).

Quantitative RT-PCR

Hearts were obtained from randomly fed 4- and 15-wk-old *db/db*, *ob/ob* mice and their age-matched controls, placed in RNAlater (Ambion, Austin, TX) and then frozen at −80 C. Tissues were homogenized, and total RNA was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Chloroform was added to the homogenate, and the RNA-containing aqueous phase

was further purified using the RNAeasy total RNA isolation kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Three micrograms of each total RNA were synthesized to cDNA using Superscript TMII RNase H-reverse transcriptase (Invitrogen) using the manufacturer's protocols and oligo dT primers. Quantitative real-time PCR was performed with 8 ng cDNA as the template as previously described by our group (18). Transcript levels for the constitutive housekeeping gene product cyclophilin were also quantitatively measured in each sample and used to normalize the transcript data obtained. Data are expressed as the fold change relative to values obtained in age-matched wild-type mice. The transcripts that were analyzed and their respective primer sequences (forward primer and reverse primer) are summarized in Table 1.

Statistical analysis

Data are expressed as mean ± SEM. All differences except for mRNA levels were analyzed by ANOVA, and significance was assessed by Fisher's protected least significant difference test or the unpaired *t* test. Gene expression data were analyzed using the nonparametric Mann Whitney *U* test because gene expression levels were not always normally distributed. 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 Inc., Cary, NC).

Results

Systemic metabolic parameters in ob/ob and db/db mice

At 4 wk of age, fasting serum glucose concentrations of *db/db* and their genetic C57BLKsJ controls were similar (103 ± 4 and 119 ± 6 mg/dl, respectively, $P = 0.1$). By 8 wk of age, *db/db* mice were severely hyperglycemic with fasting serum glucose concentrations of 277 ± 40 *vs.* 81 ± 19 mg/dl in controls ($P < 0.0001$). In contrast, fasting glucose concentrations in 4-wk-old *ob/ob* mice, although significantly higher than their age-matched controls (130 ± 13 *vs.* 57 ± 8 mg/dl), did not achieve diabetic levels. We have also previously shown that at 8 wk of age, fasting glucose concentrations in *ob/ob* mice (120 ± 6 mg/dl) are similar to values in 4-wk-old mice (9). However, by 15 wk of age, *ob/ob* mice were frankly hyperglycemic with fasting plasma glucose concentrations of 220 ± 26 *vs.* 146 ± 5 mg/dl ($P < 0.05$). The glucose tolerance test results as summarized in Fig. 1 also confirm the transition from normal glucose tolerance to severe diabetes in *db/db* mice between 4 and 8 wk of age. Glucose tolerance was modestly impaired in 4-wk-old *ob/ob*, and the 4-wk glucose tolerance test results are similar to those previously reported by us in 8-wk-old *ob/ob* mice (9). Severe hyperglycemia is present at 15 wk in *ob/ob* mice, indicating a more delayed onset of hyperglycemia relative to *db/db* mice. Table 2 summarizes the body weights, fed and fasting concentrations of insulin, triglycerides, and FFAs in both groups of mice at 4 and 15 wk of age. It is evident from these data that in relation to their genetic controls, obesity and hyperinsulinemia are present in both groups of mice as early as 4 wk of age and become progressively more severe as they age. FFA and triglyceride concentrations were not increased in 4-wk-old *ob/ob* and *db/db* mice. However, at 15 wk, fed concentrations of serum triglycerides were significantly increased in both strains of mice relative to their age-matched controls. Moreover, triglyceride concentrations in *db/db* mice were 2.2-fold higher in 15-wk *db/db* mice *vs. ob/ob* mice of similar age. FFA concentrations were increased in 15-wk *db/db* relative to age-matched controls but were not elevated in 15-wk-old *ob/ob* mice.

TABLE 1. Transcripts analyzed and sequences of primers used

Transcript	Primer sequence (5′–3′)
α-MHC	tggagacattggccagtaca
α-MHC	tcccaagttgtgtttttctgc
β-MHC	gacgaggcagagcagatcgc
β-MHC	gggcttcacaggcatccttaggg
PPAR-α	actacggagttcacgcatgtg
PPAR-α	ttgtcgtacaccagcttcagc
PGC1-α	atgtgtcgccttcttgctct
PGC1-α	atctactgcctggggacctt
PDK4	ttcacaccttcaccacatgc
PDK4	aaagggcggttttcttgatg
MCAD	tggcatatgggtgtacaggg
MCAD	ccaaatacttcttcttctgttgatca
LCAD	tcctcccgatgttctcattc
LCAD	tttctctgcgatgttgatgc
VLCAD	tatctctgcccagcgacttt
VLCAD	tgggtatgggaacacctgat
UCP2	tcatcaaagatactctcctgaaagc
UCP2	tgacggtggtgcagaagc
UCP3	tgctgagatggtgacctacga
UCP3	ccaaaggcagagacaaagtga
Cyclophylin	agcactggagagaaaggatttgg
Cyclophylin	tcttcttgctggtcttgccatt

Forward primer is listed first and reverse primer second.

Substrate metabolism, myocardial oxygen consumption, and cardiac performance in isolated working hearts from ob/ob and db/db mice

Substrate metabolism and cardiac performance were determined in hearts from 4-wk-old *ob/ob* and *db/db* mice at a point in time when *db/db* mice were entirely normoglycemic and *ob/ob* were mildly glucose intolerant. Studies were then repeated in 15-wk-old mice at a time when *db/db* mice would have been severely hyperglycemic for at least 8 wk and *ob/ob* mice for a shorter period. In 4-wk-old mice, rates of palmitate oxidation were significantly increased by 1.54- and 1.62-fold in *ob/ob* and *db/db* hearts, respectively, relative to their genetic controls (Fig. 2A). Palmitate oxidation rates were not statistically different between 4-wk-old *ob/ob* and *db/db* mice. Insulin-stimulated rates of glucose oxidation were reduced by 28% relative to controls in the hearts of *ob/ob* mice and by 37% in *db/db* hearts (Fig. 2B). Rates of glycolysis were similar (Fig. 2C). These changes in substrate metabolism were associated with significantly elevated rates of myocardial oxygen consumption in *ob/ob* and *db/db* mice relative to their respective controls (Fig. 3A), leading to a significant reduction in myocardial efficiency (Fig. 3B). Metabolic changes in 15-wk-old *ob/ob* mice were similar to those observed at 4 wk of age (Figs. 2 and 3). This was also true for *db/db* mice (Figs. 2 and 3) with the exception of palmitate oxidation rates that were 1.5-fold higher in 15-wk-old *db/db* mice *vs.* 4-wk-old *db/db* mice ($P < 0.002$, Fig. 2A). Also, although MVO_2 remained increased in 15-wk *db/db* mouse hearts relative to age-matched controls, the extent of the increase was greater in younger mice. In 4-wk-old mice, cardiac power was increased in *ob/ob* and *db/db* mice relative to their age-matched controls, but this relative increase in cardiac performance was no longer apparent at 15 wk (Table 3).

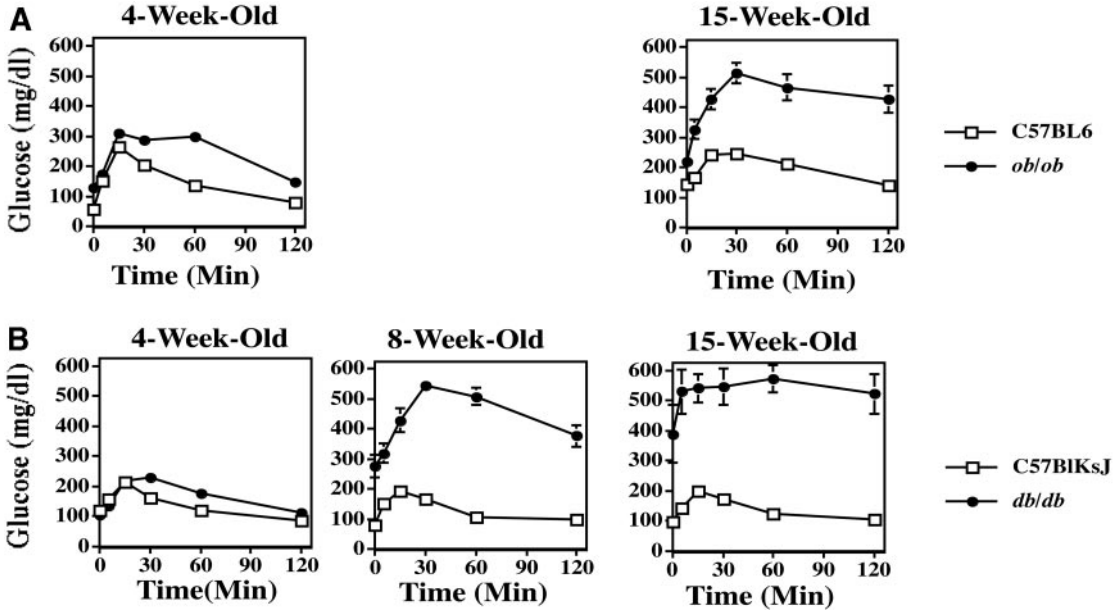

FIG. 1. Glucose tolerance tests in *ob/ob* and *db/db* mice. A, Glucose tolerance tests in *ob/ob* and C57BL6/J mice were performed at the ages as indicated. In 4-wk-old mice, glucose concentrations were significantly higher ($P < 0.05$) in *ob/ob* mice at times 0, 30, 60, and 120 min but not at 5 or 15 min. In 15-wk-old mice, glucose concentrations were significantly higher at all time points with $P < 0.05$ *vs.* controls at time 0 and $P < 0.001$ *vs.* controls at all other time points. Numbers of animals: 4 wk −*ob/ob* = 10, C57BL6/J = 9; and 15 wk −*ob/ob* = 8, C57BL6/J = 8. B, Glucose tolerance tests in *db/db* and C57BL/KsJ mice were performed at the ages as indicated. In 4-wk-old mice, glucose concentrations were significantly higher than controls ($P < 0.05$) at 30, 60, and 120 min but not at the other time points. In 8- and 15-wk-old mice, glucose concentrations were significantly higher ($P < 0.0001$) at all time points. Numbers of animals: 4 wk −*db/db* =10, C57BL/KsJ = 6; 8 wk −*db/db* = 10, C57BL/KsJ = 10; and 15 wk −*db/db* = 6, C57BL/KsJ = 6.

In vivo cardiac performance

To determine the relationship between *in vivo* cardiac function and *ex vivo* measurements of cardiac function and metabolism, we directly measured LV pressures in anesthetized mice (Table 4 and Fig. 4). In *ob/ob* mice, LV systolic pressures were increased at 4 and 15 wk, compared with controls, and there were no significant changes in LV end diastolic pressures. The rate of increase in LV pressure during systole (+dP/dt) was significantly increased in *ob/ob* mice at all ages. The rate of LV relaxation (−dP/dt) was also greater in 4-wk-old *ob/ob* hearts *in vivo*. In contrast, LV systolic pressure in *db/db* mice tended to be higher at 4 wk and was significantly increased at 8 wk but tended to be reduced in 15-wk-old mice. An increase in +dP/dt was present at 4 and 8 wk in *db/db* mice, but LV contractility decreased in 15-wk-old *db/db* mice. Similarly, whereas LV relaxation was enhanced in 4-wk-old *db/db* mice, there was a progressive reduction over time, and −dP/dt was significantly reduced in 15-wk-old *db/db* mice relative to age-matched controls (Table 3 and Fig. 4). Thus, *db/db* mice exhibit initial hypercontractility followed by a decline in LV function that appears to parallel the earlier onset of hyperglycemia. In contrast, *ob/ob* mice maintain a hyperdynamic state up to 15 wk of age. The function of *ob/ob* hearts gradually decline and become similar to that observed in control animals between 30 and 40 wk of age (data not shown).

Analysis of gene expression in hearts of ob/ob and db/db mice

We finally sought to determine the transcriptional adaptations of *ob/ob* and *db/db* mouse hearts in the transition from insulin resistance and normoglycemia to overt hyperglycemia, focusing primarily on MHC isoform expression and the expression of genes that are regulated by PPARα. In the adult mouse heart, αMHC represents the predominant isoform and the ratio of αMHC to βMHC expression is greater than 1 (19). In both models, myosin isoform expression maintained a fetal pattern with predominant expression of the βMHC isoform. Thus, αMHC expression was reduced at 4 and 15 wk in both groups of animals. βMHC expression was increased at both ages relative to controls in *db/db* hearts and did not change as the animals aged. The increase in βMHC expression in *ob/ob* mice was most noticeable in 4-wk-old animals (Fig. 5A), and although the expression apparently fell at 15-wk, the ratio of βMHC to αMHC was still reversed because of the greater reduction in αMHC expression.

With regard to PPARα-regulated genes [PDK4, medium-chain acyl-coenzyme A (acyl CoA) dehydrogenase (MCAD), long-chain acyl CoA dehydrogenase (LCAD), very long-chain acyl CoA dehydrogenase (VLCAD), UCP2, and UCP3], there was relatively little change in mRNA levels in 4-wk-old mice, indeed for some transcripts such as PDK4, MCAD, and UCP3, expression levels were actually lower in 4-wk-old *ob/ob* mice relative to age-matched controls (Fig. 5, B–D). However, there was a progressive increase in the expression levels of all genes in *db/db* mice and most genes in *ob/ob* relative to their controls as the animals aged. In general, the degree of transcriptional up-regulation of genes was greater in 15-wk *db/db* mice than in 15-wk −*ob/ob* mice, indicating a more robust degree of PPARα activation in *db/db* mice that parallels the earlier onset and greater severity of hyperglycemia and higher concentrations of FFAs and triglycerides.

TABLE 2. *In vivo* metabolite concentrations

	ob/ob		*ob/+*		*db/db*		*db/+*	
	4 wk	15 wk	4 wk	15 wk	4 wk	15 wk	4 wk	15 wk
Insulin, fast	0.47 ± 0.05 (6)	1.20 ± 0.34^{a,b} (6)	0.05 ± 0.01 (8)	0.03 ± 0.01 (5)	0.72 ± 0.18^{a} (5)	0.76 ± 0.25^{a} (4)	0.06 ± 0.01 (5)	0.05 ± 0.01 (4)
Insulin, fed	1.19 ± 0.35^{a} (8)	4.76 ± 0.23^{a,b} (6)	0.05 ± 0.01 (9)	0.22 ± 0.06 (5)	2.39 ± 0.13^{a} (4)	1.78 ± 0.41^{a} (5)	0.10 ± 0.02 (5)	0.15 ± 0.03 (4)
FFA, fast	1.01 ± 0.22 (6)	0.97 ± 0.10 (6)	1.07 ± 0.04 (8)	0.79 ± 0.04 (5)	1.25 ± 0.08 (5)	1.27 ± 0.07^{a} (10)	1.04 ± 0.08 (5)	0.66 ± 0.05^{b} (10)
FFA, fed	1.04 ± 0.05 (8)	0.76 ± 0.06 (6)	1.20 ± 0.09 (9)	0.68 ± 0.11^{b} (5)	1.05 ± 0.14 (4)	1.52 ± 0.14^{a,b} (11)	1.37 ± 0.26 (4)	0.81 ± 0.07^{b} (10)
TG, fast	1.33 ± 0.12 (6)	1.20 ± 0.13 (6)	1.58 ± 0.15 (8)	1.23 ± 0.09 (5)	1.45 ± 0.09 (5)	1.82 ± 0.10 (10)	1.57 ± 0.05 (5)	1.35 ± 0.08 (10)
TG, fed	2.19 ± 0.29 (8)	4.87 ± 1.15^{b} (6)	2.95 ± 0.30 (9)	3.32 ± 0.51 (5)	2.46 ± 0.09^{a} (4)	9.08 ± 0.96^{a,b} (11)	4.39 ± 0.56 (4)	3.83 ± 0.37 (10)
Body weight	21.9 ± 0.8^{a}	61.3 ± 1.0^{a,b}	16.0 ± 0.9	27.0 ± 0.7^{b}	16.6 ± 1.0^{a}	53.7 ± 0.9^{a,b}	12.0 ± 0.4	25.3 ± 0.7^{b}

Units: Insulin, nM; FFA, mM; triglycerides (TG), mM. Numbers of samples/animals are in *parentheses*. For body weights, n = 12–17.

^{a} $P < 0.05$ *vs.* age-matched control.

^{b} $P < 0.05$ *vs.* 4 wk of same genotype (ANOVA).

FIG. 2. Substrate metabolism in isolated working hearts from 4- and 15-wk-old *ob/ob* and *db/db* mice. Rates of palmitate oxidation (A), glucose oxidation (B), and glycolysis (C) were measured in *ob/ob* mouse hearts (*left panels*) and *db/db* mouse hearts (*right panels*) and their respective controls as indicated (n = 4–5 hearts per genotype). *, $P < 0.05$ *vs.* age-matched controls; †, $P < 0.001$ *vs.* 4-wk-old *db/db* mice; ‡, $P = 0.06$ *vs.* age-matched controls (ANOVA); **, $P < 0.04$ *vs.* age-matched controls (unpaired *t* test). gdw, Gram dry heart weight.

PPARα expression tended to increase in both models, but the degree of induction was less than that observed for PPARα-regulated genes, consistent with increased activation of PPARα by FA ligands. PPARγ coactivator (PGC) 1α expression was increased in 4-wk-old *db/db* mouse hearts but not at 15 wk, and its expression was not increased in *ob/ob* mouse hearts at any age (Fig. 5E).

Discussion

In the present study, we have demonstrated that altered myocardial substrate use clearly precedes the onset of hyperglycemia in two independent models of leptin-deficiency/resistance that initially develop obesity and insulin resistance and then progress to hyperglycemia. These hearts

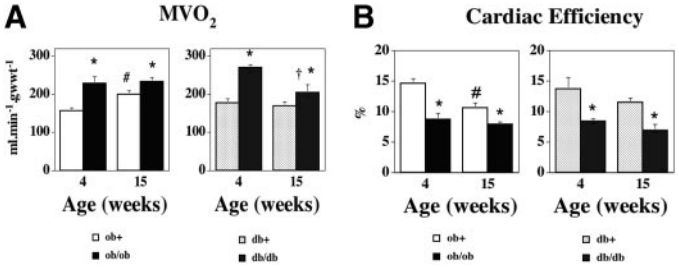

FIG. 3. Oxygen consumption and cardiac efficiency in isolated working hearts from 4- and 15-wk-old *ob/ob* and *db/db* mice. MVO_2 (A) and cardiac efficiency (B) were determined in *ob/ob* mouse hearts (*left panels*) and *db/db* mouse hearts (*right panels*) and their respective controls as indicated (n = 4–5 hearts per genotype). *, $P < 0.04$ *vs.* age-matched controls; #, $P < 0.01$ *vs.* 4-wk *ob/+*; †, $P < 0.0001$ *vs.* 4-wk *db/db* (ANOVA). gwwt, Gram wet heart weight.

are characterized by increased rates of FA oxidation and decreased rates of glucose oxidation. MVO_2 is increased and cardiac efficiency is decreased. The onset of hyperglycemia is associated with relatively modest additional changes in metabolism in these hearts, with the exception of augmented rates of FA oxidation in *db/db* mouse hearts. Of interest, cardiac function (as measured by cardiac power in isolated hearts and dP/dt *in vivo*) is actually increased at this early age. A decline in cardiac performance was observed over time in *db/db* mice, relative to *ob/ob* mice. This may be due in part to the earlier onset of hyperglycemia in *db/db* mice. The increase in FA use and the reciprocal decline in glucose use at an early age in these mice are not associated with evidence for transcriptional up-regulation of many PPARα-regulated genes. Indeed, the up-regulation of PPARα-mediated gene expression appears to occur in relation to the onset of hyperglycemia and hyperlipidemia.

The metabolic milieu of both *ob/ob* and *db/db* mice at 4 wk of age is characterized by hyperinsulinemia. In *db/db* mice there was no change in *in vivo* glucose homeostasis, and glucose homeostasis was marginally perturbed in 4-wk-old *ob/ob* mice. Concentrations of triglycerides and FFAs were not significantly different from controls in both strains. Despite the relatively normal concentrations of serum lipids, rates of FA oxidation are increased and glucose oxidation decreased in a PPARα-independent fashion. Insulin acutely increases glucose use and decreases FA oxidation in the heart. So it is possible that impaired insulin signaling in cardiomyocytes of 4-wk-old *ob/ob* and *db/db* mice could have resulted in increased rates of FA oxidation. However, cardiac insulin signaling (as evidenced by the ability of insulin to phosphorylate Akt) was normal in 4-wk-old *ob/ob* and *db/db* mice but was impaired in 15-wk-old animals (data not shown). Based on these observations, it might be difficult to invoke myocardial insulin resistance as a cause for these early metabolic changes. However, increased FA use in these hearts could be associated with increased acyl CoA synthetase activity that could lead to insulin resistance on the basis of protein acylation for example. Thus, the possibility remains that other aspects of insulin's action on cardiac metabolism, which could be independent of Akt signaling, could be impaired, and these were not formally evaluated in this study.

FFAs are ligands for PPARα signaling. Increased PPARα signaling has been suggested to be an important underlying mechanism that may be responsible for the metabolic changes in the diabetic heart (16), mediating its effects by increasing the expression of enzymes involved in FA mitochondrial β-oxidation (*e.g.* carnitine palmitoyl transferase-1 and acyl CoA dehydrogenases) and increasing the expression of UCPs, which may increase mitochondrial FA flux. In the present study, evidence for increased PPARα signaling was present in 15-wk-old but not 4-wk-old animals. The increase in the expression of PPARα-regulated genes parallels increasing concentrations of FFA in *db/db* mice, increased triglycerides (which are the major source of FFAs to the heart *in vivo*) in both strains, and the development of hyperglycemia. Thus, whereas enhanced PPARα signaling may contribute to increased FA oxidation in the 15-wk animals, it is less likely that this is the case at 4 wk of age. The focused transcriptional analysis that we performed highlights the independence of the metabolic alterations of hearts obtained from 4-wk-old *db/db* and *ob/ob* mice from changes in the expression of PPAR-regulated genes. However, we cannot rule out the involvement of other transcriptional pathways, and future studies using global gene analysis approaches could shed additional mechanistic insights.

Two additional mechanisms that could be responsible for the early changes in substrate metabolism in these models warrant further discussion, namely increased expression or sarcolemmal localization of FA transporters and mitochondrial uncoupling. Insulin has been shown to acutely increase

TABLE 3. Contractile parameters in isolated working hearts

	ob/ob		*ob/+*		*db/db*		*db/+*	
	4 wk	15 wk	4 wk	15 wk	4 wk	15 wk	4 wk	15 wk
Dry Ht Wt	22.67 ± 1.60	37.06 ± 1.34[b]	24.99 ± 1.25	33.41 ± 2.68[b]	20.01 ± 1.40	29.29 ± 1.59[b]	15.51 ± 1.60	30.15 ± 1.95[b]
LVSP	95.50 ± 3.41	99.50 ± 3.40	91.96 ± 3.64	95.46 ± 4.69	80.72 ± 2.05	92.42 ± 3.58[b]	89.04 ± 3.68	93.91 ± 4.44
LVDP	63.59 ± 3.34	61.28 ± 3.87	62.21 ± 3.90	64.11 ± 5.07	49.47 ± 3.11[a]	61.08 ± 4.13[b]	63.04 ± 3.63	64.34 ± 4.24
LVdevP	31.91 ± 1.27	38.22 ± 1.00[a,b]	29.75 ± .95	31.36 ± 1.70	31.25 ± 1.47[a]	31.33 ± 1.88	26.00 ± 1.01	29.56 ± .71
Coronary flow	4.24 ± .19	4.52 ± .18	4.43 ± .42	4.13 ± .17	4.47 ± .29[a]	4.45 ± .24	3.49 ± .20	4.30 ± .12[b]
Aortic flow	6.64 ± .27	7.88 ± .46[b]	6.65 ± .35	8.03 ± .55[b]	6.76 ± .25[a]	6.84 ± .32[a]	5.25 ± .30	8.57 ± .24[b]
Cardiac output	10.88 ± .30	12.39 ± .57[b]	11.08 ± .49	12.16 ± .52	11.23 ± .50[a]	11.29 ± .42[a]	8.73 ± .46	12.87 ± .27[b]
Cardiac power	34.93 ± 1.89	28.42 ± 1.52[b]	29.92 ± 1.78	28.22 ± 2.51	41.42 ± 2.99[a]	27.84 ± 2.19[b]	32.94 ± 1.99	29.05 ± 1.48

Units: Wet heart weight (Ht wt) and dry heart weight, mg; LV systolic pressure (LVSP), LV diastolic pressure (LVDP), LV developed pressure (LVdevP), mm Hg; coronary flow, aortic flow, cardiac output, ml/min; cardiac power, mW/g dry Ht Wt.

[a] $P < 0.05$ *vs.* age-matched control.

[b] $P < 0.05$ *vs.* 4-wk of same genotype (ANOVA).

TABLE 4. *In vivo* hemodynamic characterization of *ob/ob* and *ob+* hearts (*top*) and *db/db* and *db+* hearts (*bottom*)

	4 wk		8 wk		15 wk	
	ob/+ (9)	*ob/ob* (10)	*ob/+* (9)	*ob/ob* (9)	*ob/+* (4)	*ob/ob* (3)
Body weight (g)	19.4 ± 0.3	29.5 ± 0.9[a]	24.3 ± 0.6	47.9 ± 1.0[a]	30.0 ± 1.0	58.9 ± 1.3[a]
Heart weight (mg)	109 ± 2	107 ± 5	130 ± 5	160 ± 7[a]	143 ± 9	154 ± 3
Heart rate (min^{-1})	505 ± 34	531 ± 18	413 ± 16	460 ± 39	525 ± 29	500 ± 40
LVSP (mm Hg)	84 ± 1	97 ± 5[a]	88 ± 3	94 ± 4	94 ± 3	112 ± 6[a]
LVEDP (mm Hg)	8.4 ± 0.7	8.0 ± 1.3	8.0 ± 0.8	12.5 ± 1.8	9.8 ± 2.9	9.3 ± 2.7
+ dP/dt (mm Hg/msec)	6727 ± 381	9834 ± 1049[a]	6158 ± 560	8300 ± 943[a]	8023 ± 578	11915 ± 886[a]
− dP/dt (mm Hg/msec)	−6141 ± 341	−7697 ± 765[a]	−6297 ± 425	−6634 ± 674	−7286 ± 283	−8725 ± 1032

	4 wk		8 wk		15 wk	
	db/+ (6)	*db/db* (8)	*db/+* (9)	*db/db* (8)	*db/+* (5)	*db/db* (6)
Body weight (g)	21.1 ± 0.7	28.1 ± 0.4[a]	26.2 ± 0.3	44.4 ± 1.0[a]	27.4 ± 0.6	50.0 ± 1.0[a]
Heart weight (mg)	114 ± 2	108 ± 2	137 ± 3	142 ± 4	137 ± 5	142 ± 2
Heart rate (min^{-1})	387 ± 26	480 ± 14[a]	460 ± 18	491 ± 13	492 ± 29	410 ± 18[a]
LVSP (mm Hg)	90 ± 4	99 ± 4	91 ± 2	104 ± 4[a]	99 ± 3	91 ± 4
LVEDP (mm Hg)	9.1 ± 0.9	15.7 ± 2.4[a]	6.4 ± 1.7	13.5 ± 2.4[a]	9.8 ± 4.2	14.0 ± 2.5
+ dP/dt (mm Hg/msec)	6339 ± 714	9760 ± 694[a,b]	7147 ± 284	9241 ± 514[a]	8691 ± 1002	7226 ± 686
− dP/dt (mm Hg/msec)	−5572 ± 635	−7144 ± 517[b,c]	−6626 ± 307	−6820 ± 376	−7814 ± 445	−5237 ± 448[a]

LVSP, LV systolic pressure; LVEDP, LV end diastolic pressure; +/− dP/dt, positive and negative first derivative of LV contraction and relaxation, respectively. Numbers of animals are in *parentheses*.

[a] $P < 0.04$ *vs.* age-matched control.

[b] $P < 0.02$ *vs.* 15-wk db/db.

[c] $P = 0.056$ *vs.* age-matched control (ANOVA).

the expression and membrane localization of the fatty acid translocase (FAT)/CD36 via a phosphatidyl inositol-3 kinase/Akt-mediated mechanism (20, 21). In Zucker fatty rats that were normoglycemic but hypertriglyceridemic, FAT/CD36 was constitutively localized to the sarcolemmal membrane, and basal rates of FA uptake were increased (22). In contrast, in lean animals FAT/CD36 was predominantly located in intracellular vesicles under basal conditions. Whereas insulin acutely increased sarcolemmal content of FAT/CD36 in cardiomyocytes from lean animals, it is believed that the persistent hyperinsulinemia in the obese animals causes permanent translocation of these vesicles (22). Myocardial insulin signaling is not impaired in the hearts of 4-wk-old *ob/ob* and *db/db* mice (data not shown); thus, increased FA uptake, on the basis of increased translocation of FA transporters, is a plausible and testable hypothesis to account for increased FA use in these models.

The increased MVO_2 that characterizes the hearts of 4-wk-old *ob/ob* and *db/db* mice raises the possibility that the mitochondria of *ob/ob* and *db/db* mice are uncoupled. Expression levels of UCPs were not increased in the hearts of 4-wk-old mice of both strains. However, recent data suggest that mitochondrial uncoupling can be activated by increased FA flux and even more potently by superoxides (23, 24). Moreover, recent studies in rodents and humans have shown that obesity *per se* is associated with increased oxidative stress (25). Increased UCP3 activity in skeletal muscle *in vivo* is also associated with increased FA oxidation rates (26). Thus, future studies will be needed to evaluate for the potential role of activation of mitochondrial UCPs in altering myocardial substrate use in obesity before development of hyperglycemia.

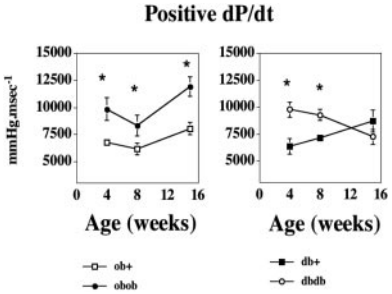

FIG. 4. *In vivo* myocardial contractility in 4-, 8-, and 15-wk-old *ob/ob* and *db/db* mice. Data were obtained in anesthetized mice after LV catheterization with a 1.4 Fr Millar catheter. Numbers of animals for ages 4, 8, and 15 wk, respectively: *ob/ob* n = 10, 9, and 3; *ob/+* n = 9, 9, and 4; *db/db* n = 8, 8, and 6; *db/+* n = 6, 9, and 5. *, $P < 0.04$ *vs.* age-matched controls (ANOVA).

Transcriptional analyses revealed a persistent fetal pattern of MHC gene expression in *ob/ob* and *db/db* mouse hearts that precede the development of hyperglycemia. Thus, obesity *per se* or leptin deficiency can lead to myosin isoform switching in the rodent heart. Similar changes in myosin gene expression have been described in insulin-deficient diabetes (14), hypothyroidism (27, 28), pressure overload hypertrophy and heart failure (27), and cardiac atrophy (29) and were also noted in the hearts of mice with cardiomyocyte-restricted deletion of insulin receptors (17). There is no clear unifying mechanism that can account for the change in myosin isoform expression in these diverse situations. It is possible that myosin isoform switching represents a stereotypic adaptation of the heart to various neurohormonal or metabolic stressors. A common feature of all reported causes of altered myocardial myosin isoform expression are significant changes in myocardial substrate use. Thus, it will be important in future to studies to determine the metabolic or bioenergetic signals that may regulate myosin isoform expression in the heart.

The other striking phenotype of the hearts of young *ob/ob* and *db/db* mice was evidence of increased myocardial con-

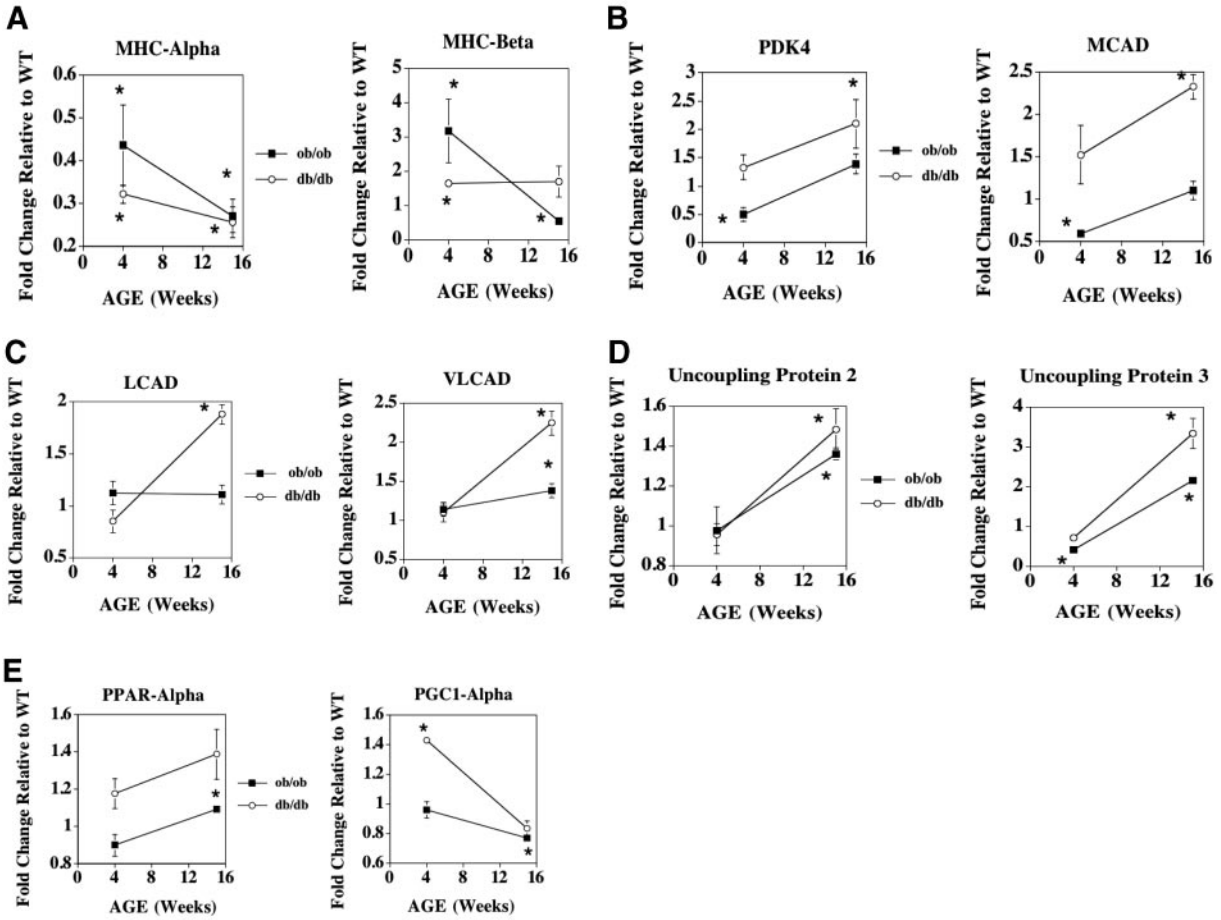

FIG. 5. Expression levels of myosin isoform mRNAs and PPARα-regulated genes. A, Fold change relative to age-matched controls in the expression of the αMHC and βMHC genes at the ages as shown. B, Fold change relative to age-matched controls in the expression of PDK4 and MCAD. C, Fold change relative to age-matched controls in the expression of LCAD and VLCAD. D, Fold change relative to age-matched controls in the expression of the UCP2 and UCP3 genes at the ages as shown. E, Fold change relative to age-matched controls in the expression of the PPARα and PGC1α genes at the ages as shown. For all transcripts *ob/ob* and *db/db* data are represented as *black squares* and *open circles*, respectively. *, $P < 0.05$ *vs.* age-matched controls (Mann Whitney *U* test). Transcript analysis was performed in triplicate in five to six hearts per genotype. WT, Wild type.

tractility. The basis for these cardiovascular changes are likely multifactorial. However, we believe that a likely cause is the myocardial adaptation to increased intravascular volume, which has been shown to occur in obesity (30). Activation of the sympathetic nervous system could also contribute to these changes. However, studies in leptin-deficient rodents suggest that the *ob/ob* mice have reduced central activation of the sympathetic nervous system (31, 32). The increased MVO_2 in 4-wk-old mice indicates that myocardial efficiency is decreased. The implication of this observation is the possibility that myocardial reserve might be decreased. As such it will be of interest to determine whether the hearts of *ob/ob* mice will exhibit an impaired response to inotropic or hypertrophic stressors. Indeed, studies of 10-wk-old *ob/ob* mice revealed a modest reduction in LV systolic function relative to controls after a single ip injection of dobutamine (33). The onset of hyperglycemia is clearly associated with loss of these adaptations in *db/db* mice between 8 and 15 wk and similar changes in *ob/ob* mice after 30 wk of age (data not shown). Although the onset of hyperglycemia is associated with normalization of LV hypercontractility in obese and insulin-resistant animals, it must be emphasized that even before the onset of hyperglycemia, the increased MVO_2 and decreased cardiac efficiency and myosin isoform switch are clear indicators of a vulnerable myocardium. Recent studies in women with morbid obesity revealed remarkably similar results to those described in our study, namely increased cardiac output, increased FA oxidation, increased MVO_2, and decreased cardiac efficiency (34). Taken together, these studies indicate that in obesity and insulin-resistant states, cardiac energy metabolism is compromised before the development of diabetes, and it is likely that efforts to reverse obesity and insulin resistance may reduce the future risk of adverse cardiovascular outcomes.

In summary we have shown that changes in myocardial FA and glucose use and myosin isoform switching are the earliest defects that occur in the context of obesity, insulin resistance, and leptin deficiency/resistance. The hearts are initially hypercontractile, but increased MVO_2 indicates that myocardial efficiency is reduced. Although these early changes are largely independent of PPARα signaling, it is likely that enhanced PPARα signaling will exacerbate these

metabolic disturbances after the onset of hyperglycemia. These early changes may create a vulnerable condition in which additional stresses or insults to the heart might lead to accelerated cardiac decompensation.

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