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Persistence of the effect of insulin on pyruvate dehydrogenase activity in rat white and brown adipose tissue during the preparation and subsequent incubation of mitochondria

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1. Increases in the amount of the active non-phosphorylated form of pyruvate dehydrogenase in rat epididymal adipose tissue, as a result of incubation with insulin, persist not only during the preparation of mitochondria but also during subsequent incubation of coupled mitochondria in the presence of respiratory substrates. 2. No effect on insulin was found if the hormone was added directly to mitochondria in the presence or absence of added plasma membranes. 3. Concentrations of several possible regulators of pyruvate dehydrogenase kinase (ATP, ADP, NADH, NAD^+, acetyl-CoA, CoA and potassium) were measured in rat epididymal-adipose-tissue mitochondria incubated under conditions where differences in pyruvate dehydrogenase activity persist as a result of insulin action. No alterations were found, and it is suggested that inhibition of the kinase is not the principal means by which insulin activates pyruvate dehydrogenase. The intramitochondrial concentration of magnesium was also unaffected. 4. Differences in pyruvate dehydrogenase activity in interscapular brown adipose tissue associated with manipulation of plasma insulin concentrations of cold-adapted rats were also shown to persist during the preparation and subsequent incubation of mitochondria in the presence or absence of GDP. 5. It is pointed out that the persistence of the effect of insulin on pyruvate dehydrogenase in incubated mitochondria will facilitate the recognition of the mechanism of this action of the hormone. Evidence that the short-term action of insulin involves an increase in pyruvate dehydrogenase phosphate phosphatase activity rather than inhibition of that of pyruvate dehydrogenase kinase is discussed.

Exposure of rat epididymal white adipose tissue to insulin leads to a marked increase in PDH_a within a few minutes (Jungas, 1970; Denton *et al.*, 1971; Weiss *et al.*, 1971). The effect is an important component of the means whereby insulin stimulates the conversion of glucose into fatty acids, and has been studied intensively in several laboratories (for reviews, see Denton *et al.*, 1975; Denton & Hughes, 1978; Wieland, 1983). Evidence has also been obtained that insulin increases PDH_a in other tissues that are important sites of fatty acid synthesis, namely liver, brown adipose tissue and lactating mammary gland (Topping *et al.*, 1977; McCormack & Denton, 1977; Baxter *et al.*, 1979; Assimacopoulos-Jeannet *et al.*, 1982).

By using isolated rat epididymal fat-cells, it has been demonstrated directly that the activation of PDH is the result of dephosphorylation of the α-subunits of the complex (Hughes *et al.*, 1980). Studies on purified preparations of the heart and kidney PDH complexes have indicated that the tightly bound ATP-linked protein kinase phosphorylates the α-subunits on three different serine residues, but that phosphorylation of only one of these (site 1) correlates well with diminution of catalytic activity (Barrera *et al.*, 1972; Yeaman *et al.*, 1978; Sugden *et al.*, 1979; Sale & Randle, 1981). Evidence has been obtained that the three sites are phosphorylated within intact fat-cells to similar ex-

Abbreviations used: PDH, pyruvate dehydrogenase; PDH kinase, pyruvate dehydrogenase kinase; PDHP phosphatase, pyruvate dehydrogenase phosphate phosphatase; PDH_a, the active non-phosphorylated form of pyruvate dehydrogenase; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $k_{0.5}$, concentration of effector required for half-maximal response.

tents and that insulin causes the approximately parallel dephosphorylation of all three (Hughes *et al.*, 1980).

There is much evidence to suggest that the properties of the protein kinase and phosphatase responsible for the regulation of the PDH system by reversible phosphorylation are broadly similar in all mammalian tissues (for reviews, see Denton *et al.*, 1975; Denton & Hughes, 1978; Randle, 1981; Reed, 1981; Wieland, 1983). The activity of the kinase is regulated by the concentration of a number of metabolites, including inhibition by pyruvate and increases in the [ADP]/[ATP] ratio (especially in the presence of high K^+) and activation by increases in [NADH]/[NAD^+] and [acetyl-CoA]/[CoA] ratios (Linn *et al.*, 1969; Roche & Reed, 1974; Cooper *et al.*, 1974, 1975; Pettit *et al.*, 1975). In contrast, the activity of the phosphatase seems to be relatively insensitive to changes in metabolite concentrations, but is activated by Mg^{2+} ($k_{0.5} \simeq 1$ mM) and Ca^{2+} ($k_{0.5} \simeq 1\ \mu$M) (Denton *et al.*, 1972; Siess & Wieland, 1972; Hucho *et al.*, 1972; Severson *et al.*, 1974).

Several hypotheses have been put forward for the mechanism whereby insulin might bring about the dephosphorylation of PDH (for reviews, see Denton *et al.*, 1981; Wieland, 1983). These have included inhibition of the kinase by decreases in the mitochondrial [ATP]/[ADP] or [acetyl-CoA]/[CoA] ratios (Wieland *et al.*, 1974; Paetzke-Brunner *et al.*, 1978), as well as activation of the phosphatase by increases in intramitochondrial Ca^{2+} (Denton *et al.*, 1978; Denton & Hughes, 1978) or by a low-M_r mediator, possibly a peptide, released from the plasma membrane after occupancy of the insulin receptors (Jarett & Seals, 1979; Seals & Czech, 1980; Kiechle *et al.*, 1981). A role for peroxides has also been suggested (Mukherjee & Lynn, 1977; May & de Haan, 1979; Paetzke-Brunner *et al.*, 1980).

Earlier studies in our laboratory have indicated that the effect of insulin on PDH_a persists during the preparation of mitochondria from rat epididymal adipose tissue (Severson *et al.*, 1976). The present studies are concerned with establishing the conditions under which the effect persists during subsequent incubation of the mitochondria from both white and brown adipose tissue, and with the assay of the mitochondrial content of some potential regulators of PDH kinase and PDHP phosphatase under conditions where the effect persists.

Experimental

Chemicals and biochemicals

These were from either Boehringer Corp. (London), Lewes, East Sussex BN7 1LG, U.K., Sigma (London) Chemical Co., Poole, Dorset BH16 7NH, U.K., or BDH Chemicals, Poole, Dorset BH12 4NN, U.K., except as follows: the calcium ionophore A23187 was kindly given by Dr. R. L. Hamill, Eli Lilly and Co., Indianapolis, IN, U.S.A. Bovine serum albumin (Sigma) was defatted by the method of Chen (1967) before use.

Source of tissue

Epididymal fat-pads were obtained from male rats (170–220g) fed *ad libitum* on a stock laboratory diet (Breeding diet: Oxoid, Basingstoke, Hants, U.K.) and housed at 23–25°C. Brown adipose tissue was taken from the interscapular region of female rats (200–300g) fed on the same diet and cold-adapted for 3–6 weeks at 5°C.

Incubation of epididymal fat-pads

All incubations were carried out at 37°C with shaking in bicarbonate medium (Krebs & Henseleit, 1932) containing 1.25mM-$CaCl_2$ (unless otherwise stated) and gassed with O_2/CO_2 (19:1) plus other additions as indicated. Incubations were performed with 5–8ml/g of tissue and were preceded by a preincubation period of 15–30min in the same medium but without additions.

Preparation, incubation and extraction of mitochondria

Details were essentially as given by McCormack & Denton (1980). Tissue was disrupted in 4ml of sucrose-based extraction medium (250mM-sucrose, 20mM-Tris/HCl, 30mg of defatted albumin/ml, 7.5mM-reduced glutathione, 2mM-EGTA, pH7.4)/g, and mitochondrial fractions were prepared by differential centrifugation. In most experiments, mitochondria were suspended in 250mM-sucrose/20mM-Tris/HCl/2mM-EGTA, pH7.4, to give a concentration of about 20mg of protein/ml, and stored at 0°C before use (up to 2h). In a few experiments, the mitochondrial fraction was further purified by centrifugation in a Percoll gradient (Belsham *et al.*, 1980).

Incubations of mitochondria were performed at 30°C at a concentration of 0.5–1mg of mitochondrial protein/ml in air-saturated KCl-based medium (see the legend to Table 1) at pH7.3 or 7.2, containing additions as stated. Uptake of O_2 was monitored with an oxygen electrode (McCormack & Denton, 1980). For the assay of pyruvate dehydrogenase and glutamate dehydrogenase, samples of mitochondria (0.5–1mg of protein) were sedimented by centrifugation for 15–30s at 10000–15000*g* in a mini-centrifuge; the pellet was immediately frozen in liquid N_2 and subsequently extracted by freezing and thawing either three times in 500μl of 100mM-potassium phosphate buffer (pH7.0) containing 1mM-dithiothreitol, 2mM-

EDTA and 50μl of rat serum/ml (to prevent proteolysis), or twice in the same medium plus 0.1% (v/v) Triton X-100. For the assay of adenine nucleotides, NAD^+, CoA and acetyl-CoA, a sample (100–250μl) of mitochondria and medium was removed at the appropriate time and mixed vigorously with an equal volume of 2% (w/v) $HClO_4$. For the assay of NADH, mitochondrial incubations were terminated by the addition of 0.6 vol. of 1M-KOH in ethanol. Potassium was extracted from mitochondria prepared and suspended in potassium-free sucrose-based medium by vigorous sonication in distilled water (1 ml/mg of protein), followed by deproteinization with 5% $HClO_4$. Magnesium was extracted by dissolving sedimented mitochondria (1 mg) in 1 ml of 1M-NaOH at 75°C for 5 min and, after cooling, diluting 10-fold with $LaCl_3$ (1 mM)/EDTA (4 mM). Potassium and magnesium were then assayed by atomic-absorption spectrophotometry.

Assay of pyruvate dehydrogenase and glutamate dehydrogenase activities

These were assayed as described by Stansbie *et al.* (1976) and McCormack & Denton (1980). Total pyruvate dehydrogenase activity was taken as that present after incubation of extracts with pig heart PDHP phosphatase, 25 mM-$MgCl_2$, and 1 mM-$CaCl_2$ for 15 min at 30°C (McCormack & Denton, 1980).

Assay of mitochondrial contents of adenine nucleotides, NAD^+, NADH, acetyl-CoA and CoA

These metabolites were assayed by using luciferase-linked techniques as described by Kerbey *et al.* (1976, 1977).

Expression of results

Enzyme activities are given in units (1μmol of substrate converted/min) measured at 30°C. Glutamate dehydrogenase is a mitochondrial enzyme; the activity of this enzyme was used in some experiments as a convenient index of recovery of mitochondria. Mitochondrial fractions prepared from epididymal fat-pads contain about 0.25 unit of glutamate dehydrogenase/mg of protein and about 0.08 unit of total pyruvate dehydrogenase activity/mg of protein. Protein was determined in samples of mitochondria sedimented from albumin-free media by a modified biuret method (Gornall *et al.*, 1949) standardized with bovine serum albumin. Results are given as means $\pm$ S.E.M. and compared by using Student's *t* test. Kinetic constants were calculated by fitting data with a non-linear least-squares regression program written for a Hewlett–Packard 8495 computer by Dr. Paul England of this Department (McCormack & Denton, 1980).

Results

Persistent increases in PDH_a in mitochondria from rat epididymal adipose tissue previously exposed to insulin

Typically, insulin increased PDH_a in epididymal fat-pads incubated in medium containing glucose, from about 30–40% to 70–90% of total activity (Stansbie *et al.*, 1976). This increase is largely maintained during the preparation of mitochondria by Polytron homogenization followed by simple differential centrifugation in sucrose-based medium (Severson *et al.*, 1976; Table 1). No consistent differences in the recovery of mitochondria or in the total activity of PDH (expressed in terms of either mitochondrial protein or glutamate dehydrogenase) are observed. In the experiments carried out in the present study, the overall means for the ratio of total PDH activity to that of glutamate dehydrogenase were 0.31 ± 0.017 in control mitochondria and 0.31 ± 0.016 in mitochondria from tissue previously exposed to insulin (results are means $\pm$ S.E.M. for 45 independent observations).

It should also be emphasized that there was no consistent difference between the rate of O_2 uptake by the two types of mitochondria with oxoglutarate/malate or isocitrate/malate as respiratory substrates. Mean respiratory control ratios (as defined by Chance & Williams, 1956) were 3.2 ± 0.2 and 3.3 ± 0.2 for each substrate respectively in mitochondria from control tissue. The corresponding values for paired preparations of mitochondria from insulin-treated tissue were 3.1 ± 0.2 and 3.3 ± 0.2. The mean maximum rates of oxidation of oxoglutarate and isocitrate by these mitochondria in the presence of ADP were 101 ± 3.8 and 106 ± 3.3% respectively of the rates in mitochondria from control tissue. (Results are given as means $\pm$ S.E.M. for 12–15 observations in all cases.)

Effects of incubation with and without oxidizable substrates other than pyruvate

Incubation of the mitochondria at 30°C in KCl-based media containing 0.1 mM-EGTA in the presence of oxoglutarate/malate, succinate or palmitoylcarnitine resulted in little change in PDH_a, and the effects of pretreatment of the pads with insulin clearly persisted for at least 10 min (Table 1). Under these conditions, the intramitochondrial ATP is maintained. However, if the mitochondria were incubated under conditions where the intramitochondrial ATP content falls, such as in the absence of added oxidizable substrate or in the presence of the uncoupler FCCP, then the PDH_a increases in mitochondria from both control and insulin-treated tissue to values that are close to the total activity, because of the

Table 1. *Persistent increases in PDH_a during preparation and incubation of mitochondria from rat epididymal white adipose tissue previously exposed to insulin*
Pads (in paired groups of 8–12) were incubated in bicarbonate-based medium containing glucose (2mg/ml) with or without insulin (50nM) for 30min at 37°C. Mitochondria were prepared (see the Experimental section) and samples taken for measurements of enzymes and ATP before and after incubation at 30°C for 5 or 10min in KCl-based medium (125mM-KCl, 20mM-Tris/HCl, 5mM-potassium phosphate and 0.1mM-EGTA, pH7.3) plus other additions as indicated. The ratio of total PDH activity to that of glutamate dehydrogenase was in the range 0.25–0.35 for all experiments and was not altered by insulin exposure. Results are given as means ± S.E.M. for the numbers of separate preparations of mitochondria shown. *$P < 0.01$ for effect of insulin.

Mitochondria	Additions to mitochondrial incubation media	Incubation time (min)	PDH (as % of total activity) in mitochondrial from tissue exposed to:		ATP (as nmol/unit of glutamate dehydrogenase) in mitochondria from tissue exposed to:		No. of observations
			No hormone	Insulin	No hormone	Insulin	
Freshly prepared	—	—	33.2±4.2	60.9±4.9*	12.2±2.1	12.6±1.51	12
Incubated	Oxoglutarate (5mM)+ malate (0.5mM)	5	29.7±2.1	57.7±5.7*	11.1±0.75	11.6±1.10	8
		10	29.1±3.7	53.6±6.0*	8.7±1.16	11.8±1.50	9
	As above plus EGTA (5mM)	5	15.8±1.4	34.9±2.3*	11.5±0.97	12.4±1.32	7
	Succinate (5mM)	5	25.5±2.9	54.5±4.7*	9.8±0.70	11.4±0.92	4
		10	26.3±2.7	55.3±4.5*	7.4±0.35	9.7±1.05	4
	Palmitoylcarnitine (50μM)+ malate (0.5mM)	5	19.2±1.5	34.6±3.7*	10.2±1.05	11.5±1.55	8
		10	24.5±3.1	46.3±6.1*	8.9±1.40	8.3±0.96	8
	No additions	5	84.0±2.8	96.0±4.4	3.6±0.75	3.1±0.83	10
	FCCP (0.5μM)+MgATP (5mM)+ EGTA (5mM)+oligomycin (5μg/ml)	5	16.0±2.5	27.0±2.1*	—	—	5
	As above plus A23187 (5μM) and valinomycin (1μg/ml)	5	12.1±1.3	14.2±2.0	—	—	5

diminution in PDH kinase activity. Subsequent studies showed that increasing the concentration of EGTA to 5mM caused a decrease in PDH_a in mitochondria from both control and insulin-treated tissue (Table 1 and Fig. 1). This was the first indication that the presence of low concentrations of extramitochondrial Ca^{2+}, especially in the absence of added Na^+ or Mg^{2+}, could increase PDH_a in these mitochondria (Marshall *et al.*, 1984). Again, the effect of exposing epididymal adipose tissue to insulin on PDH_a persists provided that an oxidizable substrate is added. A 2-fold increase from about 10–20% to 25–35% of total activity has been observed not only with oxoglutarate/malate (Table 1) but also with citrate/malate, isocitrate/malate, succinate and palmitoylcarnitine/malate. Values after 10min of incubation were not appreciably different from those obtained after 5min, suggesting that the ratio of kinase to phosphatase activity remains constant in each case, and so a steady-state PDH_a value is maintained (Fig. 2). In a few experiments, changes in PDH_a were monitored in mitochondria incubated with oxoglutarate/malate and 5mM-EGTA over 20min. Although differences between PDH_a in the mitochondria from control and insulin-treated tissues persisted, the actual values for both conditions increased during the second 10min period. This was associated with a 10–30% decrease in

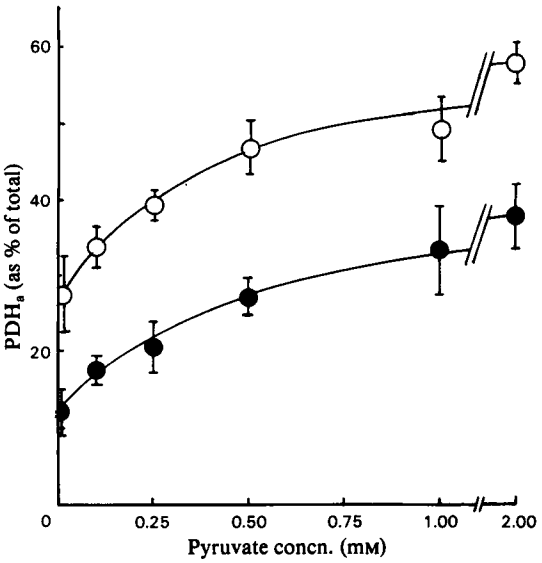

Fig. 1. *Effects of increasing concentrations of pyruvate on PDH_a in mitochondria from control (●) and insulin-treated epididymal adipose tissue (○)*

Experimental details are as given in Table 1. Mitochondria were incubated for 5min at 30°C in the KCl-based medium containing 5mM-EGTA, 5mM-oxoglutarate and 0.2mM-malate and various concentrations of pyruvate. Results are shown as means ± S.E.M. for values obtained in three separate experiments.

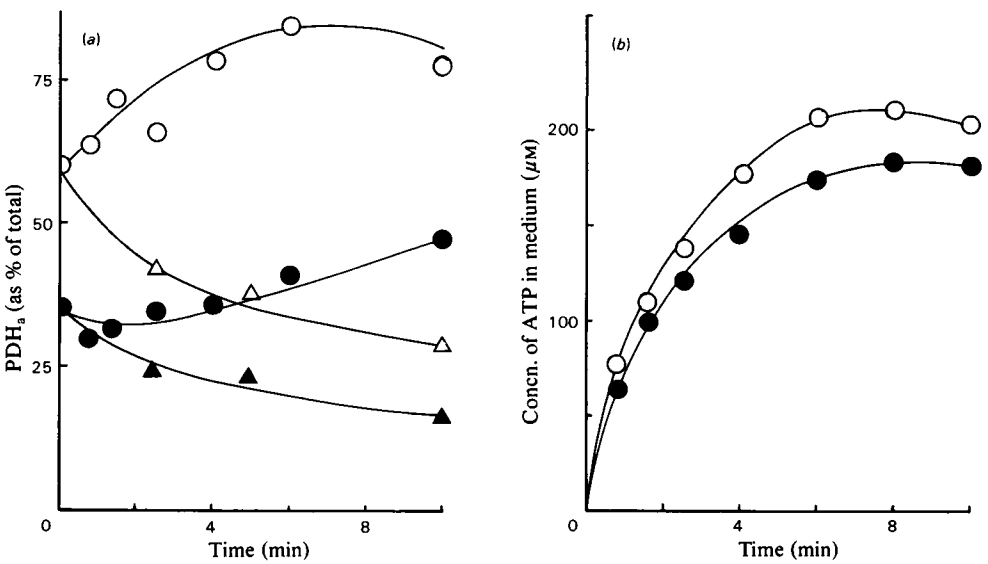

Fig. 2. *Effects of ADP on (a) PDH_a and (b) ATP synthesis in mitochondria incubated in medium containing low phosphate*

Mitochondria were from control (closed symbols) or insulin-treated (open symbols) epididymal adipose tissue and were incubated in KCl-based medium with 5mM-EGTA, 5mM-oxoglutarate and 0.5mM-malate as in Fig. 1, except that the concentration of potassium phosphate was 0.2mM (▲, △), or under the same conditions plus 0.5mM-ADP added at zero time (●, ○). Results shown are the means of three separate experiments. PDH_a in mitochondria from insulin-treated pads was significantly greater ($P<0.05$) at all times; there were no statistically significant differences in the concentration of ATP in the medium at any time.

mitochondrial ATP content, which is perhaps the result of a decline in O_2 concentration.

All subsequent experiments were done (unless otherwise stated) with 5min incubations in KCl-based medium, pH7.3, at 30°C in the presence of 5mM-EGTA and with oxoglutarate/malate as the oxidizable substrate. The increase in PDH_a in mitochondria from insulin-treated tissue under these conditions was not altered appreciably on storage of mitochondria for up to 4h at 0°C before incubation (results not shown). It was also unaltered if mitochondrial fractions were further purified by Percoll-gradient centrifugation (as described in the Experimental section). Values of PDH_a obtained with eight pairs of such preparations of mitochondria from control and insulin-treated tissue which were then incubated under the standard conditions were 19.4 ± 3.8 and $34.6 + 6.5\%$ of total activity respectively (means $\pm$ S.E.M. for eight observations). Incubation for 5min in KCl-based medium at pH6.9 or in medium at pH7.3 in which KCl was replaced by 250mM-sucrose resulted in decreases in initial PDH activity to about 9% of total activity in control mitochondria, but values were at least 2-fold greater than this in mitochondria from insulin-treated tissue under the same conditions (results not shown).

Effects of incubation with uncoupler and ATP

Addition of ATP (and oligomycin) to control fat-pad mitochondria incubated in the presence of the uncoupler FCCP results in only 16% of the total mitochondrial PDH being present as PDH_a (Table 1). Under these conditions the value in mitochondria from insulin-treated tissue is significantly increased, but the increase may be smaller than that observed in coupled mitochondria incubated with oxoglutarate/malate. The further addition of the ionophores valinomycin and A23187 resulted in the near disappearance of the effect of insulin on PDH_a.

Effects of incubation with pyruvate

Pyruvate is an inhibitor of PDH kinase and thus increases PDH_a in mitochondria when added to incubation medium (Fig. 1). Very similar responses to pyruvate were observed in mitochondria from control and insulin-treated tissue; the calculated $k_{0.5}$ values ($\pm$S.E.M.) for the effect of pyruvate from data summarized in Fig. 1 were 0.75 ± 0.11 and 0.57 ± 0.18mM respectively, and the calculated maximum increases in PDH_a in response to pyruvate were 35 ± 2.3 and $38 \pm 4.6\%$ of total PDH activity (degrees of freedom = 16 in all cases). The effects of insulin on PDH_a was apparent at all pyruvate concentrations. Very similar responses to dichloroacetate, which also inhibits PDH kinase (Whitehouse *et al.*, 1974), were observed (results not shown).

Effects of ADP

Addition of 5mM-ADP to mitochondria incubated under the standard conditions caused a substantial increase in O_2 uptake and in PDH_a, presumably because of inhibition of PDH kinase. After 5min, PDH_a values were in the range 55–85% of total PDH activity, and were only slightly higher in mitochondria from insulin-treated pads. Addition of 0.2mM-ADP caused marked but rather variable transient increases in PDH_a, followed by low steady-state values, as ADP was converted into ATP and the rate of O_2 uptake returned to State-4 values. The effect of insulin was still apparent after such an ADP cycle; PDH_a in mitochondria from control and insulin-treated tissue measured 2–3min after State-4 rates of O_2 uptake were attained were 14 ± 1 and $33 \pm 6\%$ of total activity respectively (means $\pm$ S.E.M. for five separate mitochondrial preparations).

Experiments reported in Fig. 2 were designed to allow changes in PDH_a to be monitored while a steady-state extramitochondrial value of [ADP]/[ATP] of about 1.5 was attained. This was achieved by incubating the mitochondria with oxoglutarate/malate in the initial presence of 0.5mM-ADP and 0.2mM-P_i. Over the first 6min, extramitochondrial ATP concentration increased to reach a steady-state value approaching 0.2mM. The rate of ATP synthesis and the final ATP concentration attained were not significantly different in mitochondria from control and insulin-treated tissue. PDH_a increased slightly in both types of mitochondria (in contrast with the decreases observed in the absence of added ADP). Nevertheless, PDH_a remained at least 2-fold higher in the mitochondria from insulin-treated tissue, demonstrating that the response to changes in extramitochondrial [ADP]/[ATP] ratios is very similar in the two preparations of mitochondria.

Effects of insulin

Direct addition of insulin (at concentrations in the range 0.1nM–0.1μM) to mitochondria incubated under standard conditions was without appreciable effect on PDH_a (results not shown; see also Martin *et al.*, 1972). Studies were also performed under conditions similar to those used by Kiechle *et al.* (1981) and Seals & Czech (1981), in which evidence for the activation of PDH in a cell-free system containing fat-cell plasma membranes and mitochondria was obtained. Mitochondria prepared from control tissue with or without Percoll-gradient purification were frozen and thawed and then preincubated for 5min at 37°C at a concentration of about 0.5mg of mitochondrial

protein/ml in 50mM-potassium phosphate buffer (pH7.4) containing 50μM-$CaCl_2$, 50μM-$MgCl_2$ and 250μM-ATP, followed by incubation for 2–5min in the same medium plus added plasma membranes (0.2–0.5mg of protein/ml) prepared as described by Belsham *et al.* (1980) with or without added insulin in the range 0.1nM–0.1μM. PDH_a was then determined after addition of Triton X-100 (0.1%) to the complete incubation mixture. No consistent effects of insulin or addition of membranes were observed; PDH_a was between 10 and 20% of total activity in all experiments. It was evident under these conditions that the mitochondria were not intact; for example, more than 50% of glutamate dehydrogenase and citrate synthase activities were not sedimented at the end of the incubation period by centrifugation at 10000*g* for 1min. In contrast, over 90% of both activities were sedimented after incubation of unfrozen mitochondria under the standard conditions used in the present study. On the other hand, the addition of Triton X-100 at the end of incubations under the conditions used by Kiechle *et al.* (1981) and Seals & Czech (1981) was found to be necessary if full expression of PDH_a was to be routinely attained.

Concentrations of possible regulators of PDH kinase and PDHP phosphatase in mitochondria from control and insulin-treated rat epididymal adipose tissue (Table 2)

Except for potassium, measurements of mitochondrial content were made after incubation in KCl-based medium containing EGTA with oxoglutarate/malate as the respiratory substrate. In all experiments PDH_a was significantly greater in the mitochondria from insulin-treated tissue. There was no evidence that the increase could have been brought about by a decrease in either [ATP]/[ADP] or [NADH]/[NAD^+] ratios. In fact, these ratios tended to be slightly higher in the mitochondria from insulin-treated tissue, but the increases were not statistically significant. A decrease in the [acetyl-CoA]/[CoA] ratio could also have resulted in lower activities of PDH kinase. On average, a small decrease was found, but the change was not statistically significant. Moreover, it seems unlikely that it is relevant to the regulation of PDH_a, as incubation of mitochondria in the presence of palmitoylcarnitine (25 or 50μM) and malate (200μM) increased the [acetyl-CoA]/[CoA] ratio above 2 in mitochondria from both control and insulin-treated tissue without greatly altering PDH_a (Table 1).

The total concentration of magnesium in fat-pad mitochondria incubated with oxoglutarate/malate was 43nmol/unit of glutamate dehydrogenase, which corresponds to about 10nmol/mg of mitochondrial protein. This value was the same in mitochondria from either control or insulin-treated tissue.

Table 2. *Effect of prior exposure of rat epididymal adipose tissue to insulin on the mitochondrial content of possible regulators of PDH kinase and PDHP phosphatase in isolated mitochondria incubated with oxoglutarate and malate*

Epididymal fat-pads were incubated and mitochondria prepared as given in the legend to Table 1. Except where K^+ content was to be measured, mitochondria were incubated in the KCl-based medium with addition of EGTA (2mM), oxoglutarate (5mM) and malate (0.5mM) for 5min at 30°C; for the measurement of potassium content a K^+-free sucrose-based medium was used (see the Experimental section). In all experiments PDH_a was also determined after the 5min incubation. The value expressed as a percentage of total PDH activity was increased by prior exposure of tissue to insulin by more than 80% in each individual experiment. Overall mean values (±S.E.M.) were 21.5±1.67 and 34.7±2.34% of total activity for 26 paired preparations from control and insulin-treated tissue respectively. All values below are given as means±S.E.M. Content values are expressed as nmol/unit of glutamate dehydrogenase. Effects of prior treatment of tissue on the parameters measured were not statistically different in all cases.

Parameter	Mitochondria from tissue exposed to: No hormone	Insulin	
ATP content	10.5±0.6	11.5±1.5	(5)
ADP content	8.40±1.0	8.3±1.4	(5)
[ATP]/[ADP] ratio	1.26±0.12	1.39±0.13	(5)
NADH content	11.3±1.8	13.5±2.6	(6)
NAD^+ content	14.4±3.1	9.4±2.0	(6)
[NADH]/[NAD^+] ratio	0.9±0.17	1.27±0.26	(6)
Acetyl-CoA content	1.6±0.23	1.4±0.29	(5)
CoA content	3.6±0.52	3.9±0.43	(5)
[Acetyl-CoA]/[CoA]	0.53±0.15	0.39±0.11	(5)
Magnesium content	43±6.6	43±7.1	(9)
Potassium content	313±27	249±21	(6)

The inhibition of PDH kinase by ADP may be enhanced by K^+ (Roche & Reed, 1974); thus the possibility arises that an increase in mitochondrial K^+ could lead to an increase in PDH_a. However, no evidence for any increase in potassium content was found in mitochondria prepared from insulin-treated tissue. Indeed, if anything, there was a small decrease.

Persistent changes in PDH_a in mitochondria from interscapular brown adipose tissue from cold-adapted rats injected with anti-insulin serum or glucose

In our hands, PDH_a within preparations of rat brown adipose tissue *in vitro* is close to 100% of total activity, probably because of very low concentrations of ATP in such preparations (McCormack

& Denton, 1977). However, manipulations of plasma insulin concentrations *in vivo* by injections of anti-insulin serum and glucose result in marked changes in PDH_a in cold-adapted rats (McCormack & Denton, 1977). The value of PDH_a after the lowering of circulating insulin concentrations with anti-insulin serum is about 13% of total activity, compared with a value of 30–35% in tissue from animals in which the circulating insulin had been increased within the physiological range by an injection of glucose. During preparation of mitochondria these differences are maintained (Table 4), as previously found with mitochondria from control or insulin-treated epididymal white adipose tissue. There was no change in total PDH activity; the overall mean value was 164 ± 9 m-units/mg of mitochondrial protein, which corresponds to 173 ± 13 m-units/unit of glutamate dehydrogenase activity, for 40 observations.

Mitochondria from rat interscapular brown adipose tissue, like those from other sources of brown adipose tissue, are only coupled if incubated with medium containing both albumin and a purine nucleotide such as GDP (Cannon & Lindberg, 1979; Nicholls, 1979; McCormack & Denton, 1980). Changes in PDH_a during incubation of mitochondria with or without GDP illustrate this point strikingly (Table 3). If mitochondria from tissues of untreated animals are incubated in KCl-based medium containing EGTA and albumin with succinate as respiratory substrate, PDH_a increases to some 70% of total activity within 10 min, while the ATP content remains very low. However, if 0.5 mM-GDP is present, the ATP content greatly increases and PDH_a falls sharply within 5 min to about 5% of total activity. Similar very low values of PDH_a together with high ATP content were found in mitochondria incubated in the presence of albumin and GDP with other respiratory substrates such as oxoglutarate/malate and isocitrate/malate (Table 3). However, in the absence of GDP the mitochondria incubated with either oxoglutarate/malate or isocitrate/malate contained about 1 nmol of ATP/mg of protein, presumably synthesized from GTP formed by succinate thiokinase. This value was unaltered on the further addition of the uncoupler FCCP and is thus also an indirect indication of the very low ATPase activity in uncoupled brown-adipose-tissue mitochondria (see Nicholls, 1979). Under these conditions, PDH_a was about 15% of total activity. This value is similar to that found in coupled epididymal white-adipose-tissue mitochondria from control tissue incubated under similar conditions (Table 1), whereas addition of uncoupler to white-adipose-tissue mitochondria increases PDH_a to near 100% of total activity.

Incubation of brown-adipose-tissue mitochondria from both anti-insulin-serum- and glucose-treated rats in medium containing albumin and GDP plus oxoglutarate/malate as substrate gives PDH_a activity which is less than 3% of the total (Table 4 and Fig. 3). The activity of PDH kinase seems to be considerably greater than that of PDHP phosphatase under these conditions. If the activity of the kinase is inhibited either by the lowering of ATP in the absence of GDP or by addition of pyruvate, PDH_a increases in both sets of mitochondria, but the increase is considerably greater in the mitochondria from glucose-treated rats. Thus the effects of prior exposure of the tissue to insulin on PDH_a do persist during the incubation of brown-adipose-tissue mitochondria and can be observed when the activity of PDH kinase is limited. Results of experiments with dichloroacetate, which is a potent inhibitor of PDH kinase (Whitehouse *et al.*, 1974), support this view (Fig. 3). In these studies, mitochondria were incubated

Table 3. *Effects of GDP on PDH_a in brown-adipose-tissue mitochondria incubated with various substrates*

Mitochondria were prepared from cold-adapted rats which had been killed by decapitation and incubated in 125 mM-KCl/20 mM-Tris (pH 7.2)/5 mM-potassium phosphate/2 mM-EGTA/albumin (1 mg/ml) with other additions as indicated for 10 min at 30°C. PDH_a and ATP were determined as given in the Experimental section, and values are expressed as means ± S.E.M. for three or four different mitochondrial preparations. Values were also measured after incubation for 5 min, but did not differ significantly from the 10 min values given. *$P < 0.01$ for effect of GDP.

Additions to incubation media	PDH_a (as % of total activity) in mitochondria incubated in media containing:		ATP content (as nmol/mg of protein) in mitochondria incubated in median containing:	
	No GDP	0.5 mM-GDP	No GDP	0.5 mM-GDP
10 mM-Succinate	69 ± 4.2	5.1 ± 1.0*	0.02 ± 0.01	3.1 ± 0.29*
10 mM-Oxoglutarate + 1 mM-malate	18 ± 1.6	1.8 ± 0.3*	1.32 ± 0.19	2.8 ± 0.18*
3 mM-*threo*-D_S-Isocitrate + 1 mM-malate	15 ± 1.2	2.1 ± 0.3*	1.10 ± 0.13	2.9 ± 0.18*

Table 4. *Persistent changes in PDH_a during the preparation and incubation of mitochondria from interscapular brown adipose tissue of cold-adapted rats injected with either anti-insulin serum or glucose*

Rats were anaesthetized with Sagatal (60mg/kg) and either injected intravenously with anti-insulin serum (0.3ml) or intraperitoneally with glucose (200mg in 0.5ml of 0.9% NaCl). Tissue was removed 15min after injections and mitochondria were prepared (see the Experimental section). PDH_a and ATP content were determined before and after incubation of the mitochondria for 5min at 30°C in KCl-based medium (125mM-KCl, 20mM-Tris/HCl, 5mM-potassium phosphate and 2mM-EGTA, pH7.2) containing defatted albumin (1mg/ml) and other additions as indicated. Results are given as means ± S.E.M. for the numbers of separate preparations of mitochondria indicated: $^{**}P < 0.01$, and $^{*}P < 0.05$, versus values in mitochondria from anti-insulin-serum-treated rats. No significant differences in the total activity of PDH were apparent; the overall mean value was 164 ± 9m-units/mg of mitochondrial protein for 20 separate determinations.

Mitochondria	Additions to mitochondrial incubation media	PDH_a (as % total activity) in mitochondria from tissue of rats injected with: Anti-insulin serum	PDH_a (as % total activity) in mitochondria from tissue of rats injected with: Glucose	ATP (as nmol/mg of mitochondrial protein) in mitochondria from tissue of rats injected with: Anti-insulin serum	ATP (as nmol/mg of mitochondrial protein) in mitochondria from tissue of rats injected with: Glucose	No. of observations
Freshly prepared		12.9±0.9	25.0±1.3**	0.21±0.04	0.19±0.04	10
Incubated	Oxoglutarate (10mM)+malate (1mM)	7.4±0.7	14.0±1.1**	0.95±0.11	0.85±0.09	10
	As above+GDP (0.5mM)	2.8±0.30	2.9±0.3	1.84±0.08	1.74±0.11	8
	As above+GDP (0.5mM) and pyruvate (5mM)	13.4±2.4	20.0±1.6*	2.01±0.09	1.94±0.08	6
	Pyruvate (5mM), malate (1mM) and GDP (0.5μM)	19.7±2.1	30.5±2.6**	1.53±0.11	1.64±0.12	6

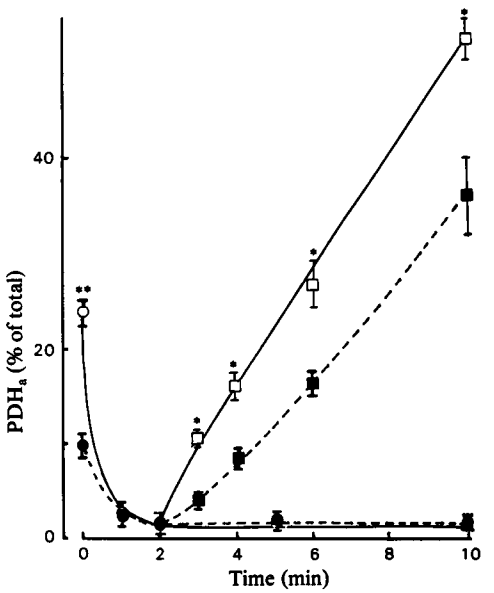

Fig. 3. *Time course of the effects of dichloroacetate on PDH_a in mitochondria from interscapular brown adipose tissue of cold-adapted rats injected with either anti-insulin serum (closed symbols) or glucose (open symbols)*

Experimental details are as given in Table 4. Mitochondria were incubated in the KCl-based medium containing defatted albumin (1mg/ml), 10mM-oxoglutarate and 1mM-malate (●, ○). At 2min, 2mM-dichloroacetate was added to certain samples (■, □). Results are given as means ± S.E.M. for observations on four to ten separate preparations of mitochondria; $^{*}P < 0.05$ and $^{**}P < 0.01$ for the effects of insulin treatment. There were no significant differences in mitochondrial ATP concentrations.

with oxoglutarate/malate in the presence of albumin and GDP. After 2min, when PDH_a had reached about 3% of total activity in both sets of mitochondria, a high concentration of dichloroacetate was added; PDH_a was then increased in both sets of mitochondria, but the initial rate of increase was significantly greater in the mitochondria from glucose-treated rats. Similar results were obtained when the dichloroacetate was added after a 5min incubation (results not shown).

Discussion

The present results clearly demonstrate that the effect of insulin on PDH_a in rat epididymal adipose tissue persists not only during the preparation of mitochondria but also during the subsequent incubation of coupled mitochondria in the presence of a variety of respiratory substrates. Similar findings were obtained with brown adipose tissue from cold-adapted rats, but in this case persistent changes in PDH_a were only apparent during incubation of the mitochondria under conditions where

the activity of PDH kinase was partially inhibited, either by a modest decrease in ATP content or by the addition of pyruvate or dichloroacetate. It can thus be concluded that the mechanism of insulin action is such that the alterations responsible for the increase in PDH_a, whether it is via effects on either or both of the interconverting enzymes, PHDP phosphatase and PDH kinase, are maintained in incubated mitochondria. This property should certainly facilitate the final recognition of the means whereby insulin regulates PDH, since it allows studies to be performed on isolated mitochondria preparations and thus lessens problems of compartmentation associated with studies on whole cells or tissue preparations.

The studies reported in this paper indicate that alterations in the total intramitochondrial concentration of a number of kinase regulators are unlikely to form the basis of the effect of insulin on the PDH system. Decreases in the ratios [ATP]/[ADP], [NADH]/[NAD^+] or [acetyl-CoA]/[CoA] could result in an inhibition of PDH kinase activity. However, no significant changes were found and, if anything, the values of the first two ratios were slightly increased in mitochondria from insulin-treated tissue. In addition, changes in pyruvate concentration would also not appear to play a role, since the increase in PDH_a remained evident in these mitochondria when they were incubated either in the absence of pyruvate or in the presence of various concentrations of pyruvate up to saturating concentrations. The similarity of response of PDH_a in mitochondria from control and insulin-treated tissue to pyruvate (Fig. 1) is in marked contrast with the greatly diminished response observed under comparable incubation conditions with heart mitochondria from starved or alloxan-diabetic rats compared with normal fed controls (Kerbey *et al.*, 1976; Kerbey & Randle, 1981; Randle, 1981; McCormack *et al.*, 1982). A similar diminished response is also evident in fat-pad mitochondria from starved or alloxan-diabetic rats (Denton, 1975; J. G. McCormack & S. E. Marshall, unpublished work). The long-term consequences of insulin deficiency associated with starvation and alloxan-diabetes on the PDH system in adipose tissue appear to be quite different from the short-term effects of insulin, since the longer-term effects cannot be corrected by short-term incubation *in vitro* of tissue from such animals with insulin (Stansbie *et al.*, 1976). Studies on rat heart suggests that the decreases in PDH_a in starvation and diabetes are initiated by an increase in PDH kinase activity, probably involving changes in protein synthesis (Kerbey & Randle, 1981, 1982; Sale & Randle, 1982). In contrast, all evidence indicates that the increase in white- and brown-adipose-tissue PDH_a brought about by short-term exposure to insulin is principally through an increase in PDHP phosphatase activity. Not only was no evidence for any appropriate change in the intramitochondrial concentration of any kinase regulator obtained in the present studies, but in an earlier study we found that the rate of phosphorylation of PDH was actually increased in mitochondria prepared from insulin-treated epididymal adipose tissue (Hughes & Denton, 1976). The simplest interpretation of this work was that insulin increased the rate of dephosphorylation of PDH and that this in turn led to an increased turnover of the PDH phosphorylation–dephosphorylation cycle under the steady-state conditions studied. If the increase in PDH_a had been the result of a decrease in PDH kinase activity, a decrease in turnover would be expected. The results with brown-adipose-tissue mitochondria in Fig. 3 also point to the activation of the phosphatase activity by insulin. In these experiments, mitochondria were initially incubated in media containing albumin and GDP with oxoglutarate/malate as the respiratory substrate. No effects of the prior manipulation of plasma insulin concentrations were evident until after the blocking of the activity of PDH kinase by addition of dichloroacetate. Then the rate of increase in PDH_a and thus of dephosphorylation was significantly higher in the mitochondria prepared from rats with high circulating concentrations of insulin.

The only known regulators of PDHP phosphatase are Ca^{2+}, Mg^{2+} and possibly also changes in [NAD^+]/[NADH] ratio (Denton *et al.*, 1972; Hucho *et al.*, 1972; Siess & Wieland, 1972; Pettit *et al.*, 1975). Since no increases in either mitochondrial Mg^{2+} or [NAD^+]/[NADH] ratio were found in the present study (Table 2), the possibility that changes in mitochondrial Ca^{2+} are important remains to be considered (Marshall *et al.*, 1984).

We have been unable to obtain any effects of insulin on PDH_a when the hormone is added directly to our mitochondrial preparations. In particular, we have not found any changes when the mitochondria are supplemented with various amounts of plasma membranes and incubations are performed under similar conditions to those used by Kiechle *et al.* (1981), Seals & Czech (1981) and Begum *et al.* (1982), under which they report a direct effect of the addition of insulin on the release of $^{14}CO_2$ from [1-^{14}C]pyruvate. Our lack of success may be related to the fact that our studies were performed with mitochondria and plasma membranes prepared by different procedures and a different assay for PDH. Nevertheless, it should be noted that the experiments of Jarett, Seals and others (Kiechle *et al.*, 1981; Seals & Czech, 1981; Saltiel *et al.*, 1981, 1982; Begum *et al.*, 1982) all employ mitochondria which were usually first frozen and

thawed, and then incubated in phosphate buffer. This is not very different from the procedure that we use for the extraction of intramitochondrial enzymes from mitochondria. It seems certain that only a fraction of the mitochondria would be in any way intact. If changes in PDH_a within fat-cell (and other) mitochondria are to be used as a reliable measure of a putative mediator of insulin action, it would seem important that incubations are first carried out under conditions where the mitochondria remain intact and preferably fully coupled; PDH_a should then be assayed after complete extraction from the mitochondria, for example with Triton X-100 under conditions where the interconversion of phosphorylated and non-phosphorylated forms of PDH is inhibited by addition of EDTA.

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