

Effects of fatty acid oxidation on glucose utilization by isolated hepatocytes

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We have studied the inhibitory action of long- and short-chain fatty acids on hepatic glucose utilization in hepatocytes isolated from fasted rats. The rates of hepatic glucose phosphorylation and glycolysis were determined from the tritiated products of [2-³H] and [6-³H]glucose metabolism, respectively. The difference between these was taken as an estimate of the 'cycling' between glucose and glucose-6-phosphate. In the presence of 40 mM glucose this cycling was estimated at 0.68 $\mu\text{mol/min/g}$ wet wt. Glucose phosphorylation was unaffected during palmitate and hexanoate oxidation to ketone bodies but glycolysis was inhibited. The rate of glucose cycling was increased during this phase to 1.25 $\mu\text{mol/min/g}$. Following the complete metabolism of the fatty acids, glycolysis was reinstated and cycling rates returned to control levels. Hepatic glucose cycling appears to be an important component of the glucose/fatty acid cycle.

Glucose utilization; Glucose/fatty acid cycle; Glucose/glucose 6-phosphate futile cycle; Isolated hepatocyte; Glycolysis

1. INTRODUCTION

The inhibition of glycolysis during fatty acid oxidation is well documented in muscle [1–3] where it brings about a 'glucose sparing' effect. This decrease in glucose utilization is explained in part by the depression of 6-phosphofructo-1-kinase activity by citrate together with a feedback inhibition of hexokinase as a result of accumulation of glucose 6-phosphate [4–6]. More recently, palmitate has been shown to inhibit glycolysis in liver by a mechanism also associated with glucose sparing [7]. The inhibitory effects of fatty acid oxidation on hepatic glycolysis and glucose utilization have been attributed to an associated decrease in the cellular concentration of fructose 2,6-bisphosphate [7], the potent allosteric activator of 6-phosphofructo-1-kinase [8,9]. However, the major enzyme responsible for glucose phosphorylation in liver is glucokinase (hexokinase D), which is insensitive to glucose 6-phosphate [10] but can be inhibited by the interaction of fructose 6-phosphate with a specific regulatory protein [11]. Hence, it is necessary to postulate an alternative mechanism to account for the glucose sparing associated with hepatic fatty acid oxidation.

One possibility, advanced by Hue et al. [7] is that the catalysis of glucose phosphorylation by glucokinase is inhibited by long-chain acyl CoA [12,13]. However, in attempting to repeat their work we observed that oxida-

tion of short-chain fatty acid is equally effective in impairing glycolysis. This was unexpected as short-chain acyl CoA derivatives are not formed in the cytoplasm and, in any case, have been shown not to inhibit glucokinase [13]. Accordingly, we sought other possible mechanisms to account for depression of hepatic glucose utilization during fatty acid oxidation. A well-recognized process by which glucose sparing can come about is embodied in the theory of 'futile' or 'substrate cycling' [14–16]. This postulates that some of the glucose 6-phosphate formed by glucose phosphorylation is immediately reconverted to glucose. Various estimates have been made of the magnitude of such cycling in liver, but generally it is regarded as not being of major quantitative importance in normal fed or fasted rats [17]. Nevertheless, the proponents of the theory have argued that it might play a critical role in modulating flux through metabolic pathways such as glycolysis, although a definitive mechanism for the regulation of cycling has not been established [17]. In this paper we provide evidence that the inhibition of hepatic glycolysis during fatty acid oxidation is associated with substantial enhancement of flux through the glucose/glucose 6-phosphate cycle, and consequent glucose sparing.

2. MATERIALS AND METHODS

Isolated liver cells from male Hooded Wistar rats (250–280 g b.wt.), starved for 24 h to deplete liver glycogen, were prepared by a modification [18] of the method of Berry and Friend [19], in which 1 mM Ca^{2+} was added to the washing media. The hepatocytes were incubated under standard conditions [20,21] with 40 mM glucose in the presence of 1 μCi [2-³H]glucose or [6-³H]glucose (New England Nuclear, USA)

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that had been purified by HPLC. Palmitic and hexanoic acids (Sigma, USA) were neutralized and dissolved in isotonic salt solutions containing 9% (w/v) bovine serum albumin, defatted according to [22], and were present where indicated at initial concentrations of 2 mM and 4 mM, respectively. At the completion of the incubation period, samples were taken for the analysis of glycogen and measurement of isotope in the products of glucose metabolism. The remaining portion was deproteinized with an equal volume of cold 1 M perchloric acid and neutralized before metabolites were measured by standard enzymatic techniques [23]. Glycogen was measured as previously described [24]. Fructose 2,6-bisphosphate was stabilized by mixing 0.3 ml of the contents of the incubation vessel with 0.3 ml 0.1 M NaOH and the mixture heated at 80°C for 10 min [25]. Samples were stored at 4°C until assayed. All extracts were diluted 10-fold with 10 mM NaOH before assay according to Van Schaftingen et al. [25].

For the determination of radioactive metabolites, samples (0.5 ml) were deproteinized in 1.5 ml of ethanol at 4°C and centrifuged. The metabolites in a 1.0 ml portion of each supernatant were separated by ion-exchange chromatography [14,26]. For measurement of the rate of intracellular glucose phosphorylation, hepatocytes were incubated with [2-³H]glucose and the release of tritium into water was measured. The amount of tritium incorporated into glycogen was also determined in order to reduce the error associated with the incomplete equilibration between glucose 6-phosphate and fructose 6-phosphate, which generally results in only about 70% of the tritium being liberated into water [14,27]. To measure tritium incorporation into glycogen the cell pellet was digested in KOH [24] and isotopically labelled glycogen co-precipitated with ethanol in the presence of carrier glycogen (20 µg). The glycogen pellet from the second ethanol precipitation was dissolved in water and counted in ACS II scintillation cocktail (Amersham, Australia).

Rates of glycolysis were determined by measuring the accumulation of tritium, derived from [6-³H]glucose, in lactate, pyruvate, amino acids and water [28]. Throughout the incubation most of the tritium was recovered as water, however, over the early incubation period (up to 30 min) substantial quantities of tritium were present in lactate and pyruvate. Negligible quantities of tritium, derived from [6-³H]glucose, were found in other metabolic products. Pentose phosphate pathway metabolism, determined according to [29], was found to account for less than 15% of glucose metabolism, either in the presence or absence of added fatty acid (data not shown). Relatively high concentrations of glucose were used for hepatocyte incubations because of the elevation of the K_m for glucose of glucokinase that occurs in *in vitro* preparations [30].

3. RESULTS

After a short lag period, hepatocytes incubated with 40 mM glucose demonstrate approximately linear rates of glucose phosphorylation, glycolysis and glycogen accumulation for at least 35 min, as previously reported by Katz et al. [26]. The rates of these processes, determined during this linear phase, together with the calculated and percentage rates of glucose cycling during this interval are given in Table I. An estimate of the quantity of glucose phosphorylated, but then cycled back to glucose, was obtained by measuring the tritium transferred from [2-³H]glucose to water and incorporated into glycogen, and subtracting the amount of tritium from [6-³H]glucose found in water, lactate, pyruvate and amino acids. The results in Table I show that between the 10 and 30 min time points, 35% of the glucose entering the hepatocyte is de-phosphorylated and returned to the extracellular medium.

When palmitate (2 mM) is also included in the incubation medium, fatty acid oxidation mainly to ketone bodies is initiated (Fig. 1). Under these conditions glycolysis is strongly depressed, but glucose phosphorylation and glycogen synthesis are unaffected (Table I). During the incubation, the added palmitate is consumed and the rate of ketone body formation, which is a function of the fatty acid concentration [31], gradually declines (Fig. 1). The cessation of fatty acid oxidation to ketone bodies, as a consequence of complete removal of the added palmitate (data not shown), is associated with substantial enhancement of the rate of glycogen synthesis and a reinstatement of glycolysis, which reaches a rate approaching the initial rate of glycolysis under conditions where glucose is the sole added substrate (Table I). In contrast, the rate of glucose phosphorylation is unaltered whether or not fatty acid oxidation to ketone bodies is taking place, remaining virtually constant

Table I
Effects of fatty acids on hepatic glucose utilisation

Incubation period (min)	Palmitate	Hexanoate	Rate of glucose metabolism (µmol/min/g)			Glucose cycling		Fructose 2,6-bisphosphate (nmol/kg)	Glucose 6-phosphate (µmol/g)
			Glucose phosphorylation	Glycolysis	Glycogen synthesis	%	Rate (µmol/min/g)		
10-30	-	-	1.94 ± 0.19	1.26 ± 0.05	0.23 ± 0.02	35.3	0.68	22.4 ± 0.84	0.213 ± 0.015
	+	-	1.88 ± 0.15	0.63 ± 0.06	0.26 ± 0.04	66.5	1.25	11.8 ± 0.38	0.224 ± 0.014
	-	+	1.76 ± 0.14	0.47 ± 0.06	0.21 ± 0.05	73.3	1.28	9.8 ± 0.81	0.221 ± 0.017
60-90	+	-	1.72 ± 0.24	1.02 ± 0.04	0.37 ± 0.08	40.7	0.70	20.0 ± 0.42	0.217 ± 0.013
	-	+	1.68 ± 0.22	0.83 ± 0.08	0.36 ± 0.08	50.9	0.85	19.4 ± 0.62	0.225 ± 0.015

Hepatocytes from fasted rats were incubated with 40 mM [2-³H]glucose (1 µCi) or 40 mM [6-³H]glucose (1 µCi) in the absence and presence of palmitate or hexanoate, added at initial concentrations of 2 mM and 4 mM, respectively. Glucose phosphorylation represents tritium recovered from [2-³H]glucose in water and glycogen. A measure of the rate of glucose glycosylated is provided by the tritium released from [6-³H]glucose as water plus that retained in lactate, pyruvate and amino acids. The percentage of glucose cycled is derived from the differences between the rates of glucose phosphorylation and glucose glycosylated and is expressed as {(Glucose Phosphorylation - Glycolysis)/Glucose Phosphorylation} × 100. Glycogen synthesis was determined enzymatically as described in Materials and Methods. The data for glucose and glucose + palmitate are presented as the mean ± S.E.M. of four experiments, whereas the data for glucose + hexanoate are from two experiments and given as the mean ± 0.5 difference between the individual experiments. All data are standardized on a g wet wt basis.

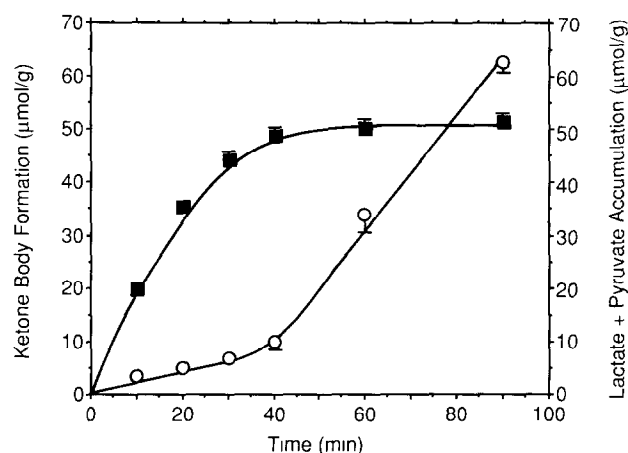


Fig. 1. Hepatocytes from fasted rats were incubated with 40 mM glucose plus 2 mM palmitate as described in Materials and Methods. Ketone body formation (■) (acetoacetate and 3-hydroxybutyrate) and lactate plus pyruvate accumulation (○) are expressed as $\mu\text{mol/g}$ wet wt. The data for each time point are the mean $\pm$ S.E.M. of 4 experiments.

throughout the length of the incubation. The pattern of glucose metabolism determined using $[2\text{-}^3\text{H}]$ and $[6\text{-}^3\text{H}]\text{glucose}$ in the absence or presence of palmitate is shown in Fig. 2. This reveals that the initial rate of glycolysis from glucose alone is not maintained over the entire incubation period. From 40 min, where tritium is recovered mainly in water, the glycolytic rate is lower and corresponds to the establishment of steady-state levels of glycolytic products (data not shown).

During the period of fatty acid oxidation to ketone bodies, there is almost a doubling of the glucose/glucose 6-phosphate cycling rate. However, when ketogenesis ceases and glycolysis accelerates, the rate of cycling falls to levels similar to those observed in the presence of glucose alone (Table I). These relationships are also apparent when hexanoate rather than palmitate is added (Table I). The presence of fatty acid has no effect on the steady-state levels of glucose 6-phosphate or fructose 6-phosphate (Table I) but, as reported by [7], depresses fructose 2,6-bisphosphate concentrations, which are restored when the rate of fatty acid oxidation declines (Table I).

4. DISCUSSION

These findings demonstrate that stimulation of oxidation of fatty acid, following its addition to hepatocytes, increases the rate of turnover of the glucose/glucose 6-phosphate cycle. The exact mechanism of this linkage remains to be explored, but the cycling rate appears to be correlated temporally with the inhibition of glycolysis associated with fatty acid oxidation to ketone bodies. Because the effects are demonstrable with a short-chain as well as a long-chain acid, it appears that the phenom-

enon is unlikely to be related to the accumulation of an intermediary metabolite of fatty acid metabolism. Significant changes in ATP or ADP concentrations are not observed in the presence of fatty acid, although there are small rises in citrate levels, which may bring about an allosteric inhibition of phosphofructokinase [32]. The decline in fructose 2,6-bisphosphate concentration, induced by fatty acid oxidation [7] has been confirmed by our studies. This decline, which has been attributed to an inhibition of 6-phosphofructo-2-kinase [7] brought about by the increase in citrate levels, also may contribute to the lowering of flux through phosphofructokinase. However, the possibility that the decrease in levels of fructose 2,6-bisphosphate is a consequence of the inhibition of glycolysis, rather than the cause of it, has not been excluded. In pancreatic islet cells, a tissue also possessing glucokinase as the major glucose phosphorylating enzyme, glycolysis can be impaired by inhibition of the α -glycerophosphate shuttle [33,34]. Hence, it is feasible that fatty acid oxidation, by limiting flux of reducing-equivalents into the mitochondria, may inhibit glycolysis and, by a mechanism yet to be elucidated, stimulate cycling.

Our results differ from those of Hue et al. [7] in that we observed no depression of glucose phosphorylation as a result of exposure of hepatocytes to fatty acid. These authors also used $[2\text{-}^3\text{H}]\text{glucose}$ and in three experiments noted a 20% depression of glucose phosphorylation. They employed 20 mM glucose, whereas we used double that concentration. However, the main point of difference between their work and ours was that we measured tritium release at shorter intervals during the incubation, whereas they took a single measurement at 45 min. As Fig. 1 illustrates, oxidation of 2

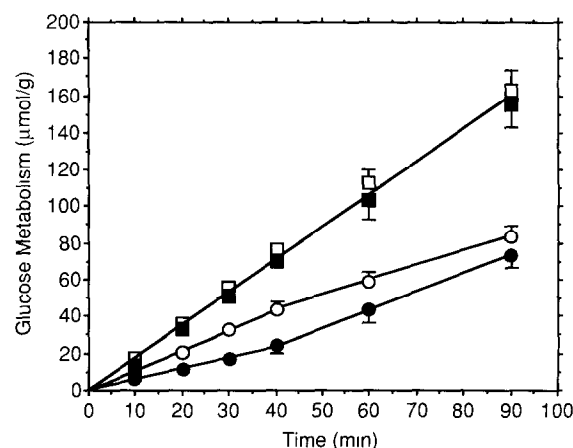


Fig. 2. Hepatocytes were incubated with 1 μCi 40 mM $[2\text{-}^3\text{H}]\text{glucose}$ (□, ■) or 1 μCi 40 mM $[6\text{-}^3\text{H}]\text{glucose}$ (○, ●) in the absence (open symbol) or presence (filled symbol) of 2 mM palmitate. The tritium from $[2\text{-}^3\text{H}]\text{glucose}$ appearing in water and glycogen is represented as glucose phosphorylation and tritium from $[6\text{-}^3\text{H}]\text{glucose}$ measured in lactate+pyruvate+amino acids+water provides a measure of the glucose glycolysed. The data for each time point are the mean $\pm$ S.E.M. of 4 experiments.

mM palmitate by 100 mg (wet wt) of hepatocytes is virtually complete within 40 min. Since Hue et al. [7] employed 1 mM palmitate, it can be assumed that this had been completely metabolized well before the end of the 45 min incubation period.

An interesting and unexpected observation in our studies was the finding that the inhibition of glycolysis in the presence of fatty acid oxidation was not associated with any change in the steady-state level of glucose 6-phosphate or in the rate of glycogen synthesis. This implies that certain molecules of glucose 6-phosphate are destined for glycolysis and that when this is depressed the fate of these molecules is immediate de-phosphorylation. It is difficult to explain this phenomenon without invoking the concept that the glucose 6-phosphate pool is not homogenous [35,36] but is segregated into glycolytic and glycogenic moieties. Even if this were to be the case, it does not explain by what mechanism glucose 6-phosphate surplus to glycolysis is detected and channelled towards de-phosphorylation. The glucose 6-phosphatase system is now known to be far more complex than has been previously recognized [37] and it is evident that the regulation of glucose/glucose 6-phosphate cycling warrants further study. In work to be published elsewhere, we have observed that other agents that inhibit glycolysis, such as ethanol, fluoride or iodoacetate, have a stimulatory effect on hepatic glucose cycling.

Our data also reveal a considerable discrepancy between the rate of glycolysis as measured from accumulation of lactate plus pyruvate compared to the measurement employing [6-³H]glucose. This discrepancy, reflected by a greater glycolytic rate when determined by the isotopic method, is especially noticeable during the period of fatty acid oxidation to ketone bodies as well as towards the end of the incubation period when levels of lactate and pyruvate approach steady state. We interpret this as an indication for a re-cycling of lactate back to glucose, an observation we have confirmed using [¹⁴C]lactate (unpublished observations). Thus, under these experimental conditions hepatocytes exhibit both an abbreviated substrate cycle between glucose and glucose 6-phosphate and an extensive one involving the entire glycolytic/gluconeogenic sequence. This phenomenon is in keeping with the many studies that have demonstrated that much of the glycogen synthesized from glucose is formed indirectly via lactate or other 3-carbon derivatives [38–41].

The studies reported here support the suggestion [7] that interactions of glucose and fatty acid in liver contribute to the glucose/fatty acid cycle [42], but demonstrate that the action of fatty acid in depressing glucose utilization is achieved by enhancing glucose cycling rather than by depressing glucose phosphorylation. It might be considered that the obligatory turnover of ATP associated with cycling between glucose and glucose 6-phosphate, and between glucose and lactate, rep-

resents an inefficient way of controlling carbohydrate flux. However, the association of cycling with fatty acid oxidation provides a potential mechanism for hepatic thermogenesis, and may account for the oxidation of fatty acid by liver in the absence of overt ATP demand [43,44].

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