Effects of protein phosphatase inhibitors on the regulation of insulin-sensitive enzymes within rat epididymal fat-pads and cells

Guy A. RUTTER,* Andrew C. BORTHWICK and Richard M. DENTON

Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, U.K.

1. The effects of the protein phosphatase inhibitors okadaic acid and microcystin LR on the regulation by insulin of pyruvate dehydrogenase and acetyl-CoA carboxylase have been studied in rat epididymal fat-pads and isolated cells. These inhibitors both completely blocked the phosphatase activity (against phosphorylase *a*) present in extracts of epididymal fat-pads, with half-maximal effects in the nanomolar range. 2. Okadaic acid treatment of pads and cells lowered the activity of acetyl-CoA carboxylase assayed in tissue extracts, both before and after treatment of the extracts with the activator, citrate. Further, okadaic acid treatment abolished the 2–3-fold difference in activity observed between extracts from control and insulin-treated tissues, assayed without prior treatment with citrate. 3. Incubation of pads with [³²P]P_i, sufficient to label the intracellular pool of ATP, demonstrated that okadaic acid increased the overall phosphorylation of acetyl-CoA carboxylase on a number of distinct sites, as judged by two-dimensional mapping of tryptic peptides. These included the 'I-peptide' [Brownsey & Denton (1982) *Biochem. J.* **202**, 77–86], the phosphorylation of which may be associated with the stimulation of the activity of the enzyme by insulin, as well as inhibitory phosphorylation sites. 4. Incubation with 1 μM-okadaic acid had no effect on the basal level of active pyruvate dehydrogenase apparent after tissue extraction, but abolished the 2–3-fold increase in this parameter which was elicited by insulin in the absence of okadaic acid. However, okadaic acid treatment did not affect the persistent increase in active pyruvate dehydrogenase levels which was apparent in mitochondria subsequently isolated from insulin-treated pads and re-incubated with an oxidizable substrate. It is concluded that the effects of okadaic acid are exerted through changes in metabolite concentrations rather than some direct action on the signalling pathway whereby insulin stimulates pyruvate dehydrogenase. 5. Microcystin LR did not mimic the effects of okadaic acid on intact cells and pads described above.

INTRODUCTION

Many of the effects of insulin on adipose-tissue metabolism result from a change in the extent of serine and threonine phosphorylation of key enzymes (Denton, 1986). Of particular importance is the net dephosphorylation, and consequent 2–3-fold activation, of the pyruvate dehydrogenase (PDH) complex, an enzyme located exclusively within the mitochondrial matrix. This effect, which persists in subsequently isolated and re-incubated mitochondria, appears to involve a change in the activity of PDH phosphatase, and to be independent of any changes in the levels of low-*M_r* regulators of PDH kinase and phosphatase (Denton *et al.*, 1984; Thomas *et al.*, 1986; Thomas & Denton, 1986; Denton *et al.*, 1989).

Treatment of epididymal fat-pads with insulin also increases 2–3-fold the activity of acetyl-CoA carboxylase (ACC) apparent in extracts isolated in the absence of citrate (Halestrap & Denton, 1973, 1974; Brownsey & Denton, 1987). This stimulation may result from increased phosphorylation of a serine residue(s) located within a particular tryptic peptide, termed the 'I-peptide' (Brownsey & Denton, 1982; Borthwick *et al.*, 1987, 1990). By contrast, hormones which increase cellular cyclic AMP appear to enhance phosphorylation of a group of distinct sites (Brownsey & Denton, 1982, 1987; Munday *et al.*, 1988; Haystead *et al.*, 1990a), leading to inhibition of the enzyme. Each of these changes in ACC activity may be linked to alterations in the proportion of the enzyme in a relatively inactive, dimeric, form (*M_r* 460 000), and a more active, polymerized, form [*M_r* (10–40) × 10⁶ (Borthwick *et al.*, 1987)], which is stabilized by citrate.

However, the sequence of events between the binding of insulin to the cell and these contrasting changes in the phosphorylation state and activity of target enzymes is poorly understood. One hypothesis is that insulin-receptor occupancy, which stimulates a tyrosine kinase activity intrinsic to the receptor, leads to the initiation of a cascade of kinase (and subsequently phosphatase) activations (Denton, 1986; Czech *et al.*, 1988; Weiel *et al.*, 1990). This hypothesis has received increasing experimental support recently with the identification of insulin-stimulated kinases (Denton, 1990; Borthwick *et al.*, 1990; Anderson *et al.*, 1990; Blackshear *et al.*, 1990; Haystead *et al.*, 1990b), including one which appears to phosphorylate and activate protein phosphatase 1 (Dent *et al.*, 1990).

We have endeavoured to investigate the importance of changes in the phosphorylation of putative intermediates in such a cascade by using two recently identified and potent phosphoprotein phosphatase inhibitors. These are okadaic acid (Bialojan & Takai, 1988; Cohen *et al.*, 1990), which is isolated from the marine sponge *Halochondria okadaei*, and microcystin LR (Mackintosh *et al.*, 1990), obtained from the cyanobacterium *Microcystis aeruginosa*. Previous studies with isolated hepatocytes and adipocytes (Haystead *et al.*, 1989) have indicated that okadaic acid dramatically increases the overall phosphorylation of a number of phosphoproteins *in vivo*, including ACC and ATP citrate-lyase (ACL) in adipocytes. Further, this compound stimulated lipolysis in these cells, consistent with the increased phosphorylation and activation of triacylglycerol lipase. Okadaic acid also stimulated glucose transport, suggesting a role for protein phosphorylation in the regulation of this process (Haystead *et al.*, 1989).

Abbreviations used: PDH, pyruvate dehydrogenase; PDH_a, proportion of the PDH complex in the active dephosphorylated form; ACC, acetyl-CoA carboxylase; ACL, ATP citrate-lyase; PP1 etc, protein phosphatase 1 etc.; I-I_{frag}, active fragment of inhibitor 1.

* To whom correspondence should be addressed.

Of the four major cytosolic protein phosphatases [PP1, PP2A, PP2B, PP2C (Cohen, 1989; Cohen & Cohen, 1989)], okadaic acid inhibits purified PP2A with an inhibition constant (K_i) below 0.1 nM, and PP1 with $K_i > 10$ nM, whereas the K_i value for PP2B is in the micromolar range, and PP2C is unaffected (Bialojan & Takai, 1988; Cohen *et al.*, 1990). Microcystin LR inhibits both PP2A and PP1 with $K_i < 0.01$ nM, and, like okadaic acid, is only a poor inhibitor of PP2B and is completely ineffective against PP2C (Mackintosh *et al.*, 1990). Significantly, okadaic acid has been shown to be without effect on isolated PDH phosphatase and does not affect the level of active PDH (PDH_a) in isolated mitochondria incubated with a respiratory substrate (Haystead *et al.*, 1989; G. A. Rutter, unpublished work). Hence, changes in the level of PDH_a observed in an intact tissue in response to okadaic acid might be expected to be related to changes in the activity of the putative 'phosphorylation cascade' activated by insulin. Evidence that okadaic acid can mimic some of the effects of insulin in the activation of specific kinases has been obtained recently (Haystead *et al.*, 1990b).

EXPERIMENTAL

Materials

Okadaic acid was from Fujisawa Chemical Co., Tokyo, Japan, or from Calbiochem Corp. [through Novabiochem (U.K.) Ltd., Nottingham, U.K.]. Microcystin LR was obtained from Calbiochem Corp., and collagenase from Worthington Diagnostic Systems (Freehold, NJ, U.S.A.). The active fragment of protein phosphatase inhibitor 1 (I-1_{frag}) was given by Dr. C. J. Proud (this Department). Other reagents were from the sources given previously (Rutter *et al.*, 1989; Borthwick *et al.*, 1987).

Methods

Isolation and incubation of fat-cells. These were obtained from epididymal fat-pads of 150–180 g male Wistar rats. Collagenase digestion was performed as described by Holman *et al.* (1981), except that the buffer was gassed thoroughly with O₂/CO₂ (19:1), and was supplemented with 5 mM-NaHCO₃. After washing, cells were maintained at 37 °C and 20–30 % cytocrit in buffer further modified to contain 1 % (w/v) dialysed BSA and 11 mM-glucose. Incubations were initiated by adding 0.5 ml of this cell suspension to 0.5 ml of fresh medium, plus additions as required. Incubations were terminated by removing the infranatant, and transferring the incubation vessel plus the cells into liquid N₂. The infranatant was routinely acidified with 1 % (v/v) HClO₄ and stored at –70 °C for assay of glycerol.

Incubation of fat-pads. These were obtained from 95–105 g rats. Pads were incubated individually at 37 °C in 1 ml of bicarbonate-buffered medium (Krebs & Henseleit, 1932), gassed with O₂/CO₂, for 15 min, and then transferred to 1 ml of fresh medium, plus the additions indicated in the Tables. At the end of the incubation period, pads were removed from the medium, lightly and rapidly blotted, and frozen in liquid N₂. Media were acidified with HClO₄ as described above for the assay of glycerol.

Pads were incubated with [³²P]P_i, for subsequent analysis of phosphopeptides, essentially as described by Brownsey & Denton (1982). In this case, the tissue was immediately disrupted at the end of the incubation with a Polytron PT-10 tissue homogenizer (setting 3, 2–3 s) into 250 mM-sucrose/20 mM-Mops/10 mM-Tris/5 mM-EGTA/7.5 mM-GSH, containing 2 mM-benzamidine and 1 µg each of pepstatin, antipain and leupeptin/ml and adjusted to pH 7.2 with KOH.

Isolation and incubation of mitochondria. Mitochondria were obtained from fat-pads, incubated as described above, by dif-

ferential centrifugation as described by Denton *et al.* (1984). These were then re-incubated in duplicate at 1–2 mg of mitochondrial protein/ml for 5 min at 30 °C in 20 mM-Mops/Tris (pH 7.3)/125 mM-KCl/5 mM-NaCl/5 mM-KH₂PO₄/5 mM-EGTA, containing 0.3 % BSA, 5 mM-2-oxoglutarate plus 1 mM-L-malate. At the end of this period, the mitochondria were sedimented (20 s, 10000 g) and frozen on liquid N₂.

Extraction of cells, pads and mitochondria, and assay of enzymes. Frozen cells and pads were disrupted at approx. 0.1 g of tissue/ml in 100 mM-KH₂PO₄/2 mM-EDTA/1 mM-dithiothreitol plus 50 µl of rat serum/ml by using a Polytron PT-10 tissue homogenizer (setting 8, 10 s) and finally re-frozen on solid CO₂. Mitochondrial pellets were extracted in 0.2 ml of phosphate/EDTA-based medium (above), by freezing and thawing three times (Denton *et al.*, 1984). All extracts were thawed and centrifuged (1 min, 10000 g) immediately before enzyme assays.

Initial PDH activity and total PDH activity (after treatment with pig heart PDH phosphate phosphatase) were determined as described by Denton *et al.* (1984), by using a linked spectrophotometric assay of acetyl-CoA production. ACC activity was assayed as described by Borthwick *et al.* (1987). One unit of enzyme activity is that which converts 1 µmol of substrate/min at 30 °C.

Other methods. Phosphoproteins were analysed by SDS/PAGE and radioautography (Brownsey *et al.*, 1984). Immunoprecipitation of ACC with antiserum raised in sheep, and mapping of tryptic peptides by two-dimensional t.l.c., were as described by Brownsey & Denton (1982).

Lipolysis in pads and cells was determined by measuring the release of glycerol into the medium. Glycerol present in the acidified incubation media (see above) was assayed as described by Garland & Randle (1962).

Phosphatase activity towards phosphorylated glycogen phosphorylase was determined in pad extracts by the method of Cohen *et al.* (1989).

Table 1. Effects of okadaic acid, microcystin and insulin on the phosphorylation of ACL and ACC in epididymal fat-pads preincubated in the presence of [³²P]P_i

Details of the incubation with [³²P]P_i are given in the Experimental section. Individual pads (from four animals, paired with or without insulin) were incubated for 15 min in the presence of the indicated additions, but in the absence of insulin. Insulin was then added as indicated, and the incubations were continued for a further 15 min, before extraction of the tissue and analysis of the phosphoproteins by SDS/PAGE on 6 % (w/v) acrylamide gels. Incorporation of ³²P was estimated by densitometric scanning of the Coomassie-Blue-stained gels and radioautographs. Data, which are representative of a further three separate experiments, indicate the percentage effect of each addition relative to control (no insulin), or relative to the presence of no addition except insulin (in parentheses).

Addition	Relative ³² P incorporation into:			
	ACC		ACL	
	Control	Insulin	Control	Insulin
None	100	130 (100)	100	220 (100)
1 µM-okadaic acid	250	278 (213)	328	310 (142)
1 µM-microcystin LR	125	156 (119)	134	245 (113)

Fig. 1. Effect of okadaic acid on the incorporation of $[^{32}\text{P}]\text{P}_i$ into tryptic phosphopeptides of ACC

Details of the incubation of fat-pads with $[^{32}\text{P}]\text{P}_i$, the isolation of ACC by immunoprecipitation, tryptic digestion and analysis of phosphopeptides by two-dimensional t.l.c. are given in the Experimental section. The key indicates the direction of electrophoresis (1) and chromatography (2), and the major tryptic phosphopeptides derived from ACC ('C', 'D', 'A' and 'I' peptides). After preincubation with $[^{32}\text{P}]\text{P}_i$ (90 min), pads were incubated for a further 30 min in the absence of further additions (a) or with $1\text{ }\mu\text{M}$ -okadaic acid (b). The maps (c) and (d) were obtained after incubation of pads as for (a) and (b) respectively, except that 83 nM -insulin was also present for the final 15 min of the incubation.

RESULTS

Effects of okadaic acid and microcystin LR on phosphatase activity in extracts and intact tissues

Microcystin LR inhibited all of the apparent phosphatase activity against phosphorylase *a* in extracts of white adipose tissue at a concentration of 10 nM . By contrast, 10 nM -okadaic acid inhibited the apparent activity by approx. 50%; 75% inhibition was achieved at 100 nM -okadaic acid, and 100% at 1000 nM -okadaic acid. In the absence of other additions, the presence of 20 nM - I-I_{frag} inhibited the apparent phosphatase activity in extracts by about 25%. Since this agent inhibits only PP1, this is consistent with a ratio of PP1:PP2A in extracts of rat

adipose tissue of about 1:3, close to earlier estimates of the ratio for rabbit adipose tissue (Ingebritsen *et al.*, 1983) and fat cells (Haystead *et al.*, 1990a). However, this ratio does depend on the substrate chosen (Haystead *et al.*, 1990a). In the additional presence of 200 nM - I-I_{frag} , 10 nM -okadaic acid inhibited the apparent phosphatase activity > 90%. These data are thus consistent with inhibition constants (K_i values) for microcystin LR against rat white-adipose-tissue PP2A and PP1, and for okadaic acid against PP2A, in the nanomolar range.

As observed by Haystead *et al.* (1989) with isolated cells, we observed that saturating concentrations of okadaic acid ($1\text{ }\mu\text{M}$) increased the overall phosphorylation of a number of phosphoproteins, including ACC (M_r 230000) and ACL (M_r 120000) in

Table 2. Effects of okadaic acid, microcystin LR, isoprenaline and insulin on lipolysis by isolated cells and intact fat-pads

Isolated cells or individual pads were incubated for 15 min in a total volume of 1 ml as described in the Experimental section in the presence of the additions shown, but in the absence of insulin. Insulin (10 nM or 83 nM, respectively) was then added as indicated, and the incubations were continued for a further 15 min. Pads from the same animal were paired with and without insulin. Data are the means ($\pm$ S.E.M.) of either three (cells) or four (pads) observations. Statistical significance was determined by unpaired Student's test: $\dagger\dagger P < 0.01$, $\dagger\dagger\dagger P < 0.001$ for the effects of additions relative to the appropriate (without or with insulin) control.

Preparation	Additions	Glycerol output (μ mol/30 min per g dry wt.)	
		Control	Insulin
Isolated cells	None	1.6 $\pm$ 0.04	1.6 $\pm$ 0.02
	1 μ M-okadaic acid	4.1 $\pm$ 0.35 $\dagger\dagger$	4.0 $\pm$ 0.24 $\dagger\dagger\dagger$
	2 μ M-microcystin LR	1.6 $\pm$ 0.13	1.8 $\pm$ 0.03 $\dagger\dagger$
	10 μ M-isoprenaline	7.8 $\pm$ 0.12 $\dagger\dagger\dagger$	7.5 $\pm$ 0.12 $\dagger\dagger\dagger$
Fat-pads	None	0.73 $\pm$ 0.01	0.67 $\pm$ 0.04
	1 μ M-okadaic acid	1.41 $\pm$ 0.16 $\dagger\dagger$	1.41 $\pm$ 0.2 $\dagger\dagger$

intact pads. These increases could be estimated from changes in the ratio of radioactivity to protein, after separation of the phosphoproteins by SDS/PAGE and scanning of Coomassie-Blue-stained gels and radioautographs. Results of a typical experiment are given in Table 1. Okadaic acid increased the apparent level of phosphorylation of both ACC and ACL to a much greater extent than microcystin LR did, particularly in the absence of insulin. As shown in Fig. 1, the increase in the phosphorylation of ACC elicited by okadaic acid was associated with an increase in the labelling of all the major phosphoproteins separated by two-dimensional t.l.c., including both 'A-' and 'D-peptides', the phosphorylation of which occurs in response to β -adrenergic agonists, and an 'I-peptide', which is phosphorylated in response to insulin (Brownsey & Denton, 1982, 1987).

The effects of okadaic acid and microcystin LR on the extent of phosphorylation of triacylglycerol lipase were estimated by measuring the stimulation of lipolysis induced by these agents

(Table 2). In both isolated cells and intact pads, 1 μ M-okadaic acid induced a 2–3-fold increase in lipolysis, consistent with an increase in the extent of phosphorylation of the lipase. This compares with the 5–10-fold increase induced by the β -adrenergic agonist isoprenaline. By contrast, the effects of microcystin LR on this parameter were barely detectable.

Effects of okadaic acid, microcystin, β -adrenergic agonists and insulin on the activity of ACC in isolated cells and intact pads

As shown in Table 3, incubation with 1 μ M-okadaic acid lowered significantly both the initial activity of ACC and the 'total' activity (apparent after incubation of the extracts with citrate). The most pronounced effect was that on the initial activity in pads or cells incubated in the presence of insulin. As a result, the increase in this parameter elicited by insulin in the absence of other additions was entirely abolished. By contrast, incubation of pads or cells with microcystin LR had no statistically significant effect on any of these parameters.

In isolated cells, isoprenaline also lowered the initial and total ACC activities, but the effects (1.5–2.0-fold) were considerably smaller than those of okadaic acid. Furthermore, the increase in initial activity in extracts from isolated cells incubated with insulin was also largely retained in the presence of isoprenaline.

The somewhat greater ACC activity observed in the intact pads in these studies compared with that in the isolated cells is probably largely due to the much smaller rats used in the latter case.

Effects of okadaic acid, microcystin LR and β -adrenergic agonists on the level of PDH_a in isolated fat-cells and intact tissue

Table 4 illustrates the effects of the protein phosphatase inhibitors and a β -adrenergic agonist on the level of PDH_a in pads and cells. With both preparations, okadaic acid blocked the increase in PDH_a elicited by insulin, but had no significant effect on the basal PDH_a level. Although isoprenaline also abolished the effect of insulin, treatment with this hormone was also associated with a significant increase in the basal level of PDH_a. Microcystin LR was entirely without effect on either basal or insulin-stimulated PDH_a levels. The total PDH activity was not affected significantly by any of these treatment (results not shown).

Table 3. Effects of okadaic acid, microcystin LR, isoprenaline and insulin on the activity of ACC in isolated cells and intact fat-pads

Details of the incubations are given in the legend to Table 2, and the assay of ACC is described in the Experimental section. Data are the means ($\pm$ S.E.M.) of the following numbers of separate incubations: 3 (cells), 8 (pads; control and insulin with no further addition) or 4 (pads; remaining conditions). Initial and total activities refer respectively to the values before or after a 20 min incubation of extracts at 30 °C with 20 mM-citrate. Statistical significance was determined by Student's *t* test: * $P < 0.05$, ** $P < 0.01$ for the effect of insulin; $\dagger P < 0.05$, $\dagger\dagger P < 0.01$, $\dagger\dagger\dagger P < 0.001$ for the effect of other additions.

Preparation	Addition	ACC activity (m-units/g of tissue)			
		Control		Insulin	
		Initial	Total	Initial	Total
Isolated cells	None	18.4 $\pm$ 3.6	95.3 $\pm$ 4.3	45.9 $\pm$ 3.4**	127.0 $\pm$ 11.9
	1 μ M-okadaic acid	5.6 $\pm$ 0.72 $\dagger$	27.7 $\pm$ 3.8 $\dagger\dagger$	5.0 $\pm$ 0.15 $\dagger\dagger\dagger$	26.4 $\pm$ 4.7 $\dagger\dagger\dagger$
	2 μ M-microcystin LR	13.4 $\pm$ 2.3	91.1 $\pm$ 2.9	45.4 $\pm$ 5.2**	96.5 $\pm$ 9.2
	10 μ M-isoprenaline	10.6 $\pm$ 0.14	65.7 $\pm$ 4.6 $\dagger$	18.6 $\pm$ 2.4 $\dagger$	71.9 $\pm$ 6.5 $\dagger$
Fat-pads	None	62.4 $\pm$ 8.3	267.0 $\pm$ 14.2	96.9 $\pm$ 13.0**	285.0 $\pm$ 21.9
	1 μ M-okadaic acid	12.6 $\pm$ 1.8 $\dagger\dagger$	91.0 $\pm$ 12.2 $\dagger\dagger$	12.3 $\pm$ 1.4 $\dagger$	86.5 $\pm$ 13.2 $\dagger\dagger$
	1 μ M-microcystin LR	46.1 $\pm$ 6.7	237.5 $\pm$ 24.0	75.1 $\pm$ 9.7*	258.1 $\pm$ 9.9

Table 4. Effects of okadaic acid, microcystin LR and isoprenaline on the increase elicited by insulin in PDH_a levels of isolated fat-cells and -pads, and in subsequently isolated and re-incubated mitochondria

Details of the incubations of cells and intact pads are given in the legend to Table 2 and in the Experimental section. Data are the means ($\pm$ S.E.M.) of three observations (cells) or six observations (pads). Data obtained for isolated mitochondria were from three separate mitochondrial preparations, each involving two sets of pads, paired without and with insulin. Details of the mitochondrial preparations and incubations are given in the Experimental section. Statistical significance was determined by Student's *t* test: **P* < 0.05, ****P* < 0.001 for the effect of insulin; †*P* < 0.05, ††*P* < 0.01, †††*P* < 0.001 for the effect of other additions relative to the appropriate (without or with insulin) control.

Preparation	Addition to intact tissue	PDH _a (% of total PDH)	
		Control	Insulin
Isolated cells	None	29.3 $\pm$ 1.5	71.6 $\pm$ 3.2***
	1 μ M-okadaic acid	27.2 $\pm$ 4.5	26.5 $\pm$ 1.8†††
	2 μ M-microcystin LR	31.5 $\pm$ 4.8	65.0 $\pm$ 7.0*
	10 μ M-isoprenaline	42.8 $\pm$ 4.4†	49.7 $\pm$ 3.7††
Fat-pads	None	30.5 $\pm$ 3.8	58.9 $\pm$ 2.2***
	1 μ M-okadaic acid	35.7 $\pm$ 1.8	42.8 $\pm$ 2.9†††
Isolated and re-incubated mitochondria	None	14.6 $\pm$ 1.2	22.9 $\pm$ 2.1*
	1 μ M-okadaic acid	12.3 $\pm$ 1.8	21.2 $\pm$ 1.4*

In order to investigate further the basis of the apparent blockage by okadaic acid of the effect of insulin on PDH_a levels, we sought to determine whether this agent blocked the persistent effect of insulin which is apparent in mitochondria subsequently isolated and re-incubated in the presence of a respiratory substrate (Denton *et al.*, 1984). As observed in our previous studies, PDH_a levels in mitochondria isolated from insulin-treated fat-pads were 1.5–2.0-fold above that in mitochondria from corresponding control pads (Table 4). Strikingly, however, PDH_a levels in mitochondria from control or insulin-treated pads were unaltered even when the pads had been incubated in the additional presence of 1 μ M-okadaic acid (Table 4), and the extent of the difference between PDH_a in mitochondria from control or insulin-treated tissue was identical.

DISCUSSION

The present studies confirm and extend the earlier studies by Haystead *et al.* (1989) on the effects of okadaic acid on the metabolism of rat epididymal fat-cells. It is evident that okadaic acid causes a complex array of intracellular changes compatible with the potent inhibitory effects of the compound on PP2A and, to a lesser extent, PP1; examples include the effects of okadaic acid on the phosphorylation of ACL and on the rate of lipolysis. Although microcystin LR is at least as potent an inhibitor of protein phosphatase activity in adipose-tissue extracts as is okadaic acid, microcystin LR was found to have much smaller effects on the metabolism of intact epididymal fat-pads or isolated cells. This was unexpected, as both inhibitors have marked cytotoxic effects on liver cells, and indeed marked increases in the activity of phosphorylase have been observed at concentrations as low as 0.1 μ M (Runnegar *et al.*, 1987; Haystead *et al.*, 1989). It seems that, although both okadaic acid and microcystin LR can gain entry to the cytosol of liver cells, the fat-cell plasma membrane is able to exclude microcystin LR, but not okadaic acid.

Insulin causes the parallel activation of PDH and ACC in

epididymal fat-cells or -pads through mechanisms which involve contrasting changes in the phosphorylation of the enzymes (see the Introduction for references). It is therefore of considerable interest that okadaic acid effectively blocks the action of insulin on both enzymes while having an insulin-like effect on glucose transport (Haystead *et al.*, 1989).

With ACC, okadaic acid causes substantial increases in the phosphorylation state of many different sites, including sites within the peptides 'A', 'D' and 'I'. Increased phosphorylation of peptides 'A' and 'D' is associated with the inhibition of enzyme activity apparent in cells or tissue incubated with β -adrenergic agonists, whereas that of the 'I' peptide is associated with activation by insulin (Brownsey & Denton, 1982, 1987). The overall effect of okadaic acid is a marked inhibition of ACC activity, which indicates, for the first time, that extensive phosphorylation of inhibitory sites is capable of over-riding the activatory effects of increased phosphorylation with the 'I' peptide.

In contrast with ACC, PDH activity was unaffected by okadaic acid in the absence of insulin. This is in agreement with the lack of inhibition of PDH phosphate phosphatase by okadaic acid. It also indicates that any changes in the levels of metabolites which might influence the activity of PDH kinase, such as pyruvate, acetyl-CoA, CoA, NAD⁺, NADH, ATP and ADP, are insufficient to cause an appreciable change in the activity of the kinase. It has recently been demonstrated that okadaic acid can mimic the activatory effects of insulin on a number of cytoplasmic serine protein kinases, including kinases acting on 'S6 peptide' (RRLSSLRA) and myelin basic protein (Haystead *et al.*, 1990b). The lack of effect of okadaic acid in the absence of insulin suggests that these kinases are probably not involved in the effect of insulin on PDH activity.

Okadaic acid causes the near-complete obliteration of the effects of insulin on the activity of PDH in both fat tissue and isolated cells. However, such an obliteration is not apparent if mitochondria from these tissues or cells are subsequently isolated and re-incubated with oxidizable substrates. It thus appears that the action of okadaic acid does not involve any direct interference with the signalling system through which insulin stimulates PDH phosphate phosphatase. Rather, it seems that okadaic acid causes some metabolic change, maybe involving PDH kinase regulators, which counters the stimulatory effects of insulin on the phosphatase and which is lost when mitochondria are subsequently isolated and incubated. The nature of this metabolic change, which appears not to occur (or to have no effect) in the absence of insulin, clearly deserves further investigation.

This work was supported by grants from the Medical Research Council (U.K.) and the British Diabetic Association. We are grateful to Ms. Avril Clark and Dr. G. Holman (University of Bath) for advice on the isolation of fat-cells, and Mr. N. J. Edgell and Dr. S. K. Moule (this Department) for assistance with the ACC assays. We are also grateful to Professor P. Cohen (University of Dundee) for suggesting that we explore the effects of okadaic acid on the activation of PDH by insulin, and for the gift of some okadaic acid.

REFERENCES

- Anderson, N. G., Maller, J. L., Tonks, N. K. & Sturgill, T. W. (1990) *Nature* (London) **343**, 651–653
- Bialojan, C. & Takai, A. (1988) *Biochem. J.* **256**, 283–290
- Blackshear, P. J., Haupt, D. M. & App. H. (1990) *J. Biol. Chem.* **265**, 11231–11234
- Borthwick, A. C., Edgell, N. J. & Denton, R. M. (1987) *Biochem. J.* **241**, 773–782
- Borthwick, A. C., Edgell, N. J. & Denton, R. M. (1990) *Biochem. J.* **271**, 795–801
- Brownsey, R. W. & Denton, R. M. (1982) *Biochem. J.* **202**, 77–86

- Brownsey, R. W. & Denton, R. M. (1987) *Enzymes*, 3rd Ed. **18**, part B, 123–146
- Brownsey, R. W., Edgell, N. J., Hopkirk, T. J. & Denton, R. M. (1984) *Biochem. J.* **218**, 733–743
- Cohen, P. (1989) *Annu. Rev. Biochem.* **58**, 453–508
- Cohen, P. & Cohen, P. T. W. (1989) *J. Biol. Chem.* **264**, 21435–21438
- Cohen, P., Klumpp, S. & Schelling, D. L. (1989) *FEBS Lett.* **250**, 596–600
- Cohen, P., Holmes, C. F. B. & Tsukitani, Y. (1990) *Trends. Biochem. Sci.* **15**, 98–102
- Czech, M. P., Klarlund, J. K., Yagaloff, K. A., Bradford, A. P. & Lewis, R. E. (1988) *J. Biol. Chem.* **263**, 11017–11020
- Dent, P., Lavoinne, A., Nakielnny, S., Caudwell, F. B., Watt, P. & Cohen, P. (1990) *Nature (London)* **348**, 302–308
- Denton, R. M. (1986) *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **20**, 293–342
- Denton, R. M. (1990) *Nature (London)* **348**, 286–287
- Denton, R. M., McCormack, J. G. & Marshall, S. E. (1984) *Biochem. J.* **217**, 441–452
- Denton, R. M., Midgley, P. J. W., Rutter, G. A., Thomas, A. P. & McCormack, J. G. (1989) *Alpha-Keto Acid Dehydrogenase Complexes: Organization, Regulation, and Biomedical Ramifications*; *Ann. N.Y. Acad. Sci.* **573**, 285–296
- Garland, P. B. & Randle, P. J. (1962) *Nature (London)* **196**, 987–988
- Halestrap, A. P. & Denton, R. M. (1973) *Biochem. J.* **132**, 509–517
- Halestrap, A. P. & Denton, R. M. (1974) *Biochem. J.* **142**, 365–377
- Haystead, T. A. J., Sim, A. T. R., Carling, D., Honnor, R. C., Tsukitani, Y., Cohen, P. & Hardie, D. G. (1989) *Nature (London)* **337**, 78–81
- Haystead, T. A. J., Moore, F., Cohen, P. & Hardie, D. G. (1990a) *Eur. J. Biochem.* **187**, 199–205
- Haystead, T. A. J., Weiel, J. E., Litchfield, D. W., Tsukitani, Y., Fischer, E. H. & Krebs, E. G. (1990b) *J. Biol. Chem.* **265**, 16571–16580
- Holman, G. D., Pierce, E. J. & Rees, W. D. (1981) *Biochim. Biophys. Acta* **646**, 382–388
- Ingebritsen, T. S., Stewart, A. A. & Cohen, P. (1983) *Eur. J. Biochem.* **132**, 297–309
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Mackintosh, C., Beattie, K. A., Klumpp, S., Cohen, P. & Codd, G. A. (1990) *FEBS Lett.* **264**, 187–192
- Munday, M. R., Campbell, D. G., Carling, D. & Hardie, D. G. (1988) *Eur. J. Biochem.* **175**, 331–338
- Runnegar, M. T. C., Andrews, J., Gerdes, R. G. & Falconer, I. R. (1987) *Toxication* **25**, 1235–1239
- Rutter, G. A., Midgley, P. J. W. & Denton, R. M. (1989) *Biochim. Biophys. Acta* **1014**, 263–270
- Thomas, A. P. & Denton, R. M. (1986) *Biochem. J.* **238**, 93–101
- Thomas, A. P., Diggle, T. A. & Denton, R. M. (1986) *Biochem. J.* **238**, 83–91
- Weiel, J. E., Ahn, N. G., Seger, R. & Krebs, E. G. (1990) *Adv. Second Messenger Phosphoprotein Res.* **24**, 182–195

Received 20 December 1990/18 February 1991, accepted 22 February 1991