Altered Lipolytic Response to Glucagon and Adenosine Deaminase in Adipocytes from Starved Rats

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1. Adipocytes isolated from epididymal adipose tissue of fed or 24 h-starved rats were incubated with a range of glucagon concentrations in the presence and absence of adenosine deaminase (4 munits/ml). 2. With adenosine deaminase present, the lipolytic response to low concentrations of glucagon (1–6 ng/ml) was considerably enhanced in cells from starved rats. 3. The effect of adenosine deaminase on basal lipolysis was altered after starvation. 4. D-3-Hydroxybutyrate (5 mM) decreased the sensitivity of lipolysis to glucagon. 5. The possible involvement of glucagon-stimulated lipolysis in the regulation of ketogenesis is briefly discussed.

The lipolytic response of adipose tissues *in vitro* to several hormones, including glucagon, is extensively documented (see review by Hales *et al.*, 1978). Although an important role is recognized for glucagon in the rapid regulation of hepatic ketogenesis [see reviews by McGarry & Foster (1977) and McGarry (1979)], this hormone is not generally assigned a significant role in the promotion of adipose-tissue triacylglycerol mobilization, which must of necessity accompany any sustained increase in ketogenesis. Part of the evidence supporting this conclusion comes from studies using rat epididymal fat-pieces or adipocytes, where it is found that lipolysis maximally stimulated by glucagon is considerably lower than that seen with maximally effective concentrations of adrenaline, noradrenaline or corticotropin. Furthermore, very high concentrations of glucagon are necessary to elicit this limited response (Vaughan, 1960; Hagen, 1961; Blecher *et al.*, 1969; Fernandez & Saggerson, 1978).

Fernandez & Saggerson (1978) showed that glucagon-stimulated lipolysis in rat epididymal adipocytes is particularly sensitive to the previous adrenal status of the donor animals. Addition of adenosine deaminase to adipocyte incubations corrected the lesion in glucagon responsiveness seen after adrenalectomy (Fernandez & Saggerson, 1978). In addition, and rather surprisingly, this addition of adenosine deaminase also raised the maximum rate of glucagon-stimulated lipolysis to the same as that seen with optimum doses of noradrenaline, corticotropin or theophylline. It was concluded from that study that the functioning of glucagon as a lipolytic agent is particularly sensitive to the effects of adenosine. Adenosine is released in

small amounts in adipocyte incubations (Schwabe *et al.*, 1973) and considerably alters the response of the cells to hormones (Fain, 1973; Ebert & Schwabe, 1973; Schwabe *et al.*, 1974; Fain & Wieser, 1975; Wieser & Fain, 1975; Schwabe *et al.*, 1975). This raises the possibility that the relative insensitivity of adipocytes to glucagon is an artefact of the system *in vitro* in which adenosine released by the cells might accumulate.

Accordingly, in the present study we have examined the lipolytic response of rat adipocytes to a range of glucagon concentrations in the presence of adenosine deaminase to see to what extent removal of adenosine from the cell incubation increases the sensitivity of the adipocytes to glucagon. It was found that low doses of adenosine deaminase, which alone only slightly increased basal lipolysis, rendered the cells more sensitive to glucagon. This effect was particularly apparent in cells from starved animals. The effects of L-lactate and D-3-hydroxybutyrate on the glucagon-responsiveness of adipocytes was also investigated, since it has been shown (Green & Newsholme, 1979) that these agents increase the sensitivity of adipocytes to insulin.

Materials and Methods

Chemicals

Nucleotides and collagenase (type I from *Clostridium histolyticum*) were obtained from International Enzymes Ltd. (Windsor, Berks., U.K.). Other enzymes were from Boehringer Corp. (London) Ltd. (Lewes, Sussex, U.K.). L-Noradrenaline bitartrate, DNA (type V, sodium salt, highly

polymerized; from calf thymus) and L-lactic acid were from Sigma (London) Chemical Co. (Kingston-upon-Thames, Surrey, U.K.). Before use, lactic acid was diluted to approx. 1M concentration in water, boiled for 15–20 min, neutralized (pH 7.0) with NaOH and standardized enzymically (Hohorst, 1965). Bovine plasma albumin powder (fraction V) was also from Sigma and was subjected to a defatting procedure (Chen, 1967) with minor modifications (Saggerson, 1972). Bovine glucagon was from Calbiochem (Hereford, U.K.). Sodium D-3-hydroxybutyrate, resolved from the racemic mixture by the method of Lehninger & Greville (1953), was a gift from Professor A. L. Greenbaum.

Animals

Male Sprague–Dawley rats (160–180 g body wt.) were maintained on GR3-EK cube diet (E. Dixon and Sons, Ware, Herts., U.K.). Animals were killed between 11:30 and 12:30 h. Starved animals had had their food withdrawn at approx. 12:00 h on the day before their use. Water was always available.

Preparation of adipocytes

The pooled epididymal fat-pads of three rats were disaggregated as described by Rodbell (1964). The cells were washed twice with Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) containing fatty acid-poor albumin (10 mg/ml) and then suspended in 18 ml of the same medium. If both fed and 24 h-starved rats were used on the same day, two parallel preparations were carried out.

Incubation of adipocytes

Portions (1 ml) of the stock cell suspension were added to 25 ml silicone-treated flasks and incubated with shaking for 1 h at 37°C in a final volume of 4 ml of Krebs–Ringer bicarbonate buffer containing 1.27 mM-CaCl₂, fatty acid-poor albumin (40 mg/ml) and 5 mM-glucose. The flask contents were continuously gassed with O₂/CO₂ (19:1). Other additions to flasks were as indicated in individual Tables and Figures.

Analytical methods

Immediately after incubation, flasks were plunged into ice and the contents deproteinized by addition of 0.5 ml of ice-cold 45% (w/v) HClO₄. The mixtures were centrifuged (10 min at 1000 *g*_{av}), 0.25 ml of 1 M-triethanolamine hydrochloride was added to the supernatants and these were then neutralized with saturated K₂CO₃. These extracts were assayed for glycerol by the method of Garland & Randle (1962). Before use, stock adenosine deaminase [EC 3.5.4.4; 2 mg/ml in 3.2 M-(NH₄)₂SO₄] was diluted 1:100 with 0.9% NaCl and dialysed overnight at 4°C against 200 vol. of the same to remove (NH₄)₂SO₄. The dialysed enzyme was standardized spectro-

photometrically at 265 nm and 25°C by the method of Kalckar (1947) on the morning of the experiment. A unit of adenosine deaminase is the amount of enzyme needed to deaminate 1 μ mol of adenosine/min at 25°C in this assay. The DNA content of 2.0 ml portions of the stock adipocyte suspension was determined as described by Saggerson (1972).

Statistical methods

Statistical significance was determined by Student's *t* test.

Results and Discussion

Effect of adenosine deaminase

Fernandez & Saggerson (1978) showed that the lipolytic response to glucagon (250 ng/ml) was considerably increased when relatively large amounts of adenosine deaminase (45–50 munits/ml) were added to adipocyte incubations. Adenosine deaminase itself, presumably by removing adenosine, increases basal lipolysis (Schwabe & Ebert, 1974; Fain & Wieser, 1975; Fernandez & Saggerson, 1978). This is a disadvantage when investigating the lipolytic response to other agents (e.g. glucagon) in the presence of adenosine deaminase. Therefore the effect of adenosine deaminase on basal lipolysis was first investigated with the objective of establishing a concentration of adenosine deaminase that would have minimal effect on basal lipolysis but yet would expand the maximal response of the process to glucagon. This was achieved with adenosine deaminase at 4 munits/ml. This concentration of the enzyme gave only a small (approx. 2½-fold) increase in basal lipolysis (Fig. 1) in cells from fed or starved rats, but permitted glucagon at 100 ng/ml to elicit lipolysis rates comparable with those obtained with 1 μ M-noradrenaline (Fig. 2); 1 μ M was the maximally effective dose of noradrenaline in our hands for stimulation of lipolysis.

An interesting difference between cells from fed and starved animals emerged from the experiment shown in Fig. 1. With adipocytes from fed rats, concentrations of adenosine deaminase above 8–10 munits/ml gave constant high basal rates of lipolysis (Fig. 1). This is essentially the same as the effect of adenosine deaminase on basal lipolysis observed by Fernandez & Saggerson (1978) with cells from fed adrenalectomized or fed sham-adrenalectomized rats. However, the response to higher concentrations of adenosine deaminase in cells from 24 h-starved rats was strikingly different (Fig. 1). In these cells adenosine deaminase had a pronounced optimum at 8 munits/ml in its effects on basal lipolysis. There is at present no explanation for this finding, which is rather difficult to reconcile with the conclusion of Londos *et al.* (1978) that adenosine effects on adipocyte metabolism are solely due to

Fig. 1. Effect of adenosine deaminase on basal lipolysis in adipocytes from fed (O) and starved (●) rats

Adipocytes were isolated from fed or 24 h-starved rats and incubated as described in the Materials and Methods section with the indicated amounts of adenosine deaminase. The results are means of four experiments. The bars indicate S.E.M. and where these are not shown they lie within the symbol. The mean adipocyte DNA per ml of flask contents was 9.9 and 11.6 μ g for the fed and starved states respectively.

inhibition of adenylate cyclase. The findings imply that low concentrations of adenosine can stimulate lipolysis in the starved state. Presumably this could be at the level of a hormone receptor linked to some effector system, which need not be adenylate cyclase.

Fig. 2 summarizes an experiment in which the lipolytic response to glucagon was compared in cells from fed and starved rats incubated with and without adenosine deaminase (4 munits/ml). In the absence of adenosine deaminase, glucagon was relatively ineffective as a lipolytic agent, in accord with previous observations (Fernandez & Saggerson, 1978). In other experiments (results not shown) it was established that 100 ng of glucagon/ml gave the maximum lipolytic response with this hormone. However, in the absence of adenosine deaminase this was only 30–40% of the response found with 1 μ M-noradrenaline. Also, in the absence of adenosine deaminase, no pronounced differences were seen between cells from fed and starved rats in their lipolytic response to glucagon. By contrast, adenosine deaminase enhanced the response of cells from fed animals to doses of 8–100 ng of glucagon/ml and dramatically enhanced the response to all tested concentrations of glucagon in the starved state. Prior starvation therefore leads to a distinct enhancement in the lipolytic response to low doses of glucagon, but this effect is only unmasked when adenosine

Fig. 2. Effect of glucagon on the absence and presence of adenosine deaminase on lipolysis in adipocytes from fed and starved rats

Adipocytes were isolated from fed and 24 h-starved rats and incubated as described in the Materials and Methods section with the indicated concentrations of glucagon. Where indicated, adenosine deaminase was present at 4 munits/ml. The results are means of four experiments. The bars indicate S.E.M. and where these are not shown they lie within the symbol. The mean adipocyte DNA per ml of flask contents was 11.8 and 13.0 μ g for the fed and starved states respectively. O, Fed; □, fed + adenosine deaminase; ●, starved; ■, starved + adenosine deaminase. The open and filled histograms refer to the fed and starved states respectively. In the presence of adenosine deaminase, lipolysis rates were significantly higher in the starved than in the fed state at glucagon concentrations of 0.5, 1, 2, 4, 6 and 8 ng/ml ($P < 0.01$, 0.01, 0.01, 0.001, 0.001, 0.05 respectively), but were not significantly different with 10 or 100 ng of glucagon/ml.

deaminase is added. For example, in the fed state 6 ng of glucagon/ml gave only 8% of the maximal lipolytic response obtainable with 100 ng of the hormone/ml. On the other hand, 6 ng of glucagon/ml gave 64% of the maximal response in the starved state. Fig. 2 also shows that the maximum lipolytic response to glucagon (100 ng/ml) was unaffected by prior starvation in the presence of adenosine deaminase and that this response was now similar to that obtained with 1 μ M-noradrenaline (at this concentration of noradrenaline, adenosine deaminase has no additional effect on lipolysis; see Fig. 4 of Fernandez & Saggerson, 1978).

Effect of D-3-hydroxybutyrate

Green & Newsholme (1979) have shown that 10 mM-DL-3-hydroxybutyrate or 10 mM-lactate

increases the sensitivity to insulin of adipocytes from fed rats. Fig. 3 shows that, conversely, the sensitivity to glucagon of cells from fed rats was decreased in the presence of 5 mM-D-3-hydroxybutyrate when adenosine deaminase was present. When D-3-hydroxybutyrate was added, lipolysis was significantly decreased with 10 ng of glucagon/ml ($P < 0.01$) and 20 ng of glucagon/ml ($P < 0.05$). Although D-3-hydroxybutyrate lowered basal lipolysis to some extent, this effect was not consistently observed and this was not statistically significant. A significant lowering of basal lipolysis by 10 mM-DL-3-hydroxybutyrate was noted by Green & Newsholme (1979), but this was observed in the presence of a very high concentration of adenosine deaminase (2 units/ml). In a single supplementary experiment it was found that various concentrations of D-3-hydroxybutyrate decreased lipolysis in the presence of glucagon (10 ng/ml) by 7, 24, 34, 41 and 51% with 0.5, 1, 2, 5 and 10 mM-D-3-hydroxybutyrate respectively. If glucagon does play a role in the control of lipolysis *in vivo* (see the discussion below), then it is possible that the antilipolytic effect of ketone bodies reported by Madison *et al.* (1964)

and Hawkins *et al.* (1971) could arise both from a diminution in sensitivity to the lipolytic agent glucagon as well as from an increase in sensitivity to the antilipolytic agent insulin (Green & Newsholme, 1979).

In other experiments (results not shown; experimental details the same as in Fig. 3) 10 mM-L-lactate significantly decreased the lipolytic response to glucagon (10 ng/ml) by 44% ($P < 0.01$) without significantly decreasing basal lipolysis (mean of four experiments).

General considerations

It is not known to what extent adenosine accumulates in or around adipocytes *in vivo* compared with the situation *in vitro*. The results presented here suggest that prevention of adenosine accumulation increases the lipolytic response of adipocytes to glucagon and reveals an increase in sensitivity to glucagon after brief starvation. It is suggested that brief starvation primes the adipocyte to respond more fully to the elevations of glucagon concentrations expected in starvation. In the longer term, therefore, glucagon might contribute to stimulation of ketogenesis under these conditions both at the hepatic and at the adipose-tissue level. It is at present unclear which of several possible factors make the main contribution to the onset of lipolysis in starvation (see Hales *et al.*, 1978). Also, as discussed above, a damping of glucagon stimulation of lipolysis by D-3-hydroxybutyrate could be part of a feedback-control system linking ketogenesis and lipolysis.

If these suggestions have validity, there should either be broad comparability between the concentrations of glucagon eliciting lipolysis in isolated cells and those measured in plasma, or any discrepancies between these should be explicable. Mean glucagon concentrations in human plasma in various conditions are reported as being between 50 and 500 pg/ml (Cahill, 1976). A slightly higher range of values has been reported for the rat (80–2000 pg/ml; Meier *et al.*, 1972; Lefebvre, 1975; Saccà *et al.*, 1976). Glucagon concentrations in man rise in starvation (Unger *et al.*, 1963; Marliss *et al.*, 1970), but we are unaware of any corresponding studies in the rat. The concentrations of glucagon eliciting appreciable stimulation of lipolysis in the present study can be as low as 1–2 ng/ml, provided that adenosine deaminase is added and the cells are obtained from a starved rat (Fig. 2). These concentrations of glucagon are rather high compared with likely physiological concentrations of the hormone. However, several other factors may have to be considered. Firstly, although some attempt has been made to minimize adenosine accumulation in the present experiments, other substances could accumulate in the cell incubations and diminish

Fig. 3. Effect of D-3-hydroxybutyrate on the lipolytic response of adipocytes to glucagon

Adipocytes were isolated from fed rats and incubated as described in the Materials and Methods section with adenosine deaminase (4 munits/ml) and the indicated concentrations of glucagon. The results are means of four experiments. The bars indicate S.E.M., and where these are not shown they lie within the symbol. The mean adipocyte DNA per ml of flask contents was 14.3 μg. O, Without D-3-hydroxybutyrate; ●, with 5 mM-D-3-hydroxybutyrate.

responsiveness to glucagon. For example, any prostaglandin accumulation is likely to lead to damping of the response to this hormone (Lefebvre & Luyckx, 1974). Secondly, it is possible that addition of higher concentrations of adenosine deaminase might further increase responsiveness to glucagon. Thirdly, the biological potency of the commercial glucagon used here is unknown. If this is lower than expected, the sensitivity of the cells to glucagon would be underestimated. Fourthly, degradation of glucagon by incubated adipocytes may be considerable (Desbuquois & Laudat, 1974; Sonne & Gliemann, 1977).

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