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Effect of nutritional status on insulin sensitivity *in vivo* and tissue enzyme activities in the rat

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The hyperinsulinaemic-glucose-clamp technique, in combination with measurement of glucose turnover in conscious unrestrained rats, was used to assess the effects of nutritional status on insulin sensitivity *in vivo* and glucose metabolism. Liver, heart and quadriceps skeletal-muscle glycogen content and activities of pyruvate dehydrogenase (PDH) and glycogen synthase were measured both basally and at the end of a 2.5 h glucose clamp (insulin 85 munits/h) in rats 6, 24 and 48 h after food withdrawal. Clamp glucose requirement and glucose turnover were unchanged by fasting. Activation of glycogen synthase and glycogen deposition in liver and skeletal muscle during the clamps were also not impaired in rats after a prolonged fast. By contrast with skeletal muscle, activation of cardiac-muscle glycogen synthase and glycogen deposition during the clamps were markedly impaired by 24 h of fasting and were undetectable at 48 h. Skeletal-muscle PDH activity fell with more prolonged fasting (6 h, $15.3 \pm 3.4\%$; 24 h, $4.7 \pm 0.7\%$; 48 h, $4.3 \pm 0.6\%$ active; $P < 0.005$), but at 24 and 48 h was stimulated by the clamp to values unchanged by the duration of fasting. Stimulation of cardiac PDH activity by the clamp was, however, impaired in rats fasted for 24 or 48 h. Basal hepatic PDH did not change significantly with fasting (6 h, $5.3 \pm 1.1\%$; 24 h, $4.6 \pm 0.7\%$; 48 h, $3.9 \pm 0.5\%$), and, although it could be partly restored at 24 h, very little stimulation occurred at 48 h. Hepatic pyruvate kinase and acetyl-CoA carboxylase activity were both stimulated by the clamps, and this was not impaired with more prolonged fasting. During the glucose clamps, blood concentrations of lactate, pyruvate and alanine were increased to a greater extent in rats fasted for 24 and 48 h than in rats studied 6 h after food withdrawal. The findings suggest that, although sensitivity to insulin of whole-body glucose disposal is unchanged with fasting, there may be qualitative differences in the metabolism of glucose.

INTRODUCTION

Glucose intolerance after energy deprivation is well recognized [1,2], but the biochemical and hormonal mechanisms are not entirely clear. Although decreased insulin secretion in response to oral or intravenous glucose is clearly important [2–4], there is evidence that tissue insensitivity to insulin may also play a part [1,2,5,6]. Liver and skeletal muscle are quantitatively the most important tissues for disposal for an oral glucose load [7,8]. However, adipose-tissue insensitivity to insulin in fasted man and animals might also contribute indirectly to glucose intolerance by increasing the plasma concentrations of non-esterified fatty acids (NEFA) and resultant ketone bodies which might inhibit glucose utilization by skeletal muscle [9–12]. Indeed, resistance to insulin has been demonstrated in adipocytes isolated from fasted rats [13–15], but whether skeletal muscle itself becomes less sensitive to insulin is unclear. Decreased glucose transport and responsiveness to insulin have been demonstrated in rat heart and diaphragm after energy deprivation [16]. These muscles are, however, highly specialized and constantly contracting, and may not be representative of the skeletal-muscle mass [17,18]. Surprisingly, perhaps, studies with isolated perfused rat hind-limb [17,19] and the isolated mouse soleus muscles [20] have suggested that fasting actually increases the sensitivity to insulin of glucose uptake.

To address the issue of tissue sensitivity to insulin after a period of fasting we have used the hyperinsulinaemic-glucose-clamp technique [21] in combination with measurement of glucose turnover in rats 6, 24 and 48 h after food withdrawal. As a measure of the action of insulin on liver, heart and quadriceps skeletal muscle in these animals, we have measured the concentrations of glycogen and the activities of a number of insulin-regulated enzymes in these tissues both basally and at the end of the glucose-clamp studies.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 300–380 g were used. Rats were maintained on standard laboratory chow and were handled daily for 6 weeks before studies *in vivo*. Studies were performed in rats 6, 24 or 48 h after withdrawal of food.

Radioisotopic-glucose turnover and euglycaemic-clamp studies

Insulin sensitivity was assessed by the euglycaemic-clamp technique [21]. Jugular- and femoral-venous cannulae were implanted under diethyl ether anaesthesia 24 h before study, and all blood samples were taken from conscious unrestrained animals. The duration of the fast

Abbreviations used: NEFA, non-esterified fatty acid; PDH, pyruvate dehydrogenase.

(6, 24 or 48 h) was timed to the end of the 2.5 h glucose-clamp study and the time of tissue removal from the animals.

After the basal blood samples were taken for glucose, insulin and intermediary-metabolite concentrations, a primed continuous infusion of [3-^{3}H]glucose (0.04 μCi/min) diluted in Haemaccel (Polygeline) was begun, and continued for 60 min. Blood samples (100 μl) were taken from the femoral-venous cannula at 30, 50, 55 and 60 min for determination of plasma glucose concentration and plasma glucose specific radioactivity.

In rats fasted for 24 or 48 h, from +60 min highly purified human insulin (Actrapid; Novo Industri) diluted in Haemaccel was infused (85 munits/h) for 2.5 h through one limb of a double-lumen cannula (Miles, Stoke Poges, Bucks., U.K.) connected to the jugular-venous cannula. This solution also contained glucose tracer to give an infusion rate of 0.12 μCi of [3-^{3}H]glucose/min throughout the clamp. In a separate group of six 24 h-fasted rats, insulin was infused as above at the rate of 255 munits/h for 2.5 h.

Blood samples (30 μl) for glucose measurement were taken at 5–10 min intervals from the femoral-venous cannula and replaced with 0.15 M-NaCl. Blood samples were taken for measurement of insulin at 150, 180 and 210 min (100 μl), of intermediary metabolites (150 μl) at 180 min, and of glucose specific radioactivity (80 μl) at 10 min intervals between 180 and 210 min, and were replaced with an equal volume of fresh washed rat erythrocytes in 0.15 M-NaCl. Blood glucose concentrations were maintained at 6.0 mM by a variable infusion of 500 g of glucose/l in water through the other arm of the double-lumen cannula. As glucose radioactivity (d.p.m.) was measured in plasma, plasma rather than blood glucose concentration was measured during turnover studies. Owing to the very low content of erythrocyte glucose transporters, blood glucose in rats is significantly lower than plasma glucose (35 % at 8.5 mM).

Basal glucose turnover and glucose turnover during a clamp were also measured in post-absorptive rats. These studies commenced 5 and 3.5 h after food withdrawal respectively, measurements of turnover, insulin sensitivity and tissue studies thus being made at 6 h after food withdrawal.

At the end of the clamp (+210 min) rats were anaesthetized by intravenous injection of 9 mg of pentobarbitone, resulting in surgical anaesthesia within 3 s. While the infusions of insulin and glucose were continued, tissue from liver, quadriceps skeletal muscle and heart was freeze-clamped within 15 s, ground under liquid N_2 and stored at −70 °C until assayed for glycogen content, glycogen synthase and PDH activities. Liver pyruvate kinase and acetyl-CoA carboxylase were also measured. Tissue was also obtained from rats that were anaesthetized in the basal state.

For determination of glucose specific radioactivity, plasma was immediately deproteinized with $Ba(OH)_2$/$ZnSO_4$ [22] and the neutral extract passed down a column of AG2-X8 anion-exchange resin. The column was eluted with deionized water, and the eluate freeze-dried. Radioactivity was determined in a Beckman liquid-scintillation counter, an external standard being used to correct for efficiency and overlapping. Minimum recovery after deproteinization, neutralization, passage through the column and freeze-drying was 91 %. Triplicate samples of the basal and clamp infusion fluids were processed in a manner identical with that for the plasma samples. Steady-state glucose specific radioactivity was reached by 30 min during the basal turnovers and also by 180 min during the clamps. The mean of the last three plasma glucose specific-radioactivity determinations in each period was therefore used in the calculation of glucose turnover. Glucose turnover was estimated from the formula:

$$\text{Glucose turnover} = \frac{\text{tracer infused (d.p.m./min)}}{\text{glucose sp. radioactivity (d.p.m./}\mu\text{mol)}}$$

Enzyme assays

Glycogen synthase activity was measured as previously described [23]. After extraction of tissues in the appropriate manner, PDH (active and total) were measured as described by McCormack *et al.* [24], pyruvate kinase as in [25], and acetyl-CoA carboxylase as in [26]. As similar results were obtained by expression of PDH activity relative to mitochondrial glutamate dehydrogenase activity, PDH data are expressed per unit wt. of tissue.

Other analyses

Glycogen was determined by the amyloglucosidase method [27]. Blood for determination of intermediary metabolites was deproteinized with 0.6 M-$HClO_4$ and the extract assayed for lactate, pyruvate, alanine, glycerol and 3-hydroxybutyrate by automated enzymic fluorimetric methods [28]. Plasma insulin was determined by radioimmunoassay [29], by using a rat or human insulin standard (Novo Industri) as appropriate and guinea-pig antiserum to pig insulin. The antibody used reacts less well with rat than with human insulin; thus 40 % displacement of ^{125}I tracer in the assay was achieved by 1.7 μg of human insulin/l and by 2.8 μg of rat insulin standard/l. Blood glucose was measured by a glucose oxidase method (Yellow Springs Glucose Analyser; Clandon Scientific, Aldershot, Hants., U.K.).

Statistical methods and calculations

Results are presented as means ± S.E.M. Significant differences between groups were assessed by Student's paired or unpaired t test or analysis of variance as appropriate. Correlations were sought by Pearson's least-squares method.

Materials

Biochemical reagents were purchased from Sigma or BDH (both of Poole, Dorset, U.K.). Auxiliary enzymes for the assays were from either Sigma or Boehringer (Mannheim, Germany). [3-^{3}H]Glucose was from Amersham International (Amersham, Bucks., U.K.). AG2-X8 anion-exchange resin was from Bio-Rad (Richmond, CA, U.S.A.). Insulin was from Novo Industri (Bagsvaerd, Denmark). Haemaccel was from Hoechst (Frankfurt am Main, Germany).

RESULTS

Glucose kinetics and blood intermediary-metabolite concentrations

Under basal conditions, plasma glucose concentrations and glucose turnover fell with duration of fasting (Table 1), accompanied by a fall in plasma insulin concentrations (6 h, 0.70 ± 0.07 μg/l; 24 h, 0.50 ± 0.05 μg/l; 48 h, 0.44 ± 0.04 μg/l; $P < 0.05$ by analysis of variance). This

Table 1. Glucose kinetics in rats fasted for 6, 24 or 48 h and during a glucose clamp

Values are given as means ± S.E.M. See the Materials and methods section for details. $^{**}P < 0.01$, $^{***}P < 0.001$ for increasing duration of fast (by analysis of variance); †††$P < 0.001$ compared with 24 h-fasted rats infused with 85 munits of insulin/h.

	Duration of fast (h)	*n*	Plasma glucose (mmol/l)	[^{3}H]Glucose turnover (μmol/min per kg)	Clamp glucose requirement (μmol/min per kg)	Residual hepatic glucose output (μmol/min per kg)
Basal	6	6	6.9 ± 0.2	64 ± 3	–	–
	24	7	5.8 ± 0.2	52 ± 4	–	–
	48	7	4.9 ± 0.2***	42 ± 4**	–	–
End of clamp (85 munits of insulin/h)	6	6	8.8 ± 0.1	197 ± 15	199 ± 12	−2 ± 5
	24	6	8.9 ± 0.1	196 ± 16	198 ± 22	−3 ± 6
	48	6	8.8 ± 0.1	176 ± 8	174 ± 11	2 ± 4
End of clamp (255 munits of insulin/h)	24	6	8.8 ± 0.0	364 ± 19†††	380 ± 22†††	−15 ± 5

Table 2. Blood intermediary-metabolite and plasma NEFA concentrations in rats fasted for 6, 24 or 48 h and during a glucose clamp

Values are given as means ± S.E.M. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$ compared with animals in the basal state; $^{**}P < 0.01$, $^{***}P < 0.001$ for increasing duration of fast (by analysis of variance); †$P < 0.05$, ††$P < 0.01$ compared with 24 h-fasted rats infused with 85 munits of insulin/h.

	Basal			Clamp (85 munits of insulin/h)			Clamp (255 munits of insulin/h)
Duration of fast (h)...	6	24	48	6	24	48	24
Metabolite *n*...	12	12	8	6	6	6	6
Lactate (μmol/l)	609 ± 66	569 ± 48	648 ± 69	1169 ± 58^c	1926 ± 201^c	1910 ± 152**^{c}	2748 ± 146††^{c}
Pyruvate (μmol/l)	71 ± 6	77 ± 7	80 ± 9	106 ± 14^a	258 ± 19^c	267 ± 30***^{c}	346 ± 27†^{c}
Alanine (μmol/l)	320 ± 18	230 ± 12	198 ± 6***	300 ± 26	383 ± 43^b	373 ± 12^c	474 ± 20^c
Glycerol (μmol/l)	120 ± 12	187 ± 14	255 ± 17***	51 ± 6^c	71 ± 6^c	99 ± 8***^{c}	69 ± 7^c
3-Hydroxbutyrate (μmol/l)	104 ± 30	717 ± 65	1463 ± 120***	18 ± 2^c	21 ± 2^c	29 ± 4^c	19 ± 2^c
NEFA (mmol/l)	0.51 ± 0.04	0.96 ± 0.07	1.43 ± 0.06***	0.10 ± 0.02^c	0.12 ± 0.02^c	0.14 ± 0.02^c	0.08 ± 0.02^c

was associated with unchanged blood lactate and pyruvate concentrations but falling blood alanine concentrations (Table 2, $P < 0.001$). As expected blood glycerol, 3-hydroxybutyrate and plasma NEFA concentrations rose over the period of starvation (Table 2).

Plasma glucose concentrations were successfully maintained during the clamp studies and were not different in the three study groups infused at 85 munits of insulin/h (Table 1). Individual coefficients of variation of plasma glucose calculated for each animal were 6.4 ± 1.7 (S.D.)%, 6.2 ± 1.8% and 7.1 ± 1.5% for the rats fasted for 6, 24 and 48 h respectively. Plasma insulin concentrations (human insulin standard) at the infusion rate of 85 munits/h were not different in the three groups studied, with an overall mean concentration of 122 ± 8 munits/l (4.22 ± 0.28 μg/l). This is known to be submaximal for whole-body glucose disposal and for stimulation of glucose uptake by skeletal muscle [21,30,31], and thus any changes in tissue sensitivity with fasting should be revealed. Glucose turnover increased 3–4-fold during the clamp, and the absolute rate was not different with more prolonged fasting (Table 1). The glucose infusion rate required to maintain the desired blood glucose concentration during the clamp was also similar in the three groups of rats (Table 1). Thus residual hepatic glucose production rate during the clamp, calculated as the difference between glucose turnover rate and the rate of glucose infusion at steady state, was also not different between the experimental groups (Table 1).

In all groups, blood lactate and pyruvate concentrations were increased, whereas glycerol and 3-hydroxybutyrate concentrations were decreased, during the clamp compared with the basal state (Table 2). Blood alanine concentrations were increased by the clamp in 24 h- and 48 h-fasted rats, but not in rats fasted for 6 h (Table 2). Blood lactate and pyruvate concentrations during the clamp rose with the duration of fasting up to 24 h, but no further change was seen after 24 h. Clamp blood glycerol concentrations rose over the 48 h, however ($P < 0.001$), and, although 3-hydroxybutyrate concentrations were suppressed during all insulin infusions, the small change at 48 h was also statistically significant ($P < 0.05$) compared with rats fasted for 6 h.

Plasma glucose concentrations were successfully maintained at the same concentration (8.8 mM) during the high-dose clamp (after 24 h of fasting), and insulin concentrations reached a plateau at 368 ± 29 munits/l. At this value, near-maximal rates of whole-body glucose disposal would be expected in the rat [30]. Steady-state glucose turnover was then 364 ± 19 μmol/min per kg.

Table 3. Hepatic glycogen concentration and enzyme activities in rats fasted for 6, 24 or 48 h and at the end of a glucose clamp

Glycogen is expressed as μmol of glucose residues after complete hydrolysis by amyloglucosidase. For glycogen synthase, PDH and pyruvate kinase, 1 unit of enzyme activity is that which converts 1 μmol of substrate into product/min at 30 °C. One munit of acetyl-CoA carboxylase activity represents 1 nmol of malonyl-CoA formed/min at 35 °C. Citrate-activated acetyl-CoA carboxylase was measured after incubation of extracts for 30 min with 20 mM-citrate at 30 °C. Values are expressed as means ± S.E.M.: $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ compared with animals in the basal state; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ for increasing duration of fast (by analysis of variance); $\dagger P < 0.05$, $\dagger\dagger P < 0.01$ compared with 24 h-fasted rats infused with 86 munits of insulin/h.

	Basal			Clamp (85 munits of insulin/h)			Clamp (255 munits of insulin/h)
Duration of fast (h)...	6	24	48	6	24	48	24
n...	6	7	7	6	6	6	6
Glycogen (μmol/g wet wt.)	174±18	7±2	8±2***	282±18^{b}	90±8^{c}	86±7***^{c}	132±8††^{c}
Glycogen synthase (units/g wet wt.)							
GS_a	0.44±0.06	0.23±0.01	0.21±0.02***	0.61±0.09	0.55±0.10^{b}	0.55±0.06^{c}	0.80±0.08^{c}
GS_T	0.90±0.13	0.71±0.04	0.54±0.07*	1.16±0.09	1.13±0.10^{b}	1.07±0.10^{b}	1.24±0.05^{c}
PDH (units/g wet wt.)							
PDH_T	1.7±0.1	1.5±0.2	2.1±0.2	1.9±0.2	1.7±0.1	1.7±0.2	1.7±0.1
PDH_a%	5.3±1.1	4.6±0.7	3.9±0.5	12.0±0.5^{c}	10.3±1.3^{b}	5.2±1.3***	6.8±0.3^{a}†
Pyruvate kinase							
K_m (mM-phosphoenolpyruvate)	1.79±0.16	1.45±0.08	1.58±0.06	1.13±0.15^{a}	1.20±0.08^{a}	1.30±0.05^{b}	1.02±0.02†^{c}
$V_{max.}$ (units/g of protein)	97.7±20.2	57.7±9.8	63.3±5.5	76.8±16.8	104.2±17.6	46.0±8.3	72.7±9.4
Acetyl-CoA carboxylase (munits/g wet wt.)							
−citrate	100±4	102±9	104±4	171±7^{c}	195±13^{c}	160±7^{c}	123±10
+citrate	383±7	255±18	193±10***	392±28	373±16^{c}	262±14***^{b}	393±20

Apparent hepatic glucose production during the last 30 min of the clamp was −15±5 μmol/min per kg, not significantly different from that with the lower-dose clamps. Blood lactate ($P < 0.01$) and pyruvate ($P < 0.05$) concentrations were further elevated by the high-dose clamp, with no significant change in blood alanine concentration. No change was found in the already suppressed blood glycerol and 3-hydroxybutyrate concentrations (Table 2).

Hepatic glycogen concentration and enzyme activities

Hepatic glycogen was completely depleted by 24 h without food (Table 3). The glucose clamp significantly increased hepatic glycogen concentration, with a similar change at different intervals without food. The high-insulin-dose clamp at 24 h of fasting resulted in a higher hepatic glycogen concentration compared with the low-insulin-dose clamp (Table 3, $P < 0.005$).

Basal total liver glycogen synthase decreased ($P < 0.05$) throughout the 48 h study period, whereas glucose 6-phosphate-independent glycogen synthase activity (GS_a) showed a major decline by 24 h, and little further change thereafter (Table 3, $P < 0.001$). Hepatic glycogen synthase activity (GS_a) was increased by the clamp (Table 3) and possibly further increased at 24 h of fasting by the higher insulin dose (Table 3, $t = 2.029$; $P < 0.1$, not significant). Glycogen synthase measured in the presence of 10 mM-glucose 6-phosphate (GS_T) was also increased during the clamps in 24 h- and 48 h-fasted rats (Table 3), so that activity at the end of the clamp did not differ with duration of fasting.

Hepatic total PDH (PDH_T) did not change with duration of fasting, or with the clamps (Table 3). Basal pyruvate dehydrogenase activity (PDH_a%) was not significantly different with duration of fasting, but stimulated activity at the end of the clamps decreased markedly (Table 3, $P < 0.001$). Thus at 48 h of fasting there was no detectable activation by the clamp. Activation of hepatic PDH in the 24 h-fasted rats was not enhanced by the high-insulin-dose clamp. Thus, although PDH_a% was increased by the 255 munits-of-insulin/h clamp compared with the basal state (Table 3, $P < 0.05$), activity at the end of the clamp was lower than during the 85 munits-of-insulin/h clamp (6.8±0.3 versus 10.3±1.3 units/g wet wt., $P < 0.05$).

Hepatic pyruvate kinase activity measured at 6 mM-phosphoenolpyruvate ($V_{max.}$) was erratic and not different between experimental groups. The K_m of pyruvate kinase for phosphoenolpyruvate did, however, decrease between basal and clamp conditions ($P < 0.005$ by analysis of variance), this activity being independent of duration of fasting. In 24 h-fasted rats the K_m for phosphoenolpyruvate was decreased by the high-insulin-dose clamp compared with the 85 munits-of-insulin/h clamp (1.02±0.02 mM- versus 1.20±0.08 mM-phosphoenolpyruvate, $P < 0.05$).

Citrate-stimulated hepatic acetyl-CoA carboxylase fell with duration of fasting in both the basal and insulin-

Table 4. Quadriceps-muscle glycogen concentration and enzyme activities in rats fasted for 6, 24 or 48 h and at the end of a glucose clamp

See the Materials and methods section and the legend to Table 3 for details. Values are expressed as means ± S.E.M: $^{b}P < 0.01$, $^{c}P < 0.001$ compared with animals in the basal state; $^{**}P < 0.01$, $^{***}P < 0.001$ for increasing duration of fast (by analysis of variance); ††$P < 0.01$, †††$P < 0.001$ compared with 24 h-fasted rats infused with 85 munits of insulin/h.

	Basal			Clamp (85 munits of insulin/h)			Clamp (255 munits of insulin/h)
Duration of fast (h)...	6	24	48	6	24	48	24
n...	6	7	7	6	6	6	6
Glycogen (μmol/g wet wt.)	33±3	17±1	15±1***	51±4^{b}	35±4^{c}	41±3^{c}	71±6†††^{c}
Glycogen synthase (units/g wet wt.)							
GS_a	0.41±0.03	0.42±0.03	0.35±0.04	0.68±0.04^{c}	0.69±0.06^{c}	0.78±0.04^{c}	0.93±0.05††^{c}
GS_T	2.27±0.12	2.17±0.09	1.99±0.13	2.30±0.05	2.22±0.16	2.44±0.11	2.12±0.05
PDH (units/g wet wt.)							
PDH_T	1.5±0.2	1.7±0.1	1.3±0.1	1.7±0.2	1.7±0.1	1.6±0.2	1.1±0.1
PDH_a%	15.3±3.4	4.7±0.7	4.3±0.6**	16.1±1.3	12.3±1.4^{c}	13.0±2.2^{b}	27.9±2.4†††^{c}

Table 5. Cardiac-muscle glycogen concentration and enzyme activities in rats fasted for 6, 24 or 48 h and at the end of a glucose clamp

See the Materials and methods section and the legend to Table 3 for details. Values are expressed as means ± S.E.M.; $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ compared with animals in the basal state; $^{**}P < 0.01$, $^{***}P < 0.001$ for increasing duration of fast by analysis of variance; †††$P < 0.001$ compared with 24 h-fasted rats infused with 85 munits of insulin/h.

	Basal			Clamp (85 munits of insulin/h)			Clamp (255 munits of insulin/h)
Duration of fast (h)...	6	24	48	6	24	48	24
n...	6	7	7	6	6	6	6
Glycogen (μmol/g wet wt.)	28±4	26±4	22±2	41±3^{a}	33±4	20±2**	58±3†††^{c}
Glycogen synthase (units/g wet wt.)							
GS_a	0.22±0.03	0.20±0.01	0.23±0.01	0.35±0.03^{b}	0.24±0.02	0.21±0.02**	0.43±0.03†††^{c}
GS_T	1.44±0.06	1.45±0.08	1.50±0.08	1.52±0.05	1.42±0.08	1.42±0.08	1.36±0.05
PDH (units/g wet wt.)							
PDH_T	4.3±0.4	3.1±0.2	3.9±0.3	4.2±0.3	4.3±0.4	4.0±0.6	4.3±0.2
PDH_a%	16.2±3.0	4.4±0.6	3.7±0.3**	22.7±4.4	11.2±2.4^{a}	11.2±4.1***	13.4±2.5^{b}

infused rats (Table 3, both $P < 0.001$). However, activity in the absence of citrate remained unchanged with fasting, in the basal state. Acetyl-CoA carboxylase activity measured in the absence of citrate was stimulated by the 85 munits-of-insulin/h clamp (Table 3, $P < 0.001$), with a similar change at different intervals without food.

Skeletal-muscle glycogen concentration and enzyme activities

Quadriceps-muscle glycogen concentration was partially depleted by 24 h of fasting (Table 4, $P < 0.001$ versus 6 h), with little change thereafter. Muscle glycogen concentrations were increased ($P < 0.001$ by analysis of variance) at the end of the clamps, but were not significantly different between experimental groups ($F = 4.583$, $0.05 < P < 0.1$). Glycogen deposition was further stimulated by the high-insulin-dose clamp (Table 4).

Quadriceps-muscle total glycogen synthase did not change with duration of fasting or with the clamps. Glycogen synthase activity (GS_a) was also unchanged with fasting in the basal or clamp states, and was similarly stimulated by the clamp itself at all durations of fasting (Table 4, $P < 0.001$). Glycogen synthase (GS_a) activity was increased to a greater extent by the high-insulin-dose clamp (0.93±0.05 versus 0.69±0.06 unit/g wet wt., $P < 0.01$). At the high insulin-infusion rate a strong correlation was found between clamp glucose requirement and quadriceps-muscle glycogen content ($r = 0.86$, $P < 0.05$) and possibly glycogen synthase (GS_a) activity

($r = 0.79$, not significant) at the end of the clamp. The relationships between end-of-clamp muscle glycogen content and clamp glucose requirement at the lower insulin-infusion rate were not statistically significant (6 h, $r = 0.69$; 24 h, $r = 0.62$; 48 h, $r = 0.79$).

Total muscle PDH activity was unchanged with duration of fasting, or with the clamps. However, PDH_a% fell markedly in the basal state with fasting (Table 4, $P < 0.005$), a change completed by 24 h. Although no stimulation of PDH_a% occurred with the low-insulin-dose clamp at 6 h of fasting, significant stimulation occurred from the depressed basal values at 24 and 48 h, so that activity at the end of the clamp did not differ with duration of fasting. In the 24 h-fasted rats activation of PDH was greater during the high-insulin-dose clamp (PDH_a% 27.9 ± 2.4% versus 12.3 ± 1.4%; $P < 0.001$).

Cardiac-muscle glycogen concentration and enzyme activities

No change in cardiac-muscle glycogen concentration in the basal state was found with fasting (Table 5). The glucose clamp increased cardiac-muscle glycogen concentrations ($P < 0.05$ by analysis of variance), but with a lesser effect with duration of fasting ($P < 0.005$). Thus at 48 h of fasting there was no glycogen deposition during the clamp (Table 5). Glycogen deposition was enhanced by the high-insulin clamp in rats fasted for 24 h (Table 5, $P < 0.001$ versus low-dose clamp).

Heart total glycogen synthase did not change with duration of fasting or with the clamps (Table 5). GS_a was also unchanged with fasting in the basal state, but stimulation by the clamp, though statistically significant overall (Table 5, $P < 0.02$), was markedly impaired by 24 h of fasting, and was undetectable at 48 h. Clamp GS_a was accordingly significantly lower with duration of fasting ($P < 0.01$). As with glycogen deposition, activation of glycogen synthase was enhanced by the high-dose clamp in rats fasted for 24 h (Table 5, $P < 0.001$).

Total cardiac-muscle PDH was unchanged with duration of fasting, or with the clamps (Table 5). As was the case with skeletal muscle, PDH_a% decreased markedly in the basal state with fasting (Table 5, $P < 0.005$). Significant stimulation of PDH_a% by the clamp was seen in rats fasted for 24 and 48 h ($P < 0.001$ by analysis of variance). Activity at the end of the clamp was, however, still lower with the longer durations of fasting than with the short period without food ($P < 0.001$). In contrast with skeletal muscle, activation of PDH was not enhanced by the higher insulin infusion rate.

DISCUSSION

Although impaired glucose tolerance after a period of energy deprivation is well recognized, it is unclear whether tissue insensitivity to insulin compounds the defect in insulin secretion. Early studies employing the intravenous insulin-tolerance test suggested insensitivity to insulin in 48 h-fasted mice [6]. Similarly, DeFronzo and colleagues [5], using the glucose-clamp technique, were able to demonstrate whole-body insensitivity to insulin in obese man fasted for 3 or 14 days. As skeletal muscle is the major tissue responsible for glucose disposal under conditions of a glucose clamp [30], these studies *in vivo* are at variance with the finding of increased sensitivity to insulin of glucose uptake by isolated mouse soleus muscle [20] or perfused rat hind-limb muscles [19]. A possible explanation for this apparent discrepancy is that in the studies *in vivo* plasma NEFA concentrations would be elevated and might be expected to impair glucose utilization by peripheral tissues [10,11]. Thus, in the obese fasted subjects, lipolysis from an expanded adipose-tissue mass would be enhanced, and suppression of lipolysis woud not be expected throughout the time interval (+20 to +120 min) of the glucose clamp during which glucose disposal was quantified [5].

In the current study, to assess the effects of insulin on peripheral tissues independently of alterations in plasma NEFA concentrations, the insulin infusion rate and duration of the clamp were chosen to ensure marked suppression of plasma NEFA concentrations. Glucose turnover and clamp glucose requirement were quantified during the last 30 min of the 2.5 h clamp, by which time plasma NEFA concentrations were suppressed to similar low values in all experimental groups (Table 2) and a steady state for glucose disposal had been attained. Under these circumstances insulin-stimulated whole-body glucose disposal was not decreased after a 48 h fast (Table 1). This is consistent with the finding of normal insulin-stimulated whole-body glucose disposal in non-obese malnourished man [32]. Our findings contrast with those of Penicaud and colleagues [33], who reported decreased glucose utilization during a glucose clamp in anaesthetized rats fasted for 84 h. However, when insulin-stimulated glucose metabolic rate in that study was expressed per unit body weight, there was little difference between fasted and post-absorptive rats. Unfortunately, in the study of Penicaud and colleagues, fasted and post-absorptive rats were clamped at their differing basal blood glucose concentrations, and thus any hypoglycaemic stimulus to counter-regulatory hormone secretion in the fasted rats might be expected to be operative during the clamps. The combination of a lower blood glucose concentration and counter-regulatory hormone secretion might also explain the failure to suppress hepatic glucose production during the clamps, despite the very high plasma insulin concentrations (5000 munits/l) achieved [33]. In the current study hepatic glucose production was completely suppressed during the clamps in all experimental groups, and thus it is not possible from the current study to ascertain whether the sensitivity of this pathway to insulin (in terms of a shift in the dose–response curve) is affected by fasting. It is possible that at submaximally effective insulin concentrations a difference might have been demonstrated.

Although the livers of the experimental animals were not weighed in our study, to decrease warm-ischaemia time, it may be assumed that they were 9–11 g. This implies that less than 16% of the infused glucose was stored as hepatic glycogen during the clamps, and this value was not affected by nutritional state. Even at the higher insulin-infusion rate in 24 h-fasted rats, the increased liver glycogen deposition in this group accounted for only 9% of the infused glucose. Since under glucose-clamp conditions little glucose carbon is incorporated into liver lipids [30], our findings are consistent with previous studies demonstrating that at high insulin doses whole-body glucose disposal is largely determined by skeletal muscle [23,30,31].

Consistent with our findings of normal whole-body

sensitivity to insulin in 48 h-fasted animals, we were unable to demonstrate any impairment of glycogen synthesis in muscle in these studies. Thus, although end-of-clamp muscle glycogen concentrations appeared lower in fasted rats ($F = 4.58$, not significant), consideration of the marked difference in basal concentrations in the experimental animals indicates that glycogen deposition in 48 h-fasted rats is not impaired. This conclusion is further supported by estimating the proportion of the infused glucose during the clamps that is accounted for by storage as glycogen. Thus, assuming that a rat is 45% muscle by wt. [34], and that the skeletal muscle sampled was typical in terms of glycogen storage, then the results suggest that, in rats fasted for 48 h, 58% of the infused glucose is accounted for by muscle glycogen, whereas in rats fasted for 6 or 24 h the value is around 35%. Interpretation is, however, complicated by the unpaired nature of the study. Consistent with studies in man [35], muscle glycogen deposition was proportionately greater (53% of infused glucose) during the 255 munits-of-insulin/h clamps in 24 h-fasted rats. The muscle glycogen data are in keeping with the lack of effect of fasting on the activation of skeletal-muscle glycogen synthase activity (Table 4).

Most of the glucose taken up by skeletal muscle and not incorporated into glycogen will enter the glycolytic pathway with the production of C_3 intermediates, lactate, pyruvate and alanine. Pyruvate may undergo oxidative decarboxylation to acetyl-CoA and further oxidation to CO_2 in the tricarboxylic acid cycle. During the glucose clamps, blood concentrations of lactate, pyruvate and alanine were increased. This increase would be consistent with a stimulation of glycolysis by insulin in peripheral tissues and with a decrease in hepatic uptake of these C_3 intermediates, as gluconeogenesis would be expected to be partially inhibited under the clamp conditions. In rats fasted for 24 and 48 h, blood concentrations of these gluconeogenic precursors were increased to a greater extent than in rats clamped 6 h after food withdrawal (Table 2). Since gluconeogenesis is not completely suppressed after a glucose load [36,37] and would be expected to be increased in the 24 h- and 48 h-fasted rats compared with the 6 h-fasted animals, it is likely that this difference with fasting is not due to decreased hepatic uptake of these gluconeogenic precursors relative to 6 h-fasted rats.

Assuming skeletal muscle to be an important source of these C_3 intermediates during the clamp, then the unchanged total glucose turnover and muscle glycogen deposition in fasted rats implies increased production owing to decreased oxidation by PDH, rather than any increase in glycolytic flux in this tissue. This would be consistent with the normal peripheral glucose disposal but impaired capacity for glucose oxidation found in protein/energy malnutrition [32]. However, although quadriceps-muscle PDH activity was decreased in the basal state with fasting, activity at the end of the clamps was not significantly different from that of rats fasted for only 6 h (Table 4). It is possible that examination of more oxidative muscle types such as gastrocnemius and soleus would have demonstrated an impairment of stimulation during the clamps, as suggested by the studies by French and colleagues [38], who reported delayed re-activation of PDH in these muscles after refeeding of 48 h-fasted rats. Gastrocnemius and soleus muscles have a predominance of slow-twitch oxidative fibres and, with respect to regulation of PDH and fatty acid oxidation, share some features with cardiac muscle. Indeed, in heart we were able to confirm the decreased basal PDH activity and impaired stimulation in response to insulin [38,39] in rats fasted for 24 or 48 h (Table 5). If regulation of muscle PDH depends to some extent on fibre type, it would appear that decreased activity of this enzyme in more oxidative fibre types might explain the increased clamp blood lactate, pyruvate and alanine concentrations with fasting.

Impaired stimulation of PDH activity by the clamp was also found in the livers of the 48 h-fasted rats. Our findings in heart and liver are thus consistent with previous studies showing that starvation decreases the proportion of complex in the dephosphorylated active form [40,41], and that this effect is not immediately reversed on administration of insulin and glucose [42]. Studies by Randle and colleagues have indicated that this slowly reversible starvation-induced decrease in active PDH is due to activation of PDH kinase, and that this is mediated by an increase in the specific activity of kinase activator protein induced by starvation [40,41]. Although the acute activation of PDH during the clamps probably reflects both a decrease in PDH kinase (secondary to inhibition of fatty acid and ketone-body supply and hence decreased mitochondrial acetyl-CoA/CoA and $NADH/NAD^+$ concentration ratios) [43] and an increase in PDH phosphatase [44,45], the latter does not appear to play a role in the longer-term starvation-induced changes in PDH activity [46]. Delayed re-activation of PDH in both liver and peripheral tissues in response to re-feeding or insulin and glucose administration may be important for promoting carbon flux through gluconeogenesis and thus resynthesis of hepatic glycogen by the indirect pathway [37,39].

As previous work has shown that lipogenesis remains depressed after the administration of carbohydrate to fasted rats [47], we also measured the activation of liver pyruvate kinase and acetyl-CoA carboxylase. These two enzymes regulated by insulin are also important in determining the direction of carbon flux. Acetyl-CoA carboxylase catalyses the first committed step in fatty acid synthesis and is considered to play an important role in the regulation of this pathway. For high rates of fatty acid synthesis in liver, increased activity of the non-equilibrium enzymes of glycolysis such as pyruvate kinase, and decreased activity of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase, are also important, in that diversion of phosphoenolpyruvate to glucose is prevented. In contrast with liver PDH_a%, pyruvate kinase was clearly stimulated by the insulin/glucose infusions, independently of nutritional status. Similarly, although citrate-activated acetyl-CoA carboxylase activity decreased with fasting (Table 3), insulin stimulation of the enzyme measured in the absence of citrate was not impaired in fasted rats.

Although our findings do not support the hypothesis that decreased whole-body sensitivity to insulin plays a role in the glucose intolerance associated with fasting, they do confirm the sparing of glucose utilization in the fasted state [48,49]. Thus, in the basal state, 48 h-fasted rats had decreased plasma glucose concentrations, and [3-^{3}H]glucose turnover was decreased by 35% compared with post-absorptive rats (Table 1). This percentage decrease in glucose turnover with fasting is similar to that found by others [48,49] and the absolute values for

glucose turnover are also comparable when allowance is made for the effect of anaesthesia, which has been shown to decrease glucose turnover in rats [50].

Insulin concentrations in the range found in the basal state would not be expected to have a direct effect on skeletal-muscle glucose utilization [33]. By contrast, adipose-tissue lipolysis is very sensitive to small changes in insulin concentration within this range [51], and thus the decrease in basal insulin concentrations found in the fasted rats would be expected to impair glucose utilization indirectly by increasing the availability of NEFA and ketone bodies (Table 2) [9–12].

The insulin-infusion rate used in the present study resulted in peripheral plasma insulin concentrations similar to peak values after an oral glucose load [52], and it remains possible that impaired clamp glucose disposal with fasting might have been demonstrated at insulin concentrations which failed to suppress plasma NEFA concentrations. Nevertheless, although insulin-stimulated whole-body glucose disposal was unchanged, the blood intermediary-metabolite responses to the clamp and the tissue PDH data suggest that fasting is associated with a qualitative difference in the metabolism of glucose. Clearly further studies are required to address the possibility of heterogeneity in the response of individual skeletal muscles to fasting.

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