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Insulin processing and action in adipocytes: evidence for generation of insulin-containing vesicles by leupeptin and monensin

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Incubation of adipocytes with ^{125}I-insulin plus leupeptin or monensin, but not chloroquine, resulted in the appearance of a novel peak of ^{125}I-insulin (modal density about 1.20 g/ml) on density gradient centrifugation; the appearance of the peak depended on the presence of specific insulin receptors on the cell surface. The fractions comprising this peak contained vesicles, probably originating from the Golgi apparatus, and dit not appear to be contaminated with lysosomes, mitochondria or plasma membrane. Entrapment of insulin in these vesicles per se did not prevent the activation of glucose transport, acetyl-CoA carboxylase or pyruvate dehydrogenase by insulin.

Insulin processing *Adipocyte* *Leupeptin* *Monensin* *Glucose transport* *Acetyl-CoA carboxylase*

1. INTRODUCTION

The mechanisms of insulin processing and action in adipocytes are incompletely understood. Insulin binds to a receptor on the plasma membrane, is subsequently internalized by receptor-mediated endocytosis and some at least is transported by endosomes (vesicles of Golgi origin) to the lysosomes and degraded [1–4]. Insulin stimulates glucose transport and activates some enzymes in adipocytes, but the nature of the signals and their sites of generation are unknown; some signals are probably generated at the plasma membrane but this is not certain [1–3,5]. We report on the effects leupeptin, a weakly basic thiolprotease inhibitor; monensin, a monovalent cationic ionophore and chloroquine, a lysomotrophic agent, on the processing and action of insulin in adipocytes.

2. EXPERIMENTAL

The preparation and characterisation of ^{125}I-labelled insulin (>85% monoiodinated) and the measurement of its specific and non-specific binding to adipocytes have been described [6].

Parametrial adipocytes, prepared from 150–200 g female Wistar rats [6], were incubated at 37°C in M199 [Medium 199, Gibco-Biocult, Paisley, Scotland (pH 7.4) containing Earle's salts, L-glutamine, 25 mM Hepes, 20 mM Tricine, 1% albumin (bovine, essentially fatty acid-free, Sigma, Poole, England), 0.2 mM penicillin and 60 μM streptomycin] for 1 h with ^{125}I-insulin (2 ng/ml). Cells were then washed with 2 μg/ml unlabelled insulin for 5 min to remove ^{125}I-insulin bound specifically to the cell surface receptors (this procedure did not displace non-specifically bound ^{125}I-insulin from the cell surface) and homogenized by standing in ice-cold homogenisation buffer (0.25 M sucrose, 10 mM Tris-HCl, 2 mM EDTA, pH 7.4) for 1 h. Defatted homogenates were centrifuged at 60000 rpm (255000 × g) for 30 min and the pellet was resuspended in 400 μl of homogenization buffer. The suspension was placed at the bottom of a discontinuous sucrose gradient and centrifuged at 27000 rpm (85000 × g) for 15 h. Fractions (1 ml) were collected from the

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gradients and analysed for radioactivity, alkaline phosphodiesterase I, succinate dehydrogenase, 5′-nucleotidase, acid phosphatase [7] and galactosyl transferase [8] or prepared for electron microscopy [7].

3. RESULTS AND DISCUSSION

Incubation of adipocytes with 500 μM leupeptin increased significantly ($p < 0.01$) by 20% the amount of ^{125}I-insulin retained by the cells (internalized insulin plus that bound non-specifically to the cell surface, not shown). Examination of such cells by electron microscopy suggested the presence of increased numbers of enlarged vesicles following incubation with leupeptin (not shown) while fractionation of cells by discontinuous sucrose gradient centrifugation showed that the distribution of ^{125}I-insulin was markedly altered: a peak of ^{125}I-insulin with a modal density of about 1.15 g/ml was found with cells incubated in the absence or presence of leupeptin, but in the presence of leupeptin, a novel peak with a modal density of about 1.2 g/ml was also present (fig.1). The distribution of 4 marker enzymes (alkaline phosphodiesterase, succinate dehydrogenase, 5′-nucleotidase and acid phosphatase) (fig.1) was not affected by leupeptin, but the distribution of a fifth, galactosyl transferase (a marker for Golgi membranes) was altered in cells incubated with leupeptin, with a much enlarged peak of modal density about 1.2 g/ml. The fractions 2–4, which contained the peak with a modal density of 1.2 g/ml, were essentially free of the other enzymes, suggesting that these fractions are relatively free of lysosomal, mitochondrial and plasma membrane fractions. Incubation of adipocytes with monensin (50 μM) also resulted in the appearance of a peak of ^{125}I-insulin with a modal density of about 1.2 g/ml, but, in contrast, no such peak was seen with cells incubated with chloroquine (500 μM) (fig.1).

Pre-treatment of adipocytes with trypsin (100 μg/ml) for 15 min followed by several washes with M199 containing 100 μg/ml trypsin inhibitor (Sigma) eliminated essentially all specific (but not non-specific) binding of ^{125}I-insulin. When such adipocytes were incubated with ^{125}I-insulin in the presence and absence of leupeptin (500 μM), and then fractionated, the peak of ^{125}I-insulin with a

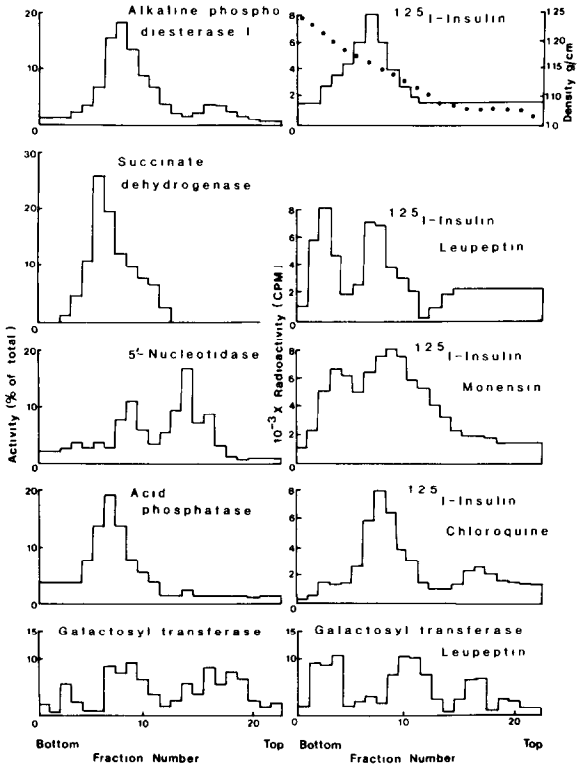

Fig.1. Discontinuous sucrose gradient centrifugation of adipocyte homogenates. Adipocytes were incubated in M199 at 37°C for 60 min with ^{125}I-insulin (2 ng/ml), in the absence or presence of leupeptin (500 μM), monensin (50 μM) or chloroquine (500 μM), and subsequently fractionated as described in the text. Marker enzyme activities are expressed as percentages of the total activity recovered from the gradient (85–95% of that applied). Distributions of ^{125}I-insulin on gradients are given as 10^{-3} × radioactivity (cpm) per fraction. Marker enzyme and radioactivity distributions are representative of a series of experiments in which each treatment was carried out at least twice.

modal density of about 1.15 g/ml was again observed, but not the peak with a modal density of 1.2 g/ml (not shown).

Electron microscopic analysis of fractions 6–9 (density 1.15 g/ml) from sucrose density gradients of untreated and treated cells (fig.1) showed a heterogeneous population of vesicles. Fractions 2–4 from leupeptin- and monensin-treated cells, but not untreated cells, also contained vesicles.

These results suggest that leupeptin and monensin cause the accumulation of insulin in vesicles,

possibly a form of endosome as they were associated with the Golgi marker, galactosyl transferase and appeared to be essentially free of the lysosomal, mitochondrial and plasma membrane markers. The appearance of the peak of insulin in fractions 2–4 seems to be dependent on the presence of high-affinity (specific) binding sites for insulin on the cell surface. As this peak of insulin was found only in the presence of leupeptin and monensin, it would appear that the vesicles are normally transient, and/or that leupeptin and monensin alter their properties (e.g., making them more dense). Both are possible: the insulin-containing endosomes identified by Suzuki and Kono [9] using density centrifugation had a modal density of about 1.06 g/ml while monensin has been shown to inhibit the transfer of epidermal growth factor, and other substances into lysosomes, causing their accumulation in endosome-like vesicles [10]. A small peak of radioactivity with a modal density of about 1.06 (fractions 16–18) was found after incubation with chloroquine (fig.1). The identity of the peak of insulin with a modal density of 1.15 g/ml (fractions 6–8, fig.1) is less clear: acid phosphatase, a lysosomal marker, also peaked here but the fractions were contaminated with other marker enzymes.

Monensin, but not leupeptin, markedly decreased the basal and insulin-stimulated uptake of 2-deoxyglucose into adipocytes ($p < 0.05$) (fig.2). Similar results were obtained using an insulin concentration of 20 ng/ml (not shown). In contrast, leupeptin, but not monensin inhibited ($p < 0.05$) insulin-induced activation of acetyl CoA carboxylase (ACC) (fig.2), while neither impaired the activation of pyruvate dehydrogenase (PDH) by insulin. Leupeptin did not prevent stimulation of glucose oxidation or activation of glycogen synthetase by insulin in adipocytes [11]. Insulin promotes the translocation of Golgi vesicles carrying the glucose translocase to the plasma membrane [12], and monensin has been shown in other systems to disrupt the intracellular transport of Golgi vesicles [3]. So, as the apparent entrapment of insulin in some form of endosome by leupeptin did not prevent insulin stimulation of glucose transport or activation of PDH, while monensin did not prevent activation of ACC, the results provide further evidence that the insulin-induced

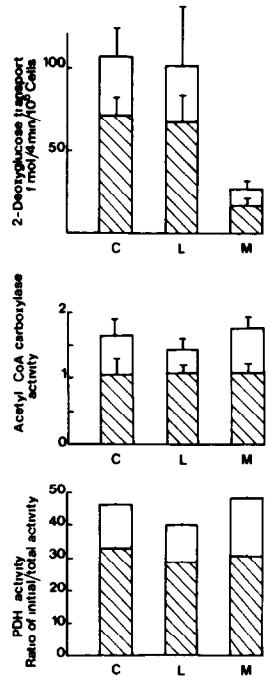

Fig.2. Effects of leupeptin and monensin on insulin stimulation of glucose transport and activation of acetyl CoA carboxylase (ACC) and pyruvate dehydrogenase (PDH). Adipocytes were incubated in either Krebs' Ringer phosphate buffer (pH 7.4) containing 1% albumin (glucose transport) or M199 (ACC and PDH) at 37°C for 60 min in the absence (C) or presence of 500 μM leupeptin (L) or 50 μM monensin (M), with ▭ or without ▭) insulin (2 g/ml). Glucose transport was measured using 3 nM 2-deoxy-D-[1-^{3}H]glucose [13]. For enzyme assay, adipocytes were centrifuged through 100 l of dinonylphthalate oil and frozen in liquid nitrogen. The initial activity of ACC (i.e., in the absence of citrate and without preincubation with citrate) was measured as described previously [14] except that the incubation time was decreased to 3 min, and is expressed as a fraction of the activity of lactate dehydrogenase (LDH) $\times 10^{-3}$ of the preparation. PDH was assayed spectrophotometrically [15]. Results are the means and SE from 3, 4 and 1 separate experiments each using pooled adipocytes from 4 or 5 rats for glucose transport, ACC and PDH, respectively.

signals stimulating these processes are produced at an early stage of the interaction of insulin with the adipocyte, prior to the formation of the putative endosome. Furthermore, as monensin disrupts the movement of vesicles within the cell [12], transmission of the signal from the plasma membrane to

the mitochondria to activate PDH does not appear to involve transport in such intracellular vesicles.

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