Application of *O*-(4-diazo-3-[^{125}I]iodobenzoyl)sucrose for the detection of the catabolic sites of low density lipoprotein

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The sites of degradation of human low density lipoprotein (LDL), are analyzed using the novel labelling compound *O*-(4-diazo-3-[^{125}I]iodobenzoyl)sucrose (D^{125}IBS). The decay from rat serum of D^{125}IBS-labelled LDL is identical to the serum decay of conventionally iodinated (ICl method) LDL. The radioactivity derived from D^{125}IBS-labelled LDL accumulates predominantly in the liver after intravenous injection and the hepatic radioactivity remains associated with the lysosomal compartment for an extended period of time, when compared to the radioactivity derived from conventionally iodinated LDL. It is concluded that the D^{125}IBS labelling procedure is an interesting new tool to study the sites of catabolism of serum lipoproteins.

Low density lipoprotein *Catabolic site* *Methodology* *Liver* *Iodination*
O-(4-Diazo-3-[^{125}I]iodobenzoyl)sucrose

1. INTRODUCTION

Recently a number of labelling compounds were developed specifically designed to study the catabolic sites of serum proteins [1–4]. These compounds contain either a sucrose [1,2], a raffinose [3] or a cellobiose [4] moiety, are not or only slowly degraded by lysosomal enzymes and do not readily pass through lysosomal and cellular membranes [5]. Thus, when such a radioactive molecule is attached to a serum protein, and the labelled protein is subsequently injected intravenously, radioactivity will accumulate within the cells catabolizing this protein. Here, the application of the D^{125}IBS label for the study of the sites of catabolism of LDL in the rat is evaluated.

2. MATERIALS AND METHODS

LDL was isolated from human plasma by sequential ultracentrifugation in a Beckman 50.2 Ti rotor in the non-protein solvent density interval of 1.019–1.063 g/ml, washed once and dialyzed extensively against saline, containing 1 mM EDTA (pH 7.4), prior to radioiodination with ^{131}I using the ICl method as in [7]. *O*-(4-Aminobenzoyl)-sucrose was radioiodinated as in [2]. 500 μl of a solution of *O*-(4-amino-3-[^{125}I]iodobenzoyl)sucrose (containing 6.5 nmol) was converted to D^{125}IBS by mixing with 20 μl of 1 M $NaNO_2$ and 20 μl of 1 N HCl for 1 min at 0°C. The reaction was stopped by the addition of 50 μl of 1 M $NaHCO_3$. 500 μl of this mixture was added to 300 μl LDL containing 0.15 mg protein in 0.5 M Na-borate buffer (pH 10.0) and kept at 0°C for 1 h. The coupling reaction was stopped by addition of 5 μl of 2% NaN_3 solution. After 1 h at 37°C, the LDL-bound D^{125}IBS was separated from the mixture by chromatography on a Sephadex G-50 column equilibrated with 0.15 M NaCl containing 8 mM phosphate, pH 7.4.

In all metabolic studies both D^{125}IBS-labelled LDL and conventionally iodinated LDL (not the same molecule), labelled with ^{131}I using the ICl method, were investigated simultaneously. The

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Abbreviations: LDL, low density lipoprotein; DIBS, *O*-(4-diazo-3-iodobenzoyl)sucrose

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rats (male, Wistar), weighing 250–300 g, maintained on standard rat chow, were fasted for 18–24 h prior to each experiment. All radioactive LDL preparations were 'screened' in vivo before use in metabolic studies. For this purpose 18–24 h fasted rats were injected intravenously with 1–2 ml radioiodinated LDL (0.15–0.30 mg protein). The blood was collected 1 h after injection, serum was isolated and used immediately for the actual experiment. At different time intervals after injection of the labelled proteins the blood was collected and a number of tissues (heart, lungs, liver, spleen, kidneys, jejunum, fat pad, muscle, hide, adrenals and testes) were excised, weighed and counted for radioactivity.

After bleeding of the rats a 20% (w/v) liver homogenate was made in 0.25 M sucrose using a Potter-Elvehjem homogenizer with a Teflon pestle and the homogenate was fractionated by differential centrifugation according to [8]. Average recovery of cathepsin activity, protein and radioactivity in the subcellular fractions from the homogenate were 109, 93 and 98%, respectively. Cathepsin activity was measured from the rate of production of radioactive amino groups from HDL-apoprotein at pH 3.8 [9]. The lipid-associated radioactivity of iodinated LDL was 5.8 ± 2.9% or 4.7 ± 1.4% (means ± SD for 3 separate preparations) for conventionally iodinated or D^{125}IBS-labelled LDL, respectively. Screening decreased these values by about 50%. The remaining LDL radioactivity was quantitatively precipitated using tetramethylurea. Na^{125}I (350–600 mCi/ml) and Na^{131}I (40 mCi/ml), both carrier free, were obtained from Amersham International, Amersham, England.

3. RESULTS

Monoexponential serum decays were observed after intravenous injection of both D^{125}IBS- and ^{131}I-labelled human LDL (not shown). The serum half-lives of both radiolabelled LDL preparations were identical (9.5 h; average value for two experiments for each label).

In fig.1 the tissue accumulation of radioactivity derived from the DIBS-labelled and the conventionally iodinated LDL are shown for the liver and the kidneys. The kidneys are an example of a tissue in which the percentage of the injected dose of

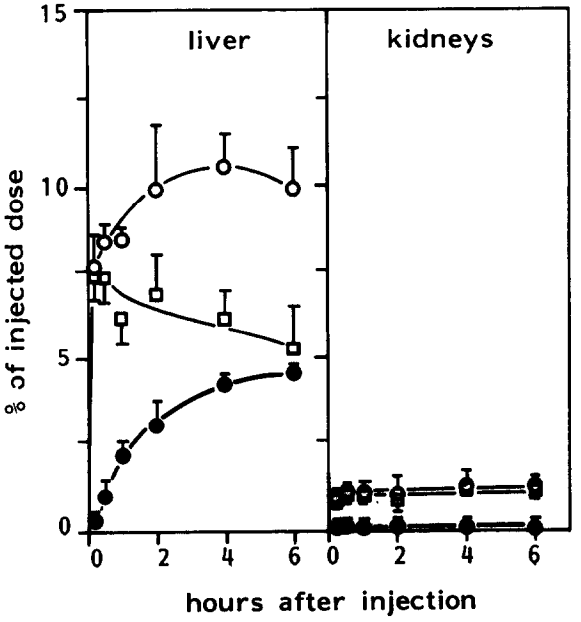

Fig.1. Accumulation of intravenously injected D^{125}IBS-labelled LDL (○) and ^{131}I-iodinated LDL (□) in the liver and the kidneys. At different time intervals after injection, blood was collected and a number of tissues, among others the kidneys and the liver, were excised, weighed and counted for radioiodine. The DIBS-dependent accumulation of radioactivity (●) in each tissue at each individual time-point was calculated as described in section 3. Mean values ± SD for 3 experiments are presented.

D^{125}IBS- and ^{131}I-labelled LDL is not statistically different at any time point. However, in the liver we see, after an initial sharp rise for both labels, a decrease of the radioiodine derived from the conventionally labelled LDL, while the radioactivity derived from the DIBS-labelled LDL continues to increase. Subtraction of the hepatic radioactivity derived from the conventionally iodinated LDL (% of injected dose) from the % of the injected dose coming from DIBS-labelled LDL results in a calculated value which we have called the DIBS-dependent accumulation of radioactivity. The only tissue besides the liver that showed any DIBS-dependent accumulation of LDL radioactivity was the spleen. 6 h after injection this tissue 'accumulated' only 0.6 ± 0.1% of the injected dose, while 4.6 ± 0.2% accumulated in the liver.

To test if the DIBS-dependent accumulation is a measure of radioiodine present within the

lysosomal compartment, the liver was analyzed by subcellular fractionation 30 min and 4 h after injection of labelled LDL. As is evident from fig.2, most of the radioactivity (derived from both D^{125}IBS and ^{131}I) was recovered in the soluble fraction of the liver shortly (30 min) after injection. In contrast, 4 h after injection of labelled LDL a considerable part of the radioactivity derived from D^{125}IBS-labelled LDL was recovered in the lysosomal fraction, while little radioiodine derived from the conventionally iodinated LDL was found in this fraction. The .DIBS-dependent accumulation 4 h after injection of the labelled LDL (fig.2, bottom) therefore occurred intracellularly in association with the lysosomal compartment. Fig.2 also shows the distribution of a lysosomal marker enzyme (cathepsin) for comparison. Other

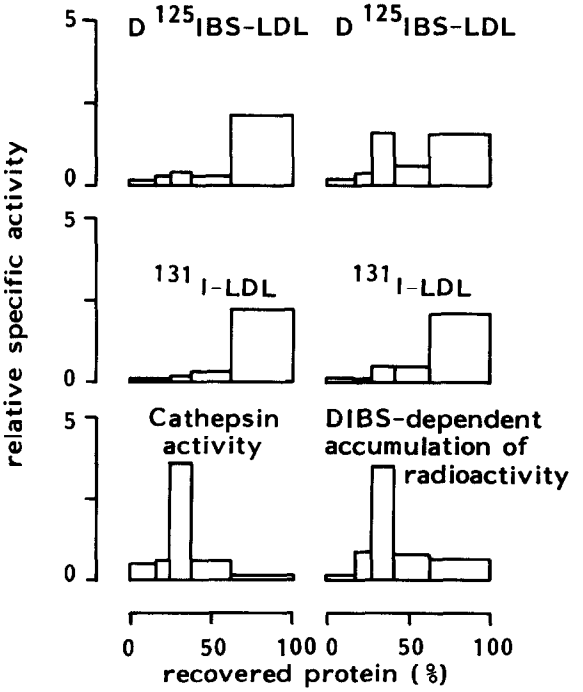

Fig.2. Subcellular fractionation of the liver 30 min (left panel) and 4 h (right panel) after intravenous injection of D^{125}IBS-LDL and ^{131}I-LDL. Blocks from left to right represent subcellular fractions in the order in which they were isolated: nuclear, mitochondrial, light mitochondrial or lysosomal, microsomal and final supernatant fractions. The relative protein content is given on the abscissa. The ordinate gives the relative specific activity (% of total recovered activity divided by % of total recovered protein).

lysosomal marker enzymes, e.g., acid phosphatase and deoxyribonuclease, showed a similar distribution as cathepsin, while the distribution of the microsomal (glucose-6-phosphatase), mitochondrial (glutamate dehydrogenase) and plasma membrane (5′-nucleotidase) marker enzymes were identical to those described in [8] (not shown).

4. DISCUSSION

Here the DIBS-labelling technique was evaluated for the study of the tissue sites of catabolism of LDL in the rat. In our hands the DIBS labelling was a simple and reliable procedure with an efficiency of labelling of all preparations of approx. 10%. The serum decay of DIBS-labelled LDL in the rat was identical to the serum decay of LDL labelled with the ICl method, indicating that the DIBS label does not influence the in vivo turnover of LDL. The serum decay of human LDL in the rat has a half-life of approx. 9.5 h. As a consequence a large fraction of LDL is still present in the circulation 4–6 h after injection. Moreover, the intravenously injected LDL will equilibrate with the extravascular space. To study the intracellular accumulation of radioiodine in tissues it is essential to correct for all extracellular label, including that present in interstitial fluid and bound to the outside of the plasma membrane. It is however extremely difficult to remove all serum proteins from, e.g., the liver, even by extensive perfusion of this organ [10,11]. It was therefore decided to apply a mathematical correction for the radioiodine present extracellularly. The D^{125}IBS-derived tissue radioactivity represents the sum of extracellular radioactivity and intracellularly accumulated radioactivity. The ^{131}ICl-derived label represents predominantly extracellular radioactivity. By subtracting the ^{131}I value from the ^{125}I value (both as % of the injected dose) one obtains a value for the intracellular accumulation of degradation products of the labelled serum protein. This was named the DIBS-dependent accumulation. This value may underestimate the intracellular accumulation to some extent since a small amount of ^{131}I is present inside the cells and since a small amount of ^{125}I leaks out of the cells during a 4–6 h experiment. Because serum LDL is bound in a reversible way to plasma membrane receptors, it can be expected that LDL present in

contaminating blood, as well as LDL bound extracellularly, will be recovered in the soluble fraction after subcellular fractionation. The hepatic radioactivity derived from labelled serum LDL was indeed almost quantitatively recovered in the soluble fraction if measured 30 min after injection (see fig.2), indicating that the radioactivity was not associated with any intracellular organelle. 4 h after injection the fraction of the radioactivity derived from the D^{125}IBS-labelled LDL equivalent with the DIBS-dependent accumulation was associated with the lysosomal compartment (fig.2). This indicates that the calculated value called the DIBS-dependent accumulation of radioactivity represents the lysosomal accumulation of the catabolic products of injected DIBS-labelled LDL.

Previously LDL catabolism has been studied using [^{14}C]sucrose label [1,12]. It was found that the liver was the most active organ for LDL degradation. This study provides further evidence for the important role of the liver in LDL catabolism. A major advantage of the DIBS-labelling procedure for the study of catabolic sites is the radioisotope used. Radioiodine is generally used for the labelling of serum lipoproteins and the metabolism of conventionally ^{131}I-iodinated and D^{125}IBS-labelled proteins can be compared directly in a double label experiment.

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