Proportion of active dephosphorylated pyruvate dehydrogenase complex in heart and isolated heart mitochondria is decreased in obese hyperinsulinaemic mice

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The proportion of active, dephosphorylated, pyruvate dehydrogenase complex was decreased in the mouse heart by obesity (by 56%), and this decrease in enzyme activity persisted during preparation and extraction of heart mitochondria. Phosphorylation and inactivation of pyruvate dehydrogenase may be a major factor in mediating the inhibitory effects of obesity on glucose oxidation in muscle, and this may represent an important mechanism in the development and/or expression of cellular insulin-resistance.

Hyperinsulinaemia and insulin-resistance are characteristic features of human and animal obesity (Crettaz *et al.*, 1980; Olefsky, 1980). In obese hyperinsulinaemic animals, glucose transport and oxidation in skeletal muscle and adipose tissue are markedly decreased compared with lean controls (LeMarchand-Brustel *et al.*, 1978*b*; Olefsky, 1980). The mechanism of these effects is not completely understood, but recent work has indicated that an insulin-receptor-complex abnormality may not be the primary determinant of insulin-resistance in obesity and a defect(s) distal to insulin-receptor interaction may be responsible for the observed alterations in glucose metabolism (Crettaz *et al.*, 1980; Olefsky, 1980).

The major determinant of glucose disposal in animal cells is the activity of the intramitochondrial pyruvate dehydrogenase complex. It is converted into inactive pyruvate dehydrogenase phosphate complex by pyruvate dehydrogenase kinase and re-activated by pyruvate dehydrogenase phosphate phosphatase (Linn *et al.*, 1969). The kinase and phosphatase reactions constitute a cycle, with the proportions of pyruvate dehydrogenase and pyruvate dehydrogenase phosphate complexes depending on their relative rates. The kinase reaction is activated by increasing ATP/ADP, NADH/NAD⁺ and acetyl-CoA/CoA concentration ratios (Cooper *et al.*, 1975; Kerbey *et al.*, 1976). *In vivo*, insulin deficiency (starvation and alloxan-diabetes) in the rat markedly diminishes the rate of pyruvate oxidation (and hence glucose oxidation) in many tissues, including muscle, and this is

brought about by a decrease in the proportion of active, dephosphorylated, pyruvate dehydrogenase complex (Kerbey *et al.*, 1976; Caterson *et al.*, 1982).

It would seem important to ascertain whether similar changes in dehydrogenase activity occur in muscle in animal models of obesity and to evaluate the potential role of this enzyme complex in the development and/or expression of cellular insulin-resistance. We now report a decrease in pyruvate dehydrogenase activity in heart muscle from gold thioglucose-treated obese hyperinsulinaemic mice that persists during preparation and extraction of heart mitochondria. This study also includes an appraisal of the effects of starvation (48 h) in lean control mice on pyruvate dehydrogenase activity in heart and isolated mitochondria.

Materials

Male mice (6 weeks of age; 20–25 g) were of the inbred CBA/T6 strain provided by the Blackburn Animal House, Department of Immunology, University of Sydney, N.S.W., Australia, and were allowed free access to food (Rat and Mouse Kubes; Allied Feeds, Rhodes, N.S.W., Australia) and water. In experiments where the effects of starvation were studied, food was removed 48 h before the animals were killed. Obesity was induced by a single intraperitoneal injection of gold thioglucose (Sigma Chemical Co., St. Louis, MO, U.S.A.) (0.5 mg/g body wt.). Control mice of the same age and weight received 0.9% NaCl. Mice were used 6–8 weeks later, when the gold thioglucose-injected group had increased their body weight by approx. 50% above that of lean control mice.

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Pyruvate dehydrogenase phosphate phosphatase was extracted from frozen ox heart and partially purified (Hutson *et al.*, 1978). Sources of other chemicals and biochemicals are as given by Kerbey *et al.* (1976).

Methods

Experiments in vivo

For details of induction of anaesthesia, removal of heart muscle and extraction of pyruvate dehydrogenase (active and inactive forms) and citrate synthase from frozen tissue, see Kerbey *et al.* (1976).

Mouse heart mitochondria: preparation, incubation and extraction

Mouse heart mitochondria were prepared by the method of Kerbey *et al.* (1976). The yield was 1–2 mg of mitochondrial protein/heart [protein determined by the method of Gornall *et al.* (1949), with bovine serum albumin as standard]. For measurement of initial pyruvate dehydrogenase activity, mitochondria (0.5–1.0 mg of mitochondrial protein) were added to 0.5 ml of KCl medium (0.12 M-KCl/20 mM-Tris/HCl/5 mM-potassium phosphate/2 mM-EGTA, pH 7.4) and separated immediately by centrifugation (Kerbey *et al.*, 1976). For measurement of total pyruvate dehydrogenase activity (sum of active and inactive forms), mitochondria were incubated in KCl medium for 10 min at 30°C (to convert inactive phosphorylated complex into active dephosphorylated complex) before centrifugation. Frozen mitochondrial pellets were extracted by freezing (liquid N₂) and thawing (30°C) (three times) in 250 µl of 50 mM-Tris / HCl / 5 mM-EDTA / 1 mM-dithiothreitol, pH 7.0 (2–4 mg of mitochondrial protein/ml).

Assay of pyruvate dehydrogenase complex and citrate synthase

Pyruvate dehydrogenase complex (active form) and citrate synthase in extracts of heart or heart mitochondria were assayed spectrophotometrically (Kerbey *et al.*, 1976). Total pyruvate dehydrogenase activity was assayed similarly in extracts of heart after incubation with pyruvate dehydrogenase phosphate phosphatase (Whitehouse *et al.*, 1974). One unit of enzyme activity converts 1 µmol of substrate into product/min at 30°C.

Assay of glucose and insulin

Immediately after removal of the heart, a sample of blood was withdrawn from the chest cavity. Blood samples were centrifuged (10000g, 15 min, 4°C), and serum was used for determination of glucose (Bergmeyer & Bernt, 1974) and immunoreactive insulin by radioimmunoassay (double-antibody method), with human insulin standards.

Results and discussion

Effects of gold thioglucose injection and 48 h starvation on body weight and serum glucose and insulin concentrations in CBA/T6 mice

When injected intraperitoneally with a single dose of gold thioglucose, CBA/T6 mice became obese within 6–8 weeks. In the present study, obese animals are defined as those that had a body weight 30% above that of lean controls after gold thioglucose injection. Body weight was increased in the gold thioglucose-injected group from 20.97 ± 0.23 g to 41.14 ± 0.66 g (for 90 observations) compared with an increase in lean control mice from 24.51 ± 0.40 g to 28.41 ± 0.40 g (for 66 observations) (for difference between final weights, $P < 0.001$). The body weight of lean control mice that had been starved (48 h) before they were killed was 24.06 ± 0.67 g (for 30 observations).

Table 1. *Effects of obesity and 48 h-starvation on pyruvate dehydrogenase-complex activity in mouse heart in vivo* For details of feeding of mice, induction of obesity, extraction and assay of pyruvate dehydrogenase complex (active and inactive forms) and citrate synthase see the Methods section. Experiments with the three groups of animals were performed in parallel and completed in one day. The results are given as means $\pm$ S.E.M. for the numbers of hearts shown in parentheses. Statistical significance of differences between experimental groups and controls: * not significant; † $P < 0.001$ against appropriate control.

Mice	Enzyme activities				
	Total pyruvate dehydrogenase (units/g dry wt.)	Citrate synthase (units/g dry wt.)	Pyruvate dehydrogenase (active form)		
			(units/g dry wt.)	(% of total)	(munits/unit of citrate synthase)
Lean control	13.89 ± 0.71 (10)	608.6 ± 15.9 (25)	2.93 ± 0.23 (43)	21.42 ± 1.63 (43)	4.83 ± 0.38 (43)
Obese	19.58 ± 0.99 (10)†	630.1 ± 18.0 (26)*	1.28 ± 0.13 (38)†	6.55 ± 0.64 (38)†	2.16 ± 0.23 (38)†
48 h-starved	13.29 ± 0.89 (10)*	646.6 ± 16.3 (16)*	0.54 ± 0.06 (36)†	4.05 ± 0.46 (36)†	0.83 ± 0.09 (36)†

Serum glucose concentrations (means $\pm$ S.E.M. for at least 24 mice in each group) were 16.15 ± 0.39 mM (lean control), 19.25 ± 0.81 mM (gold thioglucose-treated) and 10.88 ± 0.70 mM (48 h-starved). These values are qualitatively similar to but higher than those obtained in previous studies (LeMarchand-Brustel *et al.*, 1978b; Caterson & Taylor, 1982). These higher concentrations probably reflect some degree of stress induced during administration of anaesthetic before removal of tissue. This was confirmed in control experiments with non-anaesthetized mice, where serum glucose concentrations in samples of blood collected from a tail vein were significantly lower. The values (means $\pm$ S.E.M. for six mice in each group) were 8.02 ± 0.31 mM (lean controls) and 9.80 ± 0.94 mM (gold thioglucose-treated).

In agreement with previous studies (LeMarchand-Brustel *et al.*, 1978b; Caterson & Taylor, 1982), serum insulin concentrations showed some variability in gold thioglucose-treated mice, but were generally elevated with respect to lean controls. The values (in μ units/ml of serum; means $\pm$ S.E.M. for at least 24 mice in each group) were 24.27 ± 3.08 for lean controls and 82.08 ± 11.67 for gold thioglucose-treated mice (for difference, $P < 0.001$). Starvation decreased serum insulin concentration to 16.03 ± 1.52 μ units/ml (for 11 mice) (difference not significant against lean control).

Pyruvate dehydrogenase activity in mouse heart in vivo and in isolated heart mitochondria: effects of obesity and 48 h starvation

Control experiments validating the methods used for extraction and assay of pyruvate dehydrogenase complex (active and inactive forms) and

citrate synthase in heart muscle and heart mitochondria have been described previously (Whitehouse *et al.*, 1974; Kerbey *et al.*, 1976).

As shown in Table 1, total pyruvate dehydrogenase activity in heart muscle was not changed significantly by starvation (48 h), but was increased (by 41%) in obese animals. The reason for this increase has not been established in the present study, but may reflect increased enzyme synthesis as a consequence of hyperinsulinaemia before the development of insulin-resistance (LeMarchand-Brustel *et al.*, 1978a). Citrate synthase (a mitochondrial enzyme marker) activity was not significantly different between experimental groups. In hearts from lean control mice, the concentrations of total dehydrogenase complex, active dehydrogenase complex and the proportion of active dehydrogenase complex were qualitatively similar to but lower than those obtained in hearts from lean control rats (Kerbey *et al.*, 1976; Caterson *et al.*, 1982).

Obesity in the mouse significantly decreased the proportion of active, dephosphorylated, pyruvate dehydrogenase complex in heart muscle by 56% (compared with lean controls) regardless of whether activities were expressed on a dry-weight basis, as a percentage of total complex activity or per unit of citrate synthase. Control experiments with gold thioglucose-treated mice on restricted food intake (3 g/day per mouse) gave values for active pyruvate dehydrogenase that were similar to those obtained in the present study with lean control mice (2.69 ± 0.43 units/g dry wt.; mean $\pm$ S.E.M. for 13 observations), indicating that decreased dehydrogenase activity in gold thioglucose-treated animals is associated with the development of obesity and not gold thioglucose injection as such. In hearts of

Table 2. *Pyruvate dehydrogenase activity in mitochondria prepared from hearts of lean control, obese and 48 h-starved mice*

Mitochondria (0.5–1 mg) were added to 0.5 ml of KCl medium and, in any one experiment, either separated immediately by centrifugation and pellets frozen in liquid N₂ (for measurement of initial pyruvate dehydrogenase-complex activity) or incubated in the absence of respiratory substrate (10 min, 30°C) before centrifugation. Control experiments (results not shown) indicated that 10 min no-substrate values approximated total pyruvate dehydrogenase-complex activity. For details of preparation of mitochondria, composition of KCl medium, extraction and assay of pyruvate dehydrogenase complex and citrate synthase see the Methods section. Standard errors were computed from values obtained with at least four preparations of mitochondria and each preparation was made from at least eight hearts. Experiments comparing different batches of mitochondria were carried out side by side and completed on the same day. Enzyme activities (unless stated otherwise) are expressed per mg of mitochondrial protein and results are given as means $\pm$ S.E.M. for numbers of observations shown in parentheses. Statistical significance of differences between experimental groups and controls: * not significant; † $P < 0.001$ against appropriate control.

Mice	Citrate synthase activity (units/mg)	Pyruvate dehydrogenase activity		Initial pyruvate dehydrogenase activity	
		Initial (munits/mg)	10 min no-substrate (munits/mg)	% of no-substrate incubation	(munits/unit of citrate synthase)
Lean control	1.23 ± 0.030 (57)	21.21 ± 1.92 (38)	45.96 ± 1.95 (48)	46.15 ± 4.18 (38)	17.21 ± 1.55 (38)
Obese	1.28 ± 0.026 (39)*	9.95 ± 1.16 (33)†	51.94 ± 3.66 (30)*	19.16 ± 2.23 (33)†	7.78 ± 0.91 (33)†
48 h starved	1.32 ± 0.040 (24)*	6.07 ± 0.71 (18)†	46.99 ± 3.78 (20)*	12.92 ± 1.52 (18)†	4.60 ± 0.54 (18)†

lean control mice that were starved (48 h) before they were killed, the proportion of active pyruvate dehydrogenase complex was decreased by 82% (compared with fed lean controls). The effects of starvation on mouse heart pyruvate dehydrogenase activity are similar to those described in previous studies for rat heart (Sale & Randle, 1981; Caterson *et al.*, 1982).

As shown in Table 2, there were no significant differences in pyruvate dehydrogenase activity in mitochondria prepared from hearts of lean control, obese or 48 h-starved mice when mitochondria were incubated for 10 min in the absence of respiratory substrate. These values correspond closely to total complex activity in mouse heart mitochondria (results not shown; see Kerbey *et al.*, 1976), but are moderately lower than those obtained with rat heart mitochondria (Kerbey *et al.*, 1976; Hutson *et al.*, 1978). There were no significant differences in the activity of citrate synthase in extracts of mitochondria of lean control, obese or 48 h-starved mice, and values were comparable with those obtained for rat heart mitochondria (Kerbey *et al.*, 1976).

The proportion of active pyruvate dehydrogenase complex in freshly prepared mitochondria (referred to as initial pyruvate dehydrogenase activity) was markedly decreased by obesity (by 58%) and starvation (by 72%). The values for initial dehydrogenase activity (as a percentage of no-substrate incubation) in heart mitochondria from lean control, obese and 48 h-starved mice are moderately higher than the corresponding values obtained in the present study for the percentage of active pyruvate dehydrogenase complex in heart muscle *in vivo* (Table 1). This may be due to partial dephosphorylation and re-activation of pyruvate dehydrogenase during preparation and extraction of mitochondria.

General discussion and conclusions

In experiments with isolated tissue preparations it has been demonstrated that a specific inhibition of pyruvate dehydrogenase complex is involved in the impairment of glucose oxidation during starvation, in alloxan-diabetes and by the oxidation of fatty acids and ketone bodies in cardiac muscle (Randle *et al.*, 1978). In fact, the contribution of pyruvate dehydrogenase to the overall inhibition of glucose oxidation under these physiopathological conditions can be substantial. The observation that glucose oxidation in skeletal muscle and adipose tissue is markedly impaired in animal models of obesity, despite prevailing hyperinsulinaemia (Crettaz *et al.*, 1980; Olefsky, 1980), suggested the possibility that inhibition of pyruvate dehydrogenase complex may be a major factor

leading to diminished rates of glucose oxidation in these tissues. Furthermore, it seemed conceivable that the mechanisms that operate in experimental and possibly human obesity to regulate pyruvate dehydrogenase activity (and hence rates of glucose oxidation) may be related to those that are known to operate in animal tissues in starvation and diabetes, and in isolated tissue preparations oxidizing fatty acids or ketone bodies (see below).

In the present study, heart muscle was used in preference to skeletal muscle (a tissue used extensively to investigate insulin-resistance in obesity) for a number of reasons. Firstly, like skeletal muscle, the heart contributes to homeostatic control mechanisms in the whole animal by oxidizing glucose after carbohydrate intake, but glucose is spared and fatty acids and ketone bodies are oxidized at other times (Randle *et al.*, 1978). Secondly, the mechanisms that operate in heart muscle in physiological and pathological conditions (such as starvation and diabetes) to regulate pyruvate dehydrogenase activity have been clearly defined (for review see Randle *et al.*, 1981). Thirdly, it is difficult to freeze skeletal muscle *in situ* rapidly, and, because it contracts during excision, interconversion of pyruvate dehydrogenase between the active and inactive forms may occur.

The results of the present study have shown that the proportion of active, dephosphorylated, pyruvate dehydrogenase complex in heart muscle *in vivo* is substantially decreased in gold thioglucose-treated obese hyperinsulinaemic mice and that this difference in dehydrogenase activity persists during preparation and extraction of heart mitochondria. Starvation in the mouse also facilitated phosphorylation and inactivation of pyruvate dehydrogenase in heart muscle *in vivo* and in isolated heart mitochondria, but the effect was more marked than that observed in obesity. The effect of starvation in mouse heart and heart mitochondria is similar to that described previously with rat heart (Hutson *et al.*, 1978; Caterson *et al.*, 1982), and it seems reasonable to suggest that similar mechanisms may operate in both tissues to regulate interconversion of pyruvate dehydrogenase between the active and inactive forms. However, this remains to be established experimentally. In rat heart muscle in starvation and diabetes, three mechanisms are known that facilitate phosphorylation and inactivation of pyruvate dehydrogenase. (1) The kinase reaction is accelerated by increased concentration in mitochondria of protein factor(s) that may be pyruvate dehydrogenase kinase or an activator of the kinase (Kerbey & Randle, 1981, 1982). (2) Fatty acid oxidation may also activate the kinase reaction by increasing mitochondrial acetyl-CoA/CoA and NADH/

NAD⁺ concentration ratios (Kerbey *et al.*, 1976, 1977). (3) Re-activation of pyruvate dehydrogenase phosphate is inhibited by multi-site phosphorylation (Sugden *et al.*, 1978; Sale & Randle, 1981). The mechanism(s) that operates in mouse heart in obesity to facilitate phosphorylation and inactivation of pyruvate dehydrogenase is not understood. Previous studies with obese rats have demonstrated increased concentrations of circulating and muscle triacylglycerols, increased utilization of endogenous fatty acids by muscle and increased concentrations in muscle of citrate, glucose 6-phosphate and glycogen (Crettaz *et al.*, 1980), which may reflect intracellular inhibition of pyruvate and glucose oxidation as a result of increased lipid utilization. Further studies will be necessary to evaluate the contribution of β -oxidation of fatty acids to phosphorylation and inactivation of heart muscle pyruvate dehydrogenase complex in gold thioglucose-treated obese mice. Furthermore, the molecular mechanisms that may regulate interconversion of pyruvate dehydrogenase in mouse heart mitochondria in obesity are unknown and need investigation. Such studies should provide some insight into the role of the pyruvate dehydrogenase complex in the development and/or expression of cellular insulin-resistance in muscle in experimental and possibly human obesity.

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