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LOCALIZATION OF [^{125}I]-INSULIN IN SUBCELLULAR PARTICLES OF PERFUSED RAT LIVER

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SUMMARY: Rat livers were cyclically perfused for 30 min at 23°C with physiological concentrations of [^{125}I]-insulin, the mitochondrial-lysosomal fraction was isolated and subjected to sucrose density gradient centrifugation. A peak of radioactive material, similar in size to native insulin, co-migrated with the peak of lysosomal marker enzyme on density gradients. The uptake of this material was blocked at low temperatures and Triton WR-1339 pretreatment failed to alter its distribution on the gradient. These findings indicate that the radioactive material in the peak represents [^{125}I]-insulin, (or large fragments) that is associated with vesicles which compare with lysosomes in density but differ in Triton-storing capability.

INTRODUCTION: The synthesis and secretion of insulin as well as its interaction with the cell membrane have been studied intensively while much less attention has been given to the mechanisms for disposal of the hormone. Mortimore and Tietze (1,2) originally suggested that insulin was internalized by liver cells by a process separable from membrane binding. This was based on the observations that a 10 min lag period exists between the initial exposure of the perfused liver to insulin and the appearance of degradation products in the perfusate and that insulin degradation but not binding was completely abolished at 0°C. Terris and Steiner (3) have recently confirmed the presence of this lag period. In addition, the former authors found that the rate of insulin degradation was inversely related to the degree of tissue integrity.

Abbreviations: EDTA--ethylenediaminetetraacetic acid; TCA--trichloroacetic acid

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We were interested in pursuing these initial studies by following the fate of labeled insulin in the perfused rat liver exposed to physiological concentrations of the hormone. The results have shown that radioactive material, which co-chromatographs with intact insulin, is associated with intracellular particles having a density similar to lysosomes.

During the course of this work several publications have appeared which have demonstrated, by use of electron microscopic autoradiography (4,5) and fluorescence microscopy (6,7), the cellular internalization of insulin. In addition, the former reports have found insulin associated with lysosomes or lysosome-like structures.

MATERIALS AND METHODS:

Perfusion: Male rats of the Lewis strain (Microbiological Associates), maintained on regular laboratory chow and water ad libitum and weighing 120 to 135 g were used. Livers were cyclically perfused in situ as described earlier (7) with a medium consisting of a 27% suspension of washed bovine red cells (v/v) in a solution of Krebs-Ringer bicarbonate buffer and 4% bovine plasma albumin. Perfusion were carried out at 23°C unless otherwise noted. Following a 5 min period of temperature equilibration, [^{125}I]-insulin (New England Nuclear, Boston, MA) was added to the perfusate in concentrations which ranged from .56 to .99 nM (85 to 150 μU/ml, 0.34-0.59 μCi/ml) depending upon the measured final perfusate volume. The perfusion was continued 30 min after which the liver was flushed for 2.5 min with an ice-cold solution of 0.25M sucrose-1 mM EDTA. Other experimental modifications are described in the figure legends.

Tissue Fractionation: The liver was rapidly excised and weighed and portions then homogenized in 9 volumes of ice-cold sucrose-EDTA solution, pH 7.0 with a Dounce homogenizer. The nuclear (N) fraction was removed by centrifuging the homogenate to 6000 g·min. The nuclear pellet was rehomogenized and centrifuged. The supernatants were combined and the mitochondrial-lysosomal fraction prepared by centrifuging to 160,000 g·min. The mitochondrial-lysosomal pellet was resuspended in sucrose-EDTA solution, layered on a linear sucrose density gradient (1.350 to 1.021 g/ml), and centrifuged to 1.542 x 10^6 g·min.

Analytical Procedures: For measurement of insulin degradation during perfusion aliquots of perfusate were collected at timed intervals and trichloroacetic acid (TCA) added. The final concentration of TCA was 10%. TCA-soluble radioactivity was determined using a Packard Autogamma counting system. Gradient fractions (0.5 ml) were collected into either iced 0.10% Triton X-100 for enzyme assays or 20% TCA for determination of TCA precipitable radioactivity (10% TCA, final concentration). *N*-acetyl-β-D-glucosaminidase was assayed according to the method of Barrett (8) as modified by Woolen et al (9). Glucose-6-phosphatase was measured by the procedure of Nordlie and Arion (10), citrate synthase by the method of Shepard and Garland (11), 5'-nucleotidase by the method of Morre' (12) and protein as described by Lowry et al (13).

RESULTS AND DISCUSSION:

The work presented here represents preliminary experiments aimed at determining the subcellular distribution of [^{125}I]-insulin using physiological concentrations of insulin. Figure 1 shows that the perfused liver rapidly degrades [^{125}I]-insulin at 37°C, but, as previously noted (1), there is a distinct lag in the initial appearance of TCA-soluble label. At 23°C the lag

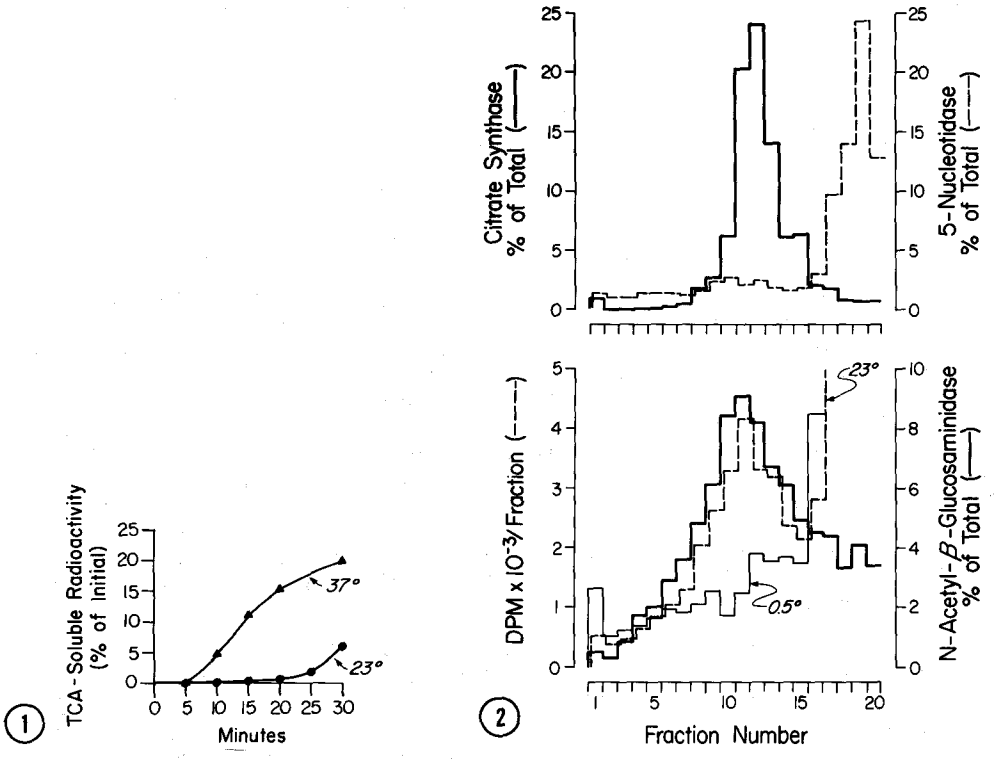

Figure 1. [^{125}I]-insulin degradation by the perfused rat liver perfused at 23° and 37°. At t_0 [^{125}I]-insulin was added to the perfusate in amounts calculated to achieve perfusate insulin concentrations of 6.7 x 10^{-10} M. Aliquots were collected at the indicated times and the intact insulin precipitated in 10% TCA. The results are expressed as the percent of the initial (t_0) TCA-precipitable radioactivity solubilized.

Figure 2. Top panel - Distribution of marker enzymes for mitochondria (citrate synthase) and plasma membrane (5'-nucleotidase) on sucrose density gradients. Data expressed as percent of total enzyme activity on the gradient. Bottom panel - Distribution of lysosomal marker enzyme (β-*N*-acetyl-glucosaminidase) and TCA-precipitable radioactivity on sucrose density gradients. Enzyme activity is expressed as a percent of total enzyme activity on the gradient. Radioactivity is expressed as disintegrations per min x 10^{-3} per fraction. The dashed line represents the distribution of radioactivity observed following perfusion of livers with 6.7 x 10^{-10}M[^{125}I]-insulin at 23°C. The lower thin solid line denoted 0.5° represents the distribution of radioactivity seen when livers are perfused at a temperature of 0.5°C.

is greatly increased and the overall rate of degradation diminished. However, the time-courses are qualitatively similar indicating that [^{125}I]-insulin had reached intracellular sites of degradation in both conditions (1-3). When M+L fractions from livers perfused at 23°C for 30 min were subjected to sucrose density gradient centrifugation, a peak of TCA-precipitable radioactivity was associated with the peak of lysosomal marker enzyme (Figure 2, bottom panel). Since this peak was not as reproducible at 37°C, 23°C was used for the initial characterization of the gradient. Experiments employing different perfusion times and temperatures are now in progress.

Figure 2 top panel, shows the distribution of marker enzymes for mitochondria (cytochrome oxidase) and plasma membrane (5'-nucleotidase) on linear sucrose density gradients. Glucose-6-phosphatase, a marker enzyme for rough and smooth endoplasmic reticulum, gave a distribution pattern similar to 5'-nucleotidase (data not shown). The lower panel of Figure 2 indicates the distribution of trichloroacetic acid (TCA) precipitable radioactivity on these gradients following perfusion of rat livers with physiological concentrations (85-125 μU/ml) of ^{125}I-insulin. At a perfusion temperature of 23°C, a peak of TCA-precipitable radioactivity was observed which coincides with the peak of lysosomal marker enzyme. When the perfusion was carried out at 0.5°, a temperature at which insulin binding occurs but cellular internalization does not (1), the radioactive peak was abolished without affecting the distribution of lysosomal enzyme. In addition, in separate control experiments we have determined that [^{125}I], either added during perfusion as [^{125}I] NaI or to homogenates as [^{125}I]-insulin, does not associate with the particulate fraction on subsequent sucrose density centrifugation. These experiments demonstrate that following the uptake of [^{125}I]-insulin by intact cells in the perfused liver, radioactive material becomes closely associated with what are presumably vesicles having a density corresponding to lysosomes. Furthermore, perfusion with [^{125}I]-NaI ruled out the possibility of a transfer of the [^{125}I] to other proteins associated with structures in the peak region of the gradient.

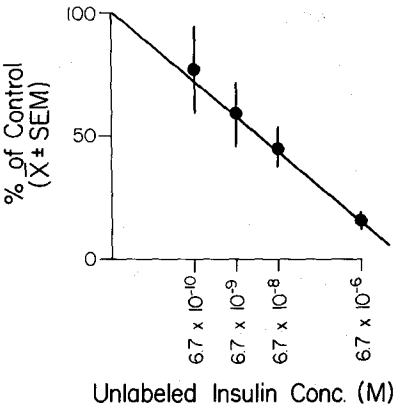

Figure 3. Competitive effect of unlabeled insulin additions on radioactivity peak height of sucrose gradients. Control represents perfusates containing 6.7×10^{-10}M[^{125}I]-insulin. Data are expressed as a percent of the peak area (dpm/fraction) of the control.

Figure 3 shows that the additon of increasing concentrations of unlabeled insulin to perfusate containing a constant amount of labeled insulin results in a corresponding decrease in the amount of radioactivity in the peak region of the gradient. This suggests that one or more steps in the uptake process is saturable and possibly limited by receptor binding (3). Preliminary identification of the radioactive material found in the gradient peak was undertaken using Sephadex chromatography of extracts of the appropriate fractions (Figure 4). Most of the recovered radioactive material co-migrated with the insulin marker, but the uneveness of the trailing edge of the peak suggests the presence of some intermediate-size products. Although we have not assessed the biological activity of the extracts, it would appear that we are dealing with a population of vesicles that does not possess significant insulin degrading activity.

Another finding which brings lysosomal involvement into question is presented in Figure 5. Animals were pretreated with Triton WR-1339, a detergent which accumulates within lysosomes and decreases their density (15). We found as have others (16) that more than 90% of the lysosomal marker is shifted to the extreme upper fractions of the sucrose gradient under these conditions (data not shown). Suprisingly, however, the radioactive peak did not move

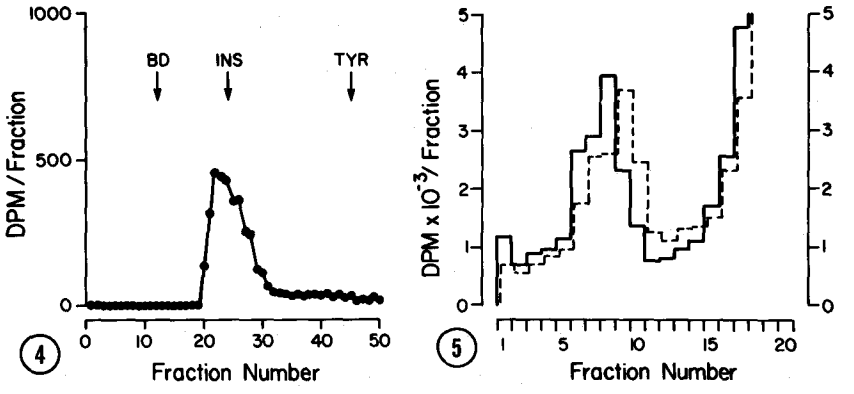

Figure 4 (left). Gel filtration of radioactive material extracted from gradient fractions 8,9 and 10. Pooled fractions were extracted in 6 M urea, 3 M acetic acid and 0.15 M NaCl and gel-filtrated over a 0.9 x 50 cm Sephadex G-50 column (Pharmacia Fine Chemical, Inc., Piscataway, N. J.) at flow rates of 8-10 ml/hr. The columns were calibrated with blue dextran (BD) as a void volume marker, native insulin (INS) and tyrosine (TYR) to indicate internal volume.
Figure 5 (right). Distribution of TCA-precipitable radioactive on sucrose density gradients following perfusion of livers with 6.7 x 10^{-10}M[^{125}I]-insulin at 23°C. The solid line represents control and the dashed line Triton WR-1339 pretreated animals. Sucrose densities were lowered to accomodate the expected decrease in lysosomal density.

(Figure 5). This would be so if the radioactive material were associated with vesicular structures that had not yet fused with lysosomes. Alternatively, given the heterogeniety of lysosomes (17,18), the radioactivity may be associated with a sub-population of lysosomes which have not accumulated Triton WR-1339 and in which proteolytic activity has not been fully expressed. Davies (19) has in fact reported that newly formed vesicles do not appear to fuse with Triton WR-1339 containing lysosomes.

At the present time there is a considerable amount of evidence favoring the internalization of the insulin molecule following its binding to the cell membrane (1-7), but there is a difference of opinion as to the localization of the molecule once it is within the cell. Bergeron and colleagues (5) report that insulin is localized primarily in Golgi vesicles whereas Carpentier and co-workers (4) found insulin in association with lysosomal elements. Our data indicate that insulin, or insulin-derived materials, appears in a vesicular structure having a density corresponding to lysosomes, but which does not accumulate Triton WR-1339. Also the fact that much of the extracted material has a molecular size comparable to native insulin suggests

that the insulin is not within a particle capable of degrading insulin rapidly as would be expected of the typical lysosome. The work of Pertoft et al (17) and Rome et al (18) is of interest in that they have shown a time-dependent redistribution of protein taken up by receptor-mediated endocytosis within lysosomal particles of lesser to those of greater density. If indeed endocytotic vesicles rapidly fuse with the GERL apparatus as has been proposed (20), one might expect insulin to be associated initially with Golgi-like vesicles that also have some lysosomal characteristics. It is obvious that we are dealing with a highly complex system of organelles and considerable caution will have to be used in interpreting the identity of any of the constituents.

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