Inhibition of acetyl-CoA carboxylase activity in isolated rat adipocytes incubated with glucagon

Interactions with the effects of insulin, adrenaline and adenosine deaminase

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1. Adipocytes isolated from epididymal fat-pads of fed rats were incubated with different concentrations of glucagon, insulin, adrenaline and adenosine deaminase, and the effects of these agents on the 'initial' activity of acetyl-CoA carboxylase in the cells were studied. 2. Glucagon (at concentrations between 0.1 and 10 nM) inhibited acetyl-CoA carboxylase activity. Maximal inhibition was approx. 70% of the 'control' activity in the absence of added hormone, and the concentration of hormone required for half-maximal inhibition was 0.3–0.5 nM-glucagon. 3. Incubation of cells with adenosine deaminase resulted in a similar inhibition of acetyl-CoA carboxylase activity. Preincubation of adipocytes with adenosine deaminase did not alter either the sensitivity of carboxylase activity to increasing concentrations of glucagon or the maximal extent of inhibition. 4. Adrenaline inhibited acetyl-CoA carboxylase to the same extent as glucagon. Preincubation of the cells with glucagon did not alter the sensitivity of enzyme activity to adrenaline or the degree of maximal inhibition. 5. Insulin activated the enzyme by 70–80% of 'control' activity. Preincubation of the cells with glucagon did not alter the concentration of insulin required to produce half the maximal stimulatory effect (about 12 μ units of insulin/ml). The effects of insulin and glucagon appeared to be mediated completely independently, and were approximately quantitatively similar but opposite. These characteristics resulted in the mutual cancellation of the effects of the two hormones when they were both present at equally effective concentrations. 6. The implications of these findings with regard to current concepts about the mechanism of regulation of acetyl-CoA carboxylase and to the regulation of the enzyme *in vivo* are discussed.

The role of glucagon in the regulation of fatty acid metabolism in adipose tissue has been considered to be relatively minor (for review see Hales *et al.*, 1978). Although it has been known for a considerable time that the hormone has a lipolytic effect on adipose-tissue preparations *in vitro*, the concentrations of glucagon required to elicit the relatively modest enhancement of lipolytic rate were much higher than the range of circulatory glucagon concentrations *in vivo* (Vaughan, 1960; Hagen, 1961). More recent work has indicated that the sensitivity to glucagon of triacylglycerol hydrolysis in adipocytes *in vitro* was enhanced in cells isolated from starved rats, especially if the concentration of adenosine in the incubation medium was lowered by addition of adenosine deaminase (Honnor & Saggerson, 1980). However, these latter studies also suggested that the lipolytic rate in adipocytes

isolated from fed rats was much less sensitive to glucagon. Although these observations could provide an explanation for the increased triacylglycerol mobilization from adipose tissue in the starved state, they do not exclude the possibility that in the fed animal glucagon could be involved in the regulation of the rate of fatty acid synthesis, i.e. lipogenesis.

It is widely accepted that acetyl-CoA carboxylase is a key regulatory enzyme in the lipogenic pathway in adipose tissue *in vivo* and that its 'initial' activity (measured in the absence of citrate and without preincubation) varies proportionately with the rate of fatty acid synthesis in the tissue (see, e.g., Stansbie *et al.*, 1976).

The 'initial' activity of the enzyme is regulated by the degree of phosphorylation at two sites (or groups of sites): insulin and adrenaline increase the degree of phosphorylation of the enzyme on two separate

sites, but with opposing effects, namely activation by insulin and inhibition by adrenaline (see Denton *et al.*, 1981; Brownsey, 1981). However, the effects of glucagon on 'initial' activity of the enzyme in adipose tissue have not been investigated. In the liver glucagon inhibits acetyl-CoA carboxylase activity (Geelen *et al.*, 1978; Witters *et al.*, 1979), and by analogy it would be expected to affect lipogenesis in the same manner in adipose tissue.

In the present work the effects of physiological concentrations of glucagon on the 'initial' activity of acetyl-CoA carboxylase (i.e. activity measured in the absence of any preincubation or addition of citrate in the assay mixture) in adipocytes isolated from epididymal fat-pads from fed rats were studied. In addition, the interactions of the effects of glucagon and those of insulin, adrenaline and adenosine deaminase on the activity of the enzyme were also investigated. The quantitative relationships between the opposing effects of glucagon and insulin suggest that the activity of acetyl-CoA carboxylase in adipose tissue *in vivo* could be very sensitive to changes in the circulatory glucagon/insulin concentration ratio. In addition, the results support the suggestion that activity of the enzyme is modulated through effects on two independent regulatory sites (Denton *et al.*, 1981).

Materials and methods

Animals

The source and maintenance of animals were as given previously (Zammit, 1980, 1981). Fed male Wistar rats weighing 120–170 g were used throughout.

Preparation of adipocytes

Animals were stunned with a blow on the head and killed by cervical dislocation. The epididymal fat-pads from three animals were rinsed in Krebs–Henseleit bicarbonate-buffered saline, pooled and finely minced, and adipocytes were prepared from them as described by Rodbell (1964). The tissue was incubated in Krebs–Henseleit saline (containing 1.25 mM-CaCl₂), fatty acid-poor albumin (40 mg/ml), 5 mM-glucose and 3 mg of collagenase/ml. After 60 min incubation the cells were washed twice in the same basal medium (without collagenase) and finally resuspended in a total volume of 12.5 ml. Portions (1 ml) of cell suspension were dispensed into each of twelve 25 ml plastic conical flasks, and 4 ml of basal medium was then added to each flask. The flasks were gassed with O₂/CO₂ (19:1), stoppered and incubated in a shaking water bath (160 oscillations/min). Throughout the entire procedure the tissue and cells were maintained at 37°C. For each batch of adipocytes prepared six flasks were used as controls and the other six as experimental flasks, such that

paired data for each individual condition could be obtained for a given preparation of adipocytes.

Incubation of adipocytes

In experiments in which the effects of different concentrations of glucagon were studied, the cells were incubated for 10–20 min in basal medium without any additions. Thereafter additions of glucagon, freshly dissolved in Krebs–Henseleit saline, were made at timed intervals and incubations continued for a further 20 min. At the end of the incubation period the cells were separated from the medium by centrifugation and immediately frozen in liquid N₂. When more than one hormone (or enzyme) was added to the incubation medium, the cells were incubated for 20 min in the presence of one hormone, after which the second hormone was added and incubation was again allowed to proceed for a further 20 min before separation and freezing of the cells. Control flasks to which no hormonal additions were made were run concurrently. Preliminary experiments established that the effects of hormonal treatments were maximally effective after 10 min and persisted for at least 50 min.

Preparation of cell extracts and assays of enzyme activities

The frozen cells were homogenized in 1.5 ml of ice-cold medium containing 250 mM-sucrose, 20 mM-Tris, 20 mM-Mops (4-morpholinepropanesulphonic acid), 2 mM-EGTA and 2 mM-EDTA (pH 7.4) with a Polytron tissue disintegrator fitted with probe OD 10S (Kinematika, Basle, Switzerland). Albumin (0.4 ml of a 100 mg/ml solution) was added to the extracts, which were then centrifuged at 12 000 g for 60 s in an Eppendorf 5412 centrifuge. A sample (50 µl) of the infranatant below the fat layer was immediately assayed for 'initial' acetyl-CoA carboxylase activity. This was measured (in duplicate) at 30°C as described previously (McNeillie & Zammit, 1982) except that, unless otherwise indicated, citrate was absent from the assay medium. The rate of reaction was linear with time (up to 4 min) and extract concentration over the range used. As a routine, assays were terminated after 4 min by addition of 0.1 ml of 6 M-HCl. Acid-stable ¹⁴C radioactivity was measured as described previously (Halestrap & Denton, 1973).

NADP⁺-malate dehydrogenase was measured spectrophotometrically at 30°C as described by Martin & Denton (1970).

Materials

Chemicals were obtained from sources described previously (McNeillie *et al.*, 1981). Collagenase (type I), crystalline bovine insulin, adrenaline and adenosine deaminase were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Crystalline pig

glucagon was a gift from Lilly Research Laboratories, Indianapolis, IN, U.S.A.

Glucagon and adrenaline solutions were prepared immediately before use. Stock solutions of insulin were frozen at -20°C in small portions and used as required. Adenosine deaminase was dialysed against 0.9% NaCl to remove $(\text{NH}_4)_2\text{SO}_4$, and its activity was assayed at 25°C before use as described previously (Bergmeyer *et al.*, 1974). One unit of adenosine deaminase activity refers to the utilization of $1\text{ }\mu\text{mol}$ of adenosine/min at 25°C .

Expression of results

To facilitate interpretation of the data, the 'initial' activity of acetyl-CoA carboxylase in extracts of cells exposed to different experimental treatments is expressed as a percentage of the 'control' activity in cells obtained from the same adipocyte preparation and incubated concurrently in the absence of added hormones or adenosine deaminase. As a routine, the activity of NADP^+ -malate dehydrogenase was also assayed in the extracts, and the activities were used to normalize carboxylase activities in order to allow for variations in cell disruption during homogenization and for minor losses incurred during harvesting of the cells. Preliminary experiments confirmed that the various experimental treatments did not alter NADP^+ -malate dehydrogenase activity.

Results

Effects of glucagon

Incubation of adipocytes with glucagon resulted in inhibition of 'initial' acetyl-CoA carboxylase activity (Fig. 1). The maximal inhibition observed (about 70%) was obtained at concentrations of glucagon above 10 nM . The concentration required to exert half the maximal effect was $0.3\text{--}0.5\text{ nM}$ glucagon. Incubation with glucagon also resulted in a decrease in the 'total' acetyl-CoA carboxylase activity (i.e. assayed in the presence of 20 mM -citrate after preincubation of extracts with the same concentration of citrate; see McNeillie & Zammit, 1982) (results not shown). In absolute terms the decrease in 'total' activity was similar to that observed in 'initial' activity, such that the 'initial'/'total' activity ratio declined from about 20% to 6% with increasing glucagon concentration (results not shown) (cf. results on the effects of adrenaline obtained by Brownsey *et al.*, 1979).

Effects of adenosine deaminase

Incubation of adipocytes in the presence of adenosine deaminase also resulted in inhibition of 'initial' acetyl-CoA carboxylase activity (Fig. 2). The maximal inhibition obtained was similar to that achieved with maximally effective glucagon concentrations. Half-maximal inhibition by adenosine

Fig. 1. Effects of increasing concentrations of glucagon on acetyl-CoA carboxylase activity in adipocytes. Cells were incubated in the absence (●) or in the presence (▲) of 4 munits of adenosine deaminase/ml. For full experimental details see the text. The activity of acetyl-CoA carboxylase is expressed as a percentage of 'control' activity obtained from cells incubated concurrently in the absence of any additions (see the Materials and methods section). Values are means of three to eight determinations performed on separate adipocyte preparations. Vertical bars indicate 1 S.E.M. Control activity averaged 2.5 nmol/min per adipocyte sample and the average carboxylase/ NADP^+ -malate dehydrogenase activity ratio was 7.5×10^{-3} .

Fig. 2. Effect of adenosine deaminase on acetyl-CoA carboxylase activity in adipocytes. Experimental details were as described in the legend to Fig. 1. Values are means of seven to nine determinations. Vertical bars indicate 1 S.E.M.

deaminase was obtained in the presence of 3–4 munits of adenosine deaminase/ml of incubation medium.

Preincubation of adipocytes with adenosine deaminase (4 munits/ml) before addition of glucagon resulted in a greater degree of inhibition at any given sub-optimal concentration of glucagon used (Fig. 1). However, the extent of maximal inhibition was unaltered (and the same as that in the presence of either agent added individually). Moreover, inclusion of adenosine deaminase did not increase the sensitivity of adipocytes to glucagon, since the half-maximally effective concentration of hormone was unaltered (Fig. 1).

Interactions between the effects of glucagon and insulin

Incubation of adipocytes with insulin alone resulted in activation of 'initial' acetyl-CoA carboxylase activity (Fig. 3). The concentration of insulin required to elicit half-maximal activation was about 12 μ units of insulin/ml. Addition of glucagon to the incubation medium resulted in a reversal of this activation of acetyl-CoA carboxylase activity at all concentrations of insulin tested. Two concentrations of glucagon were used, 0.5 nM, which gave approximately half-maximal inhibition when glucagon was used alone (see Fig. 1), and 10 nM, which was maximally inhibitory: in both instances glucagon exerted approximately half-maximal and maximal inhibitory effects respectively, irrespective of insulin concentration or state of activation of the enzyme. Consequently the sensitivity of enzyme activity to insulin was unaltered in the presence of glucagon. When either maximally effective concentrations of both insulin and glucagon or concentrations of both hormones that individually gave half-maximal effects were present simultaneously, the effects of the two hormones balanced each other exactly and the activity of acetyl-CoA carboxylase reverted to 'control' values (i.e. 100%; Fig. 3). The same effect was obtained in other experiments in which maximally effective concentrations of the two hormones were added simultaneously (results not shown).

Interaction between the effects of glucagon and adrenaline

The dependence of the inhibition of 'initial' acetyl-CoA carboxylase activity on adrenaline concentration is given in Fig. 4. Half-maximal inhibition was obtained with 10–30 nM-adrenaline. The maximal extent of inhibition with adrenaline was not significantly different from that with glucagon. Preincubation of adipocytes with glucagon resulted in increased inhibition of acetyl-CoA carboxylase at any given sub-optimal concentration of adrenaline used. However, there were no changes either in the maximal extent of inhibition of acetyl-CoA carb-

Fig. 3. *Activation of acetyl-CoA carboxylase in adipocytes incubated with insulin in the presence or in the absence of glucagon*

Cells were preincubated for 20 min in the absence (●) or in the presence of either 0.5 nM-glucagon (■) or 10 nM-glucagon (▲). The concentrations of insulin indicated were then added and incubation was continued for a further 20 min. For full experimental details see the text. Values are means of three to seven determinations. Vertical bars indicate 1 S.E.M.

Fig. 4. *Inhibition of acetyl-CoA carboxylase in adipocytes incubated with adrenaline after preincubation in the presence (■) or in the absence (●) of glucagon (0.5 nM)*

For full experimental details see the text. Values are means of three to seven determinations. Vertical bars indicate 1 S.E.M.

oxylase activity or in the concentration required to exert half the adrenaline-dependent component of the inhibition.

Discussion

The most significant observation of the present study was that 'initial' acetyl-CoA carboxylase activity was inhibited by glucagon. This observation raises the possibility that the rate of lipogenesis in a peripheral tissue (white adipose tissue) could be modulated by glucagon *in vivo*. The concentrations of glucagon required to elicit this inhibitory effect on adipocytes *in vitro* were slightly higher than the range of circulatory glucagon concentrations in rat plasma (100–300 pg/ml; see, e.g., Kuroshima *et al.*, 1978), but similar to the concentrations of the hormone that are required to elicit effects in liver preparations *in vitro* (see, e.g., Cherrington & Exton, 1976; Shade *et al.*, 1979) (although portal glucagon concentrations are likely to be higher than peripheral ones). In view of the fact that in the present study adipocytes were isolated from fed animals, it was surprising to find that the sensitivity of acetyl-CoA carboxylase activity in these cells to inhibition by glucagon was considerably higher than that reported previously for the effects of the hormone on triacylglycerol hydrolysis in adipocytes isolated from fed animals (Fernandez & Saggerson, 1978; Honnor & Saggerson, 1980), and this observation raises the question as to whether a degree of dissociation exists between the effects of the hormone on lipolysis and on carboxylase activity. In addition, there were two differences between the present observations on the interactions of the effects of glucagon with those of adenosine deaminase and those observed for triacylglycerol hydrolysis in adipocytes of fed rats by Honnor & Saggerson (1980). Firstly, addition of adenosine deaminase (at the same concentration, 4 munits of enzyme/ml) did not affect the degree of maximal inhibition exhibited by glucagon (Fig. 1). Secondly, the concentration of glucagon required to elicit half the hormone-dependent part of the response was unaltered in the presence of adenosine deaminase. These results, in conjunction with the observation that the maximal inhibition of the activity of acetyl-CoA carboxylase achieved with adenosine deaminase alone was similar to that observed in the presence of maximally effective glucagon concentrations alone, suggest that the effects of the two agents were exerted through the same mechanism. The same considerations apply to the results pertaining to the interactions between the effects of glucagon and adrenaline on the activity of acetyl-CoA carboxylase.

However, the interactions between the opposing effects of glucagon and insulin were of a totally

different nature in several respects. Thus, for any given concentration of either hormone, the absolute extent of inhibition (glucagon) or activation (insulin) was independent of whether the other hormone was present and of the concentration of the other hormone when this was present. Thus a maximally effective concentration of glucagon (10 nM) always produced an inhibition of about 70% of the 'control' activity of acetyl-CoA carboxylase. Similarly the extent of activation by insulin was always about 75% of the 'control' activity of the enzyme. The presence of glucagon (at either of two concentrations) did not alter the sensitivity of the adipocytes to insulin, as judged by the response of the activity of acetyl-CoA carboxylase (Fig. 3). Furthermore, since 0.5 nM-glucagon produced approximately half the maximal effect of glucagon irrespective of insulin concentration, it is presumed that insulin had no significant effect on the sensitivity of adipocytes to glucagon. This complete independence between the effects of insulin and of glucagon resulted in the observation that, when equally (and oppositely) effective concentrations of both hormones were present together in the incubation medium, the activity of acetyl-CoA carboxylase was not significantly different from 'control' activity. Thus from Fig. 3 it can be deduced that the activity of the enzyme was approximately 100% of 'control' both in the presence of 0.5 nM-glucagon plus a half-maximally effective concentration of insulin (12 μ units of insulin/ml) as well as in the presence of 10 nM-glucagon plus 100 μ units of insulin/ml (maximally effective concentrations). These observations suggest that the two hormones act on different and independent sites in the enzyme and that the regulatory potency of the two sites is quantitatively similar (but opposite). By analogy with electronic control mechanisms, the relationship of the effect of glucagon to that of insulin on acetyl-CoA carboxylase activity in adipocytes appears to be that of an 'offset' rather than a 'gain' control.

Current evidence suggests that the regulation of 'initial' activity of acetyl-CoA carboxylase in adipose tissue is achieved through phosphorylation and dephosphorylation at two sites (or groups of sites) on the enzyme (for review see Denton *et al.*, 1981). One site is phosphorylated in response to the exposure of adipose tissue to adrenaline and results in inhibition of the enzyme. The other site is phosphorylated on exposure of the tissue to insulin and results in activation. Phosphorylation of the two sites appears to be mutually independent (Brownsey, 1981). The results of the present study support this theory. Thus glucagon, adrenaline and adenosine deaminase all appeared to act through the same mechanism because of the synergism shown between their actions and the similarity of the maximal effects in the presence of either one or two of them. At

present it can only be presumed that their effects on acetyl-CoA carboxylase activity in adipocytes are mediated through an increase in the activity of cyclic AMP-dependent protein kinase, of which adipose-tissue acetyl-CoA carboxylase is a substrate (Brownsey & Hardie, 1980). In addition, it is known that the site phosphorylated in cells incubated with adrenaline is identical with the site phosphorylated on the purified enzyme *in vitro* by cyclic AMP-dependent protein kinase (Brownsey & Hardie, 1980). The observed effects of adenosine deaminase on acetyl-CoA carboxylase activity can also be explained by the known effects of adenosine in decreasing intracellular concentrations of cyclic AMP in adipocytes (for review see Trost & Stock, 1979). Thus a decrease in adenosine concentration in the adipocyte incubation medium by addition of adenosine deaminase would be expected to result in an increased intracellular concentration of cyclic AMP, activation of cyclic AMP-dependent protein kinase and inhibition of acetyl-CoA carboxylase through phosphorylation at the inhibitory site.

The identity and mechanism of regulation of the protein kinase that mediates the insulin-sensitive phosphorylation of the activatory site on acetyl-CoA carboxylase are not known, but the protein kinase involved is thought to be associated with the plasma membrane and to be independent of cyclic AMP (Brownsey *et al.*, 1981; Brownsey & Denton, 1982). The present results cannot be interpreted as providing evidence for glucagon- or insulin-induced phosphorylation of acetyl-CoA carboxylase in adipocytes. However, the independent nature of the effects of glucagon and insulin on the activity of the enzyme is what would be expected if, as suggested by Brownsey (1981), the phosphorylation of the two hormone-sensitive sites on acetyl-CoA carboxylase is mutually independent.

Physiological considerations

Although both the activatory effect of insulin and the inhibitory effect of glucagon on the activity of acetyl-CoA carboxylase appear to be modest when considered individually, the interaction of the opposing effects of the two hormones is capable of producing changes in the activity of the enzyme over a 6-fold range. The quantitatively similar effects of the two hormones, coupled to the apparently mutually independent nature of their effects, suggest that glucagon and insulin may be equally important in the regulation of acetyl-CoA carboxylase *in vivo* and that consequently the lipogenic rate of white adipose tissue could be very sensitive to changes in the circulatory glucagon/insulin concentration ratio.

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References

- Bergmeyer, H. U., Gawehn, K. & Grassl, M. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd edn., vol. 1, pp. 426–427, Academic Press, New York
- Brownsey, R. W. (1981) *Biochem. Soc. Trans.* **9**, 515–518
- Brownsey, R. W. & Denton, R. M. (1982) *Biochem. J.* **202**, 77–86
- Brownsey, R. W. & Hardie, D. G. (1980) *FEBS Lett.* **120**, 67–70
- Brownsey, R. W., Hughes, W. A. & Denton, R. M. (1979) *Biochem. J.* **184**, 23–32
- Brownsey, R. W., Belsham, G. J. & Denton, R. M. (1981) *FEBS Lett.* **124**, 145–150
- Cherrington, A. D. & Exton, J. H. (1976) *Metab. Clin. Exp.* **25**, 1351–1354
- Denton, R. M., Brownsey, R. W. & Belsham, G. J. (1981) *Diabetologia* **21**, 347–362
- Fernandez, B. M. & Saggerson, E. D. (1978) *Biochem. J.* **174**, 111–118
- Geelen, M. J. H., Beynen, A. C., Christiansen, R. Z., Lepreau-Jose, M. J. & Gibson, D. M. (1978) *FEBS Lett.* **95**, 326–330
- Hagen, J. H. (1961) *J. Biol. Chem.* **236**, 1023–1027
- Hales, C. N., Luzio, J. P. & Siddle, K. (1978) *Biochem. Soc. Symp.* **43**, 97–135
- Halestrap, A. P. & Denton, R. M. (1973) *Biochem. J.* **132**, 509–517
- Honnor, R. C. & Saggerson, E. D. (1980) *Biochem. J.* **188**, 757–761
- Kuroshima, A., Doi, K. & Ohno, T. (1978) *Life Sci.* **23**, 1405–1410
- Martin, B. R. & Denton, R. M. (1970) *Biochem. J.* **117**, 861–877
- McNeillie, E. M. & Zammit, V. A. (1982) *Biochem. J.* **204**, 273–280
- McNeillie, E. M., Clegg, R. A. & Zammit, V. A. (1981) *Biochem. J.* **200**, 639–644
- Rodbell, M. (1964) *J. Biol. Chem.* **239**, 375–380
- Shade, D. S., Woodside, W. & Eaton, R. P. (1979) *Metab. Clin. Exp.* **28**, 874–886
- Stansbie, D., Brownsey, R. W., Crettaz, M. & Denton, R. M. (1976) *Biochem. J.* **160**, 413–416
- Trost, T. & Stock, K. (1979) in *Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides* (Baer, H. P. & Drummond, G. I., eds.), pp. 379–393, Raven Press, New York
- Vaughan, M. (1960) *J. Biol. Chem.* **235**, 3049–3053
- Witters, L. A., Moriarty, D. & Martin, D. B. (1979) *J. Biol. Chem.* **254**, 6644–6649
- Zammit, V. A. (1980) *Biochem. J.* **190**, 293–300
- Zammit, V. A. (1981) *Biochem. J.* **198**, 75–83