Effects of fasting on hepatic and peripheral glucose metabolism in conscious rats with near-total fat depletion

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Experimental diabetes and fasting are both associated with hypoinsulinaemia and share several other metabolic features. We investigated hepatic and peripheral glucose metabolism in young rats after near-total depletion of their fat mass. Conscious rats were fasted for 72 h ($n = 13$), while 6 h-fasted animals ($n = 14$) served as controls. Rats were studied either during saline infusion or insulin (18 m-units/kg per min)-clamp studies. In fasting, despite a 2-fold increase in hepatic glucose-6-phosphatase (Glc-6-Pase) V_{max} (from $16 \pm 2 \mu\text{mol/g}$ of liver per min in control; $P < 0.001$), the basal hepatic glucose production (HGP) decreased by 47% [from $88 \pm 3 \mu\text{mol/kg}$ lean body mass (LBM) per min in control; $P < 0.01$]. The decreased HGP in fasting was associated with a 70% decrease in the hepatic levels of glucose 6-phosphate (Glc-6-P) (from $366 \pm 53 \text{ nmol/g}$ wet wt. in control;

$P < 0.01$). Thus Glc-6-Pase activity assayed in the presence of the Glc-6-P levels found *in vivo* was decreased by 44%. During hyperinsulinaemia, peripheral glucose uptake was decreased by 15% with 3 days of fasting (from $272 \pm 17 \mu\text{mol/kg}$ LBM per min in control; $P < 0.01$). This was completely accounted for by a 42% decrease in whole-body glycolysis ($P < 0.01$), while the rate of glycogen synthesis was unchanged. Thus fasting (after near-total fat depletion) differs from experimental diabetes because: (1) despite markedly increased Glc-6-Pase, HGP is decreased in fasting, due to a marked decrease in the substrate level (Glc-6-P) *in vivo*; and (2) the impairment in peripheral insulin sensitivity in fasting is due to a decrease in the glycolytic, and not the glycogen-synthetic, pathway.

INTRODUCTION

During fasting, the decreased supply of exogenous substrates leads to the progressive depletion of glycogen stores. Subsequently, increased lipolysis and proteolysis provide substrates for liver and muscle metabolism. In rodents, prolonged fasting is also associated with the progressive depletion of fat mass. Since some of the effects of fasting on glucose fluxes are believed to be solely the consequence of increased lipolysis, we examined hepatic and peripheral glucose metabolism after near-total fat depletion, when the effects of increased availability of non-esterified fatty acids (NEFA) and glycerol on peripheral (Randle Cycle) [1] and hepatic glucose metabolism are minimized.

The progressive decline in circulating insulin concentrations and increased reliance on endogenous energy stores, which characterize the metabolic adaptation to fasting, are also features of experimental diabetes. In particular, insulin deficiency results in the progressive loss of hepatic glucokinase (GK) activity, whereas glucose-6-phosphatase (Glc-6-Pase) activity is markedly increased [2,3]. Due to these and other similarities, several investigators have compared experimental models of diabetes and fasting [4–13]. However, since plasma glucose levels are increased in diabetes and decreased in fasting, it is important to delineate which metabolic features result in decreased glucose levels during prolonged fasting.

The liver plays a central role in maintaining plasma glucose levels during fasting. After the depletion of hepatic glycogen stores, the liver relies on the availability of gluconeogenic substrates, such as lactate, alanine, glycerol and pyruvate, to sustain hepatic glucose output. Interestingly, the ratio of Glc-6-Pase/GK, which positively correlates with hepatic glucose production (HGP) in 6 h-fasted control and diabetic rats [14], is

markedly increased in fasted rats. Paradoxically, HGP is decreased in animal and human fasting [5,15–17]. We therefore hypothesized that the availability of substrate for Glc-6-Pase [i.e., hepatic glucose 6-phosphate (Glc-6-P)] may cause the decreased HGP during fasting.

In humans, an additional feature common to fasting and diabetes is marked peripheral insulin resistance [4–7]. Studies in fasting and diabetic rodents have demonstrated similar increases in insulin binding and decreases in gene expression of GLUT1 and GLUT4 glucose transporters in fat [5–13]. However, although peripheral insulin resistance is a hallmark of prolonged fasting in humans, it has been reported in some [10,18,19], but not in all, rodent studies [13,15,20,21]. When present, insulin resistance in rodents was less severe than in humans [4–7]. A potential explanation for the different metabolic consequences of fasting in rodents and humans is the different degree of decrease in fat mass (FM). In humans, FM is not depleted, and so circulating NEFA and glycerol are markedly increased for over 1 week [4,6,17,22], and may largely account for hepatic and peripheral insulin resistance [1,23–26]. However, in young lean rats (less than 250 g), fasting depletes FM rapidly and results in a transient increase in plasma NEFA levels, which are then decreased by day 3 of fasting [15]. Thus this model may allow us to distinguish between the peripheral effects of increased NEFA during fasting, and the specific adaptation of peripheral glucose metabolism to fasting. Additionally, metabolic fluxes expressed as a function of body weight rather than lean body mass (LBM) may have underestimated the rate of disappearance of glucose (R_d) in the non-fasting state. Thus, assessing body composition is important not only to monitor the depletion of FM, but also to quantify more accurately insulin action.

Thus we assessed body composition, basal and insulin-

Abbreviations used: HGP, hepatic glucose production; Glc-6-Pase, glucose-6-phosphatase; Glc-6-P, glucose 6-phosphate; GK, glucokinase; LBM, lean body mass; NEFA, non-esterified fatty acids; FM, fat mass; R_d , rate of disappearance of glucose.

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stimulated glucose metabolism following near-total depletion of FM in rats, so that the metabolic features of fasting could be examined in the absence of increased plasma NEFA.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, U.S.A.) were used in all studies. Rats were housed in individual cages and subjected to a standard light (06:00–18:00 h)/dark (18:00–06:00 h) cycle. At 1 week before the study *in vivo*, rats were anaesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg body wt.) and indwelling catheters were inserted in the right internal jugular vein and in the left carotid artery. The venous catheter was extended to the level of the right atrium and the arterial catheter was advanced to the level of the aortic arch [27,28]. On the morning of the study, food was removed at 06:00 h, catheters were connected, and their patency was tested at ~08:00 h.

Body composition

LBM and FM were calculated from the whole-body volume of distribution of water, estimated by bolus injection of $^3\text{H}_2\text{O}$ into each experimental rat [29]. Briefly, 20 μCi of $^3\text{H}_2\text{O}$ (New England Nuclear) was injected intra-arterially at 08:00 h on the morning of the study, and five samples for measurement of plasma $^3\text{H}_2\text{O}$ were collected at 30 min intervals between 1 and 3 h thereafter. Steady-state for $^3\text{H}_2\text{O}$ specific radioactivity, in our hands, is generally achieved within 30–45 min.

Euglycaemic-clamp study

Studies were performed in awake, unstressed, chronically catheterized rats by using the euglycaemic-clamp technique in combination with $[3\text{-}^3\text{H}]\text{glucose}$ infusion as previously described [27,28]. Rats from which food was removed for 6 h (this time was selected in order to allow us to measure glucose fluxes accurately without residual intestinal absorption of nutrients) before the studies *in vivo* ($n = 13$) served as control for the 72 h-fasted rats ($n = 14$). These two nutritional states were selected since a 72 h fast in rats is associated with near-total depletion of FM, and, as a consequence, with decreases in plasma glycerol and NEFA concentrations to those observed in the post-absorptive state. Additionally, 3 days fasting is associated with marked decrease in muscle glycogen concentration, whereas the 6 h-fasted rats more closely resemble the post-absorptive state in humans, with substantial glycogen content in liver and muscle. Briefly, at 11:00 h, 5–7 days after the placement of the catheters, 6 h- and 72 h-fasted rats received infusions of $[3\text{-}^3\text{H}]\text{glucose}$ and either saline ($n = 6$ and 5 respectively) or insulin-clamp studies (I; $n = 6$ and 9 respectively) for 120 min. However, since food intake in rats is maximal at ~24:00 h, and thereafter it may decrease, it is likely that our 6 h-fasted rats decreased or interrupted food intake for some time before food removal. Thus, to examine whether the time of food removal alters peripheral insulin sensitivity, we studied an additional group of conscious rats ($n = 5$) in which food was removed shortly after their expected peak of food intake (at 03:00 h). In each rat, two insulin (18 m-units/kg per min)-clamp studies (as above) were performed at 09:00 h and at 14:00 h (approx. 6 h and 11 h after food removal). We aimed to assess the metabolic changes which developed in the transition from short to prolonged fasting/starvation.

Primed-continuous infusions of HPLC-purified $[3\text{-}^3\text{H}]\text{glucose}$ (New England Nuclear; 15–40 μCi bolus, 0.4 $\mu\text{Ci}/\text{min}$) and insulin (18 m-units/kg per min) were initiated and maintained

throughout the remainder of the study. A variable infusion of a 25% glucose solution was started at zero time and periodically adjusted to clamp the plasma glucose concentration at 6.66 mM (120 mg%). Plasma samples for determination of $[^3\text{H}]\text{glucose}$ specific radioactivity were obtained at 10 min intervals, and for determination of plasma insulin, lactate, glycerol and NEFA concentrations at 30 min intervals, before and during the study. The total volume of blood withdrawn was ~3.0 ml/study; to prevent volume depletion and anaemia, a solution (1:1, v/v) of ~4.0 ml of fresh blood (obtained by heart puncture from a littermate of the test animal) and heparinized saline (10 units/ml) was infused. At the end of the insulin infusion, rats were anaesthetized (pentobarbital 60 mg/kg body wt., intravenously), the abdomen was quickly opened, and rectus abdominal muscle and liver were freeze-clamped *in situ* with aluminium tongs precooled in liquid nitrogen [27,28]. The time from the injection of the anaesthetic until freeze-clamping of the liver was less than 45 s. All tissue samples were stored at -80°C for subsequent analysis.

The study protocol was reviewed and approved by the Animal Care and Use Committee of the Albert Einstein College of Medicine.

Whole-body glycolysis

The rate of glycolysis was estimated as previously described [30]. Briefly, the specific radioactivity of plasma $^3\text{H}_2\text{O}$ was determined by liquid-scintillation counting of the protein-free supernatant (Somogyi filtrate) before and after evaporation to dryness. Because ^3H on the C-3 position of glucose is lost to water during glycolysis, it can be assumed that plasma ^3H is present in either $^3\text{H}_2\text{O}$ or $[3\text{-}^3\text{H}]\text{glucose}$. Although ^3H may also be released during fructose 6-phosphate cycling and/or pentose phosphate cycling, these pathways account for only a small percentage of glucose turnover [31].

Glycogen formation *in vivo*

Muscle glycogen synthesis was quantified by two independent means: first, by subtracting the glycolytic rate from the R_a , and second, by determining the increment in glycogen concentration above fasting levels in several skeletal-muscle samples. Muscle glycogen concentration was determined after digestion with amyloglucosidase, as previously described [28,30]. The intra-assay and the inter-assay coefficients of variation were $<10\%$ (at 0.250 g% tissue wt.) when a muscle homogenate was assayed as multiple samples. Samples of the tissue homogenate (100 μl) were also employed to determine the amount of ^3H label in glycogen. Glycogen was precipitated by washing in 10 vol. of ethanol and then incubating for 1 h at -20°C . The procedure was repeated three times, and then the precipitate was collected, dried down and dissolved in water before scintillation counting.

Glycogen synthase activity

Muscle glycogen synthase activity was measured by a modification [28,32] of the method of Thomas et al. [33] and is based on the measurement of the incorporation of radioactivity into glycogen from UDP- $[U\text{-}^{14}\text{C}]\text{glucose}$. Tissue samples (20–30 mg) were homogenized in 2.0 ml of Tris/HCl buffer, pH 7.8, containing 10 mM EDTA, 5 mM dithiothreitol, 50 mM NaF and 2.5 g/l rabbit liver glycogen (Type III). The homogenate was centrifuged at 2000 g for 15 min (at 4°C), and the supernatant was used for glycogen synthase assay by measuring the incorporation of UDP- $[U\text{-}^{14}\text{C}]\text{glucose}$ into glycogen at 30°C . To approximate to conditions *in vivo*, synthase activity was measured

in the presence of a physiological concentration of 0.11 mM Glc-6-*P*. Total glycogen synthase D activity was measured in the presence of 7.2 mM Glc-6-*P*. For the kinetic analysis, the assay was conducted at final concentrations of 0.003, 0.017, 0.033, 0.09, 0.33 and 1.4 mM UDP-glucose; the data were linearized as Eadie-Hofstee plots and fitted by using linear regression. The K_m for UDPG is the reciprocal of the slope, whereas V_{max} is the ordinate intercept divided by the slope.

Glycogen phosphorylase activity

Muscle glycogen phosphorylase activity was measured as previously described [16,32]. This assay is based on the measurement of the incorporation of ^{14}C into glycogen from labelled glucose 1-phosphate. Glycogen phosphorylase *a*, the active phosphorylated enzyme, was assayed in the absence of AMP, and phosphorylase *b*, the total enzyme activity, was assayed in the presence of 5 mM AMP. Tissue homogenates (20–30 mg) were prepared as described above. The supernatant was used for glycogen phosphorylase assay by measuring the incorporation of [^{14}C]glucose 1-phosphate into glycogen at 30 °C in a mixture containing 33 mM Mes, 200 mM KF, 0.45 % mercaptoethanol, 15 mM glucose 1-phosphate (50 $\mu\text{Ci}/\text{mmol}$) and 3.4 mg/ml glycogen. Phosphorylase *b* was assayed in the same manner, except that the mixture contained 100 mM glucose 1-phosphate (6 $\mu\text{Ci}/\text{mmol}$), 13.4 mg/ml glycogen and 5 mM AMP.

Glc-6-Pase activity

Tissues were obtained from liver samples freeze-clamped *in situ* at the end of the studies *in vivo*. Small fragments were sampled from different parts of the liver to a total weight of approx. 100 mg/tube. Liver homogenates were prepared in 50 mM Hepes/100 mM KCl/1 mM EDTA/5 mM MgCl_2 /2.5 mM dithioerythritol. Homogenates were centrifuged at 100 000 *g* for 45 min to sediment the microsomal fraction (the supernatant was used for the GK assay). The assay performed is based on the hydrolysis of P_i from Glc-6-*P* [3,14]. The post-microsomal fraction had no detectable Glc-6-Pase activity. The microsomal fraction was incubated with 0.5, 1.0, 2.5, 5.0 and 10.0 mM Glc-6-*P*. The reaction was carried at 37 °C, and stopped after 20 min with a solution containing acid molybdate, with 2/9 vol. of 10 % SDS and 1/9 vol. of 10 % ascorbic acid. It was then incubated for 20 min at 45 °C, and the A_{820} was read. A standard curve was constructed by using different concentrations of P_i .

GK activity

Hepatic GK activity was measured by the continuous-assay spectrophotometric method as described by Davidson and Arion [34], with some modifications [14]. The post-microsomal fraction was assayed in a medium, pH 7.4 at 37 °C, containing 50 mM Hepes, 100 mM KCl, 7.5 mM MgCl_2 , 5 mM ATP, 2.5 mM dithioerythritol, 10 mg/ml BSA and 0.5 mM glucose (hexokinase) or 7, 10, 15, 18 and 50 mM glucose (total phosphorylating activity), 0.5 mM NAD^+ , 4 units of Glc-6-*P* dehydrogenase (*Leuconostoc mesenteroides*) and the equivalent of 1 mg wet wt. of liver. The reaction was initiated by the addition of ATP, and the rate of NAD^+ reduction was recorded at 340 nm. Glucose phosphorylation was determined as the change in A_{340} in the complete medium minus that in the absence of glucose under conditions in which the A_{340} was increasing linearly with time (from 20 to 40 min).

Analytical procedures

Plasma glucose was measured by the glucose oxidase method

(Glucose Analyzer II; Beckman Instruments) and plasma insulin by radioimmunoassay using rat and pig insulin standards. Plasma [^3H]glucose radioactivity was measured in duplicate in the supernatants of $\text{Ba}(\text{OH})_2$ and ZnSO_4 precipitates of plasma samples after evaporation to dryness to eliminate $^3\text{H}_2\text{O}$. Muscle Glc-6-*P* concentrations were measured spectrophotometrically as described by Michal [35]. Muscle glycogen was determined as previously described [28,30]. Plasma lactate and muscle Glc-6-*P* were measured spectrophotometrically [36,37]. Plasma NEFA concentrations were determined by an enzymic method with an automated kit according to the manufacturer's specifications (Waco Pure Chemical Industries, Osaka, Japan).

Calculations

Data for total body glucose uptake and hepatic glucose production represent the mean values during the last 30 min. The hepatic glucose production was calculated as the difference between the tracer-derived rate of appearance and the infusion rate of glucose. To take into account differences in the basal glucose concentrations between 6 h- and 72 h-fasted rats, the rate of glucose clearance (ml/kg per min) was also calculated as the rate of glucose turnover (mg/kg per min) divided by the plasma glucose concentration (mg/ml). The distribution space of water was obtained by dividing the total d.p.m. of $^3\text{H}_2\text{O}$ injected by the steady-state specific radioactivity of plasma water, which was assumed to be 93 % of the total plasma volume. LBM was calculated from the whole-body water distribution space divided by 0.73. Rates of whole-body glycolysis were estimated from the increment per unit time in $^3\text{H}_2\text{O}$ (d.p.m./ml per min) $\times$ body water mass (ml)/[^3H]glucose specific radioactivity (d.p.m./mg). All values are presented as means $\pm$ S.E.M. Comparisons between groups were made by using repeated measures of analysis of variance where appropriate. Where *F*-ratios were significant, further comparisons were made by using Student *t* tests.

RESULTS

Biochemical and metabolic characteristics of the experimental animals (Table 1)

Prolonged fasting resulted in the near-total depletion of FM. Body weight was decreased by an average of 57 g (20 % of the

Table 1 Body composition and biochemical characteristics

Values shown are for mean body wt. [BW-b (b, before fasting), BW-f (f, final time of fasting)], LBM (actual, and as percentage of BW), FM (actual, and as percentage of BW), and mean fasting levels of glucose, insulin, lactate and NEFA in 6 h- and 72 h-fasting conscious rats. Abbreviation: NS, not significant.

	6 h	72 h	<i>P</i> <
Body composition			
BW-b (g)	256 $\pm$ 10	272 $\pm$ 5	NS
BW-f (g)	254 $\pm$ 11	215 $\pm$ 4	0.001
LBM (g)	236 $\pm$ 8	213 $\pm$ 7	0.005
LBM (%)	88 $\pm$ 1	99 $\pm$ 1	0.001
FM (g)	32 $\pm$ 6	2 $\pm$ 1	0.001
FM (%)	11.6 $\pm$ 1.8	0.6 $\pm$ 0.2	0.001
Biochemical characteristics			
Glucose (mM)	7.1 $\pm$ 0.2	5.7 $\pm$ 0.3	0.001
Insulin (pM)	206 $\pm$ 12	86 $\pm$ 18	0.001
FFA (mM)	0.83 $\pm$ 0.07	0.64 $\pm$ 0.03	0.001
Glycerol (mM)	0.16 $\pm$ 0.02	0.08 $\pm$ 0.01	0.001
Lactate (mM)	0.57 $\pm$ 0.04	0.32 $\pm$ 0.03	0.01

Table 2 HGP during basal and insulin (+I)-clamp studies in 6 h- and 72 h-fasting conscious rats, expressed per LBM and per liver weight* $P < 0.01$ versus 6 h; † $P < 0.01$ versus basal infusion.

HGP	6 h	6 h+I	72 h	72 h+I
$\mu\text{mol/kg}$ of LBM per min	88 ± 3	$21 \pm 5^\dagger$	$47 \pm 3^*$	$10 \pm 5^*\dagger$
$\mu\text{mol/g}$ of liver per min	2.04 ± 0.20	$0.47 \pm 0.05^\dagger$	$1.37 \pm 0.20^*$	$0.21 \pm 0.02^*\dagger$

initial weight) in the 72 h-fasted rats. Approx. 60 % of the decline in weight was due to the almost complete lost of FM, and the remainder was accounted for by a modest (10 %) decrease in LBM. Fasting plasma glucose decreased by 20 %, insulin by 59 %, NEFA by 23 %, glycerol by 55 % and lactate by 44 %.

Hepatic glucose metabolism during fasting

HGP during basal period and insulin-clamp study (Table 2)

Basal HGP was decreased by 47 % in the 72 h- compared with the 6 h-fasted rats. Liver weight was decreased by 25 % after fasting (7.4 ± 0.5 g versus 9.9 ± 1.2 g in control). However, basal HGP was still significantly decreased by 33 % after fasting when expressed per g of liver.

Although HGP was similarly suppressed during hyperinsulinaemia to approx. 20 % of basal levels, it was significantly higher in the 6 h-fasted controls. Hepatic glycogen concentrations at the end of the insulin clamp were 23.3 ± 2.5 mg/g of liver in the 6 h- and 9.6 ± 0.8 in the 72 h-fasted rats ($P < 0.01$).

Hepatic GK and Glc-6-Pase kinetics (Table 3)

The $V_{\max}$ of Glc-6-Pase was markedly increased and that of GK was markedly decreased in the 72 h-fasted rats, whereas K_m values were not affected. A 2 h period of euglycaemic hyperinsulinaemia did not affect the $V_{\max}$ of GK in either group, but

it decreased the $V_{\max}$ of Glc-6-Pase by 25 % and 26 % in 6 h- and 72 h-fasted rats respectively. The Glc-6-Pase/GK ratio (of the group's enzymes $V_{\max}$) was increased nearly 5-fold in the 72 h-fasting rats with and without insulin infusion.

Impact of Glc-6-P concentrations *in vivo* on Glc-6-Pase activity (Table 4)

Row (A) displays the Glc-6-Pase activity measured at physiological Glc-6-P concentrations of 1 mM in both 6 h- and 72 h-fasted liver samples. These activities resembled the pattern displayed for the $V_{\max}$. In fact, the activity was nearly doubled after 72 h of fasting, and decreased in both groups at the end of the insulin clamp. Row (B) depicts an estimate of the hepatic Glc-6-P concentration *in vivo* in liver intracellular water. Hepatic concentrations of protein (180 ± 13 and 171 ± 15 mg of protein/g wet wt. of liver) and liver dry weight (26.6 ± 1.3 % and 27.1 ± 1.0 % of liver was dry weight) were similar in 6 h- and 72 h-fasted rats. Thus we assumed that 73 % of liver weight is water, and that 30 % of liver water is extracellular [37]. The hepatic Glc-6-P concentration was markedly decreased in fasting, and it was further decreased during the insulin-clamp studies in both groups. In row (C), the Glc-6-Pase activity is displayed at the Glc-6-P levels found *in vivo* to account for the effect of changes in the hepatic Glc-6-P concentration on Glc-6-Pase activity. At these Glc-6-P concentrations *in vivo*, Glc-6-Pase activity was decreased by 44 % after 72 h of fasting, and it was further decreased in both groups at the end of the insulin clamp.

Peripheral glucose metabolism during fasting

Basal and insulin-stimulated glucose disposal (Table 5)

Glucose clearance rate was decreased in 72 h- as compared with 6 h-fasted rats, whether expressed per LBM or per body weight (12.4 ± 0.4 and 8.3 ± 0.5 ml/kg of LBM per min, and 11.3 ± 0.4 and 8.2 ± 0.4 ml/kg body wt. per min in the 6 h- and 72 h-fasted rats respectively; $P < 0.01$). During the insulin-clamp studies, steady-state plasma glucose (6.7 ± 0.3 and 6.6 ± 0.1 mM) and insulin (2436 ± 159 and 2672 ± 119 pM) concentrations were

Table 3 Hepatic Glc-6-P and GK $V_{\max}$ and K_m

The kinetics were obtained from liver freeze-clamped *in situ* from control and fasted rats, with or without hyperinsulinemia (+I). The Glc-6-Pase/GK ratios are obtained from the $V_{\max}$ values.

* $P < 0.01$ versus 6 h; † $P < 0.01$ versus basal infusion.

	6 h	6 h+I	72 h	72 h+I
Glc-6-Pase ($V_{\max}$) ($\mu\text{mol/g}$ of liver per min)	15.9 ± 1.7	$12.0 \pm 1.8^\dagger$	$37.2 \pm 4.1^*$	$27.7 \pm 2.2^*\dagger$
Glc-6-Pase (K_m) (mM)	1.2 ± 0.2	1.6 ± 0.2	1.7 ± 0.3	1.4 ± 0.1
GK ($V_{\max}$) ($\mu\text{mol/g}$ of liver per min)	10.1 ± 0.9	9.6 ± 1.4	$4.9 \pm 0.5^*$	$4.7 \pm 0.5^*$
GK (K_m) (mM)	9.8 ± 1.2	$5.5 \pm 0.9^\dagger$	12 ± 2.3	9.5 ± 2.9
Glc-6-Pase/GK $V_{\max}$ ratio	1.57	1.25	7.59	5.89

Table 4 Impact of the hepatic Glc-6-P concentration on Glc-6-Pase activity

Row (A) Glc-6-Pase activity measured at equal Glc-6-P concentrations (1 mM) at the end of basal or insulin-clamp (+I) studies; (B) hepatic Glc-6-P concentrations at the end of basal or insulin-clamp studies; (C) Glc-6-Pase activity measured at the hepatic concentrations of Glc-6-P *in vivo* during basal and insulin infusion. * $P < 0.01$ versus 6 h; † $P < 0.01$ versus basal infusion.

	6 h	6 h+I	72 h	72 h+I
(A) Glc-6-Pase ($\mu\text{mol/g}$ of liver per min)	7.0 ± 0.5	$4.4 \pm 0.7^\dagger$	$13.5 \pm 1.5^*$	$11.8 \pm 1.2^*\dagger$
(B) Glc-6-P ($\mu\text{mol/ml}$ of intracellular water)	0.68 ± 0.05	$0.29 \pm 0.05^\dagger$	$0.13 \pm 0.02^*$	$0.06 \pm 0.01^*\dagger$
(C) Glc-6-Pase ($\mu\text{mol/g}$ of liver per min)	5.8 ± 0.5	$1.9 \pm 0.3^\dagger$	$3.3 \pm 0.2^*$	$1.5 \pm 0.1^*\dagger$

Table 5 Glucose uptake, glycolysis and glycogen synthesis during the insulin-clamp studies in 6 h- and 72 h-fasting rats, expressed per LBM and per body weight (BW)* $P < 0.01$ from that expressed per LBM; NS, not statistically significant.

	6 h	72 h	$P <$
Glucose uptake			
$\mu\text{mol/kg LBM per min}$	272 ± 14	233 ± 11	0.001
$\mu\text{mol/kg BW per min}$	$239 \pm 17^*$	233 ± 11	NS
Glycolysis			
$\mu\text{mol/kg LBM per min}$	130 ± 4	81 ± 5	0.001
$\mu\text{mol/kg BW per min}$	$114 \pm 4^*$	81 ± 5	0.001
Glycogen synthesis			
$\mu\text{mol/kg LBM per min}$	142 ± 9	152 ± 6	NS
$\mu\text{mol/kg BW per min}$	$125 \pm 8^*$	152 ± 6	0.001

similar in the two experimental groups. The suppression of plasma NEFA levels during hyperinsulinaemia was also similar in both groups (to plasma levels of 0.32 ± 0.03 and 0.24 ± 0.03 mM in the 6 h- and 72 h-fasted rats respectively). When the R_d was expressed per LBM, it was decreased by 15% in the 72 h- as compared with 6 h-fasted rats. However, R_d values were similar in both groups when expressed per body weight. In order to explore further the effect of early fasting on peripheral insulin sensitivity, we studied an additional group of conscious rats ($n = 5$) from which food was removed at 03:00 h (see the Materials and methods section) and insulin (18 m-units/kg per min)-clamp studies were performed at 09:00 and at 14:00 h. R_d during the insulin-clamp studies ($222 \pm 17 \mu\text{mol/kg body wt. per min}$) was similar to that previously observed in the 6 h-fasted rats (Table 5). R_d also did not change significantly between 09:00 h ($222 \pm 17 \mu\text{mol/kg body wt. per min}$) and 14:00 h ($206 \pm 22 \mu\text{mol/kg body wt. per min}$).

Rates of glycolysis and glycogen synthesis (Table 5)

Hyperinsulinaemia stimulated whole-body glycolysis in both groups, yet glycolysis was significantly decreased by 38% in the 72 h-fasted rats. Glycolysis was also significantly decreased in 72 h-fasted rats when expressed per kg body wt. Despite this marked decrease in glycolysis, plasma lactate concentrations, which during the insulin clamp increased by 37% (to

0.78 ± 0.08 mM) in control rats, increased by ~4-fold (to 1.19 ± 0.14 mM) in the fasted rats.

Glycogen formation from plasma glucose, calculated by subtracting the glycolytic rate from the R_d , was similar during hyperinsulinaemia in the 6 h- and 72 h-fasted rats (142 ± 9 and $152 \pm 6 \mu\text{mol/kg of LBM per min}$ respectively) (Table 5). However, it was significantly increased in 72 h-fasted rats during hyperinsulinaemia when calculated per unit of body wt. (125 ± 8 and $152 \pm 6 \mu\text{mol/kg body wt. per min}$ respectively) (Table 5). Consistent with the results expressed per LBM, the insulin-mediated increase in muscle glycogen concentration was similar in 6 h- and 72 h-fasted rats (133 ± 17 and $110 \pm 13 \mu\text{mol/kg of LBM per min}$ respectively) (calculated from data presented in Table 6). Finally, the [^3H]glucose radioactivity incorporated into muscle glycogen during hyperinsulinaemia was not different in the two groups (170448 ± 15360 and 176112 ± 17280 d.p.m./g of muscle, in the 6 h- and 72 h-fasted rats respectively).

Muscle glycogen and Glc-6-P concentration (Table 6)

Basal skeletal-muscle Glc-6-P concentration was decreased by 72 h of fasting. During hyperinsulinaemia, Glc-6-P concentrations were increased by over 50% in control and by nearly 3-fold in fasting. The net increase in Glc-6-P levels during hyperinsulinaemia was 100 mM more in the 72 h- than in the 6 h-fasted rats (calculated from Glc-6-P levels in Table 6). The fasting muscle glycogen concentration was 33% lower in the 72 h-fasted as compared with the 6 h-fasted rats. No significant difference was noted in the amounts of glycogen that were stored after hyperinsulinaemia, 30.8 ± 3.1 and $25.4 \pm 3.5 \mu\text{mol/g wet wt.}$ in 6 h- and 72 h-fasted rats respectively.

Muscle glycogen synthase and phosphorylase (Table 6)

The K_m of the muscle glycogen synthase for UDP-glucose was similarly decreased after insulin infusion in the two groups. The V_{max} of glycogen synthase was unchanged by fasting and hyperinsulinaemia (Table 6, part A). Muscle glycogen phosphorylase activity did not change significantly with fasting and during the insulin infusion (Table 6, part B).

DISCUSSION

This study was undertaken to define the adaptation of hepatic and peripheral glucose metabolism to prolonged fasting after near-total depletion of FM. Since the effects of increased lipolysis

Table 6 Muscle Glc-6-P, glycogen, glycogen synthase (GS) and glycogen phosphorylase (GP) from muscles freeze-clamped *in situ* at the end of saline or insulin (+I) infusions in control and fasted rats(A) K_m and V_{max} of muscle GS in the presence of 0.11 mM (physiological) Glc-6-P concentration. (B) Activity of AMP-independent and AMP-dependent forms of muscle GP. * $P < 0.001$ versus control; † $P < 0.01$ versus basal infusion.

	6 h	6 h + I	72 h	72 h + I
Glc-6-P (nmol/g wet wt.)	337 ± 28	$529 \pm 21^\dagger$	$157 \pm 14^*$	$455 \pm 18^\dagger$
Glycogen ($\mu\text{mol/g wet wt.}$)	46.4 ± 4.6	$77.2 \pm 2.9^\dagger$	$31.4 \pm 2.3^*$	$56.8 \pm 3.9^\dagger$
(A) GS				
K_m (mM)	0.32 ± 0.06	$0.12 \pm 0.01^\dagger$	0.31 ± 0.09	$0.11 \pm 0.01^\dagger$
V_{max} ($\mu\text{mol/g wet wt. per min}$)	0.71 ± 0.09	0.73 ± 0.11	0.61 ± 0.12	0.75 ± 0.10
(B) GP				
Independent ($\mu\text{mol/g wet wt. per min}$)	14.6 ± 4.2	16.7 ± 1.5	14.6 ± 3.3	12.8 ± 2.7
Dependent ($\mu\text{mol/g wet wt. per min}$)	90.2 ± 12.7	88.4 ± 12.3	65.4 ± 12.9	125.5 ± 12.9

on hepatic and peripheral glucose metabolism are blunted in this model of fasting, we examined whether it still shares common metabolic features with experimental diabetes.

The decreased HGP in fasting [5,15–17,38] may reflect the hepatic adaptation to changes in substrates available within the liver (glycogen) and/or derived from peripheral tissues (NEFA, glycerol and lactate). However, this increased mobilization of endogenous energy and substrates for gluconeogenesis and the marked increase in Glc-6-Pase activity, associated with fasting hypoinsulinaemia, are in marked contrast with the decrease in HGP. In fact, the fasting-induced increase in hepatic Glc-6-Pase activity (~ 2.3 -fold) and decrease in GK activity ($\sim 51\%$) closely resemble the changes previously reported in partially (90%) pancreatectomized diabetic rats, i.e., 2.1-fold increase in Glc-6-Pase and 57% decrease in GK activity [14]. Furthermore, these co-ordinated changes in enzyme activities resulted in a similar fold increase in Glc-6-Pase/GK ratio (8.4- and 7.6-fold in diabetes and fasting respectively), an index which is highly correlated with HGP in 6 h-fasted rats [14]. The magnitude of the changes in hepatic GK and Glc-6-Pase activities induced by a 72 h fast in the present study is similar to that previously reported [2,3,39].

In the present study, we observed a marked decrease in the hepatic Glc-6-P concentration in the 72 h-fasted rats. Decreased hepatic Glc-6-P levels were previously reported in 24 h-fasted rats and at the end of insulin-clamp studies in both conscious [40] and anaesthetized [41] rats. Thus, it is possible that this marked decline in substrate availability over-rode the increased Glc-6-Pase activity. To evaluate further the impact of the diminished availability of Glc-6-P on the activity of the hepatic Glc-6-Pase, we compared the enzyme activity in fasted and control animals in the presence of the hepatic Glc-6-P concentration measured *in vivo*. The estimate of Glc-6-P concentrations in the hepatic water *in vivo* is supported by the observation that the hepatic water and protein concentrations were similar in 6 h- and 72 h-fasted rats, in accordance with Minassian et al. [39]. Under these experimental conditions, this estimate of Glc-6-Pase activity *in vivo* was decreased by 44% in fasting, and resembled the decline in HGP. This adaptation distinguishes the control of HGP in fasting and diabetes [14,42]. Thus, prolonged fasting appears to shift the rate-determining steps for the regulation of HGP from the net rate of Glc-6-P dephosphorylation to glucose to the rate of formation of Glc-6-P. The latter conclusion may also explain the different impact of the increased availability of gluconeogenic substrates following overnight or prolonged fasting [17]. As recently suggested *in vitro* [39], the increased concentration of 2-oxoglutarate during fasting may also contribute to the decrease in Glc-6-Pase activity *in vivo*.

In the present study, a significant decrease in the basal rate of glucose clearance was also observed with fasting. Indeed, decreased glucose utilization was previously demonstrated in several tissues by the deoxyglucose method [43,44], and fasting in humans was consistently associated with the onset of peripheral insulin resistance. In most human studies, starvation lasted for over 5 days, yet body weight decreased by less than 5–8% per week, and the depletion of FM was only partial [4,5,7]. In young rats, FM is more rapidly and completely mobilized (Table 1). Thus, to account for these dramatic changes in body composition, it is important to express the rates of glucose uptake during insulin-clamp studies as a function of the LBM rather than per unit body weight. Penicaud et al. [38] reported severe peripheral insulin resistance in young fasted rats (body wt. ~ 200 g), using the insulin-clamp technique in anaesthetized rats. Importantly, they expressed their results per rat (mg/min) rather than per unit body weight, in the attempt to account partially for the impact of fasting-induced changes in FM. Conversely, Youn and

Buchanan [15] failed to demonstrate any decrease in peripheral insulin-mediated glucose uptake in conscious 48 h-fasted Wistar rats (body wt. ~ 250 g). Similarly, in the present study, R_d during the insulin-clamp study was unchanged in control and fasted rats when expressed per kg body weight. However, if the fasting-induced changes in body composition are taken into consideration in the study by Youn and Buchanan, a similar degree of peripheral insulin resistance would be demonstrated in 48 h-fasted rats [15] and in 72 h-fasted rats (the present study). Since Youn and Buchanan [15] have also demonstrated both increases and decreases in glucose uptake, depending on the kind of muscles, the regulation of glucose metabolism in fasting may be muscle-specific. Whether glucose uptake in fasting young rats is normal or decreased, the magnitude of the impairment is much less than in animal models of diabetes [14,16].

Changes in the rates of lipolysis and lipid oxidation during prolonged fasting certainly play a major role in the onset of insulin resistance in humans [17,22,45,46]. Increased NEFA levels have been shown to induce skeletal muscle insulin resistance via increased delivery of energy (NEFA) and substrates (glycerol) [1,23–26]. Whereas in young rats, due to near-total depletion of FM, plasma NEFA levels were decreased, circulating NEFA were dramatically increased in humans for over 3 and up to 14 days [4–7]. Thus increased circulating NEFA levels may be largely responsible for the development of severe insulin resistance in fasting humans. Indeed, in human fasting, the decrease in glucose utilization is over 50% [4–7]. Consistent with the above hypothesis, preventing the increase in plasma NEFA levels in fasting subjects by inhibition of lipolysis (with acipomox) increased insulin sensitivity [47]. Concomitantly with lower plasma NEFA levels, the degree of insulin resistance observed in the present study in rats is still much less than that reported in humans [4–7]. The initial FM is expected to determine the degree and the time-course of the metabolic alteration following prolonged fasting. In fact, in rats with $\sim 50\%$ more body weight than those in the present study, the circulating NEFA concentrations were increased 2-fold after 24 h and increased 3-fold after 48 h of fasting [48], and plasma NEFA and glycerol concentrations were still detectable even after 11 days of fasting [44]. Furthermore, in the latter study [44] 6 days of fasting were required to lower plasma NEFA and glycerol concentrations to the levels observed in our 72 h-fasted rats. Finally, insulin resistance was not demonstrated in fasted rats studied in muscle perfusion or isolated muscle preparations, where *in vivo* NEFA and glycerol levels were not maintained [13,15,20,21].

Concomitant with the development of peripheral insulin resistance, the intracellular pathways of glucose disposal during the insulin clamp were markedly affected by prolonged fasting. Whole-body glycolysis was significantly decreased, consistent with a recent study *in vivo* in rats [15]. Failure to demonstrate this decrease in some previous studies may be due to the use of less sensitive methodology in isolated muscle preparations or perfusion systems [13,20]. In the present study, whole-body and skeletal-muscle glycogen repletion during hyperinsulinaemia was unchanged, in agreement with previous reports in isolated muscle preparations or muscle perfusion systems [13,20,21]. Other insulin-clamp studies have shown increased rates of glycogen formation when expressed per kg body wt. [15,19]. Several lines of evidence support our expression of the result per LBM in this study. First, the insulin-induced increase in muscle glycogen was similar between groups. Second, the incorporation of ^3H into muscle glycogen from [$3\text{-}^3\text{H}$]glucose was similar in control and fasting rats after hyperinsulinaemia. Third, the kinetics of the skeletal-muscle glycogen synthase were similarly affected by hyperinsulinaemia in the two groups.

Thus, decreased glycolysis accounted for the peripheral insulin resistance in this model of fasting. Decreased glucose oxidation was probably the primary event in these changes in cellular glucose disposal during fasting. In fact, during hyperinsulinaemia, despite a marked decline in total glycolytic flux, the increment of the plasma lactate concentrations above baseline was enhanced. In this regard, it is of interest that, in addition to 12% of FM, the 72 h-fasted rats lost approx. 10% of their LBM, whereas 3 days of fasting in humans resulted in just ~3% decrease in total body mass [4,48]. The mobilization of amino acids from muscle may result in increased availability of amino acids for oxidation, which may compete with glucose oxidation similarly to lipids [49,50]. Alternatively, it is possible that the depletion of glycogen stores is the first event that signals for decrease in glucose oxidation. Indeed, a strong relationship was previously shown between initial glycogen concentration and rates of glycogen deposition and indices of glucose uptake after re-feeding of fasted rats [19]. However, the insulin-induced increase in muscle Glc-6-P concentration, which was significantly larger in the fasting group, appears to be sustained mainly via decreased flux through glycolysis. Although it could be argued that insulin clamp is a form of re-feeding, these data support a primary defect in glycolysis, since it is not totally overcome by short-term infusion of glucose and insulin resulting in increased Glc-6-P levels in fasting. This observation also suggests that glucose transport/phosphorylation is not impaired in muscle during fasting and appears to be at odds with the marked decrease in the gene expression of the GLUT4 glucose transporter previously reported in fat tissue in fasting and experimental diabetes [10–12]. However, when GLUT4 proteins and mRNA were assessed in muscle tissue, they were increased or unchanged in fasting and decreased in diabetes [9,51–54], suggesting differential regulation of glucose transport in muscle and fat during fasting. Finally, Koopmans et al. [55] have performed insulin clamp in 3-days-fasted and 3-days-diabetic rats (induced acutely by streptozotocin) to match them for the degree of catabolism and hypoinsulinaemia. They demonstrated peripheral insulin resistance in the diabetic rats compared with controls, but failed to demonstrate decreased insulin responsiveness during fasting (FM in fasting versus control was not accounted for). They also demonstrated an increase in HGP in diabetes and a decrease in fasting, in agreement with the present study in fasting and our previous studies in 90%-pancreatectomized diabetic rats [14,15]. Although we [30] have demonstrated that the peripheral insulin resistance of diabetes is characterized by a severe impairment in muscle glycogen synthesis (also in agreement with human studies), that of fasting is due to decreased glycolysis.

In summary, the present data indicate that near-total depletion of FM and decrease in plasma NEFA concentrations may explain the lower magnitude of insulin resistance during fasting in rats as compared with humans. The decline in insulin-mediated glucose uptake was completely accounted for by a severe impairment in glycolytic flux, but the rate of glycogen deposition was unchanged. Similar to experimental diabetes, hypoinsulinaemia during fasting is associated with increased Glc-6-Pase activity. However, HGP was decreased, rather than increased, due to a marked decrease in the hepatic concentration of Glc-6-P. Since fasted rats have been employed as a model of insulin resistance, it is important to recognize the differences and limitations of this model as compared with human fasting and with animal models of diabetes.

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