Variations in hepatic carbon flux during unrestricted feeding

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Previous findings have established a pivotal role for hepatic pyruvate dehydrogenase complex (PDH) in regulating hepatic carbon flux during the starved-to-fed and fed-to-starved nutritional transitions [Holness, McLennan, Palmer & Sugden (1988) *Biochem. J.* **252**, 325–330; Holness & Sugden (1990) *Biochem. J.* **268**, 77–81]. We have therefore examined liver PDH activities during the light and dark phases of the feeding cycle in the adult rat in relation to hepatic glycogenesis, fatty acid synthesis and cholesterologenesis. There was significant synchronous suppression of lipogenesis and glycogenesis during the light phase; rates were restored asynchronously during the dark (feeding) phase. Glycogen concentrations declined during the light phase and increased during the dark phase. Despite quite dramatic changes in rates of glycogen and lipid synthesis and hepatic glycogen concentrations during the light and dark phases, hepatic PDHa (active form) activity remained relatively unchanged. Qualitative and quantitative differences in the pattern of change in rates of synthesis of fatty acid and cholesterol suggested regulation at pathway-specific sites distal to PDH.

INTRODUCTION

A marked diurnal rhythm in rates of hepatic lipid synthesis is observed in mice allowed free access to standard rodent chow (Hems *et al.*, 1975; Cornish & Cawthorne, 1978). The maximum rate of lipid synthesis is observed during the period over which most of the daily food intake is ingested (the dark phase of a 12 h-dark/12 h-light cycle). There have also been reports of increases in hepatic cholesterol (Edwards *et al.*, 1972; Gibbons *et al.*, 1983) and fatty acid synthesis (Kimura *et al.*, 1970) during the dark period of the 24 h cycle in rats. It has been suggested that hepatic glycogenolysis may provide significant carbon for hepatic fatty acid synthesis in mice during the light period, whereas lactate (and related C₃ derivatives of glucose) may assume greater significance as lipogenic precursor during the dark (feeding) period (Salmon *et al.*, 1974; Clark *et al.*, 1974; Hems *et al.*, 1975). Lactate can also be used as a precursor for hepatic glycogen synthesis, particularly during the first few hours of re-feeding after prolonged starvation. In this specific situation, a low hepatic pyruvate dehydrogenase (PDH) complex activity restricts hepatic lipid (fatty acid plus cholesterol) synthesis (Holness *et al.*, 1988a) and facilitates net glycogen repletion via the indirect (gluconeogenic) pathway (Holness *et al.*, 1986).

The precise time course of diurnal changes in hepatic lipid (fatty acid and cholesterol) synthesis has been compared with that of hepatic glycogen content in lactating rats (Munday & Williamson, 1983; Gibbons *et al.*, 1983). The peak rate of lipid synthesis occurred at approx. 4 h into the dark phase of a 12 h-dark/12 h-light cycle, preceding the attainment of the peak hepatic glycogen content (Munday & Williamson, 1983). This pattern is consistent with the concept that hepatic glycogen breakdown may supply carbon for the synthesis of both fatty acid and cholesterol during the light period, when food intake is less. However, the utilization of glycogen carbon as a major precursor for hepatic lipogenesis and cholesterologenesis necessitates the conversion of glucose 6-phosphate into acetyl-CoA via glycolysis and the PDH complex. It is implied that the hepatic PDH complex retains significant activity and rates of hepatic lipid synthesis remain relatively high during periods of net loss of hepatic glycogen content. This contrasts markedly with the pattern observed during the fed-to-starved transition, where hepatic PDH-complex inactivation closely parallels net hepatic

glycogen loss (Holness & Sugden, 1989). In view of the pivotal role of hepatic PDH complex in orchestrating the lipogenic and glycogenic responses of the liver to starvation and re-feeding, we considered it important to examine hepatic PDHa (active form of PDH) activities during both light and dark phases in relation to hepatic fatty acid and cholesterol synthesis, glycogenesis and changes in hepatic glycogen content.

MATERIALS AND METHODS

Materials

Sources of materials were as in Holness *et al.* (1988a) and Holness & Sugden (1990). Standard laboratory chow (52% carbohydrate, 15% protein, 3% fat and 30% non-digestible residue) was purchased from Special Diet Services, Witham, Essex, U.K. Kits for measurement of insulin were purchased from Alpha Laboratories, Eastleigh, Hants., U.K.

Methods

Animals and diets. Female virgin albino Wistar rats (200–250 g) were provided with chow *ad libitum* and maintained on a 12 h-light/12 h-dark cycle (light from either 10:00 or 22:00 h). Rats were sampled at 3 h intervals as indicated in the legends. The total daily food intake of the rats was 22.9 ± 2.5 g of chow, of which only approx. 15% was consumed during the light period (see also Kimura *et al.*, 1970; Bruckdorfer *et al.*, 1974; Munday & Williamson, 1983). There was a dramatic decline in food consumption at the initiation of the light phase. Whereas approx. 5.3 g of chow was consumed during the last 3 h of the dark phase, only approx. 1.1 g of chow was consumed during the initial 3 h of the light phase. Food consumption remained low (with an intake of 0.5–0.9 g of chow per 3 h study period) throughout the light phase, but was rapidly restored at the beginning of the dark phase to 3.9–5.3 g of chow per 3 h period.

Measurements of rats of hepatic lipid and glycogen synthesis. ³H₂O was administered by intraperitoneal injection at 1 h before sampling at the times indicated. Rates of lipid and glycogen synthesis were estimated as ³H incorporation from ³H₂O into tissue lipid or glycogen as described in detail previously (Holness *et al.*, 1988a,b). Cholesterol (non-saponifiable lipid) and fatty acids (saponifiable lipid) were separated as described by Gibbons *et al.* (1983).

Abbreviations used: PDH, pyruvate dehydrogenase; PDHa, the active form of PDH; GUI, glucose utilization index.

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Measurement of glucose utilization index (GUI). GUI values were estimated by using 2-deoxy[^3H]glucose as described by Ferré *et al.* (1985) with conscious unrestrained rats, each fitted with an indwelling cannula (see Issad *et al.*, 1987) at least 5 days before the experiment. Details of the experimental procedures involved in the measurements of GUI values and blood glucose concentrations are given in Sugden *et al.* (1990). Despite the differences in food intake during the light and dark phases, blood glucose remained relatively constant (light, 4.92 ± 0.14 mM-glucose; dark, 5.10 ± 0.10 mM-glucose; $P > 0.05$). Mean coefficients of variance of blood glucose concentrations over each 1 h period of sampling were $< 15\%$ in each instance (results not shown). GUI values were calculated as described by Issad *et al.* (1987) by dividing the radioactivity (d.p.m.) of tissue 2-deoxy[^3H]glucose 6-phosphate by the calculated integral of blood 2-deoxy[^3H]glucose/[glucose]. As in previous studies (e.g. Issad *et al.*, 1987; Sugden *et al.*, 1990), GUI values were not corrected for the discrimination factor (lumped constant) for 2-deoxyglucose in glucose metabolic pathways. Times indicated for measurement of values of GUI refer to the time of injection of 2-deoxy[^3H]glucose rather than to the time of tissue sampling; most uptake of radiolabelled 2-deoxyglucose occurs during the first 20 min after injection (Sokoloff *et al.*, 1977; James *et al.*, 1985).

Enzyme assays. PDH complex (active form, PDHa) and citrate synthase activities were measured in freeze-clamped liver extracts as described previously (Holness *et al.*, 1988a), except that 2.5% (w/v) Triton X-100 and proteinase inhibitors [benzamidine (1 mM), leupeptin (10 μM), tosyl-lysylchloromethane (0.3 mM)] were included in the extraction medium. PDHa activities have been expressed relative to citrate synthase. This corrects for any differences in the efficiency of mitochondrial extraction. A unit of enzyme activity is defined as that which converts 1 μmol of substrate into product/min at 30 $^{\circ}\text{C}$.

Metabolite assays. Glycogen concentrations were determined by the method of Keppler & Decker (1974).

Statistics. Statistical significance of differences was assessed by Student's unpaired *t* test. Results are means $\pm$ S.E.M. for the numbers of rats indicated.

RESULTS

Hepatic glycogen and lipid synthesis

A 31% decline in the rate of lipid (fatty acid plus cholesterol) synthesis was observed as early as 3 h into the light phase (Fig. 1). Rates of lipid synthesis continued to decline progressively, such that suppression of lipid synthesis was substantial (66%) after 6 h of the light phase. The rate of lipid synthesis did not subsequently decrease (Fig. 1). In contrast, there was a precipitous (63%) decline in the rate of glycogen synthesis (measured by the incorporation of ^3H from $^3\text{H}_2\text{O}$ into glycogen) during the first 3 h of the light phase, but only a modest further decline in glycogen synthesis over the remainder of the light phase (Fig. 1). The hepatic glycogen concentration was maintained high (approx. 75 mg of glucose equivalents/g wet wt.) over the first 3 h of the light period, but significant net glycogen depletion was initiated between 3 and 6 h into the light period and glycogen concentrations continued to decline throughout the remainder of the light phase. The lowest glycogen concentration [14% of the initial (0 h-light) value] was observed at the end of the light phase (Fig. 1). The synchronous suppression of glycogenesis and lipogenesis observed over the first 3 h of the light phase was concomitant with a 28% decline in plasma insulin concentrations from an initial value of 30.4 ± 2.3 $\mu\text{units/ml}$; concentrations were further decreased after 6 h (see Table 1) and reached a

minimum (18% of initial) after 12 h. It appears likely that a decline in insulin underlies the suppression of glycogenesis and lipogenesis observed during the light phase, as is the case after acute (6 h) starvation (Munday *et al.*, 1991).

A small significant increase in the rate of glycogenesis, accompanied by a modest increase in glycogen content, was observed by the end of the first 3 h of the dark phase (Fig. 1); insulin concentrations were restored to 53% of the 0 h light

Fig. 1. Hepatic total lipid synthesis, glycogen synthesis and glycogen deposition during unrestricted feeding

Rats were provided with free access to food and water. Rates of lipid (fatty acid plus cholesterol; $\blacktriangle$) and glycogen synthesis ($\bullet$) were measured by using $^3\text{H}_2\text{O}$ at 3 h intervals during light and dark phases as indicated. Hepatic glycogen concentrations ($\circ$) were also measured. Details are given in the Materials and methods section. Results are means $\pm$ S.E.M. for 4–8 rats.

Table 1. Hepatic carbon flux, peripheral glucose utilization and circulating glucose and insulin concentrations at 6 h into the light phase in rats fed *ad libitum* or starved

Rats provided with unrestricted access to food (termed 'fed *ad libitum*') consumed 0.90 ± 0.24 g of chow (equivalent to 2.6 mmol of glucose) over the 6 h period. Statistically significant differences between the two nutritional states are indicated by: * $P < 0.01$.

	Nutritional status	
	Fed <i>ad libitum</i>	6 h-starved
Hepatic PDHa activity (m-units/unit of citrate synthase)	18.0 ± 3.4	$4.3 \pm 0.9^*$
Hepatic glycogen concn. (mg/g wet wt.)	51.8 ± 11.1	41.4 ± 10.1
Total lipid synthesis ($\mu\text{g-atom}$ of H/h per g wet wt.)	7.9 ± 1.1	8.5 ± 1.5
Muscle GUI (ng of glucose/min per mg)		
Heart	77.6 ± 9.5	85.9 ± 12.4
Diaphragm	42.5 ± 5.6	31.5 ± 3.6
Soleus	29.3 ± 4.0	20.8 ± 3.1
Adductor longus	19.0 ± 2.6	17.3 ± 2.6
Blood glucose (mM)	4.52 ± 0.18	4.68 ± 0.13
Plasma insulin (μunits)	21.7 ± 8.5	15.0 ± 1.6

Fig. 2. Hepatic fatty acid and cholesterol synthesis during unrestricted feeding

Fatty acid (●) and cholesterol (▲) synthesis rates were measured separately as described in the Materials and methods section and the legend to Fig. 1. Rates of total lipid synthesis are shown for comparison (◇). Results are means $\pm$ S.E.M. for 4–8 rats.

value over this period (results not shown). These increases preceded the first significant increase in lipid synthesis (observed after 3–6 h of darkness). The rate of glycogenesis continued to rise until 9 h into the dark phase (Fig. 1), the period associated with the most rapid rate of glycogen deposition (Fig. 1). Peak rates of hepatic lipid synthesis occurred during the last 3 h of the dark phase and, as in the study of Munday & Williamson (1983) with lactating rats, preceded the peak hepatic glycogen concentration (observed at the end of the dark period and during the first 3 h of the light period; Fig. 1).

Fatty acid and cholesterol synthesis

The patterns of changes in the individual rates of synthesis of saponifiable (fatty acid) and non-saponifiable (cholesterol) lipid are shown in Fig. 2. Fatty acid synthesis made the predominant (up to 82% of total) contribution to total lipid synthesis, particularly when rates were high (during the latter part of the dark phase). In contrast, the maximum contribution of cholesterologenesis to total lipid synthesis (up to 40% of total) occurred during the last 6 h of the light phase and the first 3 h of the dark phase when total lipid synthesis was low (Fig. 2). Consequently both the decline in hepatic lipid synthesis occurring between 3 and 6 h of the light phase and the increase in total lipid synthesis observed from 3 to 12 h into the dark phase are almost entirely accounted for by changes in rates of fatty acid synthesis. Absolute rates of cholesterol synthesis were generally higher during the dark phase and lower during the middle of the light phase (see also Edwards *et al.*, 1972; Gibbons *et al.*, 1983); however, only a 2.4-fold variation in rates of cholesterol synthesis was observed (Fig. 2). This variation is far less striking than the 3.7-fold variation in rates of fatty acid synthesis (Fig. 2).

Hepatic PDHa activity

We examined whether the changes in rates of hepatic lipid synthesis observed during the light and dark phases were paralleled by changes in hepatic PDHa activities, as is the case after acute (6 h) starvation (Holness & Sugden, 1990) and chow re-feeding after prolonged (48 h) starvation (Holness *et al.*,

1988a). Contrary to our expectation, hepatic PDHa activities were unchanged between those periods where total rates of hepatic lipid synthesis were particularly high (from 6 h-dark to 3 h-light inclusive; mean PDHa activity of 16.8 ± 2.4 m-units/unit of citrate synthase, $n = 16$) and those when they were low (from 6 h-light to 3 h-dark inclusive; mean PDHa activity of 17.6 ± 2.2 m-units/unit of citrate synthase; $n = 18$, $P > 0.05$). Mean hepatic citrate synthase activities were 10.5 ± 0.3 and 11.3 ± 0.4 units/g wet wt. of liver respectively over these periods. It can therefore be calculated that the expressed activities of PDHa are consistently adequate to permit a rate of conversion of pyruvate into acetyl-CoA sufficient to sustain even the highest observed rate of lipid synthesis (see also Holness & Sugden, 1990).

Because of the apparent disparity in the regulation of hepatic carbon flux during an acute voluntary decline in food intake (at 6 h into the light phase), as opposed to enforced and total food withdrawal (after 6 h starvation), we examined hepatic carbohydrate metabolism in these two nutritional states in a single experiment (Table 1). There was a notable failure to suppress hepatic PDHa activity during the light phase when food was available, whereas PDHa activity was markedly depressed (by approx. 75%) after 6 h of starvation. In contrast, hepatic glycogen loss and suppression of lipid synthesis was equivalent to that observed during 6 h of starvation.

DISCUSSION

One purpose of the present study was to extend earlier studies on hepatic carbon flux during enforced changes from one nutritional state to another (the fed-to-starved and starved-to-fed transitions) by examining hepatic metabolism during voluntary changes in food intake in rats permitted unrestricted access to food. The present results indicate several important distinctions.

Glucose 6-phosphate derived from hepatic glycogen can be released into the bloodstream as glucose to maintain glycaemia or enter glycolysis. Rates of uptake and phosphorylation of glucose by peripheral tissues (characterized by high rates of glucose utilization) were very similar in the 6 h-light/fed *ad libitum* and 6 h-light/6 h-starved rats (Table 1), but, despite significant carbohydrate absorption from the intestine in the former but not the latter group (see legend to Table 1), blood glucose concentrations were identical (Table 1). This suggests that, when dietary carbohydrate is available, it is likely that the predominant fate of glycogen-derived glucose 6-phosphate is entry into glycolysis with suppression of hepatic glucose output. In contrast, it would appear that after even very short-term starvation the major fate of glucose 6-phosphate is glucose output. We have previously established a close temporal correlation between loss of hepatic glycogen and inhibition of hepatic PDHa activity during the initial 6 h of the fed-to-starved transition (Holness & Sugden, 1989). This is not the case during voluntary changes in food intake. The ability to retain a relatively high PDHa activity, despite significant stimulation of hepatic glycogenolysis during the light phase (but not after 6 h starvation), may be related to pyruvate-induced suppression of PDH kinase activity (reviewed by Randle, 1986), owing to the continued generation of pyruvate via hepatic glycolysis. It is conceivable, as suggested by Munday & Williamson (1983), that the glycolytic degradation of glucose 6-phosphate derived from net glycogen breakdown could permit a minimal rate of hepatic lipid synthesis to be sustained during the latter part of the light phase by contributing precursor(s) for hepatic lipogenesis via PDH.

The regulation of hepatic PDH activity is important in the facilitation of glycogen deposition and the restriction of hepatic

lipid synthesis during the first few hours of re-feeding after prolonged (48 h) starvation (Holness *et al.*, 1988a,b). The selective stimulation of glycogenesis (but not lipogenesis) observed during the initial 3 h of the dark phase is reminiscent of that observed on chow re-feeding after prolonged starvation (Holness *et al.*, 1988a). However, there is no evidence for suppression of PDH activity over the period when rates of glycogenesis are high during the dark phase, as is the case on re-feeding after prolonged starvation (Holness *et al.*, 1988a). A low hepatic PDHa activity may only be necessary to facilitate hepatic glycogen deposition in those circumstances where the predominant precursors utilized are C₃ derivatives of glucose (i.e. where there is clear competition between glycogenesis and PDH for substrate) or when the carbohydrate supply is not necessarily assured. High rates of both lipid synthesis and glycogenesis are observed concomitantly from 6 to 9 h of darkness during unrestricted feeding.

As changes in rates of lipid synthesis are observed in the absence of changes in PDHa activity, it would appear that primary control of hepatic lipid synthesis during voluntary changes in food intake is achieved via changes in absolute rates of flux through (active) PDH (and rates of acetyl-CoA production from pyruvate) or to modulation of carbon flux at site(s) distal to PDH. Furthermore, the qualitative and quantitative differences in the patterns of change in rates of synthesis of fatty acid and cholesterol suggest specific differential regulation at the level of acetyl-CoA carboxylase and/or 3-hydroxy-3-methylglutaryl-CoA reductase (which influence the relative use of cytoplasmic acetyl-CoA for fatty acid and cholesterol synthesis respectively). The introduction of regulatory loci distal to PDH might be expected, since, under conditions of unrestricted feeding, the primary objective is not carbohydrate conservation but the optimal utilization of available carbohydrate.

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