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Insulin regulation of the activity and phosphorylation of branched-chain 2-oxo acid dehydrogenase in adipose tissue

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The activity of the intramitochondrial branched-chain 2-oxo acid dehydrogenase (BCDH), like that of pyruvate dehydrogenase, is regulated, at least in part, by interconversion between the active dephosphorylated enzyme and its inactive phosphorylated form. The stimulatory effect of insulin on BCDH activity was compared with its effect on phosphorylation of the enzyme. Intact tissues were incubated in the presence or the absence of insulin, and then mitochondria were isolated and disrupted before assaying for enzyme activity or estimating the extent of enzyme phosphorylation. Tissues were incubated in either the presence or the absence of leucine, which also stimulated BCDH activity up to 10-fold. Insulin (1 munit/ml) doubled the activity of BCDH in the absence and in the presence of leucine. Together, 1 mM-leucine and insulin appeared to stimulate BCDH activity fully. Phosphorylation of BCDH was estimated indirectly by measuring the incorporation of ^{32}P into phosphorylation sites that remained unesterified after preparing mitochondrial extracts under conditions that preserved the effect of insulin on BCDH activity. Increased incorporation of ^{32}P in these experiments implies decreased phosphorylation *in situ* when tissues were incubated with insulin and leucine. In the absence of leucine, little incorporation of ^{32}P into BCDH was detected. In the presence of leucine, however, incorporation of ^{32}P into BCDH was markedly increased, and insulin increased ^{32}P incorporation still further. The results support the hypothesis that leucine and insulin both stimulate the activity of BCDH by promoting its dephosphorylation.

INTRODUCTION

In rat adipose tissue, insulin stimulates two intramitochondrial enzyme complexes, PDH [1–5] and BCDH [6]. These complexes oxidize 2-oxo acids derived from glucose and the branched-chain amino acids, respectively, to form lipogenic precursors. Both PDH and BCDH are regulated by product inhibition and also by interconversion between their inactive phosphorylated forms and their active dephosphorylated forms [7,8]. Insulin stimulates the activity of PDH by promoting its dephosphorylation [9,10], but an effect of insulin on the phosphorylation of BCDH has not been reported. The activity of BCDH in extracts of insulin-treated tissues was twice that of controls [6], suggesting that insulin might also promote its dephosphorylation.

It is not known how the binding of insulin to its receptor on the cell surface stimulates these enzymes. It has been suggested that insulin stimulates PDH indirectly by means of a second messenger that increases the activity of PDH phosphatase [11,12], but comparable data for BCDH are not available. Two intramitochondrial phosphatases that dephosphorylate PDH [13,14] and a third phosphatase that dephosphorylates BCDH [15] have been purified from kidney mitochondria. However, insulin does not appear to stimulate the activity of PDH or BCDH in kidney mitochondria (H. M. Goodman & G. P. Frick, unpublished work), and it is not known whether any of these phosphatases might account for the effect of insulin on PDH or BCDH in adipose-tissue mitochondria.

The regulation of protein kinase and phosphatase activity can be assessed either by incubating tissues in buffer containing $[^{32}P]P_i$ and then isolating and analysing the ^{32}P-labelled phosphoproteins [16], or by isolating phosphoproteins that have not been radioactively labelled and determining which phosphorylation sites contain unlabelled phosphate [17]. We have used an approach similar to the 'back titration' method of Sale & Randle [17] to determine whether insulin promotes dephosphorylation of both PDH and BCDH in adipose tissue by isolating unlabelled phosphoproteins and incorporating ^{32}P into those sites that were not already esterified by unlabelled phosphate. Mitochondrial proteins were prepared from control and insulin-treated tissues and incubated with $[\gamma\text{-}^{32}P]ATP$ under conditions that favoured phosphorylation and inactivation of PDH and BCDH by endogenous kinases. The availability of unoccupied phosphorylation sites was estimated from the amount of ^{32}P that was incorporated into the ^{32}P-labelled enzymes. The results indicate that insulin promotes dephosphorylation of both PDH and BCDH. Studies of BCDH, which appears to be regulated by protein kinases and phosphatases that differ from the corresponding enzymes that regulate PDH, may help us to understand how insulin acts on these intramitochondrial enzymes.

METHODS

Male rats weighing 115–130 g were obtained from Charles River Breeding Laboratories (Wilmington, MA, U.S.A.) and were maintained on Purina 5008 chow (Ralston Purina, Richmond, IN, U.S.A.) for approx. 1 week before use. Rats were killed by cervical dislocation,

Abbreviations used: BCDH, branched-chain 2-oxo acid dehydrogenase (EC 1.2.4.4); PDH, pyruvate dehydrogenase (EC 1.2.4.1).

and the epididymal fat-pads were immediately excised and incubated in Krebs–Ringer bicarbonate buffer (0.12 M-NaCl, 4.7 mM-KCl, 3.6 mM-$CaCl_2$, 1.2 mM-KH_2PO_4, 1.2 mM-$MgCl_2$, 25 mM-$NaHCO_3$) that contained 10 mM-fructose and 1% bovine serum albumin (Fraction V; Reheis Chemical Co., Phoenix, AZ, U.S.A.). Pooled tissues were incubated in flasks sealed with serum stoppers and equilibrated with O_2/CO_2 (19:1). After incubation for 30 min at 37 °C, some tissues were transferred to flasks containing fresh buffer plus 1 munit of insulin/ml, and paired tissues were transferred to buffer lacking insulin for a second 30 min incubation.

Mitochondria

Mitochondria were isolated essentially as described by Belsham *et al.* [18]. The effects of leucine and insulin on the activity and phosphorylation of BCDH were preserved by chelation of bivalent cations and rapid isolation of mitochondria at 0–4 °C. When adipose-tissue mitochondria were isolated in the presence of 100 mM-NaF and 5 mM-4-methyl-2-oxopentanoate to inhibit phosphatases and BCDH kinase, respectively, the activity of BCDH was similar to that of mitochondria isolated in the absence of the inhibitors. Therefore the inhibitors did not appear to be needed to prevent activation or inactivation of BCDH during the isolation procedure, although they do appear to be needed when assaying BCDH in extracts of liver, kidney and muscle [19]. In the absence of inhibitors, both the inactive (less than 10% of maximal activity) and the active (more than 90% of maximal activity) forms of BCDH were detected after isolation of adipose tissue mitochondria without the inhibitors. Similarly, Denton *et al.* [20,21] did not find it necessary to add inhibitors in order to preserve the effect of insulin on PDH activity in adipose-tissue mitochondria.

Approx. 3 g of adipose tissue was homogenized in 30 ml of 10 mM-Hepes, pH 7.4, containing 300 mM-sucrose, 1 mM-EGTA, 1% bovine serum albumin (dialysed against 50 mM Tris buffer, pH 7.4, and then distilled water) and 1 mM-dithiothreitol. In some experiments a mixture of proteinase inhibitors (0.1 mM-phenylmethanesulphonyl fluoride, 10 μM-tosyllysylchloromethane, 10 μM-tosyl-phenylalanylchloromethane, 20 μg of leupeptin/ml and 20 μg of pepstatin/ml; all final concentrations) was added to all of the buffers in which mitochondria were isolated or incubated. Tissues were homogenized at 23 °C with an Ultra-Turrax homogenizer (Tekmar, Cincinatti, OH, U.S.A.) at approx. 50% full speed for 30 s. The homogenates were immediately chilled on ice, and subsequent steps were carried out at 0–4 °C. The homogenates were centrifuged at 1000 ***g*** for 5 min, the pellets and fat were discarded, and the aqueous portions were centrifuged at 10000 ***g*** for 10 min. The mitochondrial pellets were resuspended in 2 ml of buffer containing a 1:10 dilution of Percoll (Pharmacia, Piscataway, NJ, U.S.A.) and centrifuged for 3 min at 15000 ***g*** in 1.5 ml plastic centrifuge tubes. The supernatants were discarded and the mitochondria were resuspended in 1 ml of buffer (10 mM-potassium phosphate, pH 7.4, 150 mM-KCl, 0.1 mM-EGTA and 1 mM-dithiothreitol) and resedimented at 15000 ***g*** for 1 min. Before assay of BCDH activity or activation or inactivation of the enzymes, mitochondria were disrupted osmotically by resuspending them in a hypo-osmotic buffer (10 mM-potassium phosphate buffer plus 1 mM-dithiothreitol) at approx. 0.5 mg of protein/ml.

Both BCDH and PDH were activated by incubating the disrupted mitochondria for 20 min at 37 °C with 10 mM-$MgCl_2$. The particulate fraction was recovered by centrifugation at 15000 ***g*** for 1 min, resuspended in the hypo-osmotic buffer, and assayed for enzyme activity. No PDH or BCDH activity was detected in the supernatant after activating and sedimenting the enzymes. To estimate the extent of activation, specific activities were calculated from the initial protein concentration rather than the protein actually recovered by sedimenting the particulate fraction. Both enzymes were inactivated by incubating the disrupted mitochondria for 5 min at 37 °C with 0.3 mM-ATP plus 5 μM-oligomycin, and the particulate fraction was recovered by centrifugation and resuspended in the hypo-osmotic buffer as described above before being assayed for PDH or BCDH activity.

Assays for BCDH and PDH activity

Samples (100 μl) containing 50–100 μg of protein were assayed for BCDH activity as previously described [6]. The reaction mixture (final volume 150 μl) contained 10 mM-potassium phosphate buffer, pH 7.4, 3 mM-$MgCl_2$, 1 mM-EDTA, 1 mM-dithiothreitol, 1 mM-NAD^+, 0.1 mM-CoA, 0.1 mM-thiamin pyrophosphate, 0.1% Triton X-100 and 0.2 mM-4-methyl-2-oxo[1-^{14}C]-pentanoate (sp. radioactivity 3.5 μCi/μmol). The radioactive substrate was prepared from [1-^{14}C]leucine (Amersham, Arlington Heights, IL, U.S.A.) as previously described [6]. The reactions were terminated after 5 min by addition of 100 μl of 0.3 M-sodium citrate adjusted to pH 3.0 with H_3PO_4. The reaction vial was placed in a 40 ml plastic tube, which also held a 7 ml scintillation vial containing several drops of phenethylamine (Eastman Kodak, Rochester, NY, U.S.A.). The 40 ml tube was capped and incubated for 30 min at 37 °C to trap $^{14}CO_2$, which was measured by liquid-scintillation spectrometry. The activity of PDH was determined by the same procedure, except that 5–10 μg of protein was assayed, a solution containing [1-^{14}C]pyruvate (Amersham) plus unlabelled pyruvate (0.2 mM; 3.5 μCi/μmol) was substituted for 4-methyl-2-oxo[1-^{14}C]-pentanoate, and the reaction was terminated after 2 min.

Incorporation of ^{32}P into mitochondrial proteins

Sites that remained unphosphorylated in the isolated mitochondria were phosphorylated with [γ-^{32}P]ATP by a procedure similar to that used by Sale & Randle [17] to study PDH. Mitochondrial proteins (50–100 μg) were phosphorylated in 100 μl of a solution that contained 5 μM-oligomycin, [γ-^{32}P]ATP (Amersham) and unlabelled ATP (0.3 mM, 2 Ci/mmol). After incubation at 37 °C for 5 min, the mitochondrial proteins were denatured by addition of 100 μl of 1 M-trichloroacetic acid, sedimented at 10000 ***g*** for 1 min, rinsed with 150 μl of 95% (v/v) ethanol, and redissolved in 150 μl of 60 mM-Tris/HCl buffer, pH 6.8, containing 2% SDS, 5% mercaptoethanol, 10% glycerol and 0.001% Bromophenol Blue. The amount of protein in samples dissolved in SDS was determined by the dye-binding assay described by Schaffner & Weisman [22]. Proteins were separated by polyacrylamide-gel electrophoresis on 10%-acrylamide slab gels. The gels were fixed, stained, and dried before exposure to X-ray film (Kodak AR-5) to

detect ^{32}P-labelled proteins. The ^{32}P-labelled bands located by autoradiography were excised, and the amount of ^{32}P present in each band was determined by liquid-scintillation spectrometry. Molecular masses of ^{32}P-labelled proteins were estimated by comparing their mobilities with those of standards obtained from Sigma (St. Louis, MO, U.S.A.).

A ratio proportional to the specific radioactivity of ^{32}P-labelled BCDH was calculated from the amount of ^{32}P recovered in the 50000-Da band and the amount of protein in the sample that was applied to the gel. After activation of BCDH by $MgCl_2$ this ratio was greatly increased. Since only 69±3% (mean±S.E.M., seven experiments) of the protein was recovered when the $MgCl_2$-activated enzyme was sedimented and resuspended in fresh buffer, the increased ratio reflects both enrichment of insoluble mitochondrial proteins for BCDH and an increase in the specific radioactivity of the ^{32}P-labelled enzyme. When BCDH was activated by $MgCl_2$, the ratio was corrected for enrichment of the enzyme and then used to calculate the percentage of maximum ^{32}P incorporation. The effect of insulin on ^{32}P incorporation into BCDH was determined by comparing the percentage of maximum ^{32}P incorporation in mitochondria from control and insulin-treated tissues.

To measure the specific radioactivity of [γ-^{32}P]ATP, samples were analysed by h.p.l.c. using a C_{18} reversed-phase column as described by Taylor *et al.* [23]. The amount of ^{32}P eluted with the ATP peak was determined by liquid-scintillation spectrometry, and the amount of ATP was determined by comparing its peak height with that of a freshly prepared ATP standard (50–500 pmol). Peak heights were a linear function of the amount of ATP.

RESULTS AND DISCUSSION

To measure the effects of leucine and insulin on the activity of BCDH and on its state of phosphorylation, paired tissue segments were incubated in the absence or presence of leucine, insulin or both, and then homogenized in an iso-osmotic buffer. Mitochondria were isolated from the homogenates by centrifugation and resuspended in a hypo-osmotic buffer to disrupt them before assay for enzyme activity. As reported previously [6], leucine greatly increased the activity of BCDH, and the activation persisted throughout the procedure to isolate and disrupt mitochondria, as shown in Table 1. When the mitochondrial preparations were incubated in phosphate buffer containing 10 mM-$MgCl_2$, BCDH activity increased still further. Stimulation by leucine was concentration-dependent, and the activity of the leucine-stimulated enzyme was always less than or equal to that of the $MgCl_2$-activated enzyme. When tissues were incubated in 10 mM-leucine the activity of BCDH appeared to approach its maximum value, as determined by activation with $MgCl_2$. Since the enzyme appeared to be maximally stimulated when tissues were incubated in 10 mM-leucine, one would not expect further stimulation of leucine oxidation by insulin if the stimulatory effect of insulin were due to activation of BCDH. However, the rate at which tissue segments oxidized 10 mM-leucine was increased by more than 30% when insulin was added [24]. It is possible that the activity of BCDH may not be the only factor that limits the relatively high rate at which intact tissues oxidize high concentrations of the branched-chain amino acids. Other factors, such as the availability of amino-group acceptors, transport across the mitochondrial membrane, or product inhibition of BCDH activity, might influence the rate at which tissues oxidize 10 mM-leucine and account for some of the stimulatory effect of insulin.

Table 1. Effect of leucine on BCDH activity

Tissues were incubated in the absence or presence of leucine for 30 min before the isolation and disruption of mitochondria. One sample of each mitochondrial preparation was incubated for 20 min at 37 °C in the presence of 10 mM-$MgCl_2$ to maximize BCDH activity. The insoluble proteins in both the activated and the untreated samples were sedimented at 15000 *g* for 1 min, resuspended in fresh buffer, and assayed for BCDH activity. Results are means±S.E.M. for three experiments. After incubation with Mg^{2+}, the activity of BCDH did not differ in extracts from tissues that had been incubated at different leucine concentrations. * Differed significantly from results obtained without added leucine ($P<0.05$).

Leucine concn. (mM)	BCDH (nmol/min per mg of protein): Activity (*a*)	BCDH (nmol/min per mg of protein): Maximum activity (*b*)	Activity (% of maximum) (100×*a*/*b*)
0	0.500±0.062	6.34±0.51	7.97±0.20
0.2	1.12±0.21	5.39±0.18	20.9±4.2
1.0	2.36±0.49*	5.74±0.37	41.3±8.6*
10	4.60±0.17*	5.56±0.44	83.4±4.4*

Table 2. Effect of insulin on the activity of BCDH in extracts of tissues incubated in the absence or presence of leucine

Tissue segments were incubated for 30 min in the absence or presence of leucine, 1 munit of insulin/ml, or both. Mitochondria were isolated, resuspended in hypo-osmotic phosphate buffer and assayed for BCDH activity. Maximum BCDH activity was determined as in Table 1. Results are means±S.E.M. for the numbers of experiments indicated in parentheses. The effect of insulin was calculated from the paired results of the number of experiments indicated in parentheses, and was significant at each leucine concentration: *$P<0.05$, **$P<0.005$.

Leucine concn. (mM)	BCDH activity (nmol/min per mg of protein): Control	BCDH activity (nmol/min per mg of protein): +Insulin	Increase by insulin (% of control)
0 (7)	0.235±0.057	0.382±0.056	81±30*
0.2 (6)	0.414±0.058	0.899±0.090	146±49*
1.0 (5)	2.58±0.34	4.73±0.47	94±27*
	Activity (% of maximum): Control	**Activity (% of maximum): +Insulin**	**Increase (% of maximum activity)**
0.2 (6)	6.6±1.0	14.5±1.7	6.16±1.03**
1.0 (5)	49.2±6.0	91.8±3.7	42.7±5.4**

When tissues were incubated with insulin, the activity of BCDH in mitochondrial extracts doubled, as shown in Table 2. The 2-fold increase was seen in both the absence and the presence of 0.2 mM- and 1 mM-leucine, even though leucine alone stimulated BCDH activity by as much as 10-fold. These effects on BCDH activity can account for the nearly 2-fold increase in leucine oxidation produced by insulin when adipose-tissue segments were incubated with concentrations of leucine as high as 1 mM [24]. When tissues were incubated with 1 mM-leucine and insulin together, the activity of BCDH was 92% of its maximum value and did not differ significantly from that of the $MgCl_2$-activated enzyme.

When crude tissue extracts were assayed for BCDH activity in previous studies [6], we found the stimulatory effect of insulin only when assays were conducted at substrate concentrations less than 50 μM, and suggested that insulin might lower the K_m of the enzyme rather than increase its $V_{max.}$, as would be expected if the effect of the hormone were to promote the conversion of the inactive into the active form of BCDH. In the present study, in which isolated mitochondria were used rather than crude tissue extracts, the stimulatory effect of insulin was apparent at a concentration of 200 μM (Table 2). It is not clear why the stimulatory effect of insulin was not detected at higher substrate concentrations in the earlier studies, but the present results obtained with isolated mitochondria are consistent with the idea that insulin promotes interconversion of the inactive into the active form of the enzyme. However, the statistically significant increase in enzyme activity that was seen when tissues were incubated *in vitro* without added leucine (Table 2, line 1) is of doubtful physiological significance, since the enzyme was almost entirely inactive even in the presence of insulin. The results in Tables 1 and 2 illustrate how insulin and leucine stimulate BCDH activity individually, and suggest that together they can account for substantial activation of the adipose-tissue enzyme under physiological conditions.

To inactivate BCDH, isolated mitochondria were disrupted osmotically and then incubated with 0.3 mM-ATP plus 5 μM-oligomycin to inhibit ATPase activity. At 5 min after addition of ATP, the activities of both PDH and BCDH were decreased to less than 3% of their maximum values (Table 3). Greater inactivation could not be achieved by higher concentrations of ATP or oligomycin, by longer or shorter incubations, or by addition of NaF to inhibit phosphatase activity. Reactivation of the enzymes occurred spontaneously when the incubations were continued for 30 min, presumably because the enzymes were dephosphorylated after hydrolysis of the added ATP (results not shown).

The inactivating effect of ATP, though extensive, was incomplete, and remaining BCDH activity in mitochondria isolated from insulin-treated tissues was twice that of controls (Table 3). Similarly, when mitochondria were isolated from tissues that were incubated with leucine, the BCDH activity that remained after treatment with ATP was higher than that of controls and, as seen with insulin, was proportional to the activity seen before the ATP treatment (Fig. 1). Multiple regression analysis of the BCDH activity that remained after maximal inactivation with ATP revealed no significant differences in the efficacy of the ATP treatment in mitochondria from control, insulin-treated or leucine-treated tissues. Although the degree of its phosphorylation may determine the activity of BCDH, the failure of ATP to decrease BCDH activity to a value that was independent of its prior activity suggests that leucine and insulin may produce persistent changes that regulate the extent to which BCDH is phosphorylated. The results might reflect covalent changes in either the kinase or phosphatase that regulates BCDH activity, or they might reflect the phosphorylation of a site on BCDH other than that which regulates its catalytic activity. Two phosphorylation sites have been identified on BCDH purified from kidney mitochondria [25], and regulation of enzyme activity appears to depend on phosphorylation of only one of them. The function of the second phosphorylation site is unknown. The two sites have also been identified in BCDH from adipocytes [16].

Table 3. Effect of insulin on BCDH and PDH activity after activation by Mg^{2+} or inactivation by ATP

Tissues were incubated in buffer that contained 0.2 mM-leucine, in either the presence or the absence of 1 munit of insulin/ml for 30 min. Mitochondria were isolated and disrupted before activating BCDH and PDH by incubation with $MgCl_2$ or inactivating them by incubation with ATP. Activated, inactivated and untreated samples were sedimented and resuspended in fresh buffer before assaying for enzyme activity. Results are means ± S.E.M. for the numbers of experiments in parentheses. Significance of insulin effects: [a] $P < 0.001$ by paired t test, [b] no significant difference, [c] $P < 0.005$ by paired t test.

	Activity (nmol/min per mg of protein)	
	Control	+Insulin
BCDH (18)		
Initial activity	0.761 ± 0.059	1.44 ± 0.15[a]
Maximum activity	7.31 ± 0.32	7.52 ± 0.34[b]
Activity after inactivation	0.119 ± 0.019	0.224 ± 0.063[a]
Decline in activity (% of initial activity)	84	84[b]
PDH (3)		
Initial activity	61.5 ± 1.1	74.8 ± 0.5[c]
Maximum activity	172 ± 5	160 ± 8[b]
Activity after inactivation	9.45 ± 3.30	6.73 ± 1.42[b]
Decline in activity (% of initial activity)	85	91[b]

Inactivation of PDH by ATP was similar to that of BCDH. In the three experiments shown in Table 3, we observed no residual effect of insulin on PDH activity after addition of ATP, but a small effect might have been detected with a larger number of observations or with tissues incubated under different conditions.

In parallel experiments we measured the incorporation of ^{32}P into BCDH and PDH under the same conditions as those used to inactivate the enzymes, except that ATP was made radioactive by the addition of [γ-^{32}P]ATP. Two proteins, with molecular masses of approx. 50 and 46 kDa, were heavily labelled (Fig. 2). The band corresponding to purified BCDH was not completely resolved from the much more heavily labelled adjacent band, which corresponds to PDH [10,26], unless phosphorylation was carried out in the presence of dichloroacetate, a strong inhibitor of PDH kinase [26,27].

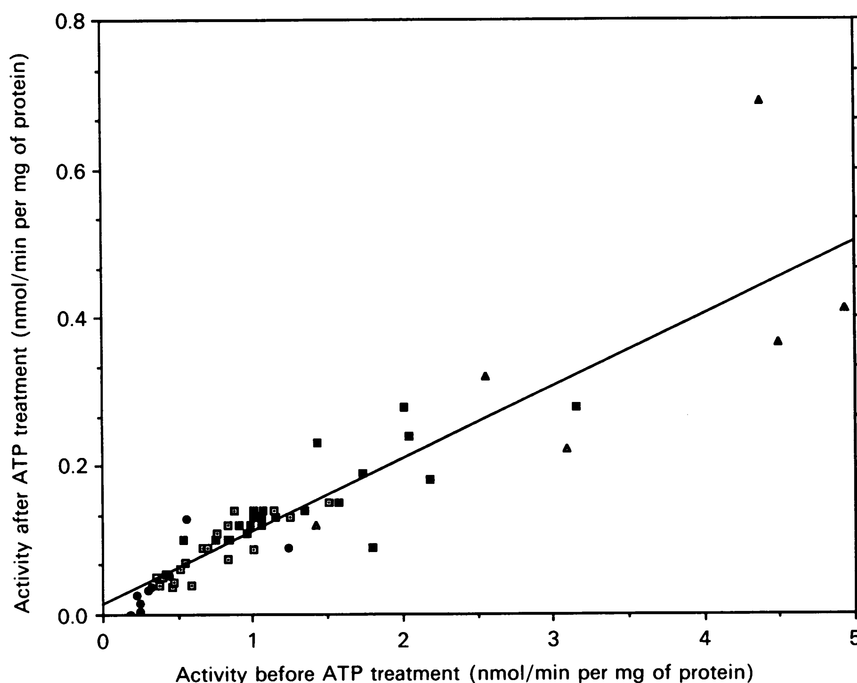

Fig. 1. Effect of ATP on BCDH activity

Mitochondria were isolated from tissues that were incubated with: ●, no additions; □, 0.2 mM-leucine; ■, 0.2 mM-leucine and 1 munit of insulin/ml; △, 1.0 mM-leucine; or ▲, 10 mM-leucine. Enzyme activity was assayed before and after treatment with 0.3 mM-ATP for 5 min. The linear regression has a slope of 0.0981 ± 0.0063 and a correlation coefficient of 0.903 (57 observations). The slope was similar to the mean value of the ratio:

(activity after ATP treatment)/(activity before ATP treatment)

which was 0.112 ± 0.005 (mean ± S.E.M.). No significant difference was seen when the efficacy of the ATP treatment was compared in mitochondria from control, insulin- or leucine-treated tissues. The effects of leucine and insulin on the activity that remained after the ATP treatment were also analysed by multiple regression, by using the following model:

$$y = a + b_1 x_1 + b_2 x_2 + b_3 x_3$$

where y = BCDH activity that remained after treatment with ATP, x_1 = BCDH activity before treatment with ATP, x_2 = leucine concentration and x_3 = insulin concentration. The coefficients estimated by analysing these results, and the probability that the coefficients differed from zero, were:

Coefficient	Estimate (mean ± S.E.M.)	*P*
b_1	0.0793 ± 0.0122	< 0.001
b_2	0.0102 ± 0.0057	Not significant
b_3	0.0197 ± 0.0167	Not significant
a	0.0233	–

We thank Dr. Robert Lew for his assistance with the statistical evaluation of these results.

In agreement with previous reports [26,28,29], dichloroacetate also inhibited BCDH kinase, but the effect was smaller; dichloroacetate decreased the incorporation of ^{32}P into BCDH by less than 2-fold, compared with a 4-fold decrease in ^{32}P incorporation into PDH. A small amount of ^{32}P was also found in proteins that had molecular masses less than 46 kDa. Since dichloroacetate also decreased ^{32}P incorporation into these proteins, it is possible that they might be proteolytic fragments of PDH or BCDH, even though proteinase inhibitors did not appear to diminish their abundance.

Incorporation of ^{32}P into BCDH was increased when extracts of leucine- and insulin-treated tissues were compared with controls, and was decreased in the presence of 4-methyl-2-oxopentanoate. These results are consistent with the hypothesis that activation of BCDH by either leucine or insulin corresponds to dephosphorylation of the enzyme, providing more unoccupied acceptor sites for ^{32}P. The virtual absence of ^{32}P incorporation in the presence of 4-methyl-2-oxopentanoate (Fig. 2) suggests that phosphorylation of these sites was catalysed by BCDH kinase, which is strongly inhibited by 4-methyl-2-oxopentanoate [26,28,29]. Our results are consistent with those obtained when incorporation of ^{32}P into BCDH was measured *in situ* after incubation of adipocytes in the presence of 4-methyl-2-oxopentanoate

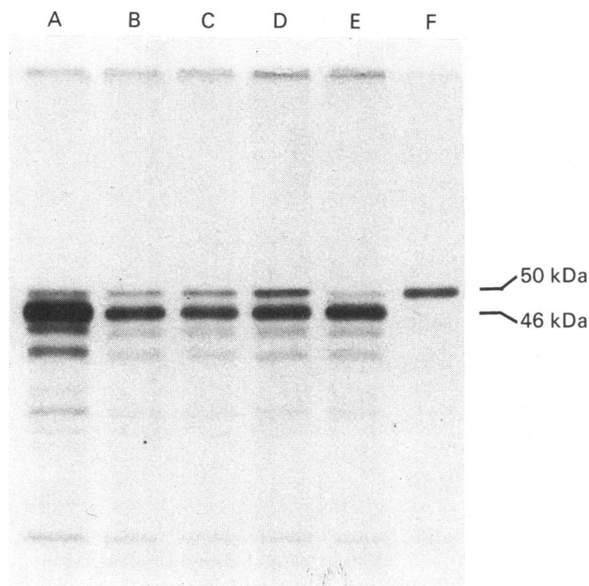

Fig. 2. Incorporation of ^{32}P into mitochondrial proteins

Mitochondria (A–E) and purified BCDH (F) were incubated in the presence of 0.3 mM-[γ-^{32}P]ATP (approx. 2 Ci/mmol) and 5 μM-oligomycin; 1 mM-dichloroacetate was also present in B, C, D and E. The mitochondria were isolated from tissues that were incubated without leucine (A, B), or with 1 mM-leucine (C–E) and 1 munit of insulin/ml (D, E). Phosphorylation of BCDH was inhibited by addition of 1 mM-4-methyl-2-oxopentanoate (E). Purified BCDH was kindly provided by Dr. S. J. Yeaman (Department of Biochemistry, University of Newcastle upon Tyne). Incubations were terminated by addition of trichloroacetic acid, and 47 μg of the radioactive protein in each sample was separated by SDS/polyacrylamide-gel electrophoresis and detected by autoradiography.

and [^{32}P]P_i. Under these conditions ^{32}P incorporation was also inhibited by 4-methyl-2-oxopentanoate [16].

In seven experiments similar to that shown in Fig. 2, insulin increased the incorporation of ^{32}P from 47 to 78% of that seen when BCDH was activated by incubation with $MgCl_2$ before addition of [γ-^{32}P]ATP (Table 4). Three estimates of the amount of ^{32}P that might contaminate ^{32}P-labelled BCDH averaged 29 ± 6% of the maximum ^{32}P incorporation into BCDH. These estimates were obtained by measuring incorporation of ^{32}P into the inactive form of BCDH obtained after treatment with unlabelled ATP or by isolating mitochondria from tissues that were incubated without added leucine or insulin, and by measuring ^{32}P incorporation in the presence of 4-methyl-2-oxopentanoate. Under these conditions, ^{32}P incorporation represents phosphorylation of the relatively inactive BCDH, background ^{32}P, and incorporation into proteins other than BCDH.

Incubation of tissues with insulin also increased the subsequent incorporation of ^{32}P into BCDH by 0.49 ± 0.14 pmol/mg of protein (mean ± S.E.M., 15 observations; $P < 0.005$) when mitochondria were isolated from tissues that were incubated with 0.2 mM-leucine. Although phosphorylation of BCDH was difficult to measure under these conditions, because ^{32}P incorporation was similar to background ^{32}P, the effect

Table 4. Incorporation of ^{32}P into the 50000 Da subunit of BCDH in mitochondrial extracts of tissues that were incubated in the presence or absence of insulin

Tissues were incubated for 30 min with 1 mM-leucine in the presence or absence of insulin, and mitochondria were isolated, disrupted, and incubated with [γ-^{32}P]ATP to incorporate ^{32}P into BCDH. The ^{32}P-labelled proteins were separated by SDS/polyacrylamide-gel electrophoresis, located by autoradiography, excised and counted for radioactivity to determine the amount of ^{32}P present. To estimate the maximum ^{32}P incorporation, mitochondria were incubated with $MgCl_2$ before incorporation of ^{32}P into BCDH. Phosphate incorporation into BCDH was calculated from the ^{32}P incorporation and the specific radioactivity of [γ-^{32}P]ATP. Results are means ± S.E.M. for seven observations: $*P < 0.05$.

Mitochondrial extract	Phosphate incorporation into BCDH (pmol/mg of protein)		
	Control	+Insulin	Insulin effect
Control (*a*)	7.84 ± 0.66	12.1 ± 1.6	4.26 ± 1.42*
Maximally activated (*b*)	18.6 ± 3.3	17.6 ± 2.8	−1.03 ± 2.16
Phosphate incorporation (% of maximum) (100 × *a*/*b*)	47.4 ± 5.7	78.0 ± 14.9	30.7 ± 10.1*

of insulin was significant and corresponded to stimulation of enzyme activity by approx. 6% of the maximum BCDH activity (Table 2). The correspondence between ^{32}P incorporation and the stimulation of BCDH activity was similar to that in the presence of 1 mM-leucine, since an increase of 4.26 pmol of phosphate/mg of protein corresponded to an increase in BCDH activity equal to 43% of its maximum activity (Tables 2 and 4). The incorporation of ^{32}P into BCDH from tissues that were incubated with 10 mM-leucine was 16 pmol/mg of protein (results not shown), which is similar to that when the enzyme was activated by $MgCl_2$ before the incorporation of ^{32}P (Table 4). Incubation of tissues with 10 mM-leucine and treatment of mitochondria with $MgCl_2$ appeared to make a similar (maximal) number of potential phosphorylation sites available for incorporation of ^{32}P and activated BCDH to a similar degree (Table 1). The incorporation of ^{32}P into PDH was also increased when mitochondria from insulin-treated tissues were compared with those of control tissues, even in the presence of dichloroacetate (Fig. 2).

When compared with the amount of BCDH activity, incorporation of ^{32}P into BCDH shown in Table 4 is similar to that seen when the purified enzyme was phosphorylated [25,28,30]. Approx. 18 pmol of phosphate/mg of protein was incorporated into proteins that had a maximum BCDH activity of approx. 6 nmol/min per mg of protein (Table 1). Therefore 3 pmol of phosphate was incorporated per munit of BCDH activity (1 munit equals 1 nmol of substrate oxidized to $^{14}CO_2$/min). For comparison, a maximum of 4.2 pmol of phosphate/munit of enzyme was incorporated into BCDH purified from rabbit liver [28], and the maximum phosphorylation of BCDH purified from bovine or rat

kidney was 2.2 or 0.7 pmol/munit respectively [25,30]. Although BCDH in adipose tissue appears to differ from the enzyme in other tissues because it is stimulated by insulin, the number of potential phosphorylation sites appears to be similar to that in other tissues.

Insulin and leucine both stimulate the oxidation of branched-chain amino acids by adipose-tissue segments, both stimulate the activity of BCDH, and both increase the availability of phosphorylation sites on the enzyme. The effect of leucine is likely to depend on its transamination and is similar to that of 4-methyl-2-oxopentanoate, which has been shown to inhibit BCDH kinase *in situ* [16]. Stimulation of BCDH activity by 10 mM-leucine was nearly complete and corresponded to complete dephosphorylation of the enzyme, as indicated by the maximal incorporation of ^{32}P into the enzyme from leucine-treated tissues. The effects of insulin and leucine were synergistic, i.e. together they stimulated BCDH activity much more than would be expected if their effects were additive. Insulin doubled BCDH activity over a wide range of leucine concentrations and promoted dephosphorylation of the enzyme by an amount that was proportional to the increase in the enzyme activity. Therefore insulin and leucine appear to activate BCDH by separate mechanisms. The effect of insulin could be due to direct inhibition of BCDH kinase or to some process that facilitates the inhibition of BCDH kinase by leucine. Alternatively, insulin might stimulate BCDH phosphatase. Since the stimulatory effects of leucine and insulin were still apparent after BCDH activity was decreased as much as possible by phosphorylating the enzyme, ligand-mediated inhibition of BCDH kinase cannot fully account for the regulatory effects of either leucine or insulin on BCDH activity.

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