Altered liver glycogen metabolism in fed genetically obese mice

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1. The cyclic AMP and glycogen concentrations and the activities of phosphorylase kinase, phosphorylase *a* and glycogen synthase *a* were not different in livers from lean or *ob/ob* mice despite increased plasma glucose and insulin in the obese group. The liver water content was decreased by 10% in the obese mice. 2. In hepatocytes isolated from lean mice and incubated with increasing glucose concentrations (14–112 mM), a sequential inactivation of phosphorylase and activation of glycogen synthase was observed. In hepatocytes from obese mice the inactivation of phosphorylase was not followed by an activation of synthase. 3. The inactivation of phosphorylase occurred more rapidly and was followed by an activation of synthase in hepatocytes isolated from both groups of mice when in the incubation medium Na^+ was replaced by K^+ or when Ca^{2+} was omitted and 2.5 mM-EGTA included. 4. The inactivation of phosphorylase and activation of synthase were not different in broken-liver-cell preparations from lean and obese animals. 5. The re-activation of phosphorylase in liver filtrates in the presence of 0.1 μM -cyclic AMP and MgATP was inhibited by about 70% by EGTA and stimulated by Ca^{2+} and was always greater in preparations from *ob/ob* mice. 6. The apparent paradox between the impairment of glycogen metabolism in isolated liver preparations and the situation *in vivo* in obese mice is discussed.

Genetically obese mice of the *ob/ob* strain are characterized by severe hyperinsulinaemia and moderate hyperglycaemia (reviewed by Bray & York, 1979). Although both glucose and insulin favour hepatic glycogen synthesis in normal livers, the liver glycogen concentration of fed obese mice was not different from that of lean littermates (Shull & Mayer, 1956; Kaplan & Fried, 1975; Yen & Stamm, 1981; Hue & van de Werve, 1982). In perfused livers of fed *ob/ob* mice, higher rates of glycogenolysis and glucose production than in lean animals have been reported (Elliott *et al.*, 1971) and glucagon-induced glucose release was not antagonized by insulin (Ma *et al.*, 1978), indicating that, in the obese animal, the impairments in hepatic glycogen metabolism may be related to an insulin-resistance. To attempt evaluation of these impairments we have measured cyclic AMP and the activities of the rate-limiting enzymes of glycogen metabolism in freeze-clamped livers from lean and obese mice. The effect of glucose on interconversion of phosphorylase and glycogen synthase was investigated in isolated hepatocytes, as well as the effect of cyclic AMP on the glycogenolytic cascade in broken-liver-cell preparations. Our results show an increased responsiveness of phosphorylase activ-

ation in the presence of cyclic AMP and an impairment of synthase activation by glucose in liver preparations from obese mice compared with those of normal controls. The data together suggest that the glycogenolytic cascade in livers of obese mice may be continuously increased and that, as a result, phosphorylase *a* is maintained at an activity presumably high enough to prevent (via its known inhibitory effect on synthase phosphatase) the activation of glycogen synthase.

Materials and methods

Animal handling

Male obese (C57BL/6J-*ob/ob*) mice and thin littermates (obtained from the Jackson Laboratory, Bar Harbor, ME, U.S.A.), 17–21 weeks old, were fed *ad libitum* with a standard laboratory chow. The animals were used between 10:00 and 11:00 h. Venous blood samples were taken for the measurement of plasma glucose (Huggett & Nixon, 1957), and immunoreactive insulin (Herbert *et al.*, 1965). For sampling of livers, mice were anaesthetized by intraperitoneal injection of pentobarbital (0.1 mg/g body wt.), and the abdomen was opened 15 min later. At 15 min after laparotomy, the liver was

freeze-clamped *in situ* with small aluminium tongs pre-cooled in liquid N₂ (Wollenberger *et al.*, 1960). The frozen livers were kept at -60°C for subsequent enzyme and metabolite measurements.

Isolation and incubation of hepatocytes

Hepatocytes were prepared as described by van de Werve (1980), except that the liver perfusion was performed at constant flow rate and variable pressure. The cells were washed and incubated either in a Krebs-Henseleit (1932) bicarbonate buffer with Ca²⁺ (Na⁺ medium) or without Ca²⁺ and with 2.5 mM-EGTA (Ca²⁺-depleted medium) or in a medium (K⁺ medium) containing 118 mM-KCl, 4.75 mM-NaCl, 2.5 mM-CaCl₂, 1.18 mM-KH₂PO₄, 1.18 mM-MgSO₄ and 25 mM-KHCO₃. All media were in equilibrium with O₂/CO₂ (19:1) at pH 7.4. The cell suspensions (about 50 mg of the cells/ml) were incubated with various glucose concentrations in stoppered vials placed in a rotating water bath kept at 37°C. To measure enzyme activities, 100 µl samples of the suspension were pipetted and immediately frozen in tubes kept in liquid N₂. Trypan Blue exclusion was not different in cells freshly isolated from lean or obese livers or incubated in the different media, and was not less than 89%.

Preparation of liver filtrates

Mice were decapitated, the liver was excised (without the gall bladder) and homogenized in a Potter-Elvehjem tube in 3 vol. of ice-cold 0.1 M-glycylglycine, pH 7.4. Liver extracts were prepared at 0°C by centrifuging the homogenate for 10 min at 8000 g. Filtrates were obtained by applying 1 ml of extract on a column containing 30 ml of packed Sephadex G-25 (medium grade) gel equilibrated and eluted with 50 mM-glycylglycine, pH 7.4, and collecting a fraction (1.5 ml) which was eluted together with haemoglobin. The filtrates were incubated at 20°C in the presence of 0.5% glycogen, 5 mM-(NH₄)₂SO₄ and 50 mM-glycylglycine, pH 7.4, and, when indicated, in the presence of 3 mM-EGTA or increasing concentrations of free Ca²⁺ (calculated as in Portzehl *et al.*, 1964). For the determination of liver water content, freeze-clamped livers were weighed before and after freeze-drying.

Measurement of enzyme activities and metabolites

The determinations of phosphorylase *a* and glycogen synthase *a* in hepatocytes (Hue *et al.*, 1975) have been described in detail previously. Active phosphorylase kinase was measured in freeze-clamped livers in the presence of 7 mM-EGTA as described in van de Werve *et al.* (1977). In one series of experiments, phosphorylase kinase was also measured in the presence of 0.3 µM-Ca²⁺. Total phosphorylase was assayed in the presence of 1 mM-AMP, 500 mM-(NH₄)₂SO₄ and 150 mM-NaF

at pH 6.5 as recommended by Stalmans & Hers (1975). For the assay of phosphorylase kinase, purified crystalline muscle phosphorylase *b*, devoid of phosphorylase kinase and phosphorylase phosphatase activities, was purchased from Dr. P. Cohen (Dundee). Phosphorylase phosphatase was measured by the release of [³²P]P_i from ³²P-labelled phosphorylase *a* (Yang *et al.*, 1980) in liver filtrates prepared as described by Doperé & Stalmans (1982) in a medium containing 50 mM-imidazole, 1 mM-dithiothreitol and 250 mM-sucrose at pH 7.4. The enzyme unit is the amount that converts 1 µmol of substrate/min under the conditions of the assay. Glycogen was determined as described by Hue & van de Werve (1982). Cyclic AMP was assayed by the method of Gilman (1970) in trichloroacetic acid extracts purified as described by van de Werve *et al.* (1974).

Reagents

UDP-glucose, bovine serum albumin (fraction V), oyster glycogen (type II), amylo-(α-1,4-α-1,6)-glucosidase (grade III), collagenase (type I), bovine heart cyclic AMP-dependent protein kinase and cyclic AMP were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Glucose oxidase and peroxidase were purchased from Boehringer, Mannheim, Germany. Glucose 1-phosphate was from Fluka A.G., Buchs, Switzerland, and sodium pentobarbital (Nembutal) from Abbott Laboratories, Zug, Switzerland. Sephadex G-25 was provided by Pharmacia, Uppsala, Sweden. Cyclic [³H]AMP and UDP-[¹⁴C]glucose were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Basal characteristics of livers from lean and obese mice

Table 1 shows some of the changes related to the genetic (*ob/ob*) obesity syndrome, i.e. increased body and liver weight and elevated plasma glucose and insulin concentrations. There was no difference in the concentrations of cyclic AMP and glycogen nor in the activities of phosphorylase *a* and synthase *a* in freeze-clamped livers of obese compared with lean mice. Phosphorylase kinase activity measured in the presence of 7 mM-EGTA was decreased in *ob/ob*-mice livers, but this difference was no more statistically significant when corrected for the 10% lower water content (a similar value was reported by Lin *et al.*, 1981) or when measured in the presence of 0.3 µM-Ca²⁺ (lean, 33 ± 3.2, and *ob/ob*, 27.6 ± 0.6 units/g of liver; *n* = 3). Otherwise, the parameters just mentioned were the same in both groups whether the water content was taken into consideration or not. The liver protein content has repeatedly been reported to be the same in

Table 1. Basal characteristics of experimental mice

Values are means $\pm$ S.E.M. for the numbers of animals shown in parentheses. Statistical significance was assessed by Student's *t* test for unpaired data comparison (n.s., not significant).

	Lean	Obese	<i>P</i>
Body wt. (g)	32 $\pm$ 0.7 (6)	61 $\pm$ 0.9 (6)	<0.0005
Plasma			
Glucose (mg/100 ml)	188 $\pm$ 4 (6)	242 $\pm$ 18 (5)	<0.01
Insulin (ng/ml)	1.3 $\pm$ 0.2 (5)	55 $\pm$ 8 (4)	<0.0005
Liver			
Wet wt. (g)	0.9 $\pm$ 0.05 (3)	2.7 $\pm$ 0.16 (3)	<0.0005
Total water content (ml/100 g)	67 $\pm$ 0.8 (8)	55 $\pm$ 0.9 (9)	<0.0005
Cyclic AMP (nmol/g)*	1.0 $\pm$ 0.1 (13)	0.86 $\pm$ 0.06 (12)	n.s.
Glycogen (mg/g)*	58 $\pm$ 3 (10)	51 $\pm$ 5 (10)	n.s.
Phosphorylase kinase (units/g)*	8.5 $\pm$ 1.0 (10)	5.7 $\pm$ 0.4 (10)	<0.01
Phosphorylase <i>a</i> (units/g)*	3.6 $\pm$ 0.4 (9)	4.4 $\pm$ 1.1 (11)	n.s.
Synthase <i>a</i> (munits/g)*	30 $\pm$ 10 (6)	29 $\pm$ 8 (8)	n.s.

* Values expressed per g wet wt. of liver.

Fig. 1. Effect of glucose concentration on the interconversion of phosphorylase and synthase in hepatocytes Liver cells from lean (O, Δ) and *ob/ob* ($\bullet$, $\blacktriangle$) mice were incubated at 37°C in the presence of glucose as indicated. Values shown are means $\pm$ S.E.M. for three experiments. O, $\bullet$, Activity of phosphorylase *a*; Δ , $\blacktriangle$, activity of synthase *a*.

livers from lean and obese mice (Willms *et al.*, 1970; Karakash *et al.*, 1976; Yen & Stamm, 1981; Kaplan & Leveille, 1981).

Effect of glucose on interconversion of phosphorylase and synthase in liver preparations from lean and *ob/ob* mice

In freshly isolated hepatocytes from both lean and obese mice, synthase was mostly in the *b* form, whereas phosphorylase was about 60% in the *a* form (total phosphorylase was 55 $\pm$ 5 and 60 $\pm$ 10 units/g of protein in hepatocytes from lean and obese mice respectively; *n* = 6, difference n.s.). Fig. 1 shows that on incubation of the cells at 37°C in the presence of increasing glucose concentrations (14–112 mM) there was a dose-dependent inactivation of phos-

phorylase in hepatocytes from both groups. Although there was a trend towards higher steady-state concentrations of phosphorylase *a* in obese-mouse hepatocytes in the presence of 56 mM- and 112 mM-glucose, these values were not statistically different (*P* > 0.1) from those in lean-mouse liver cells. The inactivation of phosphorylase was followed, after a lag period, by an activation of synthase only in the liver cells of lean mice. No activation of synthase was detectable in cells from obese mice even at the highest glucose concentration used.

The possibility that impaired synthase activation in hepatocytes from obese mice was due to a lack of synthase phosphatase was then tested on acellular liver preparations. When liver filtrates were incubated at 20°C there was no difference in the

Fig. 2. Sequential inactivation of phosphorylase and activation of synthase in liver filtrates

Liver filtrates were incubated at 20°C as described in the Materials and methods section. Symbols are as in Fig. 1.

activation of synthase, which proceeded similarly in preparations of lean or obese mice (Fig. 2). The activation of synthase occurred after a lag period of about 20 min, the time required in both groups of mice for the inactivation of phosphorylase. Comparable results were obtained when the filtrates were incubated in the presence of 28 mM-glucose or 5 mM-caffeine or with liver extracts at different dilutions (results not shown). Phosphorylase phosphatase, measured by the release of [32 P]P_i from 32 P-labelled phosphorylase *a*, was not different in liver filtrates from both groups of mice (results not shown) or after proteolytic activation of phosphorylase phosphatase by trypsin (M. Bollen, personal communication). In Fig. 3 the activities of phosphorylase *a* and synthase *a* taken from all experiments with hepatocytes (data from Fig. 1) or filtrates have been correlated. Fig. 3(a) shows that in hepatocytes from lean mice there is an inverse relationship be-

Fig. 3. Correlation between the activity of phosphorylase *a* and synthase *a* in liver preparations

Values of phosphorylase *a* from liver preparations as indicated are plotted versus the corresponding values of synthase *a*. (a) Isolated hepatocytes from experiment in Fig. 1; (b) liver filtrates; O, lean mice; ●, *ob/ob* mice.

Fig. 4. Influence of the ionic composition of the incubation medium on the interconversion of phosphorylase and synthase in hepatocytes

Cells from lean (O, Δ) and *ob/ob* (●, $\blacktriangle$) mice were incubated in the presence of 112 mM-glucose in media as indicated. Symbols are as in Fig. 1.

Fig. 5. Correlation between the activity of phosphorylase *a* and synthase *a* in hepatocytes

For details see Fig. 3. Values of all hepatocyte preparations similar to those depicted in Fig. 4 were plotted. O, Lean mice; ●, *ob/ob* mice.

Fig. 6. Activation of phosphorylase by cyclic AMP in liver filtrates: effect of EGTA and Ca^{2+}

Liver filtrates from lean (O, □) and *ob/ob* (●, ■) mice were preincubated for 60 min at 20°C as described in the Materials and methods section without (□, ■) or with (O, ●) 3 mM-EGTA and increasing concentrations of free Ca^{2+} (calculated as in Portzehl *et al.*, 1964). The filtrates were further incubated with 4 mM-ATP, 8 mM- MgSO_4 and 0.1 μM-cyclic AMP. Values shown are means $\pm$ S.E.M. for three experiments.

tween the two activities, but that, in contrast, in hepatocytes from *ob/ob* mice, low phosphorylase *a* values (down to about 10% of the total activity) were not associated with an increased proportion of synthase in the *a* form. In liver filtrates (Fig. 3b), however, the correlation was identical for both groups.

Effect of the ionic composition of the incubation medium on interconversion of phosphorylase and synthase by glucose in hepatocytes

Since the experiments just described indicated the presence of normally functioning synthase phosphatase in liver filtrates of both lean and obese animals, we attempted to reveal its activity in isolated hepatocytes by changing the ionic composition of the incubation medium. Such ionic changes, indicated below, are known to affect the interconversion of phosphorylase and synthase in rat hepatocytes (Hue *et al.*, 1975; van de Werve *et al.*, 1977). When Na^+ was replaced by K^+ (K^+ medium) or when Ca^{2+} was omitted and 2.5 mM-EGTA was included (Ca^{2+} -depleted medium) in the incubation medium, the addition of glucose provoked in either case a more profound inactivation of phosphorylase, which was followed by an activation of synthase in hepatocytes isolated from lean as well as from obese mice (Fig. 4). Fig. 5 shows the correlation between phosphorylase *a* and synthase *a* activities in hepatocytes incubated in the modified media: the elevated synthase *a* values are associated with low phosphorylase *a* values in isolated hepatocytes of both groups of mice.

Effect of cyclic AMP and Ca^{2+} on phosphorylase activation in liver filtrates

When liver filtrates were incubated in the pres-

ence of EGTA or with increasing concentrations of free Ca^{2+} , phosphorylase was inactivated at the same rate in preparations from lean or obese mice irrespective of Ca^{2+} changes (results not shown). If thereafter Mg-ATP and cyclic AMP were added, phosphorylase *a* was re-activated to variable extents. Fig. 6 shows that phosphorylase *a* formation was always considerably greater in liver filtrates from *ob/ob* mice. The re-activation of phosphorylase was inhibited by about 70% in the presence of EGTA and was optimally stimulated at concentrations of Ca^{2+} close to $0.3\ \mu\text{M}$ in both liver preparations.

Discussion

Interconversion of phosphorylase in liver filtrates

The inactivation of phosphorylase in lean-mouse liver filtrates was reportedly unaffected by EGTA or Ca^{2+} in the $0.01\text{--}1\ \mu\text{M}$ range (van de Werve, 1981); this feature was confirmed here in both lean- and obese-mouse liver preparations. When phosphorylase was then re-activated by the addition of Mg-ATP and cyclic AMP, the amount of phosphorylase *a* formed was dependent on the Ca^{2+} concentration and was optimal at $0.1\text{--}1\ \mu\text{M}\text{-Ca}^{2+}$. Rat liver phosphorylase kinase is known to be stimulated by Ca^{2+} in the micromolar range (Shimazu & Amakawa, 1975; Khoo & Steinberg, 1975; van de Werve *et al.*, 1977; Vandenheede *et al.*, 1977; Sakai *et al.*, 1979; Doorneweerd *et al.*, 1982; Chrisman *et al.*, 1982), and our present results suggest that this property is shared by mouse liver phosphorylase kinase. The apparent K_a (about 10^{-9}M) for Ca^{2+} of phosphorylase activation was not different in lean- or obese-mouse liver preparations, but more phosphorylase *a* was formed in liver filtrates from obese mice. This increased formation of phosphorylase *a* can be the result of changes at one or several steps in the glycogenolytic cascade, which have yet to be defined.

Impairment of synthase activation by glucose in hepatocytes from ob/ob mice

The effect of glucose on the sequential inactivation of phosphorylase and activation of synthase, previously observed in rat hepatocytes (Hue *et al.*, 1975), is shown in the present work to operate similarly in lean-mouse hepatocytes. In obese-mouse hepatocytes, however, the inactivation of phosphorylase was not followed by an activation of synthase. The fact that synthase activation in broken-liver-cell preparations from lean and obese mice was comparable demonstrates that synthase phosphatase is present but inhibited in the intact liver cells of *ob/ob* mice. The most obvious explanation for this inhibition is that although the steady-state activities of phosphorylase *a* in obese-mouse hepatocytes were not statistically different from those in lean-

mouse liver cells, these values may be high enough to inhibit synthase phosphatase completely, the threshold inhibitory phosphorylase concentration being close to 10% of the total activity (Stalmans *et al.*, 1971). Such an inhibition is strongly suggested in the experiments with obese-mouse liver filtrates, where the activation of synthase took place only after the almost complete disappearance of phosphorylase *a*. Moreover, the changes introduced in the ionic composition of the incubation medium of hepatocytes, which revealed synthase activation in the obese group, were associated with a further decrease in phosphorylase *a*. The removal of Ca^{2+} from the incubation medium is expected to modify the intracellular Ca^{2+} concentration in such a way that phosphorylase kinase is less active, resulting in lower phosphorylase *a* values and in de-inhibition of synthase phosphatase in obese-mouse hepatocytes. A similar explanation may be given when the incubation mixture of the hepatocytes was enriched in K^+ : in this experimental condition, an intracellular modification of univalent cations may alter mitochondrial Ca^{2+} transport and homeostasis (Judah & Ahmed, 1963; Brand & De Selincourt, 1980; Haworth *et al.*, 1980; Hughes *et al.*, 1980; Heffron & Harris, 1981; Goldstone & Crompton, 1982).

From our observations, we may hypothesize that the intracellular Ca^{2+} is maintained at a higher steady-state concentration in obese-mouse hepatocytes. This hypothesis is substantiated by the work of Fraser & Trayhurn (1983) showing that the cycling of Ca^{2+} transport was persistently activated in liver mitochondria from *ob/ob* mice; and indications (Lin *et al.*, 1981) that the intracellular ratio of Na^+ to K^+ might be somewhat elevated in *ob/ob*-mouse liver. A higher cytosolic Ca^{2+} concentration is expected to keep phosphorylase in the *a* form and/or to inhibit synthase phosphatase (van de Werve, 1981). A stimulation of a specific Ca^{2+} -dependent synthase kinase, identified in rabbit liver (Ahmed *et al.*, 1982; Payne *et al.*, 1983), is also worth considering. Higher phosphorylase *a* activities in the isolated *ob/ob*-mouse liver may explain the increased glucose production and glycogenolysis observed previously in the perfused organ (Elliott *et al.*, 1971).

Liver glycogen metabolism in ob/ob mice in vivo

In the liver of fed lean and obese mice, phosphorylase was about 30% in the *a* form, which should completely inhibit synthase phosphatase (Stalmans *et al.*, 1974). Accordingly, synthase was almost entirely in the inactive form. The elevated glycaemia in *ob/ob* mice should favour phosphorylase inactivation; on the other hand, a putative elevated hepatic intracellular Ca^{2+} concentration and an increased glycogenolytic cascade, suggested

by our results, are expected to promote phosphorylase activation: the resulting steady-state activity of phosphorylase *a* *in vivo* may therefore not be different from that of lean mice, as is actually observed.

Under our experimental conditions no difference was found in hepatic cyclic AMP concentration. This is at variance with an increase reported in livers of 8-week-old *ob/ob* mice (Lavine *et al.*, 1975) which had 2-fold higher plasma glucagon concentrations. Since hyperinsulinaemia is already present at 8 weeks (Bray & York, 1979) and glucagon is still increased in older animals (Dubuc *et al.*, 1982), of the same age as we used, it is possible that the lack of difference in hepatic cyclic AMP that we observe is an indication of an impairment of insulin's ability to counteract cyclic AMP formation by glucagon, a defect which may not yet be expressed in younger mice. Such a lack of antagonism by insulin of glucagon-promoted glucose production in the perfused liver has been documented previously in 12-week-old *ob/ob* mice (Ma *et al.*, 1978). Phosphorylase kinase was not increased in liver of *ob/ob* mice, in keeping with the unchanged hepatic cyclic AMP concentration.

The lack of detectable differences between livers from lean and obese mice when enzymes of glycogen metabolism are considered under basal conditions (Table 1) contrast with some of those observed during this study when dealing with more extreme situations (Figs. 2 and 6). This does not preclude the existence of a potential abnormal handling of glycogen in the obese-mouse liver that may occur, e.g., when glycogen synthesis is induced by glucose. Indeed, Kreutner *et al.* (1975) observed such an impairment in liver glycogen synthesis and synthase activation after glucose administration *in vivo* to 24 h-starved *ob/ob* mice.

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