¹²⁵I-Labelled Insulin Degradation by Isolated Rat Hepatocytes: The Roles of Glutathione-Insulin Transhydrogenase and Insulin-Specific Protease

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Summary. Isolated rat hepatocytes degraded ¹²⁵I-insulin with a K_m of 150 nmol/l. Degradation was stimulated by the addition of glutathione and dithiothreitol. In cells incubated with diamide, glutathione was oxidised to the disulphide. Regeneration of reduced glutathione commenced after a further 30 min incubation at 37 °C. Diamide (1 mmol/l) significantly inhibited insulin degradation by hepatocytes ($p < 0.001$). The 'apparent V_{max} ' for insulin degradation was decreased tenfold and the K_m decreased to 25 nmol/l. The diamide-insensitive degrading activity was cell-associated and produced an interme-

diate of hormone degradation that was apparently of a higher molecular weight than insulin A chain. The biological activity of the intermediate was 0.03% of that of insulin. The diamide-insensitive activity was not due to release of protease into the medium by cell lysis. We conclude that there are at least two pathways capable of degrading insulin existing in rat hepatocytes.

Key words: Insulin-hormone degradation, hepatocyte, proteases, glutathione, insulin transhydrogenase, diamide.

Various mechanisms have been proposed to explain the rapid breakdown of insulin *in vivo*. The hormone may be reduced to give separate A and B chains in a reaction catalysed by glutathione-insulin transhydrogenase (EC 1.8.4.2) with glutathione as reductant. The individual chains may then be degraded further by proteolysis. Evidence for such a mechanism has been obtained from a number of tissues including liver, kidney, muscle and fat [1]. An insulin-specific protease has also been shown to occur widely in the body, the sites including liver [2] and skeletal muscle [3, 4]. The enzyme from adipose tissue effects an initial cleavage between residues 16 and 17 of the insulin B chain, and then further proteolysis with the production of low molecular weight fragments [3]. This protease has a low K_m for insulin, whereas the transhydrogenase system exhibits 50% of its maximal activity at 1.5 μ mol hormone/l, a concentration well above that normally found *in vivo* [5].

Isolated hepatocytes have been widely used for the study of insulin degradation [6–10]. Both they and fat cells degrade insulin rapidly, although a substantial and variable proportion of this activity is found in the incu-

bation medium [9, 11, 12]. Cell-associated degradation involves hormone binding to the surface receptors [9, 10]. A role for internalisation and lysosomal uptake in hormone degradation has also been proposed [10] although this is the subject of some controversy [13].

The reagent diamide (azodicarboxylic acid bisdimethylamide) causes a rapid oxidation of glutathione to its disulphide in many tissues and cell preparations [14]. Cellular integrity is retained and glutathione is regenerated during incubations with substrate at 37 °C. Because glutathione is potentially an important factor in regulating insulin breakdown in liver, we have investigated the effects of diamide on insulin metabolism by isolated rat liver cells. The results are discussed in terms of the two mechanisms for degradation outlined below.

Materials and Methods

Measurement of Insulin Degradation

Isolated rat hepatocytes were prepared by collagenase digestion [14] from male 48 h starved rats (Sprague-Dawley, weight: 200–300 g). All incubations were performed in a modified Krebs-Henseleit bicarbonate medium containing (in mmol/l): NaCl, 119; NaHCO₃, 25; KCl, 4.8; MgSO₄, 1.2; KH₂PO₄, 1.2; CaCl₂, 1.2; pH 7.4, gassed with O₂/CO₂ (95:5%). All incubations (final volume 5–15 ml), in triplicate, contained 10⁶ cells/ml and lactate (10 mmol/l) and pyruvate (1 mmol/l) as substrates. Where appropriate, additions were made to cell incubations

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Fig. 1. Degradation of ^{125}I -labelled insulin by rat hepatocytes. Hepatocytes were separated from medium after incubation for 30 min at 37°C . Resuspended cells (□), separated medium (Δ) and control cells (●) were incubated with ^{125}I -insulin (0.1 nmol/l) at 37°C . Degradation of insulin was measured as the appearance of trichloroacetic acid-soluble radioactivity. Results are the means of at least three independent observations

Fig. 2. Hepatocytes ($\sim 10^6$ cells/ml) were incubated at 37°C with increasing concentrations of ^{125}I -insulin in the presence (●) or absence (○) of diamide (1 mmol/l). Degradation of insulin was measured between 2 and 4 min after the addition of insulin by the appearance of trichloroacetic acid-soluble radioactivity. Results are the means of at least three independent observations

before addition of hormone to permit equilibration within cells. Incubations were terminated either by rapid separation of cells from medium [16] or by addition of trichloroacetic acid (final volume 10 g/100 ml).

Monocomponent porcine insulin (Burroughs Wellcome, Dartford, Kent, UK) radio-iodinated with ^{125}I by the Chloramine-T procedure [17], was purified by gel filtration. The specific activity of the iodinated hormone was 200 Ci/mmol. At least 90% of the iodoinsulin contained only one iodine atom/insulin molecule, situated on tyrosine A_{14} , as determined by reductive cleavage of the insulin molecule into separated A and B chains [18] and the partial enzymatic hydrolysis of the A chain with chymotrypsin [19]. Iodoinsulin was $>95\%$ precipitable by trichloroacetic acid (10 g/100 ml, 4°C), and retained full biological activity as assessed by its ability to stimulate glucose incorporation into fat cell lipid [20].

The degradation of hormone was measured by determining the amount of label remaining soluble in trichloroacetic acid (10 g/100 ml, 4°C). Degradation is expressed as a percentage of the total amount of ^{125}I -insulin.

Biological Activity of Insulin-Derived Protein

The biological activity of products of insulin degradation was assessed by measurement of their ability to stimulate the incorporation of $[3\text{-}^3\text{H}]$ glucose into fat cell lipid [20]. Cell isolation and incubations were performed in Krebs-Ringer bicarbonate buffer, pH 7.4 [20], containing defatted albumin (10 mg/ml) and glucose (0.1 mg/ml). Cells were obtained by incubation of pieces of epididymal fat pad with collagenase (100 mg/ml) for 15 min at 37°C . Cells were washed three times, and were incubated in 1 ml buffer with $[^3\text{H}]$ glucose, a range of concentrations of monocomponent porcine insulin and the sample to be tested. Glucose incorporation into lipid was determined from the counts in the organic phase after extraction of incubations with scintillation fluid (2,5-diphenyloxazole (0.5 g/100 ml), protosol (3 ml/100 ml) in toluene).

Cleavage of Disulphide Bonds [18]

Freeze-dried samples were dissolved in 0.5 ml Tris buffer (1.44 mol/l, pH 8.6). Urea (0.6 g) 2-mercaptoethanol (25 μl) and 10 μl EDTA (5 g/100 ml) were added. After 4 h at room temperature an equal volume of acetic acid was added, and samples were fractionated by gel filtration through Sephadex G-75, superfine (column 40×1.6 cm, flow rate 6 ml/h).

Fractionation of Incubations

Supernatant fractions from liver cell incubations were freeze-dried, resuspended in acetic acid (1:1, v/v with water) and fractionated on Sephadex G-75, superfine (column 37×1.6 cm, flow rate 6 ml/h). Some samples were re-fractionated on Sephadex G-50, superfine, in acetic acid (1 mol/l), (column 60×0.9 cm, flow rate 10 ml/h).

Cell Viability

The metabolic integrity of hepatocytes was assessed in all preparations by measurement of ATP content and the formation of glucose from lactate (10 mmol/l) [21].

Measurement of Cellular Metabolites

Glucose [21], ATP [21] and glutathione [22] were assayed by standard methods.

Disc Polyacrylamide Gel Electrophoresis

Insulin-derived protein was analysed by disc polyacrylamide gel electrophoresis [23]. Freeze-dried samples were dissolved in HCl (0.01 mol/l) containing sucrose (20 g/100 ml) and bromophenol blue (1 mg/100 ml), and applied to acrylamide gels (7.5 g/100 ml) containing no sodium dodecyl sulphate, of diameter 0.5 cm and length 6 cm. Running conditions, staining and destaining of the gels were as described previously [23], except that SDS was omitted from the running buffer.

Results

Degradation of insulin by isolated rat hepatocytes at 37°C proceeded after a lag phase of 1–2 min (Fig. 1). At all insulin concentrations degradation proceeded to be-

Fig. 3. The effect of diamide (1 mmol/l) on the reduced glutathione (GSH) content of rat hepatocytes at 37 °C

Fig. 4. Hepatocytes were incubated at 37 °C with ^{125}I -insulin (0.1 nmol/l) for 5 min in the presence (—) or absence (---) of diamide (1 mmol/l). Cells were separated from medium by centrifugation and the supernatant fractionated on a column of Sephadex G-75 in acetic acid: water (1 : 1, v/v). The fractions indicated were pooled and refractionated on columns of Sephadex G-50

tween 65 and 70% of the total counts. Gel filtration of the supernatant fraction after incubation showed the appearance of radioactivity in the region corresponding to low molecular weight products, simultaneously with the disappearance of counts from the insulin peak. At no time during the incubation was there an accumulation of radioactivity associated with free A chain [6, 7]. Similar elution profiles were obtained using more dilute, or more concentrated suspensions of hepatocytes. After an extended period of incubation of 30 min at least 25% of the total radioactivity remained precipitable by trichloroacetic acid (10 g/100 ml, 4 °C). This is representative of intact hormone either bound to or internalised by the hepatocytes and is not free in the incubation medium. The K_m for insulin degradation by rat hepatocytes, as determined by Lineweaver-Burks analysis of the data, was 150 ± 10 nmol/l and the V_{max} 14.2 ± 0.6 ng insulin degraded/mg dry weight per min [Fig. 2]. This rate is higher than previously reported [7]; however, the experiments

reported here were performed at 37 °C and in a medium lacking albumin. The latter has been shown to inhibit protease released from cells during incubation [9, 11]. To assess the degree of leakage of degrading activity from the cells, hepatocytes were incubated for 30 min after which time cells were separated from medium by centrifugation (50 g, 2 min). The medium and the cells resuspended in fresh medium were independently incubated with 0.1 nmol/l ^{125}I -insulin at 37 °C (Fig. 1). At least 95% of the degrading activity remained associated with the cells throughout the incubation.

The rate of degradation of insulin by hepatocytes was significantly enhanced by the addition of reduced glutathione or dithiothreitol to the incubation medium. In the absence of cells neither glutathione nor dithiothreitol caused the insulin to break down.

Incubation of Hepatocytes with Diamide

Addition of diamide (1 mmol/l) to suspensions of hepatocytes at 37 °C resulted in a very rapid oxidation of hepatocyte glutathione (Fig. 3). The reappearance of glutathione was observed after further incubation of the cells at 37 °C for 20 min. After 90 min of incubation, 30% of the glutathione had been regenerated. At a concentration of 1 mmol/l, diamide did not significantly affect either the ATP content of the hepatocytes or their ability to synthesise glucose de novo from lactate (10 mmol/l) as substrate. At higher concentrations diamide caused sharp decreases in both ATP content and gluconeogenic capacity to levels indicative of cellular degeneracy and death [21].

Effect of Diamide on [^{125}I] Insulin Degradation

Glutathione is the most abundant intracellular reduced thiol and is the major physiological cofactor for glutathione-insulin transhydrogenase [9]. Purified glutathione-insulin transhydrogenase did not degrade ^{125}I -insulin alone; in the presence of added glutathione insulin was rapidly reduced. Addition of diamide, in amounts in molar excess of those of glutathione, inhibited hormone degradation completely.

^{125}I -insulin degradation by isolated rat hepatocytes was significantly inhibited by incubation with diamide (1 mmol/l) ($p < 0.001$). Diamide did not degrade ^{125}I -insulin in the absence of hepatocytes. In the presence of diamide (1 mmol/l) the K_m and 'apparent V_{max} ' (based on trichloroacetic acid solubility) for insulin degradation by liver cells, as determined by Lineweaver-Burks analysis of data, decreased from 150 to 25 nmol/l and from 14.2 to 0.9 ng insulin/mg dry weight per min, respectively (Fig. 2). The diamide-insensitive degrading activity was associated with the cells and could not be removed by repeated washing procedures.

The possibility that diamide might act by blocking a membrane-associated event rather than insulin degradation per se is excluded by observations with whole cell

Fig. 5. Polyacrylamide gel electrophoresis of purified intermediate of insulin degradation by rat hepatocytes. A = purified intermediate; B = unfractionated incubation medium

Fig. 6. An isolated intermediate of insulin degradation was subjected to conditions that cleave disulphide bonds. The elution profile is from a column of Sephadex G-75 in acetic acid (1:1, v/v with water), V_0 = void volume bovine serum albumin marker; V_{ins} = insulin marker, K_{av} = 0.31; V_A = insulin A chain marker, K_{av} = 0.46. The elution profiles are (—) before and (---) after reduction

homogenates. Although the concentrations of glutathione were lower in liver homogenates than in hepatocytes (5.2 and 8.1 $\mu\text{mol/g}$ wet weight, respectively), homogenates degraded ^{125}I -insulin at a greater rate than did an equivalent weight of cells. Diamide (1 mmol/l) completely oxidised the glutathione present in the homogenates and inhibited ^{125}I -insulin degradation by 70% ($p < 0.001$).

To investigate the diamide-insensitive degradation in more detail, the supernatant fraction after incubation was gel filtered (Sephadex G-75). After a 5 min incubation in the presence of diamide (1 mmol/l), an intermediate of insulin degradation accumulated (Fig. 4); this eluted at a volume corresponding to that expected for a molecule larger than A chain. Parallel incubations were performed containing unlabelled bovine insulin (1 $\mu\text{mol/l}$) in addition to, or instead of, labelled insulin. Fractions corresponding to the intermediate peak were pooled and twice subjected to gel filtration (G-50); this yielded a protein running as a single band on polyacrylamide gel electrophoresis (Fig. 5). The purified intermediate was subjected to conditions that cleave interchain disulphide bonds, and the reaction mixture was gel filtered again (G-75). The new labelled peak now appeared in a position associated with peptides smaller than A chain (Fig. 6; K_{av} A chain = 0.46; K_{av} reduced intermediate = 0.52), suggesting that the original peptide retained at least one of the interchain disulphide bridges.

The biological activity of the intermediate, as measured by its ability to stimulate the incorporation of glucose into adipocyte lipid relative to an insulin standard, was found to be 0.03% of that of the insulin standard. Such an activity represents an almost total inactivation of the hormone by a diamide-insensitive enzyme system, or a contamination of one part in 30,000 with insulin, a level undetectable by the techniques of analysis used.

Discussion

These studies were performed to investigate the metabolism of insulin by isolated rat hepatocytes. Hepatocytes degraded insulin after a lag phase of 1–2 min, consistent with the idea that degradation is a receptor-mediated phenomenon [7, 9, 10]. Degradation was stimulated by reduced glutathione and by dithiothreitol. Both these compounds are known to act as co-substrates for glutathione-insulin transhydrogenase with insulin as substrate. The reasons for stimulation by thiol compounds are not clear. The intracellular content of glutathione is already sufficient to saturate the transhydrogenase [5], so that the observations may be more consistent with the degrading activity being located on the external surface of the plasma membrane. In the presence of the thiol oxidant diamide, a trichloroacetic acid-precipitable fragment is produced by proteolysis. The maximum velocity of insulin degradation is decreased in conjunction with a decrease in the K_m of the system for insulin. This seemingly contradictory situation may be explained by comparing it with other systems. A proteolytic cleavage has been described previously for skeletal muscle [3] and more recently cultured human lymphocytes [24, 25]. Degradation by human lymphocytes appeared to be due to a non-receptor-mediated degrading activity [25, 26] since analysis of the radioactivity in the medium demonstrated the formation of a species that was precipitable by both

trichloroacetic acid and insulin antibody, but was unable to rebind to its receptor, representing an inactivation of the hormone by a very limited proteolysis [24, 25]. In the system employed here, limited proteolysis is not detected by trichloroacetic acid-precipitability and the $V_{\max}$ may be very much faster than the observed rates of formation of trichloroacetic acid-soluble radioactivity. Degradation of insulin by intralysosomal proteases has been suggested to be the major site of liver insulin degradation [10], but results from this laboratory [27] have shown that inhibition of intralysosomal degradation does not affect the overall rates of insulin breakdown by hepatocytes.

We conclude, therefore, in agreement with previous reports [2, 3, 7], that hepatocytes possess a low K_m protease capable of degrading insulin and that this may be the initial step in the breakdown of the hormone. Whilst having an unattractive K_m for insulin, glutathione-insulin transhydrogenase may still play a role in the degradation of insulin by hepatocytes. It is possible that rather than being the initiator of degradation, glutathione-insulin transhydrogenase may occupy the second position in the pathway of insulin degradation, serving to degrade the already proteolytically-cleaved insulin molecule and facilitate the rapid clearance of the hormone.

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