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Inhibition of Degradation of Insulin by Ophthalmic Acid and by a Bovine Pancreatic Proteinase Inhibitor

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We have previously observed that, on subcutaneous administration, a significant proportion of insulin is degraded at the site of injection. The present paper reports that the degradative activity of slices of rat adipose tissue can be inhibited *in vitro* by ophthalmic acid, a natural analogue of glutathione, and by bovine pancreatic proteinase inhibitor, whereas it is increased by the addition of reduced glutathione.

We have previously shown, by using semisynthetic [^{3}H]insulin as a radioactive tracer, that there is an appreciable degree of degradation of insulin at the site of subcutaneous injection in pigs (Halban *et al.*, 1978*a*; Berger *et al.*, 1979). This effect could reflect the destruction of the insulin subsequent to binding to its specific receptors, or it could simply be due to the liberation of enzymes from damaged cells. Cells are likely to be damaged both by the insertion of the needle and by the local changes in both hydrostatic and osmotic pressure within the tissue as the insulin suspension is introduced. In any case the degradative activity would be likely either to be proteolytic or to involve the reduction of the disulphide bridges of the hormone, or both (Mirsky, 1957; Izzo, 1975; Varandani & Nafz, 1976). The possibility that a noticeable proportion of the insulin used therapeutically in man is degraded before it even reaches the circulation has its clinical implications, and we have sought likely inhibitors of the suspected enzymic activities as a preliminary to the further biochemical characterization of the system. Since some insulin-degrading systems are proteolytic (Izzo, 1975; Duckworth *et al.*, 1975), we have studied bovine pancreatic proteinase inhibitor (Trasylol; Bayer, A.-G.). Le Cam *et al.* (1975) reported briefly that Trasylol inhibited insulin degradation by hepatocytes. Since the insulin-specific dehydrogenase activity described by Tomizawa & Halsey (1959) is glutathione-linked, we also decided to test the effect of ophthalmic acid (γ-glutamyl-2-amino-n-butanoylglycine; Waley, 1966), a naturally occurring analogue of glutathione. Ophthalmic acid lacks a thiol group, and has in its place the structurally rather similar but chemically unreactive methyl group; it might therefore act as an inhibitor of glutathione-linked transhydrogenases. Such an inhibitory activity is known in the case of glyoxalase I (EC 4.4.1.5; Cliffe & Waley, 1961).

The present paper reports the finding that both Trasylol and ophthalmic acid inhibit the degradation of insulin by pieces of isolated adipose tissue. Even though the subcutaneous injection site *in vivo* is certain to contain damaged tissue, our model system is somewhat artificial. It is, however, technically rather difficult to obtain precise numerical data in the whole animal and we decided to carry out these model experiments as a first step, to establish the best way to study the inhibition *in vivo*.

Materials and Methods

Unless otherwise stated, reagents were of analytical grade. The ophthalmic acid was either synthesized by ourselves (S. E. Hoare & R. E. Offord, unpublished work) or purchased from Bachem A.-G., CH-4416 Bubendorf, Switzerland. The proteinase inhibitor (Trasylol; Bayer A.-G., Leverkusen, Germany) was obtained in ampoules of iso-osmotic saline solution at 20000 kallikrein inhibitor units/ml (1mg is approximately equal to 7000 units). The human serum albumin was supplied by the Swiss Red Cross, and bovine serum albumin (fraction V) was from Sigma, St. Louis, MO, U.S.A.

Semisynthetic [*Phe*^{B1}-^{3}H]insulin at sp. radioactivity 1–10Ci/mmol was prepared by the method of Halban & Offord (1975) with the modifications reported by Halban *et al.* (1976). The stock solutions of insulin were, typically, at a concentration of 2×10^6d.p.m./ml (as determined in a liquid-scintillation counter) in 0.2M-glycine/HCl containing human serum albumin at 0.25% (w/v), pH8.8.

For assays of degradation, 50μl of stock solution of [^{3}H]insulin was added to 1.5ml of Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) in an incubation vial. The final concentration of [^{3}H]-insulin in the vial is 70nM. The medium contained in

addition glucose (1 mg/ml) and bovine serum albumin (0.5 mg/ml). Six pieces of fat with a total weight of 250 mg were added to the incubation mixture. The fat was removed only from the perirenal region of male rats, immediately after decapitation. The rats were of approx. 400 g weight and had been fed *ad libitum*. We were careful to cut the fat into pieces that were as nearly equal in size and shape as possible. Control experiments (M. Berger, unpublished work) showed that the fat obtained in this way was metabolically normal in its ability to release non-esterified fatty acid and glycerol, and that the release responded normally to the addition of insulin and noradrenaline. When required for the insulin-degradation assays, other substances (e.g. potential inhibitors, non-radioactive insulin, NaCl) could be added to the incubation vials, just before the [^{3}H]insulin, either as the solid or as concentrated solutions. Incubation was carried out for 60 min at 37°C with continuous gassing by O_2/CO_2 (19:1, v/v) at 2 litres/min. It was commenced immediately after the addition of the [^{3}H]-insulin. Preliminary experiments had shown that degradation approaches, but does not quite reach, its maximum extent after 60 min, and that approx. 50% of the insulin was degraded. Between 94 and 97% of the initial radioactivity remained in the supernatants.

At the end of the incubation the liquid was drawn off and frozen to await analysis for the extent of degradation of insulin. Analyses were carried out in the gel-filtration system described by Halban *et al.* (1976, 1978*b*; Berger *et al.*, 1978). This system employs a column (0.8 cm × 70 cm) of Sephadex G-50 eluted at 20 ml/h with 0.2 M-glycine/HCl containing 0.25% (w/v) human serum albumin, pH 8.8. It gives three radioactive peaks, eluting at positions corresponding to molecules substantially greater in size than insulin, to molecules of the same apparent size as insulin and to molecules substantially smaller than insulin. This pattern, and the characterization of the individual peaks, has been described previously (Halban *et al.*, 1976, 1978*b*; Davies, 1978). Briefly, the conclusions reached so far are that the large molecules cannot be converted into material of smaller molecular weight by disaggregating agents, and they lack the immunoreactivity of insulin. The material of intermediate size co-electrophoreses with insulin and has its immunoreactivity. The small molecules behave electrophoretically and chromatographically as though they were of the same size as, or only a little larger than, single amino acids. We have never found any material with the electrophoretic or chromatographic properties of the free B-chain or its oxidized homo- or hetero-polymers. The large molecules do not release B-chain on performic acid oxidation (J. G. Davies & R. E. Offord, unpulished work). These results suggest that both the large and the small molecules are the result of covalent chemical processing of the insulin, and we have taken the percentage of the total radioactivity found in these two peaks, determined by liquid-scintillation techniques, as a measure of the extent of the degradation. (Although much remains to be done in the characterization of the degradation products, the present paper is only concerned with the establishment of a system *in vitro* and its use in the search for inhibitors; for these purposes it is sufficient to establish that the method of measurement described gives a useful measure of the extent of disappearance of intact insulin.)

The [^{3}H]insulin used in these experiments gave, when the stock solutions were analysed, 98 ± 2% (mean ± S.D.) of the radioactivity in the peak corresponding to insulin; this percentage was not significantly different after blank incubations carried out exactly as above, but with the fat omitted. Glutathione (1.5 mM) does not give rise to detectable degradation in the absence of fat.

Preliminary experiments had shown that all samples of rat adipose tissue, irrespective of their anatomical origin and shape, degraded insulin in the test system and that the degradation was subject to the inhibition described below. But the precautions described in the Materials and Methods section were necessary to minimize variability in the rates of degradation from experiment to experiment.

Results and Discussion

Table 1 shows the extent of degradation observed under various conditions. We conclude from these results that Trasylol and ophthalmic acid both inhibit the degradative activity that we observe in our model system. The decrease in the extent of degradation on addition of unlabelled insulin is substantial even at a concentration of insulin markedly lower than the concentration of serum albumin that was present in all the incubations. There is some support for the idea of a glutathione-linked component in the degradative system by the observation, which parallels those of Varandani (1973) in homogenates of rat liver, that glutathione increases the degree of degradation only when the fat is present. This increase is only slightly decreased by concentrations of Trasylol that would be strongly inhibitory when present alone; at 2200 kallikrein units/ml the degradation in the presence of reduced 3 mM-glutathione is still 136% of that in the standard incubation mixture; at 4400 units/ml and 3 mM-glutathione it is 132% of that in the standard incubation mixture. By contrast, the stimulation of degradation brought about by glutathione is overcome by ophthalmic acid. The degradation in the presence of 1.5 mM-glutathione is 122% of that of the standard incubation; the value is returned to that of the standard incubation if ophthalmic acid (30 mM) is present in addition to the glutathione.

Table 1. *Degree of degradation after 1 h, expressed as a percentage of that observed in the standard incubation mixture*

The relative value of 100% corresponds to an absolute extent of degradation of approximately 50%. Thus the value in the presence of 10mM-glutathione represents almost total destruction of the insulin. The numerical values are means for two or more sets of duplicates. In no case was the spread of the values more than ±7% of the mean. In all incubations with fat present, the ratio of the radioactivity in the first peak to that in the third peak of the column effluent (see the text) was approximately constant at about 1:5 in favour of the latter peak.

Standard supplement to the incubation mixture	Degree of degradation (%)
Ophthalmic acid (15mM)	72
Ophthalmic acid (60mM)	43
NaCl (60mM)	100
Trasylol (660units/ml)	84
Trasylol (3300units/ml)	66
Trasylol (6600units/ml)	52
Reduced glutathione (0.75mM)	124
Reduced glutathione (1.5mM)	150
Reduced glutathione (3mM)	176
Reduced glutathione (10mM)	190
Unlabelled insulin (1.4μM)	46
Unlabelled insulin (7μM)	31

The way is now clear for the further biochemical characterization of the activities observed, and the further study of the inhibition. It would also be most desirable to relate these activities to the insulin-degrading systems described in isolated tissues by Mirsky (1957), Tomizawa & Halsey (1959) and subsequent authors, as well as to that described by ourselves at the site of subcutaneous injection of insulin in the whole animal (Halban *et al.*, 1978*a*; Berger *et al.* 1979).

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