Inactivation of pyruvate dehydrogenase complex in heart muscle mitochondria of gold–thioglucose-induced obese mice is not due to a stable increase in activity of pyruvate dehydrogenase kinase

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The proportion of pyruvate dehydrogenase (PDH) complex in the active dephosphorylated form was decreased (compared with fed lean control mice) in heart muscle mitochondria after the induction of obesity with gold–thioglucose (by 54 %) or starvation of lean mice for 48 h (by 81 %). The effects of obesity to inactivate PDH complex were demonstrable 4 weeks after administration of gold–thioglucose, and occurred despite significant hyperinsulinaemia in obese animals. Phosphorylation and inactivation of PDH complex in mouse heart muscle in starvation was attributed to a stable increase (2.7-fold) in the activity of PDH kinase as measured in extracts of mitochondria mediated by increased specific activity of a protein activator of PDH kinase (KAP) [Denyer, Kerbey & Randle (1986) *Biochem. J.* 239, 347–354]. In obese mice no such increase in kinase activity was observed, and we conclude that phosphorylation and inactivation of PDH complex in heart muscle in obesity is not mediated by KAP, but rather is a consequence of increased lipid oxidation.

INTRODUCTION

The activity of the intramitochondrial pyruvate dehydrogenase (PDH) complex is the major determinant of glucose oxidation in animal tissues and is subject to regulation by reversible phosphorylation catalysed by PDH kinase (MgATP-dependent; inactivating) and PDH phosphatase (activating) [1]. In rats starved for 48 h or rendered diabetic by alloxan, PDH complex is inactivated by phosphorylation in 24–48 h, leading to a decrease (up to 30-fold, depending on tissue) in percentage active form of PDH complex in muscles, liver, kidney and white adipose tissue (for reviews, see [2] and [3]). This is apparently effected by an increase in the activity of PDH kinase by two mechanisms. Oxidation of fatty acids may rapidly activate the kinase by increasing mitochondrial [acetyl-CoA]/[CoA] [4,5]; this effect is reversed rapidly by inhibitors of fatty acid oxidation (e.g. tetradecylglycidate) [6]. Longer-term effects of starvation to increase PDH kinase activity are apparently mediated by increased specific activity of a protein activator of the kinase (KAP), which may be separated from PDH complex and kinase intrinsic to the complex by gel filtration [7–10].

There is now a substantial body of evidence which indicates that in the gold–thioglucose-induced obese mouse, characterized by hyperinsulinaemia and cellular insulin resistance, PDH complex is phosphorylated and inactivated in heart muscle and isolated heart mitochondria [11–14], but not in liver [14] or brown adipose tissue [15]. We have suggested previously [11,14] that enhanced phosphorylation and inactivation of PDH

complex may be a major factor mediating the inhibitory effects of obesity on glucose oxidation in muscle, and that this may be important in the development and/or expression of cellular insulin resistance. However, the molecular mechanisms that operate in animal models of obesity to facilitate phosphorylation and inactivation of PDH complex in heart muscle are largely unknown and require investigation.

In this study, using fed and 48 h-starved mice as reference points, we have compared the effects of obesity on the activities of PDH complex (active form and total) and of PDH kinase in extracts of mitochondria prepared from heart muscle. We give evidence that, unlike starvation, obesity does not cause a stable increase in the activity of PDH kinase, and we suggest that enhanced phosphorylation and inactivation of PDH complex in heart muscle in obesity is a consequence solely of increased β -oxidation of fatty acids rather than an increase in the activity of KAP [which is known to mediate the longer-term effects of insulin-deficient states (i.e. starvation, insulin-dependent diabetes) to inactivate PDH complex in animal tissues]. The physiological implications of these findings with respect to the pathogenesis of insulin resistance in obesity is discussed.

MATERIALS AND METHODS

Animals and chemicals

Details pertaining to mice (source, feeding, induction of obesity) and sources of chemicals are as given by Kerbey *et al.* [11] and Caterson *et al.* [12].

Abbreviations used: PDH, pyruvate dehydrogenase; KAP, PDH kinase activator protein.

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Table 1. Effects of gold-thioglucose administration and 48 h starvation on mouse body weight and serum concentrations of glucose, insulin and non-esterified fatty acids (NEFA)

Mice from each group were studied 4 and 8 weeks after intraperitoneal administration of saline (lean fed, 48 h-starved) or gold-thioglucose (obese). Results are means $\pm$ S.E.M. for the numbers of animals in each group given in parentheses. For details relating to induction of obesity and assay of serum concentrations of glucose, insulin and non-esterified fatty acids, see the Materials and methods section. * $P < 0.001$ against age-matched lean control; † $P < 0.05$, †† $P < 0.01$ against 4-week values in same experimental group.

Mice	Body wt. (g)	Serum concentrations of:		
		Glucose (mM)	Insulin (μ units/ml)	NEFA (mequiv./l)
Lean control				
4 weeks	23.3 $\pm$ 0.4 (50)	13.2 $\pm$ 0.4 (45)	13.0 $\pm$ 1.2 (41)	2.49 $\pm$ 0.17 (45)
8 weeks	25.8 $\pm$ 0.6 (39)††	12.9 $\pm$ 0.5 (27)	23.7 $\pm$ 3.6 (27)†	2.95 $\pm$ 0.15 (13)
48 h-starved				
4 weeks	20.4 $\pm$ 0.4 (62)*	4.6 $\pm$ 0.3 (43)*	8.2 $\pm$ 0.9 (41)*	2.33 $\pm$ 0.17 (45)
8 weeks	20.1 $\pm$ 0.4 (39)*	3.0 $\pm$ 0.2 (27)*††	11.0 $\pm$ 1.3 (27)*	2.51 $\pm$ 0.17 (15)
Obese				
4 weeks	32.5 $\pm$ 0.7 (62)*	16.2 $\pm$ 0.7 (52)*	59.0 $\pm$ 8.0 (42)*	2.70 $\pm$ 0.14 (50)
8 weeks	39.1 $\pm$ 0.7 (42)*††	18.0 $\pm$ 0.4 (30)*	144.0 $\pm$ 21.0 (28)*††	3.12 $\pm$ 0.07 (20)

Mitochondria: preparation, incubation and extraction

Heart muscle was removed from mice (6–12 per group) immediately after killing (by stunning and cervical dislocation). Mitochondria were prepared from heart muscle [4] in isolation buffer (0.25 M-sucrose/5 mM-Tris/HCl/2 mM-EGTA, pH 7.4) in the presence (series A) or absence (series B) of 1 mM-dichloroacetate/50 mM-NaF for the assay of activities of PDH complex (active form, series A; total, series B) and of PDH kinase (series B). The yield of mitochondrial protein was 1–2 mg per heart. Mitochondria (0.5–1.5 mg of protein) were added to 1 ml of KCl medium [4] and either pelleted immediately by centrifugation (14000 g, 30 s) for series A or incubated (15 min, 30 °C) in KCl medium containing 10 μ M-carbonyl cyanide *m*-chlorophenylhydrazone before centrifugation (for series B) to convert inactive phosphorylated PDH complex into the active dephosphorylated form. Mitochondrial pellets were frozen in liquid N₂. Extracts were prepared by freezing (liquid N₂) and thawing (30 °C) twice in 50 mM-potassium phosphate buffer, pH 7.0, containing 10 mM-EGTA, 2 mM-dithiothreitol and 1 mM-tosyl-lysylchloromethane at a mitochondrial protein concentration of 5–12 mg/ml.

Enzyme assays

PDH complex activity (active form and total) in extracts of heart mitochondria was assayed spectrophotometrically by coupling to arylamine acetyltransferase [4]. For assay of PDH kinase, 212.5 μ l of extract containing 1 mM-MgCl₂, 7.5 μ g of oligomycin B and approx. 65 munits of PDH complex was preincubated for 2 min at 30 °C. A 50 μ l sample was withdrawn for assay of PDH complex (zero-time activity), followed by addition of ATP to 0.5 mM (11 μ l). Three 50 μ l samples were taken for assay of PDH complex between 15 and 90 s (the exact times depended on the expected rate of ATP-dependent inactivation determined in pilot experiments). Control experiments showed no loss of PDH complex activity over this incubation period in the absence of ATP. PDH kinase activity was expressed as the apparent first-order rate constant (K , min⁻¹) for

ATP-dependent inactivation of PDH complex, calculated from least-squares linear regression analysis of log_n (% active PDH complex) as a function of time [8].

Assay of serum glucose, insulin and non-esterified fatty acids

Immediately after removal of the heart, a sample of blood was withdrawn from the chest cavity. Blood samples were centrifuged (14000 g, 10 min), and serum was used for determination of glucose (by using hexokinase and glucose-6-phosphate dehydrogenase) [16] and insulin (double-antibody radioimmunoassay, with human insulin standards). Non-esterified fatty acids were assayed enzymically by using acyl-CoA synthetase, acyl-CoA oxidase and peroxidase, resulting in the oxidate condensation of 3-methyl-*N*- β -hydroxyethylamine with 4-aminoantipyrine to form a coloured adduct, the A_{550} of which was measured (Wako Pure Chemical Industries, Osaka, Japan).

RESULTS

Body weight and serum concentrations of glucose, insulin and non-esterified fatty acids of mice (lean fed, lean 48 h-starved and gold-thioglucose-induced obese) are given in Table 1. Body weight of obese animals was increased by 39.5 and 51.6% at 4 weeks and 8 weeks respectively after administration of gold-thioglucose. The serum concentration of glucose was significantly elevated in both groups of obese animals, as was serum insulin. Insulin concentrations were higher in both control and obese 8-week groups of animals compared with their respective 4-week group. The presence of hyperglycaemia and hyperinsulinaemia indicated that insulin resistance was present 4 weeks after the induction of obesity. There was no difference in serum non-esterified fatty acids between lean control and 48 h-starved animals, although concentrations in 8-week control and obese animals were higher than in their corresponding 4-week group.

The activities of PDH complex (percentage in the

Table 2. Activities of PDH complex (active form) and PDH kinase in extracts of mouse heart mitochondria; effects of obesity and 48 h starvation

Mice from each group were studied 4 and 8 weeks after intraperitoneal administration of saline (lean fed, 48 h-starved) or gold-thioglucose (obese). For details of preparation, incubation and extraction of mitochondria, assay of activities of PDH complex and of PDH kinase, see the Materials and methods section. The results given (means $\pm$ S.E.M.) are for a minimum of ten determinations from five separate preparations of mitochondria from at least 60 mice. Mitochondria from each group of mice were prepared in parallel and assays were performed on the same day. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ against lean control values.

Mice	PDH complex (active form)		Activity of PDH kinase (K , min^{-1})
	(munits/mg of mitochondrial protein)	(% of total)	
4 weeks			
Lean control	20.16 $\pm$ 1.70	17.00 $\pm$ 1.4	1.02 $\pm$ 0.05
48 h-starved	2.63 $\pm$ 0.03***	2.40 $\pm$ 0.4***	3.26 $\pm$ 0.42***
Obese	17.39 $\pm$ 2.79	12.60 $\pm$ 1.6*	1.00 $\pm$ 0.16
8 weeks			
Lean control	23.47 $\pm$ 2.76	19.9 $\pm$ 2.6	0.88 $\pm$ 0.08
48 h-starved	4.46 $\pm$ 0.54***	3.8 $\pm$ 0.7***	2.40 $\pm$ 0.16***
Obese	16.61 $\pm$ 1.20*	9.2 $\pm$ 1.0**	1.02 $\pm$ 0.10

active dephosphorylated form) and of PDH kinase in extracts of heart mitochondria from the three groups of animals, 4 and 8 weeks after the induction of obesity, are given in Table 2. Total PDH complex activity (sum of active and inactive forms) (means $\pm$ S.E.M. for numbers of mice in parentheses) was 121.50 $\pm$ 12.09 (16) (lean control), 108.55 $\pm$ 22.59 (8) (lean, 48 h-starved) and 124.50 $\pm$ 15.08 (10) (obese) munits/mg of mitochondrial protein 4 weeks after induction of obesity, and 130.87 $\pm$ 20.37 (13) (lean control), 155.68 $\pm$ 26.11 (10) (lean, 48 h-starved) and 224.48 $\pm$ 30.33 (14) (obese) munits/mg of mitochondrial protein 8 weeks after induction of obesity. The marked increase in total PDH complex activity in the 8-week obese group has been a consistent feature of this and also of previous studies [11,14]. The reason for this increase is not known, but may reflect increased enzyme synthesis as a consequence of hyperinsulinaemia [17].

Starvation of lean mice for 48 h decreased the percentage of PDH complex in the active form in heart muscle mitochondria by approx. 84 % in both the 4- and 8-week groups of mice, and this was attributed to a stable increase (3.2-fold and 2.7-fold respectively) in the activity of PDH kinase, which persisted through the preparation, incubation and extraction of mitochondria. The values obtained in the present study for the effects of starvation on the activities of PDH complex (active form) were qualitatively similar to, but lower than, those obtained in previous studies with rat heart (e.g. see [18]). This is almost certainly due to the inclusion in the present study of dichloroacetate and NaF (inhibitors of PDH kinase and phosphatase respectively) in sucrose buffers to prevent interconversion of PDH complex between the active and inactive forms during isolation of mitochondria. PDH kinase activities in mouse heart mitochondria were significantly lower than those obtained in previous studies with extracts of heart mitochondria from fed or 48 h-starved rats, but the ratios of activities (starved/fed) were comparable [9,19].

Induction of obesity with gold-thioglucose also

decreased the proportion of PDH complex in the active dephosphorylated form in heart mitochondria by 26 and 54 % for the 4- and 8-week groups respectively. However, unlike starvation, phosphorylation and inactivation of PDH complex in obesity was not associated with a stable increase in the activity of PDH kinase. The significance of these findings in relation to the pathogenesis of insulin resistance in obesity is discussed below.

GENERAL DISCUSSION AND CONCLUSIONS

The molecular basis for enhanced phosphorylation and inactivation of mitochondrial PDH complex in rat tissues in insulin-deficient states (starvation, alloxan-diabetes) is now well understood and apparently involves an increase in the activity of PDH kinase by two mechanisms. Oxidation of fatty acids and/or ketone bodies may activate PDH kinase by increasing mitochondrial concentration ratios of acetyl-CoA/CoA and NADH/NAD⁺. This is a shorter-term mechanism and may be rapidly reversed in heart muscle and kidney by inhibitors of fatty acid oxidation such as 2-tetradecylglycidate [6]. The extensive studies by Randle and colleagues with rat heart [4,7,9] and liver [10] have indicated that there is a further and longer-term mechanism involving PDH kinase activation, which is not mediated by short-term effects of acetyl-CoA or NADH. This is a stable mechanism which persists through preparation, incubation and extraction of mitochondria and mediated apparently by increased specific activity of KAP, which binds tightly to the PDH complex (possibly the E2 component) [10].

In the present study we wished to ascertain whether similar mechanisms operate in insulin-resistant states (obesity, non-insulin-dependent diabetes) to decrease percentage of active PDH complex in tissues. We have demonstrated clearly that, unlike starvation, increased phosphorylation and inactivation of PDH complex in heart muscle from gold-thioglucose-obese hyperinsulinaemic mice cannot be attributed to a stable increase in

the activity of PDH kinase as measured in extracts of mitochondria. It therefore seems reasonable to conclude that KAP (which is known to mediate the longer-term effects of insulin-deficient states to activate PDH kinase) is not involved in mediating the inhibitory effects of obesity on PDH complex activity in mouse heart muscle. Previous studies with obese hyperinsulinaemic mice have indicated that inactivation of PDH complex in heart muscle may be reversed *in vivo* by intravenous administration of the fatty acid-oxidation inhibitor 2-tetradecylglycidate [12]. Moreover, recent studies in man by Felber and colleagues have provided convincing evidence that increased tissue lipid oxidation is the major factor in the pathogenesis of insulin resistance in obesity and non-insulin-dependent diabetes [20]. Taken together, these findings suggest that increased phosphorylation and inactivation of PDH complex in mouse heart muscle in obesity is due exclusively to increased β -oxidation of fatty acids.

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