Regulation of Exogenous and Endogenous Glucose Metabolism by Insulin and Acetoacetate in the Isolated Working Rat Heart

A Three Tracer Study of Glycolysis, Glycogen Metabolism, and Glucose Oxidation

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Abstract

Myocardial glucose use is regulated by competing substrates and hormonal influences. However, the interactions of these effectors on the metabolism of exogenous glucose and glucose derived from endogenous glycogen are not completely understood. In order to determine changes in exogenous glucose uptake, glucose oxidation, and glycogen enrichment, hearts were perfused with glucose (5 mM) either alone, or glucose plus insulin (40 μU/ml), glucose plus acetoacetate (5 mM), or glucose plus insulin and acetoacetate, using a three tracer (^{3}H, ^{14}C, and ^{13}C) technique. Insulin-stimulated glucose uptake and lactate production in the absence of acetoacetate, while acetoacetate inhibited the uptake of glucose and the oxidation of both exogenous glucose and endogenous carbohydrate. Depending on the metabolic conditions, the contribution of glycogen to carbohydrate metabolism varied from 20–60%. The addition of acetoacetate or insulin increased the incorporation of exogenous glucose into glycogen twofold, and the combination of the two had additive effects on the incorporation of glucose into glycogen. In contrast, the glycogen content was similar for the three groups. The increased incorporation of glucose in glycogen without a significant change in the glycogen content in hearts perfused with glucose, acetoacetate, and insulin suggests increased glycogen turnover. We conclude that insulin and acetoacetate regulate the incorporation of glucose into glycogen as well as the relative contributions of exogenous glucose and endogenous carbohydrate to myocardial energy metabolism by different mechanisms. (*J. Clin. Invest.* 1997. 100:2892–2899.) Key words: citric acid cycle • NMR • isotopomer analysis

Introduction

Myocardial substrate use is tightly controlled not only by the availability of substrate and oxygen, but also by the workload imposed on the heart and the hormonal environment. While fatty acid oxidation is the major energy source for the heart under normal conditions in vivo, changes in the above factors can drastically alter the relative contributions of exogenous metabolic fuels to myocardial energy production (1). Glucose plays an important role in myocardial energy metabolism, providing ATP through both glycolysis and oxidation in the citric acid cycle. Numerous studies have demonstrated that the uptake of glucose by the heart is regulated by insulin. Further, the rate of glucose uptake is determined, in part, by the presence of competing substrates (2). Once exogenous glucose enters the cardiac myocyte, it can be metabolized to pyruvate through glycolysis, and then may either be converted to lactate or enter the citric acid cycle. Further, glucose may be used to synthesize glycogen, which, in turn, is also used for energy production (3).

Recent work has suggested that the metabolic fate of exogenous glucose may differ from that of glucose derived from myocardial glycogen stores. Specifically, glycogen is preferentially oxidized while exogenous glucose is preferentially converted to lactate (4, 5). While the amount of exogenous glucose that is used under nonischemic conditions is greater than that of glycogen, it has been estimated that glycogen still accounts for ~ 40% of the energy produced from carbohydrates (5). However, previous studies have not determined if the relative contribution of glycogen to carbohydrate energy metabolism changes with metabolic perturbations. Further, it has been suggested that glycogen acts as a substrate reservoir that is recruited in response to abrupt increases in metabolic demand (3). The energetic advantage of oxidation of glucose compared with glycolysis, combined with the ready metabolic availability of glycogen stores would explain, in part, the importance of maintaining myocardial glycogen stores.

Previous studies have demonstrated the interactions of competing substrates and hormonal stimulation on the metabolic fate of exogenous glucose in the heart (6, 7). However, it has been difficult to analyze several aspects of glucose use (i.e., glycolysis, glycogen metabolism, pyruvate dehydrogenase flux, and citric acid cycle flux) in a single heart together with the relative contributions of exogenous glucose and glucose derived from glycogen to energy production. In order to characterize the effects of different effectors of glucose metabolism, we used a three-tracer (^{3}H, ^{14}C, and ^{13}C) method to determine the metabolic fates of exogenous glucose and glycogen in the isolated heart in the presence of a competing substrate and insulin stimulation. Our findings demonstrate that both acetoacetate and insulin stimulate the incorporation of glucose into glycogen and change the relative contribution of exogenous

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glucose and endogenous carbohydrate to myocardial energy metabolism, but they most likely do so by different mechanisms.

Methods

Materials. The stable isotopes [1-^{13}C]glucose and [2,4-$^{13}C_2$]ethylacetoacetate were purchased from Cambridge Isotope Laboratories (Andover, MA). The [2,4-$^{13}C_2$]acetoacetate ester was hydrolyzed under alkaline conditions and neutralized with hydrochloric acid to form sodium acetoacetate. [2-^{3}H]Glucose and [U-^{14}C]glucose were obtained from Amersham Corp. (Arlington Heights, IL). All analytic chemicals and enzymes were obtained either from Fisher Scientific Co. (Lexington, MA), Sigma Chemical Company (St. Louis, MO), or Boehringer Mannheim Biochemicals (Indianapolis, IN).

Isolated working rat heart preparation. Hearts from fasted, male Sprague-Dawley rats (Harlan Sprague-Dawley Inc., Indianapolis, IN) were isolated after the rats were anesthetized with pentobarbital (0.1 mg/kg, i.p.) and heparinized (300 U, i.v.). Hearts were rinsed in Krebs-Henseleit bicarbonate buffer at 4°C and initially perfused retrogradely with Krebs-Henseleit buffer ([Ca^{2+}]: 0.85 mM) containing glucose (5 mM) to wash out residual blood and allow the resumption of forceful contraction. The isolated working rat heart preparation described earlier (2) was used with modifications that allow the quantitative collection of $^{14}CO_2$ released by the heart (3). Hearts were perfused at 37°C at a preload of 15 cm H_2O and an afterload of 140 cm H_2O with Krebs-Henseleit bicarbonate buffer containing 1% BSA (fraction V, fatty acid free) and equilibrated with 95% O_2/5% CO_2. Hearts were perfused for 60 min with either glucose (5 mM) alone, glucose plus insulin (40 μU/ml), glucose plus acetoacetate (5 mM), or glucose plus insulin plus acetoacetate. In experiments designed to determine the contribution of exogenous glucose to alanine and citric acid cycle flux, [1-^{13}C]glucose (99.9% abundance) was used instead of unlabeled (natural abundance) glucose. In experiments designed to determine the contribution of acetoacetate to citric acid cycle flux, [2,4-$^{13}C_2$]acetoacetate was used instead of unlabeled acetoacetate. Perfusate samples were collected every 5 min for determination of metabolite concentrations. Aortic flow, coronary flow, aortic pressure, and heart rate were measured every 10 min and used to determine cardiac power (8). At the end of the perfusion period, hearts were freeze clamped with aluminum tongs precooled in liquid nitrogen. The hearts were stored at −80°C until further analysis.

^{13}C-NMR spectroscopy of heart extracts. Approximately 1 g of the frozen heart was homogenized in 0.1 N HCl/66% ethanol/0.9 M perchloric acid. The homogenate was centrifuged at 4000 *g* for 10 min and the supernatant decanted. The supernatant was applied to a cation exchange column (AG 50W-X8 [H^+ form]; Bio-Rad Laboratories, Hercules, CA) which was then washed with 0.01 N HCl. Amino acids were then eluted from the column with ammonium hydroxide (5 N), collected and concentrated to dryness in a Speedvac (Savant Instruments, Inc., Farmingdale, NY). The samples were then resuspended in D_2O and the relative ^{13}C enrichments of the amino acids determined using ^{13}C-NMR spectroscopy.

Samples were placed in 5 mm NMR tubes and ^{13}C-NMR spectra were acquired at 125.76 MHz using a standard ^{13}C/^{1}H probe (AM 500; Bruker Instruments, Inc., Billerica, MA). Spectra were acquired using a 30° pulse, quadrature detection, digital resolution of 2.7 Hz per point and with a pulse program for inverse-gated heteronuclear WALTZ decoupling with a delay of 1 s between pulses. The C3 and C4 peaks for glutamate were used for isotopomer analysis to determine relative contribution of [1-^{13}C]glucose and [2,4-$^{13}C_2$]acetoacetate to acetyl-CoA (F_c) (9, 10). The calculation of F_c for glucose was not corrected for pyruvate dilution and therefore represents the contribution of exogenous glucose to the acetyl-CoA pool.

GC/mass spectroscopy of heart extracts. After NMR spectroscopy, a portion of the tissue extract was derivatized to form the *N*-trifluoroacetyl-*n*-butyl esters of the amino acids in order to perform gas chromatography/mass spectroscopy (11). Absolute isotopic enrichments of glutamate and alanine were determined by gas chromatography using a Hewlett-Packard 5890 gas chromatography system (HP-1 capillary column, 12 m × 0.22 mm × 0.33 μm film thickness; Hewlett-Packard Co., Palo Alto, CA). The system was interfaced to a Hewlett-Packard 5971A mass selective detector operating in the positive chemical ionization mode with methane as the reagent gas. The mass isotopomer distribution of alanine m + 1 to m + 3 was determined from the ion intensities of m/z 242–247 and the mass isotopomer distribution of glutamate m + 1 to m + 5 from the ion intensities of m/z 356–363. Because the perfusate glucose was enriched 99.9% in the C1 position with ^{13}C, the contribution of exogenous glucose to the pyruvate pool could be determined by multiplying the absolute enrichment of the C3 position of alanine by two in order to account for the two pyruvate molecules that arise from one molecule of glucose via glycolysis. In addition, relative rates of pyruvate entry into the citric acid cycle through PDH flux were also determined based on the steady state [4-^{13}C]glutamate/[3-^{13}C]alanine enrichment (C4-GLU/C3-ALA) ratio (12–14).

Metabolite determination. Rates of uptake and phosphorylation of exogenously supplied glucose were based on the production of $^{3}H_2O$ from [2-^{3}H]glucose as described previously (15, 16). Rates of $^{14}CO_2$ production were determined from exogenously supplied [U-^{14}C]-glucose by determining the accumulation of $^{14}CO_2$ in the perfusate and correcting for the amount of $^{14}CO_2$ released into the gas phase of the perfusion system (3). Lactate released into the perfusate was determined using an automated analyzer (2300 STAT; Yellow Springs Instruments, Yellow Springs, OH). Perfusate acetoacetate and β-hydroxybutyrate were determined using standard spectrophotometric assays (17). In the analysis of rates of substrate uptake and disposal, metabolite release and tissue metabolites, results from hearts perfused with [1-^{13}C]glucose and acetoacetate were combined with those from hearts perfused with glucose and [2,4-$^{13}C_2$]acetoacetate. Rates of substrate use or metabolite release are expressed as μmol per minutes per gram dry weight.

Glucose-6–phosphate and citrate were determined enzymatically from perchloric acid extracts of frozen tissue samples (17). Glycogen was extracted from a portion of the freeze-clamped heart by tissue digestion with KOH and precipitation with ethanol as previously described (18). The precipitated glycogen was extensively washed with ethanol before further analysis. Glucosyl residues in glycogen were determined after digestion of the glycogen with amyloglucosidase. An aliquot of the extracted, digested glycogen was counted for ^{14}C radioactivity to determine the incorporation of exogenous glucose into the glycogen pool. Net rates of incorporation of exogenous glucose into glycogen were determined from the specific activity of the glycogen after 60 min of perfusion and the specific activity of the perfusate glucose. Tissue metabolite content is expressed as μmol/g dry weight and net rates of incorporation of exogenous glucose into glycogen are expressed as μmol per minutes per gram dry weight.

Statistical analysis. All results are expressed as the mean±SEM. Differences were analyzed using a one-way ANOVA except for the analysis of cardiac power in which a two-way ANOVA was used. Differences between groups were determined using the Newman-Kuels test. A P value < 0.05 was considered statistically significant.

Results

Contractile performance was stable in all four groups over the entire 60 min of perfusion (Fig. 1). Although the cardiac power for hearts perfused with glucose alone tended to be lower, there were no statistically significant differences between any of the groups. These findings suggest that the energy requirements of the various groups were similar.

Rates of glucose uptake, glucose oxidation, lactate production and acetoacetate use are listed in Table I. As expected, the addition of physiologic amounts of insulin (40 μU/ml) to

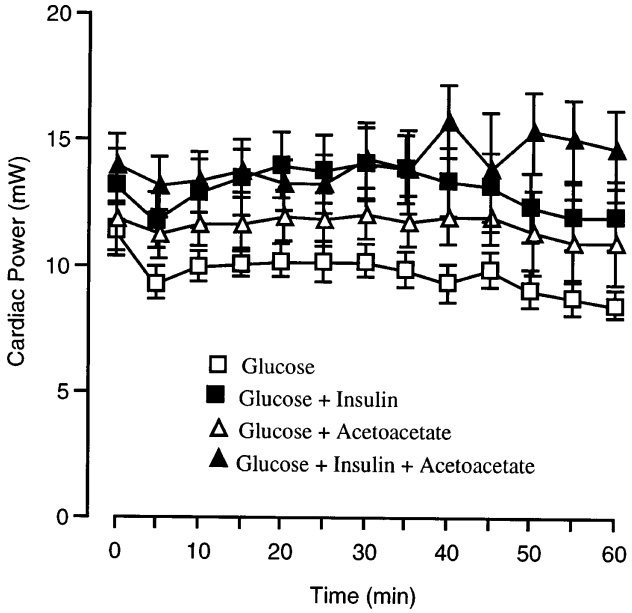

Figure 1. Cardiac power in isolated working rat hearts using glucose either alone (*open squares*), with insulin (40 μU/ml; *closed squares*), acetoacetate (5 mM; *open triangles*), or acetoacetate and insulin (*closed triangles*). Note that there are no differences in contractile activity either over the duration of the perfusions or between the various groups.

the perfusate resulted in a 54% increase in glucose uptake in hearts using glucose as the only exogenous substrate. The presence of acetoacetate as a competing substrate inhibited glucose uptake by 74% in the absence of insulin. However, the addition of insulin to hearts using both glucose and acetoacetate resulted in a twofold increase in glucose uptake compared with hearts using glucose and acetoacetate alone, although glucose uptake was still significantly less than in non–insulin-stimulated hearts using glucose alone. Lactate release increased with insulin stimulation both in hearts using glucose alone or glucose and acetoacetate. Insulin stimulation did not change the rate of exogenous glucose oxidation, as determined by $^{14}CO_2$ production, in hearts using glucose as a sole substrate (Table I). In contrast, the addition of acetoacetate as a competing substrate resulted in a striking 97% inhibition of glucose oxidation. Further, the addition of insulin to hearts using both glucose and acetoacetate did not change the rate of glucose oxidation significantly.

The metabolic fates of acetoacetate in the heart include reduction to β-hydroxybutyrate and oxidation in the citric acid cycle. The rate of acetoacetate oxidation was determined by subtracting the rate of β-hydroxybutyrate production from the rate of acetoacetate use. The rate of acetoacetate removal from the perfusate was not significantly affected by insulin stimulation (Table I). Further, the addition of insulin did not affect the rate of reduction of acetoacetate to β-hydroxybutyrate through the action of β-hydroxybutyrate dehydrogenase or the rate of acetoacetate oxidation.

With respect to the entry of exogenous glucose into the glycogen pool, the presence of insulin or acetoacetate had significant effects on both the myocardial content of glycogen as well as the rate of incorporation of exogenous glucose into glycogen. The addition of either insulin or acetoacetate in the presence of glucose resulted in a doubling of the glycogen content compared with hearts using glucose as a sole substrate (Fig. 2). This was accompanied by a twofold increase in the rate of incorporation of exogenous glucose into glycogen when compared with hearts perfused with glucose alone. The addition of both acetoacetate and insulin to hearts using glucose was additive and increased the incorporation of exogenous glucose into glycogen sixfold compared with non–insulin-stimulated hearts using glucose alone and 2.5–3-fold compared with hearts using glucose in the presence of either acetoacetate or insulin (Fig. 2). However, acetoacetate and insulin together increased glycogen content to a similar level as either insulin or acetoacetate alone.

In order to understand further the metabolic interactions that may occur with the addition of insulin and/or acetoacetate to hearts using glucose, the tissue contents of glucose-6–phosphate and citrate were determined. The addition of insulin and/or acetoacetate to hearts using glucose increased the tissue content of glucose-6–phosphate significantly when compared with hearts using glucose as a sole substrate (Fig. 3). The greatest increase in glucose-6–phosphate occurred in hearts perfused with glucose plus insulin or glucose plus insulin and acetoacetate. Insulin stimulation had no effect on the citrate content of hearts using glucose alone. In contrast, the addition of acetoacetate increased the tissue content of citrate fivefold when compared with hearts using glucose as a sole substrate. The tissue content of citrate in hearts perfused with glucose,

Table I. Rates of Glucose Uptake and Oxidation, Lactate Production, and Acetoacetate Use in Isolated Perfused Rat Hearts

			Exogenous			
	Glucose	Lactate	Glucose	Acetoacetate	β-hydroxybutyrate	Acetoacetate
Substrate(s)	*Uptake*	*Release*	*Oxidation*	*Uptake*	*Release*	*Oxidation*
Glucose ($n = 6$)	5.43±0.36	0.94±0.23	3.33±0.26	ND	ND	ND
Glucose + insulin ($n = 8$)	8.37±1.05*	6.69±1.03*	3.53±0.24	ND	ND	ND
Glucose + acetoacetate ($n = 10$)	1.46±0.35*	1.42±0.21	0.09±0.04*	16.01±2.31	1.24±0.16	13.34±2.87
Glucose + insulin + acetoacetate ($n = 8$)	3.01±0.92*	2.95±0.56*‡	0.13±0.04*	19.41±3.73	1.53±0.15	18.12±3.67

Rates expressed as μmol per minutes per glam dry weight. ND, not determined. *$P < 0.05$ compared to hearts perfused with glucose alone; ‡$P < 0.05$ compared to hearts perfused with glucose and acetoacetate.

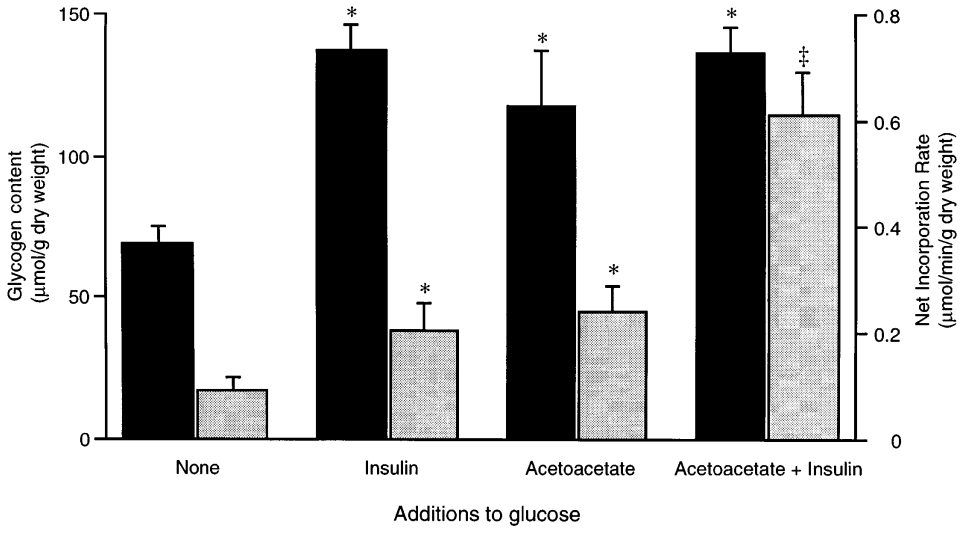

Figure 2. Myocardial glycogen content (*black bars*) and rates of incorporation of exogenous glucose in glycogen (*shaded bars*) for hearts perfused with either glucose alone or with insulin, acetoacetate or acetoacetate and insulin. $^{*}P < 0.05$ compared with hearts perfused with glucose alone; $^{\ddagger}P < 0.05$ compared with all other groups.

acetoacetate, and insulin was fourfold higher that the citrate content of hearts perfused with glucose plus insulin.

With respect to the metabolism of glucose and glycogen, the absolute ^{13}C enrichment of the C3 of alanine from [1-^{13}C]glucose can be used to estimate the relative contribution of ^{13}C-labeled glucose (i.e., exogenous glucose) to the pyruvate pool. Based on this measurement, the addition of insulin to hearts using glucose as a sole exogenous substrate increased the contribution of exogenous glucose to pyruvate by one-third (Table II). In contrast, the addition of acetoacetate as a competing substrate increased the contribution of endogenous sources of pyruvate (unlabeled glycogen, alanine, and lactate) to the pyruvate pool as evidenced by a decrease in the absolute enrichment of alanine compared with hearts perfused with glucose alone. The presence of both acetoacetate and insulin tended to increase the contribution of exogenous glucose to the pyruvate pool when compared with hearts using glucose as a sole substrate, however this difference was not statistically significant. In contrast, the contribution of exogenous glucose to the pyruvate pool was significantly greater for hearts perfused with glucose, acetoacetate, and insulin compared with hearts using glucose and acetoacetate in the absence of insulin. However, it must be kept in mind that the absolute rates of pyruvate production from both exogenous and endogenous sources of carbohydrate are decreased in the setting of ketone body oxidation.

The addition of insulin to hearts using glucose as the sole exogenous substrate did not change the relative contribution of carbohydrate to the formation of acetyl-CoA as determined by the C4-GLU/C3-ALA ratio (Table II). However, addition

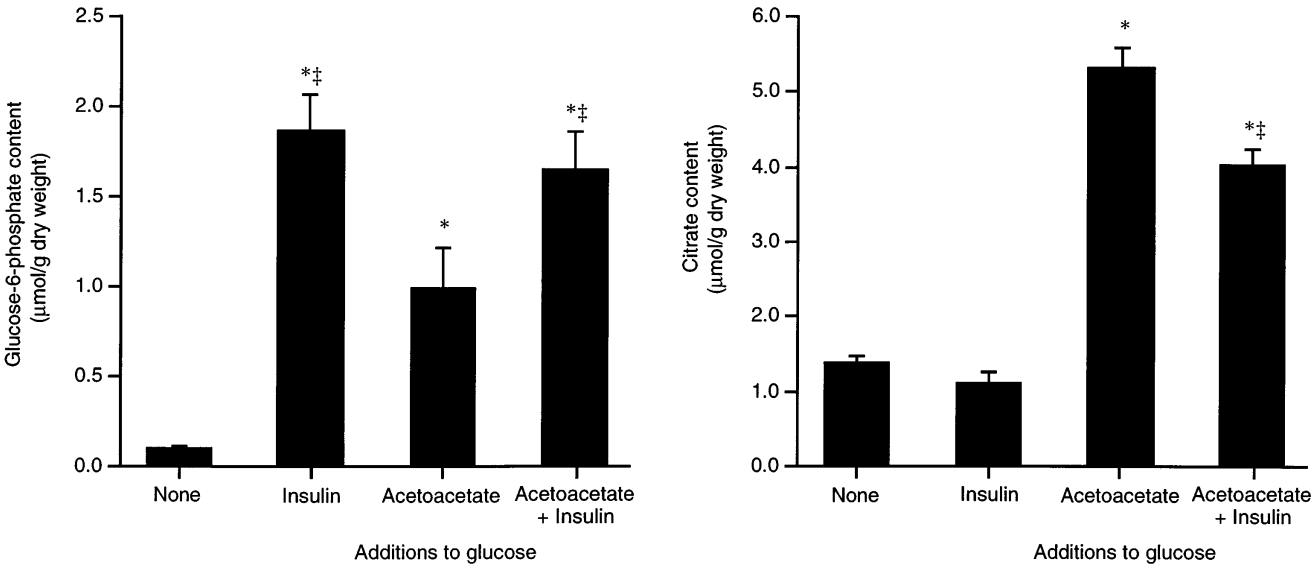

Figure 3. Tissue glucose-6–phosphate and citrate contents for hearts using glucose either alone, with insulin, with acetoacetate, or with insulin and acetoacetate. $^{*}P < 0.05$ compared with hearts perfused with glucose alone, $^{\ddagger}P < 0.05$ compared with hearts perfused with glucose and acetoacetate.

Table II. ^{13}C Enrichments of Myocardial Glutamate and Alanine in Isolated Working Rat Hearts

	C3-ALA	C4-GLU		
Substrate(s)	Enrichment (%)	C3-ALA	$F_{cGLUCOSE}$	$F_{cACETOACETATE}$
Glucose ($n = 6$)	32.1±1.6	0.34±0.02	0.39±0.01	ND
Glucose + insulin ($n = 8$)	40.8±1.6*	0.29±0.03	0.40±0.02	ND
Glucose + acetoacetate ($n = 10$)	22.1±2.2*‡	0.03±0.01*‡	ND	0.78±0.02
Glucose + insulin + acetoacetate ($n = 8$)	35.9±1.6§	0.04±0.01*‡	ND	0.80±0.06

*$P < 0.05$ compared to hearts perfused with glucose alone; ‡$P < 0.05$ compared to hearts perfused with glucose and insulin; §$P < 0.05$ compared to hearts perfused with glucose and acetoacetate. ND, not determined.

of acetoacetate as a competing substrate decreased the entry of glucose into the citric acid cycle by about 90% in either the presence or absence of insulin. It is important to note that exogenous and endogenous carbohydrate contributed only 33% to the acetyl-CoA pool in hearts using glucose as a sole substrate (Table II). This suggests that when glucose is present as the only exogenous fuel source, the heart uses a significant amount of endogenous noncarbohydrate substrates, presumably triglycerides.

Hearts using [1-^{13}C]glucose in the presence of acetoacetate had minimal glutamate enrichment because of a decreased production of glucose-derived acetyl-CoA. As a result, it is difficult to analyze the multiplet structure of the individual carbons of glutamate to determine rates of citric acid cycle turnover based on measurements derived from [1-^{13}C]glucose. Therefore, complementary experiments were performed in which hearts were perfused with unlabeled glucose and [2,4-$^{13}C_2$]acetoacetate. Because acetoacetate readily enters the citric acid cycle in mammalian hearts (19), there is a high degree of enrichment in the individual carbons of the citric acid cycle intermediates, and, by extension, a high degree of enrichment of the carbons of their transamination productions (i.e., glutamate and aspartate). Analysis of the isotopomers of glutamate derived from [2,4-$^{13}C_2$]acetoacetate demonstrated that acetoacetate contributed ~ 80% of the acetyl-CoA to the citric acid cycle (Table II). Further, the contribution of acetoacetate to the citric acid cycle was not affected by the addition of insulin.

Discussion

Myocardial glucose metabolism can be affected by many different factors (1). In these studies, we investigated the effects of insulin stimulation and use of a competing substrate on the metabolism of exogenous glucose and endogenous glucose stored in the form of glycogen. Several important new aspects of myocardial carbohydrate metabolism were characterized in these studies. First, insulin and acetoacetate have additive effects on increasing the incorporation of exogenous glucose into glycogen. Secondly, insulin stimulation and acetoacetate oxidation affect the relative contributions of exogenous glucose and endogenous carbohydrate, including glycogen, to energy metabolism. The findings also suggest that the addition of insulin and acetoacetate may increase the turnover of the myocardial glycogen pool and that an important fate of exogenous glucose is the maintenance of glycogen stores.

Effects of insulin and acetoacetate on glycogen metabolism. In this study, glycogen metabolism was evaluated both by changes in the absolute amount of glycogen as well as by the net rate of incorporation of exogenous glucose into the myocardial glycogen pool. We have shown previously that the glycogen content of hearts from fasted rats immediately ex vivo is ~ 125 μmol glucosyl units per gram dry weight (3, 6). The tissue content of glycogen decreases during the initial 5 min of retrograde perfusion with glucose as the only substrate to ~ 100 μmol glucosyl units per gram dry weight and continues to decrease at a rate of ~ 1.5 μmol glucosyl units per minutes per gram dry weight if a competing substrate or insulin is not present (3, 6). This study confirms these previous findings of net glycogen degradation in hearts perfused with glucose as a sole substrate. In addition, this study demonstrates that the addition of acetoacetate or physiologic concentrations of insulin, either separately or together, maintains glycogen stores at levels similar to those found in the heart immediately ex vivo. The maintenance of the glycogen stores can occur through increased glycogen synthesis, decreased glycogen degradation, or both.

Previous work has demonstrated that glycogen breakdown in the heart can be converted to net glycogen synthesis by the addition of either insulin and/or ketone bodies (7) or lactate (20). While the ability of different competing substrates to augment insulin-stimulated rates of net glycogen synthesis has not been studied, one ^{13}C-NMR study has suggested that the rate of glycogen synthesis in insulin-stimulated heart muscle is greater in the presence of ketone bodies than in the presence of either lactate or pyruvate (21).

The measurement of glucose incorporation into glycogen in this study is based on the accumulation of [U-^{14}C]glucose in glycogen and therefore represents net flux, not absolute rates, of synthesis. Therefore, the enhanced rate of incorporation of exogenous glucose without a significant change in the myocardial glycogen content for hearts using glucose and acetoacetate in the presence of insulin would be consistent with an increase in the rate of glycogen turnover when compared with hearts using glucose with either insulin alone or acetoacetate alone. However, our measurements do not take into account the rates of glycogen breakdown. It may be that glycogen degradation is inhibited with the addition of either insulin or acetoacetate, and that the rate of degradation is further inhibited with the addition of both insulin and acetoacetate when compared with hearts perfused with glucose alone. Therefore, in the ab-

sence of data on the rate of glycogen degradation, rates of turnover can only be estimated.

These studies also demonstrated that there was incorporation of exogenous glucose into glycogen in hearts perfused with glucose alone, in which there is evidence of net glycogen degradation. This finding is in keeping with previous in vivo and in vitro studies that have shown that exogenous glucose continues to be incorporated in glycogen in the setting of net glycogen breakdown (3, 22, 23). The finding of glycogen enrichment during glycogenolysis does not support the previously proposed last on, first off degradation of myocardial glycogen (24). Rather, our findings support earlier work demonstrating a random degradation of myocardial glycogen (3, 5).

The mechanism by which insulin promotes glycogen synthesis involves covalent and noncovalent modification of glycogen synthase activity and increased availability of intracellular glucose (25). In contrast, the mechanism by which competing substrates increase glycogen synthesis is not completely understood. Competing substrates may increase glycogen synthesis through the inhibition of phosphofructokinase by citrate (26), causing accumulation of glucose-6–phosphate that stimulates glycogen synthase activity (20). Insulin stimulation also causes glucose-6–phosphate to accumulate, also stimulating glycogen synthase activity (20). However, in contrast to hearts oxidizing acetoacetate, insulin stimulation does not cause changes in the tissue content of citrate and therefore, phosphofructokinase is most likely not inhibited. The other mechanism responsible for enhanced glycogen synthesis in hearts using a competing substrate most likely involves a decreased reliance on glucose to support energy production. Previously, it has been hypothesized that glycogen synthesis is increased when the rate of glycolysis is inhibited to a greater extent than the rate of glucose uptake and phosphorylation (21). In both groups of hearts perfused with glucose and acetoacetate, in which the glycogen content was greater than that for hearts using glucose alone, the inhibition of glucose oxidation was 10-fold greater than the inhibition of glucose uptake.

No studies have determined if the presence of a competing substrate causes covalent modification of either glycogen synthase or phosphorylase in the heart. Previous investigators have demonstrated that there is a close correlation between glucose-6–phosphate concentrations and the percentage of total glycogen synthase that is present in the activated form (27). However, skeletal muscle glycogen synthase activity has been shown to be inversely related to rates of lipid oxidation in healthy men both under basal and insulin stimulated conditions (28). Further, the provision of fatty acid decreases the fraction of activated glycogen synthase (GS_a) as well as the rate of incorporation of exogenous glucose into glycogen in euglycemic hyperinsulinemic clamp studies in humans (29). Thus there are distinct differences in the regulation of glycogen synthesis between heart and skeletal muscle.

Metabolic fate of exogenous glucose and glycogen in the heart. Recent pulse-chase experiments have shown that a greater proportion of the glycogen that enters glycolysis is eventually oxidized when compared with the proportion of exogenous glucose that is oxidized (4, 5). Given the preferential oxidation of glycogen, as well as the more favorable energetics of oxidative phosphorylation compared with substrate level phosphorylation, it is easy to understand the advantages of maintaining glycogen stores in the heart (4). The present finding that glucose derived from glycogen may account for 20–60% of the pyruvate pool is in keeping with a previous study demonstrating that glycogen metabolism accounts for $\sim$ 40% of the overall ATP produced from glucose (5). In contrast to this earlier study, this report is the first study to demonstrate that the contribution of glycogen to carbohydrate energy metabolism varies with insulin stimulation and substrate competition. It must be kept in mind that the fraction of alanine that is not labeled with ^{13}C may tend to overestimate the contribution of glycogen to the pyruvate pool because other unlabeled carbon sources may contribute to the alanine pool. However, given the estimated rates of glycogen turnover, glycogen most likely contributes significantly to the pyruvate pool.

In the setting of insulin stimulation, $\sim$ 80% of the pyruvate was derived from exogenous glucose compared with the 64% contribution of exogenous glucose in hearts using glucose without insulin, indicating that the majority of carbohydrate energy metabolism was supported by exogenous glucose. This preference for exogenous glucose over glycogen can be explained by the increase in exogenous glucose uptake caused by insulin stimulation. With respect to the effects of competing substrates, acetoacetate is known to inhibit glucose uptake in the heart (2, 19). In addition, ketone bodies inhibit glucose oxidation through inhibition of pyruvate dehydrogenase by increasing the acetyl-CoA/CoASH ratio (8, 30). As a result of decreased glucose oxidation, glucose uptake is decreased, and the contribution of glycogen to the pyruvate pool increases to almost 60%.

This study also confirms inhibition of carbohydrate oxidation in the presence of acetoacetate based on a decrease in the C4-GLU/C3-ALA ratio. Ketone bodies have also been shown to inhibit β-oxidation of long chain fatty acids in the heart (31, 32). Taken together, these findings indicate that when present, ketone bodies are a significant energy-providing substrate and therefore represent a major source of acetyl-CoA for the citric acid cycle. In this study, isotopomer analysis of the carbons of glutamate revealed that acetoacetate represented about 80% of the acetyl-CoA pool in hearts using glucose and acetoacetate in either the absence or presence of insulin. Previous studies using concentrations of glucose and acetoacetate identical to those used in the present studies, have suggested that acetoacetate may represent $>$ 70% of the oxidative fuel for the heart (19). Interestingly, that value can fall to $\sim$ 50% in the presence of nonphysiologic concentrations of insulin (19) or to 23% at acetoacetate concentrations of 0.17 mM (33).

In contrast to ketone bodies, acetyl-CoA derived from exogenous glucose and other endogenous carbohydrate sources accounted for at most 40% of the acetyl-CoA entering the citric acid cycle if glucose is the only exogenously supplied substrate. Therefore, other endogenous fuels, most likely triglycerides, accounted for 60–70% of the acetyl-CoA. The present findings are supported by previous studies which have demonstrated that endogenous triacylglyerol stores undergo β-oxidation in the isolated heart (34, 35). In contrast, when glucose and acetoacetate are present as competing exogenous substrates, $>$ 80% of the acetyl-CoA can be accounted for by the exogenously supplied substrates.

Study limitations. These studies were performed using hearts from fasted rats perfused with glucose in either the presence or absence of one competing substrate, acetoacetate. It is likely that the relative contributions of exogenous and endogenous carbohydrate would differ if similar studies were performed using hearts from fed animals. Specifically, we have

demonstrated that insulin sensitivity in the hearts from fed animals differs from that in fasted animals (6). In addition, the accumulation of glycogen in hearts from fed animals differs from that in hearts from fasted animals when hearts are perfused with glucose, insulin, and lactate (6). While the nutritional status of the animal may affect the contributions of exogenous and endogenous carbohydrate to energy metabolism, the substitution of another competing substrate, such as fatty acid, would be less likely to affect the results of our studies. Free fatty acid oxidation causes the tissue contents of acetyl-CoA and citrate to increase (26, 36), resulting in inhibition of pyruvate dehydrogenase and phosphofructokinase, respectively, in a fashion similar to ketone body metabolism. In contrast, increases in the perfusate calcium concentration would be expected to increase the activity of calcium-dependent enzymes such as pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase and would therefore increase glycolysis and the contribution of carbohydrate to the acetyl-CoA pool (37).

In this study, rates of glycogen degradation were not determined. Because absolute rates of glycogen turnover would require determining rates of degradation as well as synthesis, rates of glycogen turnover can only be estimated in this study based on the rates of net incorporation of exogenous glucose into glycogen and the fact that there were no significant differences in the tissue content of glycogen for hearts perfused with glucose plus insulin, glucose plus acetoacetate, and glucose plus insulin and acetoacetate. Because the rate of incorporation of exogenous glucose into glycogen varies in these groups, the rates of glycogen degradation would also have to vary in order to maintain similar glycogen contents in the three groups. By virtue of the fact that there is significant glycogen degradation in the hearts perfused with glucose alone, the rate of glycogen turnover must be high. However, glycogen stores are not maintained in this group and it is therefore difficult to compare the findings in these hearts with the other groups with respect to glycogen turnover. Further experiments will need to be performed in which rates of both glycogen synthesis and degradation are determined in order to determine the interactive effects of insulin and a competing substrate on glycogen turnover as well as the metabolic fate of glycogen.

An associated issue concerning glycogen metabolism involves the metabolic fate of glycogen in oxidative versus nonoxidative pathways. The absolute enrichment of C3-ALA determines the contributions of exogenous glucose and endogenous carbohydrate to the alanine pool. While there may be intracellular compartmentalization of alanine, it has been shown that the enrichment of C3-ALA reflects the enrichment of the acetyl-CoA pool entering the citric acid cycle (38). However, the enrichment of alanine does not necessarily reflect the enrichment of the intracellular lactate pool. Studies of the degradation of glycogen should help to determine the extent to which the nonoxidative metabolism of glycogen is affected by insulin and competing substrates.

In conclusion, these studies suggest that glycogen, rather than being merely a storage form for glucose in the heart, represents a dynamic pool of available carbohydrate that is constantly turning over. However, the rate at which the pool turns over is under tight metabolic and hormonal control. In turn, these factors also determine the contribution of exogenous glucose and glycogen to energy production. Based on these results, glycogen likely represents an important source for myocardial energy production, even under aerobic conditions, that must be considered in studies of energy production in the heart.

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