Pregnancy and pentobarbital anaesthesia modify hepatic synthesis of acylglycerol glycerol and glycogen from gluconeogenic precursors during fasting in rats

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1. Incorporation of gluconeogenic precursors into blood glucose and hepatic glycogen and acylglycerol glycerol was examined in 24 h-fasted virgin rats by using a flooding procedure for substrate administration. At 10 min after their intravenous injection, the conversion of alanine or glycerol into liver glycogen or acylglycerol glycerol was proportional to glucose synthesis. 2. In 24 h-fasted 21-day-pregnant rats, the incorporation of alanine and glycerol into hepatic acylglycerol glycerol was markedly enhanced compared with the control group. In addition, during fasting at late pregnancy, the proportion of substrates directed to acylglycerol glycerol as compared with the fraction incorporated into glucose was augmented. 3. In pentobarbital-treated fasted rats, the incorporation of both alanine and pyruvate into circulating glucose and into hepatic glycogen and acylglycerol glycerol was increased. Pentobarbital treatment increased the proportion of substrates incorporated into liver glycogen, compared with the fraction appearing in circulating glucose. These changes were concomitant with a marked accumulation of glycogen. 4. The data indicate that, during fasting, gluconeogenesis provides glucose as well as hepatic glycogen and acylglycerol glycerol, independently of whether the substrates enter gluconeogenesis at the level of pyruvate or dihydroxyacetone phosphate.

INTRODUCTION

Hepatic gluconeogenesis plays a central role in maintaining glucose homeostasis when exogenous glucose is not supplied [1,2]. However, gluconeogenesis also accomplishes other metabolic functions. It is the source of intermediates, such as glucose 6-phosphate, that can be used for glycogen synthesis [3–6], actively contributing to the repletion of liver glycogen stores during refeeding [7,8]. Furthermore, the conversion of pyruvate into hepatic acylglycerol glycerol during fasting has also been shown [9], suggesting that gluconeogenesis might also provide α -glycerol phosphate for fatty acid esterification.

The present study was designed to assess whether gluconeogenic incorporation into hepatic glycogen and acylglycerol glycerol depends on the nature of the gluconeogenic precursor. To this end, we have examined the pattern of incorporation *in vivo* of alanine, pyruvate or glycerol, which enter gluconeogenesis at different levels. Furthermore, this investigation was expanded to certain conditions characterized by accelerated hepatic gluconeogenic activity during fasting, such as pregnancy and pentobarbital treatment. In the fasted late-pregnant rat there is a complex array of metabolic adaptations that include the appearance of hypoglycaemia, activated gluconeogenesis and accelerated catabolism of adipose-tissue stores [10–13]. On the other hand, pentobarbital anaesthesia in fasted rats accelerates gluconeogenic activity and glycogen repletion after intravenous glucose infusion [8,14].

MATERIALS AND METHODS

Animals

Sprague–Dawley female rats were mated when they reached 160 g, and gestation was timed from the appearance of spermatozoa in vaginal smears. Sex- and age-matched virgin rats were studied in parallel. Animals were kept in collective cages in a temperature-controlled room ($23 \pm 1^\circ\text{C}$) with a 12 h-on/12 h-off light cycle, and fed *ad libitum* with Purina chow pellets for rats (Panlab, Barcelona, Spain), containing (by wt.) 17% protein, 3% lipid, 58.7% carbohydrate, 4.3% cellulose and 5% minerals, and having an energy content of 12.2 kJ (2.9 kcal)/kg. Rats fasted for 24 h were used in all studies.

Experiments *in vivo*

Pregnant rats at day 21 of gestation and their virgin controls were injected intraperitoneally with either sodium pentobarbital (33 mg/kg body wt.) or saline, and 30 min thereafter they received a tail-vein pulse injection of [U - ^{14}C]alanine, [3 - ^{14}C]pyruvate or [U - ^{14}C]glycerol (10 μCi , 0.2 mmol/200 g body wt.) (The Radiochemical Centre, Amersham, Bucks., U.K.). Animals were guillotined 10 min after tracer administration, and blood was collected from the neck wound into heparinized tubes at the same time as a liver sample was excised and immediately placed in liquid N_2 . The time elapsed between killing the animal and freezing of a liver sample was never longer than 6 s.

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Analyses and calculations

Blood samples were deproteinized with $\text{Ba(OH)}_2/\text{ZnSO}_4$ [15], and the supernatants were used for glucose determination [16] and ^{14}C glucose purification. In experiments using $[\text{U-}^{14}\text{C}]$ alanine and $[\text{3-}^{14}\text{C}]$ pyruvate, ^{14}C glucose purification was done by ion-exchange column chromatography, using anionic-cationic micro-columns, as previously described [10]. With this technique, recovery of $[\text{U-}^{14}\text{C}]$ glucose added to blood before protein precipitation was over 99%, whereas less than 0.19%, 0.22% and 0.22% of $[\text{U-}^{14}\text{C}]$ alanine, $[\text{3-}^{14}\text{C}]$ pyruvate and $[\text{U-}^{14}\text{C}]$ lactate respectively were recovered in the same fraction, when also added to blood. When $[\text{U-}^{14}\text{C}]$ alanine was the injected tracer, another blood sample, distinct from that used for glucose purification, was used to obtain plasma that was deproteinized with 10% sulphosalicylic acid in 0.1 M-HCl. The supernatants were used for alanine quantification with a Beckman 121 BM amino acid analyser and for purification of ^{14}C alanine through cation micro-columns (2.5 cm $\times$ 4 mm) (AG 50W X8, from Bio-Rad Laboratories, Richmond, CA, U.S.A.) eluted with 3 ml of distilled water and 3 ml of 2 M- NH_3 . With this technique, recovery of $[\text{U-}^{14}\text{C}]$ alanine added to plasma was over 93%, whereas that of $[\text{U-}^{14}\text{C}]$ glucose was less than 0.4%. When glycerol was used as the tracer, ^{14}C glucose and ^{14}C glycerol were separated from plasma by ascending paper chromatography [17]. With this technique, recovery of $[\text{U-}^{14}\text{C}]$ glucose and $[\text{U-}^{14}\text{C}]$ glycerol was 92% and 86% respectively. Circulating concentrations of glycerol were determined fluorimetrically [18]. Samples of frozen liver were precipitated with ethanol after alkali digestion [19], and hydrolysed with 2.5 M- H_2SO_4 for 2 h at 100 °C for

radioactivity counting and glucose analysis with glucose oxidase, as described previously [10]. Another sample of frozen liver was used for lipid extraction [20] and fractionation [21]. The neutral acylglycerol fractions were used for saponification (1 h; 100 °C; ethanolic 1 M-KOH), and the radioactivity was partitioned into fatty acids and acylglycerol glycerol as previously described [11]. Radioactivity was measured in a 2,5-diphenyl-oxazole/1,4-bis-(5-phenyloxazol-2-yl)benzene-based scintillation cocktail dissolved in xylene/Triton X-100.

Results were expressed as μmol of gluconeogenic substrate incorporated into glucose, glycogen or acylglycerol glycerol during 10 min. Thus, the incorporation of substrate into glucose was calculated by the equation:

$$\text{Incorporation } (\mu\text{mol}/10 \text{ min}) = \frac{[\text{U-}^{14}\text{C}]\text{Glucose formation (d.p.m.}/10 \text{ min})}{\text{Sp. radioactivity of substrate in plasma (d.p.m.}/\mu\text{mol})}$$

In turn, the formation of ^{14}C glucose was calculated as radioactive substrate incorporated into glucose in the glucose distribution space of the animal [22], considering the 'glucose space' as 38% of body weight [13]. Incorporation of the gluconeogenic substrate into hepatic glycogen or acylglycerol glycerol was calculated as for glucose. Half-life ($t_{1/2}$) of the administered ^{14}C alanine or ^{14}C glycerol from blood was determined by linear regression of the decay curve by the method of least squares. Statistical comparisons among the groups were done with Student's *t* test.

RESULTS AND DISCUSSION

Synthesis of glycogen and acylglycerol glycerol from gluconeogenic substrates during fasting

To assess the comparative conversion of different gluconeogenic substrates into hepatic glycogen or acylglycerol glycerol in a condition of enhanced gluconeogenic activity, 24 h-fasted rats were intravenously injected with 0.2 mmol of either $[\text{U-}^{14}\text{C}]$ alanine or $[\text{U-}^{14}\text{C}]$ glycerol and studied 10 min afterwards. We have previously shown the existence of linear incorporation of gluconeogenic substrates into blood glucose and hepatic lipids, during the first 10 min of substrate administration [13,17], and therefore this time-frame was selected for these studies. Preliminary experiments indicated that after intravenous administration of 0.2 mmol of $[\text{U-}^{14}\text{C}]$ alanine or $[\text{U-}^{14}\text{C}]$ glycerol the specific radioactivity of the injected substrate was relatively constant in blood over a 20 min period (results not shown). This occurred in spite of a rapid decrease in the concentrations of unlabelled and radioactive substrate, and in that regard the disappearance rate for glycerol was somewhat higher than for alanine, as judged from the $t_{1/2}$ values (Table 1). As measured by this bolus administration procedure, the circulating concentrations of alanine and glycerol were much higher than under physiological situations (Table 1), and the measurement of gluconeogenic flux indicates the actual intrinsic gluconeogenic capacity rather than the contributions *in vivo* of the different endogenous substrates to glucose synthesis. Thus, using this flooding procedure for substrate administration, we circumvent the question raised by Katz *et al.* [23] concerning the sites of tracer administration and sampling in the study of metabolic rates. In addition, since we used a flooding dose of unlabelled substrate, we also eliminated the significance of any possible metabolic exchange between

Table 1. Circulating glucose and substrate concentrations and substrate disappearance rate in alanine- and glycerol-injected 24 h-fasted rats

Values are means $\pm$ S.E.M. for five to seven observations/group. Fasted rats received a pulse of 0.2 mmol (10 μCi) of either $[\text{U-}^{14}\text{C}]$ alanine or $[\text{U-}^{14}\text{C}]$ glycerol through the tail vein and blood was collected at different times to estimate the substrate-disappearance rate and specific radioactivity. Circulating glucose and substrate (alanine and glycerol) concentrations before and 10 min after substrate administration are shown. Initial glucose concentration was determined from blood collected from the tail, whereas glucose concentration at 10 min was determined from blood collected from the neck. * Significant difference between the alanine- and glycerol-injected groups ($P < 0.05$).

	Alanine	Glycerol
Initial glucose concn. (mM)	4.6 $\pm$ 0.4	5.0 $\pm$ 0.3
Glucose concn. at 10 min (mM)	4.8 $\pm$ 0.3	5.3 $\pm$ 0.2
Initial substrate concn. (μM)	433 $\pm$ 43	143 $\pm$ 11*
Substrate concn. at 10 min (μM)	1150 $\pm$ 51	577 $\pm$ 51*
$t_{1/2}$ of substrate-disappearance rate (min)	2.00 $\pm$ 0.07	1.78 $\pm$ 0.08*
Circulating sp. radioactivity of administered substrate (d.p.m./ μmol)	1283 $\pm$ 77	2558 $\pm$ 143*

^{14}C and ^{12}C atoms in the oxaloacetate pool, as has been previously reported [24].

Different specific radioactivities were attained for the alanine and the glycerol groups (Table 1) and, in consequence, data for substrate incorporation into glucose or hepatic products were corrected by the circulating specific radioactivity of the substrate (see the Materials and methods section). The incorporation of substrates into blood glucose, liver glycogen or liver acylglycerol glycerol was greater for glycerol than for alanine, even after correction for the specific radioactivity of the substrate (Table 2). However, the ratios of liver glycogen to blood glucose and of liver acylglycerol glycerol to blood glucose were similar for both substrates (Table 2), indicating that there is a constancy in the amount of substrate, flowing through gluconeogenesis, that is directed into glycogen or into the glycerol moiety of acylglycerols. The results indicate that equimolar amounts of alanine and glycerol have marked differences with regard to their conversion into glucose. Our data agree with greater values of glucose synthesis obtained from dihydroxyacetone than from lactate/pyruvate in isolated hepatocytes [25]. That might be explained in light of recent studies indicating that pyruvate carboxylase plays a primary role in the control of the entire pathway of gluconeogenesis from pyruvate, a role that is quantitatively more important than the steps catalysed by phosphoenolpyruvate carboxykinase or fructose-1,6-bisphosphatase [26,27]. In this regard, alanine enters gluconeogenesis at the level of pyruvate and therefore is subjected to control by pyruvate carboxylase, whereas glycerol avoids this step, since it is initially phosphorylated by glycerol kinase, whose activity in the liver is very high [28,29], and it enters gluconeogenesis in the form of dihydroxyacetone phosphate.

Table 2. Incorporation of gluconeogenic substrates into circulating glucose and hepatic glycogen and acylglycerol glycerol in the 24 h-fasted rat

Results are means $\pm$ S.E.M. for seven to ten observations per group. Rats fasted for 24 h were intravenously injected with 0.2 mmol (10 μCi) of $[\text{U-}^{14}\text{C}]$ alanine or $[\text{U-}^{14}\text{C}]$ glycerol, and 10 min later they were killed to determine the substrate incorporation into circulating glucose and into hepatic glycogen and acylglycerol glycerol. Results are expressed as μmol of circulating substrate incorporated into product/10 min per whole liver. * Significant difference between alanine and glycerol groups ($P < 0.05$).

	Incorporation (μmol of substrate/10 min)	
	Alanine	Glycerol
Incorporation into glucose	80.7 $\pm$ 13.3	127.0 $\pm$ 8.7*
Incorporation into glycogen	0.33 $\pm$ 0.01	0.46 $\pm$ 0.06*
Incorporation into acylglycerol glycerol	1.92 $\pm$ 0.38	3.04 $\pm$ 0.32*
Glycogen/glucose ratio	0.0041 $\pm$ 0.006	0.0036 $\pm$ 0.006
Acylglycerol glycerol/glucose ratio	0.024 $\pm$ 0.004	0.024 $\pm$ 0.003

Gluconeogenesis provides substrates for the synthesis of the glycerol moiety of hepatic acylglycerols. Since triose reduction is needed in order to progress into the pathway of acylglycerol glycerol synthesis, the gluconeogenic synthesis of this compound may be facilitated in the starved condition by the higher redox state of cytosol [30,31]. The proportion of gluconeogenic substrate incorporated into acylglycerol glycerol in comparison with the proportion released as glucose was similar for various substrates, including glycerol. Provided that glycerol is directly converted into α -glycerol phosphate by a glycerol kinase activity, preferential synthesis of acylglycerols from glycerol can be proposed. The role of hepatic acylglycerols synthesized during fasting might be related to a transient storage of fatty acids and/or their incorporation into very-low-density-lipoprotein particles. Liver is considered to be the main receptor site for non-esterified fatty acids [32]. Lipolysis in adipose depots is activated during fasting, so that a large amount of fatty acids is released into circulation and reaches the liver. Herein we have shown that a certain proportion of the α -glycerol phosphate required for fatty acid esterification comes from gluconeogenesis. Since it has been reported that the availability of α -glycerol phosphate limits hepatic acylglycerol synthesis *in vitro* [33–35], modification of gluconeogenic flux would alter the availability of α -glycerol phosphate and, in consequence, the esterification rate of the acyl groups.

Effect of pregnancy on the synthesis of acylglycerol glycerol and glycogen from gluconeogenic substrates during fasting

The same protocol as described above was used in virgin and 21-day-pregnant rats which had fasted for 24 h. Circulating specific radioactivity of alanine and glycerol did not differ between control and pregnant groups (results not shown). As shown in Table 3, glucose appearance 10 min after administration of labelled alanine and glycerol was similar in virgin and pregnant rats. Although no differences in the incorporation of the substrates into liver glycogen were detected between the two groups, their conversion into hepatic acylglycerol glycerol was markedly enhanced in the pregnant group (Table 3). The ratio of liver acylglycerol glycerol to blood glucose was always significantly greater in pregnant than in virgin animals, although values did not differ between substrates. In fact, a significant linear relationship was obtained when all the individual data for precursor incorporation into blood glucose were plotted against data for precursor incorporation into hepatic acylglycerol glycerol (results not shown). The ratio of liver glycogen to blood glucose was not altered with pregnancy.

Our results during fasting in late pregnancy revealed that hepatic acylglycerol glycerol synthesis from gluconeogenic substrates is modulated independently of the intrinsic gluconeogenic activity. During fasting in late pregnancy, triacylglycerol lipolysis is greatly enhanced [11,36], causing a marked elevation in the plasma concentration of non-esterified fatty acids and their subsequent arrival in the liver. The enhanced acylglycerol glycerol synthesis from gluconeogenic substrates in the fasted late-pregnant rat agrees with the high concentrations of hepatic α -glycerol phosphate found in this situation [37], and this condition is probably brought

Table 3. Incorporation of gluconeogenic substrates into blood glucose and hepatic glycogen and acylglycerol glycerol in the 24 h-fasted virgin or 21-day-pregnant rat

Results are means $\pm$ S.E.M. for five to ten observations per group. Rats fasted for 24 h were intravenously injected with 0.2 mmol (10 μ Ci) of [U- 14 C]alanine or [U- 14 C]glycerol, and 10 min later they were killed to determine the substrate incorporation into circulating glucose and hepatic glycogen and acylglycerol glycerol. Results are expressed as μ mol of circulating substrate incorporated into product/10 min per whole liver. * Significant difference between virgin control and pregnant groups ($P < 0.05$).

Rats...	Incorporation (μ mol of substrate/10 min)			
	Alanine		Glycerol	
	Control	Pregnant	Control	Pregnant
Incorporation into glucose	82.0 $\pm$ 12.8	80.9 $\pm$ 11.2	120.0 $\pm$ 8.4	105.1 $\pm$ 8.9
Incorporation into glycogen	0.34 $\pm$ 0.2	0.37 $\pm$ 0.05	0.42 $\pm$ 0.05	0.31 $\pm$ 0.05
Incorporation into acylglycerol glycerol	1.88 $\pm$ 0.37	5.12 $\pm$ 0.12*	3.10 $\pm$ 0.31	7.65 $\pm$ 1.47*
Glycogen/glucose ratio	0.0041 $\pm$ 0.0005	0.0045 $\pm$ 0.0004	0.0035 $\pm$ 0.0005	0.0029 $\pm$ 0.0003
Acylglycerol glycerol/glucose ratio	0.023 $\pm$ 0.004	0.063 $\pm$ 0.005*	0.026 $\pm$ 0.003	0.073 $\pm$ 0.007*

about by the increased arrival of non-esterified fatty acids.

Effect of anaesthesia on gluconeogenic synthesis of glycogen and acylglycerol glycerol during fasting

Pentobarbital anaesthesia is known to stimulate gluconeogenesis in fasted rats [13], so in a further set of experiments we investigated the incorporation of radioactive alanine and pyruvate into products in 24 h-fasted pentobarbital-anaesthetized and control rats. Data were expressed as d.p.m. of 14 C-labelled substrates incorporated into product. In keeping with previous observations [13], pentobarbital anaesthesia caused a marked increase in the incorporation of alanine and pyruvate into blood glucose (Table 4). Furthermore, incorporation of [14 C]-alanine and [14 C]pyruvate into both liver glycogen and acylglycerol glycerol was also significantly enhanced by pentobarbital anaesthesia. The effect of pentobarbital anaesthesia on liver glycogen synthesis from the two substrates studied was greater than the effect on synthesis

of either glucose or liver acylglycerol glycerol (Table 4). Similar results were obtained when data from alanine-injected groups were corrected for specific radioactivity of circulating substrate (results not shown). To gain a better understanding of these effects, data were also expressed as ratios of liver [14 C]glycogen or [14 C]-acylglycerol glycerol to circulating [14 C]glucose (Table 4). Pentobarbital anaesthesia did not modify the liver [14 C]acylglycerol glycerol/blood [14 C]glucose ratio, although it did cause a large increase in the liver [14 C]glycogen/blood [14 C]glucose ratio, from both alanine and pyruvate. In addition, we found that the liver glycogen concentrations markedly increased in pentobarbital-treated rats (Table 5), and that specific radioactivity of [14 C]glycogen was lower in the anaesthetized group than in the control group (Table 5), suggesting that the net accumulation of liver glycogen caused by pentobarbital treatment was not entirely due to gluconeogenesis, but also a consequence of an accelerated glycogen synthesis via the direct pathway, i.e. glucose $\rightarrow$

Table 4. Incorporation of gluconeogenic substrates into circulating glucose and hepatic glycogen and acylglycerol glycerol in control and pentobarbital-anaesthetized rats during fasting

Results are means $\pm$ S.E.M. for five to ten observations per group. Rats fasted for 24 h, either conscious or unconscious as a result of a venous injection of sodium pentobarbital (33 mg/kg body wt.), were intravenously injected with 0.2 mmol (10 μ Ci) of [U- 14 C]alanine or [3- 14 C]pyruvate, and 10 min later they were killed to determine substrate incorporation into circulating glucose and into hepatic glycogen and acylglycerol glycerol. Results are expressed as d.p.m. of substrate incorporated into product/10 min per whole liver. * Significant difference between control and pentobarbital-treated groups ($P < 0.05$).

	$10^{-2} \times$ Incorporation (d.p.m./10 min)			
	Alanine		Pyruvate	
	Control	Pentobarbital	Control	Pentobarbital
Incorporation into glucose	1036 $\pm$ 171	2258 $\pm$ 128*	1724 $\pm$ 72	2997 $\pm$ 43*
Incorporation into glycogen	4.2 $\pm$ 1.6	12.4 $\pm$ 2.4*	6.7 $\pm$ 0.7	32.9 $\pm$ 9.0*
Incorporation into acylglycerol glycerol	24.6 $\pm$ 4.9	50.9 $\pm$ 5.6*	36.8 $\pm$ 3.0	52.9 $\pm$ 7.1*
Glycogen/glucose ratio	0.0041 $\pm$ 0.0005	0.0055 $\pm$ 0.0004*	0.0039 $\pm$ 0.0005	0.0109 $\pm$ 0.0008*
Acylglycerol glycerol/glucose ratio	0.024 $\pm$ 0.004	0.023 $\pm$ 0.003	0.021 $\pm$ 0.003	0.018 $\pm$ 0.003

Table 5. Glycogen concentration and glycogen specific radioactivity in liver after gluconeogenic-substrate administration to control and pentobarbital-anaesthetized rats during fasting

Results are means $\pm$ S.E.M. for five to ten observations per group. For details, see the legend to Table 4. At 10 min after administration of 0.2 mmol (10 μ Ci) of [U- 14 C]-alanine or [3- 14 C]pyruvate to control or pentobarbital-treated 24 h-fasted rats, the concentration and specific radioactivity of liver glycogen were assessed. * Significant difference between the control and pentobarbital groups ($P < 0.05$).

Precursor	Rats	Glycogen (μ mol/g)	Glycogen sp. radioactivity (d.p.m./ μ mol)
Alanine	Control	0.22 $\pm$ 0.06	403 $\pm$ 112
	Pentobarbital	7.22 $\pm$ 1.11*	27 $\pm$ 5*
Pyruvate	Control	0.56 $\pm$ 0.17	198 $\pm$ 46
	Pentobarbital	12.78 $\pm$ 1.11*	43 $\pm$ 12*

glucose 6-phosphate $\rightarrow$ glycogen [8]. Our data are in keeping with the report [8] of enhanced glycogen repletion after intravenous glucose infusion in the pentobarbital-anaesthetized rat, probably owing to activation of the direct pathway for hepatic glycogen synthesis. Certainly this condition represents an interesting model in which to investigate the possible regulating mechanisms of the direct and indirect pathways for glycogen synthesis in the liver, and deserves further study.

In conclusion, our results indicate that during fasting hepatic gluconeogenesis serves to synthesize glucose as well as glycogen and the glycerol moiety of acylglycerol, in a fashion that is independent of the nature of the gluconeogenic precursor studied. Thus there is a certain loss of gluconeogenic intermediates at the level of dihydroxyacetone phosphate and glucose 6-phosphate, and this channelling relies on intrinsic gluconeogenic activity. In late pregnancy there is an increased proportion of gluconeogenic substrates directed into hepatic acylglycerol, perhaps as a consequence of an increased arrival of non-esterified fatty acids from adipose depots. During pentobarbital anaesthesia, a greater proportion of gluconeogenic precursors is incorporated into hepatic glycogen, contributing to the active net synthesis of glycogen which takes place in that condition.

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