Inactivation of Rat Adipocyte Pyruvate Dehydrogenase by Palmitate

PROTECTION AGAINST THIS EFFECT BY INSULIN IN THE PRESENCE OF GLUCOSE

By Suren R. SOORANNA and E. David SAGGERSON

Department of Biochemistry, University College London, Gower Street, London WC1E 6BT, U.K.

(Received 30 April 1979)

1. Adipocytes from rat epididymal fat-pads were incubated for 30 min with 5 mM-glucose and concentrations of lactate, pyruvate and amino acids typical of those found in rat plasma. 2. PDH_a (active form of pyruvate dehydrogenase) activity was significantly increased after incubation of the cells with insulin (200 μ -i.u./ml), and decreased by incubation with palmitate (0.5–2 mM). 3. In the presence of insulin, palmitate did not decrease PDH_a activity. 4. Dichloroacetate (1 mM) increased PDH_a activity in the absence of palmitate to the same extent as did insulin. In the presence of dichloroacetate but the absence of insulin, palmitate decreased PDH_a activity. In the presence of dichloroacetate and insulin, palmitate again did not decrease PDH_a activity. 5. It is concluded that, in the presence of glucose, insulin has a strong protective action against inactivation of adipocyte PDH_a by fatty acids.

PDH_a (EC 1.2.4.1) activity in liver, heart and kidney is decreased with increasing extracellular fatty acid concentrations (Wieland *et al.*, 1971, 1972; Guder *et al.*, 1974; Kerbey *et al.*, 1976). Oleate has been observed to have a similar effect on PDH_a activity in epididymal fat-pads incubated with fructose (Taylor *et al.*, 1973), and palmitate decreased PDH_a activity in rat adipocytes incubated with fructose or without a carbohydrate substrate (Sooranna & Saggerson, 1976). Activation and inactivation of the adipose-tissue PDH complex by various stimuli is thought to be important in the regulation of fatty acid synthesis from carbohydrate (Weiss *et al.*, 1974; Denton *et al.*, 1977). With rat adipocytes, palmitate has been found to decrease fatty acid synthesis from [¹⁴C]fructose or [¹⁴C]pyruvate and, in the absence of added substrates, from ³H₂O (Sooranna & Saggerson, 1975). On the other hand, fatty acid synthesis from [¹⁴C]glucose is not decreased on incubation with palmitate (Saggerson, 1972). It was therefore decided to examine the effect of palmitate on PDH_a activity in the presence of the more physiological substrate glucose. These studies were performed with cells incubated with or without insulin in order to investigate also the possibility that the actions of palmitate in inactivating and insulin in activating PDH might have some interplay with each other. Palmitate has previously been found to alter the effect of insulin on PDH in adipocytes incubated without substrates or with fructose (Sooranna & Saggerson, 1976).

Abbreviations used: PDH, pyruvate dehydrogenase; PDH_a and PDH_b, active and inactive forms respectively of pyruvate dehydrogenase.

Materials and Methods

Chemicals

Nucleotides, sodium pyruvate and, unless otherwise stated, enzymes were obtained from Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K. Amino acids were from BDH Chemicals, Poole, Dorset, U.K. Collagenase (type I from *Clostridium histolyticum*) was from P-L Biochemicals, Milwaukee, WI, U.S.A. Thiamin pyrophosphate was from Calbiochem, San Diego, CA, U.S.A. Bovine serum albumin (fraction V) was from Sigma (London) Chemical Co., London S.W.6, U.K., and was treated to remove fatty acids by the method of Chen (1967). Calf thymus DNA ('highly polymerized' type V) was also from Sigma. Sodium palmitate, from Nu Chek Prep., Elysian, MN, U.S.A., was associated with fatty acid-poor albumin as described by Evans & Mueller (1963). Dichloroacetic acid, from BDH, was neutralized with NaOH before addition to incubation media. Bovine insulin (six times recrystallized) was obtained from Boots Pure Drug Co., Nottingham, U.K.

Animals

Male Sprague–Dawley rats weighing 160–190 g were used. These were maintained on cube diet 41B (Bruce & Parkes, 1949) and water was supplied at all times. Pigeons were obtained from Havering Animal Suppliers, Romford, Essex, U.K.

Techniques with fat-cells

Preparation. Isolated fat-cells were prepared by the method of Rodbell (1964) as previously described

(Saggerson & Tomassi, 1971). Cells from six epididymal fat-pads were suspended in a final volume of 12 ml in Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) containing fatty acid-poor albumin (10 mg/ml).

Incubation. Portions (1 ml) of this stock cell suspension were incubated in 4 ml of Krebs–Ringer bicarbonate buffer previously gassed with O_2/CO_2 (19:1) in shaken 25 ml silicone-treated Erlenmeyer flasks as described by Saggerson & Tomassi (1971). Fatty acid-poor albumin was present during incubations.

When added to incubations, sodium pyruvate, sodium L-lactate and amino acids were present initially at concentrations similar to those in rat blood: pyruvate, 100 μ M (Goodwin & Williams, 1952); lactate, 1.0 mM (Ronzoni, 1950); aspartate, 13 μ M; threonine, 212 μ M; serine, 235 μ M; glutamine, 495 μ M; glutamate, 112 μ M; proline, 112 μ M; citrulline, 56 μ M; glycine, 400 μ M; alanine, 299 μ M; valine, 169 μ M; methionine, 39 μ M; isoleucine, 101 μ M; leucine, 150 μ M; tyrosine, 86 μ M; phenylalanine, 66 μ M; ornithine, 79 μ M; lysine, 328 μ M; histidine, 64 μ M; arginine, 75 μ M; tryptophan, 60 μ M; asparagine, 38 μ M; taurine, 194 μ M; cysteine, 305 μ M (Mowbray & Last, 1974).

After incubation, the contents of incubation flasks were transferred to silicone-treated homogenizer tubes, centrifuged for 20 s at approx. 200 $g_{av.}$; the resulting infranats were removed by aspiration and the tubes containing fat-cells plunged into liquid N_2 .

Preparation of tissue extracts. Extracts from frozen fat-cells were prepared by homogenization in 1.0 ml of ice-cold 0.25 M-sucrose medium containing 1 mM-EDTA, 1 mM-dithiothreitol and 10 mM-Tris/HCl buffer (pH 7.4) in an Ultra-Turrax homogenizer at 4°C for three 10 s periods over 1 min. The extracts were centrifuged for 20 s in an Eppendorf 5412 centrifuge (8000 $g_{av.}$), and the resulting fat-free homogenates used directly for enzyme assays.

Enzyme assays

Pyruvate dehydrogenase was assayed spectrophotometrically at 25°C by coupling it with pigeon liver arylamine acetyltransferase (EC 2.3.1.5) and following the absorbance change at 460 nm due to acetylation of *p*-(*p*-aminophenylazo)benzenesulphonic acid (Coore *et al.*, 1971). Glutamate dehydrogenase (EC 1.4.1.2) was assayed as described by Martin & Denton (1970).

Analysis of incubation media

Samples of media were deproteinized, and lactate and pyruvate assayed enzymically as described by Saggerson & Greenbaum (1970). Non-esterified fatty acids were assayed as described by Sooranna & Saggerson (1975).

Measurement of fat-cell DNA

This was as described by Saggerson (1972).

Expression of results

To correct for possible incomplete recoveries of cells after incubation or for incomplete breakage of mitochondria during homogenization, PDH_a activity was expressed as μ mol of acetyl-CoA formed/min per unit of glutamate dehydrogenase. Where appropriate, statistical differences between experimental values were *t*-tested on the basis of paired differences.

Results

Preliminary experiments established that insulin increased PDH_a activity approx. 2-fold in 60 min incubations of adipocytes with 5 mM-glucose alone (no addition of palmitate). The dose-dependence of this effect was investigated and the maximum effect was observed with 200 μ -i.u. of insulin/ml (1.5 nM). Half-maximal effect was observed with approx. 20 μ -i.u./ml (150 pM). The sensitivity of the cells to insulin was therefore about 5 times higher than the sensitivity of pieces of epididymal fat-pads incubated

Fig. 1. Dependence of PDH_a activity on fatty acid concentration

Cells were incubated for 30 min in Krebs–Ringer bicarbonate containing fatty acid-poor albumin (38 mg/ml) as indicated. Sodium lactate, sodium pyruvate and amino acids were added at the concentrations listed in the Materials and Methods section. The results are the means $\pm$ S.E.M. for five experiments with five cell preparations. The mean fat-cell DNA was 6.9 μ g/ml of incubation medium. The fatty acid measurements refer to the final concentrations in incubation media. Open symbols, without insulin; closed symbols, with insulin. Initial palmitate concentrations (mM): zero ($\bullet$, $\circ$); 0.5 ($\blacksquare$, $\square$); 1.0 ($\blacktriangle$, $\triangle$); 1.5 ($\bullet$, $\circ$); 2.0 ($\blacktriangledown$, $\triangledown$).

with glucose (Weiss *et al.*, 1974) and similar to that briefly reported for adipocytes by Weiss *et al.* (1974). Therefore 200 μ -i.u. of insulin/ml was chosen as a suitable concentration to use in subsequent experiments.

Fig. 1 demonstrates the effect of palmitate on PDH_a activity in the presence and absence of insulin. Since adipocytes incubated with glucose and insulin are capable of rapidly removing fatty acids from incubation media, it was considered that, as previously (Sooranna & Saggerson, 1976), PDH_a activities should be related to the observed final fatty acid concentrations rather than to the initial additions of palmitate. For this reason also, incubations were performed for only 30 min. To create as far as possible physiological conditions, these incubations contained L-lactate, pyruvate and amino acids (see the Materials and Methods section for concentrations). In the absence of insulin, PDH_a activity was decreased by all tested concentrations of palmitate.

However, in the presence of insulin, no decrease in PDH_a activity was seen with any of the tested palmitate additions. In fact, where 0.5 mM-palmitate was added initially, there was a small, but significant, increase in PDH_a activity ($P < 0.05$ on a paired difference basis). Insulin therefore appeared to protect PDH against inactivation by palmitate. The ability of insulin to promote fatty acid uptake was also very apparent.

When adipocytes are incubated with glucose and insulin there is a considerable increase in the accumulation of pyruvate in incubation media on addition of palmitate (Saggerson, 1972). Although it is extremely difficult to ascertain whether changes in incubation medium pyruvate concentrations in any way reflect changes in the mitochondrial content of this metabolite, it is conceivable that this protective effect of insulin could result from changes in pyruvate concentration.

These might mask effects of palmitate, since pyruvate has been found to inhibit PDH_a kinase in various tissues, including adipose tissue (Linn *et al.*, 1969; Siess *et al.*, 1971; Martin *et al.*, 1972). For this reason, further experiments were performed in which dichloroacetate was added to incubations. This substance also inhibits PDH_a kinase (Whitehouse *et al.*, 1974; Cooper *et al.*, 1974) and, in incubated epididymal fat-pads, is as effective as insulin in increasing PDH_a activity, neither agent producing any further effect in the presence of the other (Whitehouse *et al.*, 1974). We have confirmed this finding with adipocytes and find 0.1–10 mM-dichloroacetate to be effective in this respect (results not shown). It was presumed that changes in PDH_a kinase activity following pyruvate accumulation would be minimized in the presence of 1 mM-dichloroacetate.

Fig. 2 shows that, in the presence of dichloroacetate, PDH_a activity was again decreased by palmitate when

Fig. 2. Dependence of PDH_a activity on fatty acid concentration in the presence of dichloroacetate

Cells were incubated as described for Fig. 1, except that 1 mM-sodium dichloroacetate was present. The results are the means $\pm$ S.E.M. for five experiments with five cell preparations, and the mean fat-cell DNA was 6.9 μ g/ml of incubation medium. Where not shown, error bars lie within the symbols. The fatty acid measurements refer to the final concentrations in incubation media. Symbols as for Fig. 1.

insulin was absent, whereas, in the presence of insulin, inactivation of PDH on addition of fatty acid was not evident. This experiment suggests differences between the actions of insulin and dichloroacetate, i.e. insulin, but not dichloroacetate, protects PDH against inactivation by fatty acids. Since the site of action of dichloroacetate is probably inhibition of PDH_a kinase, this might imply that the site of action of insulin is elsewhere, e.g. perhaps at PDH_a phosphatase, as suggested by Hughes & Denton (1976).

Discussion

Two novel findings emerge from this study. First, provided that glucose is present, insulin has the most interesting property of protecting PDH_a against inactivation by physiological concentrations of long-chain fatty acids. This may be of considerable physiological importance. As a consequence of this the percentage stimulatory effect of insulin is significantly increased by addition of fatty acid. It would be of theoretical interest to know whether insulin is capable

of activating PDH in the complete absence of fatty acid. This state is of course not obtainable in practice, since cells incubated without palmitate release a small amount of non-esterified fatty acid. Second, the effects of insulin and dichloroacetate as activating agents are clearly separable because of the inability of dichloroacetate to protect PDH against inactivation in the presence of fatty acids. This finding is relevant to any studies involving the administration of dichloroacetate *in vivo*.

The ability of insulin to protect PDH against inactivation by fatty acids might be explained by the proposal that insulin alters the relationship between intracellular and extracellular concentrations of fatty acids or decreases the intracellular free concentrations of fatty acyl derivatives (Halestrap & Denton, 1974). When adipocytes are incubated with fructose, palmitate decreases PDH_a activity in both the presence and absence of insulin (Sooranna & Saggerson, 1976). In these latter experiments some activation of PDH by insulin that was distinct from its ability to alter extracellular fatty acid concentration was apparent, but it was much smaller than the effect seen here with glucose (and pyruvate, lactate and amino acids). In the presence of fructose therefore the protective action of insulin was barely apparent. At variance with this previous finding, Taylor *et al.* (1973) found that insulin protected PDH against inactivation by 1 mM-oleate in fat-pads incubated with fructose. However, in these latter experiments a range of oleate concentrations was not tested and final concentrations of fatty acid were not measured at the time of sampling the tissue PDH activity. It is quite possible that insulin promoted the removal of all or most of the oleate under the conditions of these experiments (100–300 mg of tissue from starved/carbohydrate-refed rats incubated in 2 ml for 30 min).

In conclusion, insulin totally protects PDH against inactivation by fatty acid when glucose is present. This may be contrasted with the inability of the hormone to do this in the absence of carbohydrate substrates (Sooranna & Saggerson, 1976) or the relatively small effect of insulin on PDH that is seen in the presence of fructose and palmitate (Sooranna & Saggerson, 1976). It is possible that these differences are related to the rates of flux of palmitate from incubation media into tissue acylglycerols, which differ considerably between these conditions of incubation.

We acknowledge the skilled technical assistance of Miss C. Carpenter, and financial support by the British Diabetic Association and the Medical Research Council.

References

- Bruce, H. M. & Parkes, A. S. (1949) *J. Hyg.* **47**, 202–206
 Chen, R. F. (1967) *J. Biol. Chem.* **242**, 173–181
 Cooper, R. H., Randle, P. J. & Denton, R. M. (1974) *Biochem. J.* **143**, 625–641
 Coore, H. G., Denton, R. M., Martin, B. R. & Randle, P. J. (1971) *Biochem. J.* **125**, 115–127
 Denton, R., Bridges, B., Brownsey, R., Evans, G., Hughes, W. & Stansbie, D. (1977) *Biochem. Soc. Trans.* **5**, 894–900
 Evans, H. H. & Mueller, P. S. (1963) *J. Lipid Res.* **4**, 39–45
 Goodwin, T. W. & Williams, G. P. (1952) *Biochem. J.* **51**, 708–714
 Guder, W. G., Wieland, O. H. & Stukowski, B. (1974) *Eur. J. Biochem.* **42**, 529–538
 Halestrap, A. P. & Denton, R. M. (1974) *Biochem. J.* **142**, 365–377
 Hughes, W. A. & Denton, R. M. (1976) *Nature (London)* **264**, 471–473
 Kerbey, A. L., Randle, P. J., Cooper, R. H., Whitehouse, S., Pask, H. T. & Denton, R. M. (1976) *Biochem. J.* **154**, 327–348
 Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
 Linn, T. C., Pettit, F. H., Hucho, F. & Reed, L. J. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **64**, 227–234
 Martin, B. R. & Denton, R. M. (1970) *Biochem. J.* **117**, 861–877
 Martin, B. R., Denton, R. M., Pask, H. T. & Randle, P. J. (1972) *Biochem. J.* **129**, 763–773
 Mowbray, J. & Last, K. S. (1974) *Biochim. Biophys. Acta* **349**, 114–122
 Rodbell, M. (1964) *J. Biol. Chem.* **239**, 375–380
 Ronzoni, E. (1950) *Am. J. Physiol.* **169**, 499–505
 Saggerson, E. D. (1972) *Biochem. J.* **128**, 1057–1067
 Saggerson, E. D. & Greenbaum, A. L. (1970) *Biochem. J.* **119**, 193–219
 Saggerson, E. D. & Tomassi, G. (1971) *Eur. J. Biochem.* **23**, 109–117
 Siess, E., Wittman, J. & Wieland, O. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 447–452
 Sooranna, S. R. & Saggerson, E. D. (1975) *Biochem. J.* **150**, 441–451
 Sooranna, S. R. & Saggerson, E. D. (1976) *FEBS Lett.* **69**, 144–148
 Taylor, S. I., Mukherjee, C. & Jungas, R. L. (1973) *J. Biol. Chem.* **248**, 73–81
 Weiss, L., Löffler, G., & Wieland, O. H. (1974) *Hoppe-Seyler's Z. Physiol. Chem.* **355**, 363–377
 Whitehouse, S., Cooper, R. H. & Randle, P. J. (1974) *Biochem. J.* **141**, 761–774
 Wieland, O., Siess, E., Schulze-Wethmar, F. H., von-Funcke, H. G. & Winton, B. (1971) *Arch. Biochem. Biophys.* **143**, 593–601
 Wieland, O. H., Patzelt, G. & Löffler, G. (1972) *Eur. J. Biochem.* **26**, 426–433