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Quantification of the accumulation and degradation of β-very-low-density lipoproteins *in vivo* using a ^{19}F-containing residualizing label and n.m.r. spectroscopy

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β-Very-low-density lipoproteins (β-VLDL) were conjugated to the ^{19}F-containing residualizing label, *NN*-dilactitol-3,5-bis(trifluoromethyl)benzylamine (DLBA), to determine whether the metabolism of this lipoprotein fraction could be characterized *in vivo* with n.m.r. spectroscopy. Solution state ^{19}F high-resolution n.m.r. spectroscopy of DLBA–β-VLDL, containing either intact apoproteins or selectively enzymically digested products, demonstrated that the extent of degradation could be distinguished by differences in spin–spin relaxation times (T_2 times). DLBA–β-VLDL was injected intravenously into rabbits, and accumulation of ^{19}F in hepatic tissue was quantified non-invasively by n.m.r. spectroscopy 5 and 30 h after injection. In addition to quantifying the accumulation of DLBA–β-VLDL in heptatic tissue, a marked decrease (approx. 100 Hz) in the linewidth of ^{19}F resonance from labelled lipoproteins was observed at 30 h compared with the 5 h interval in continuously monitored animals. The change in linewidth was consistent with a decrease in molecular size that occurred during protein degradation, resulting in increased T_2 times. To demonstrate that T_2 times can be used as an index to quantify apoprotein degradation *in vivo*, relaxation measurements were performed on livers excised 20 h after injection of DLBA–β-VLDL into rabbits. Two molecular motional fractions were revealed by relaxation profiles representing either an intact or an extensively degraded form of apoprotein. The amplitudes of each component were compared with results from trichloroacetic acid precipitation of liver homogenates acquired from rabbits 20 h after injection of β-VLDL labelled with the radioiodinated analogue of DLBA, dilactitol-^{125}I-tyramine. The amount of degraded apoprotein determined by n.m.r. spectroscopy and acid precipitation was $68.6 \pm 7.0\%$ and $58.7 \pm 7.5\%$ ($n = 4$) respectively. The results of this study demonstrate that ^{19}F n.m.r. spectroscopy can be used to define the temporal characteristics of the hepatic metabolism of lipoproteins *in vivo* by quantifying both the tissue-specific accumulation and extent of apoprotein degradation. The methodology developed offers promise for the non-invasive, sequential and longitudinal evaluation of lipoprotein metabolism *in vivo*.

INTRODUCTION

Lipoprotein metabolism has been characterized extensively *in vitro* by employing cell culture, although there is much less information on mechanisms of tissue metabolism *in vivo*. The advent of radioiodinated residualizing protein labels such as dilactitol tyramine (DLT; Strobel *et al.*, 1985) and cellibiitol tyramine (TC; Pittman *et al.*, 1983) has permitted the acquisition of quantitative data for defining protein catabolism. Conventional protein labels, such as [^{3}H]leucine and ^{125}I, leak rapidly from cells following delivery by a carrier protein, which prohibits quantification of protein catabolism. In contrast, because residualizing labels remain trapped intracellularly owing to their resistance to hydrolysis, large molecular size and hydrophilicity, characterization of the sites of catabolism of lipoproteins has been possible (Pittman *et al.*, 1983; Daugherty *et al.*, 1985). Residualizing labels have been used to identify the tissue and cellular catabolic sites of a number of other proteins, including asialofetuin (Strobel *et al.*, 1985), albumin (Yedgar *et al.*, 1983; Strobel *et al.*, 1986) and immunoglobulins (Moldoveanu *et al.*, 1990). While these residualizing labels have provided a means of obtaining quantitative data, their use generally requires serial autopsy for analysis of the kinetics of tissue accumulation. Residualizing labels such as DLT and TC have been radioiodinated with either ^{131}I or ^{125}I for use with γ-radiation camera scintigraphy to permit data acquisition on tissue protein accumulation non-invasively. Although this technique can be used to demonstrate the biodistribution of protein tracers, it suffers from an inherent lack of quantification and minimal localization of the tissue of interest.

We have reported on an alternative residualizing label in which the reporter molecule contains ^{19}F, making it amenable to detection by n.m.r. spectroscopy. N.m.r. spectroscopy is a quantitative, non-radioactive and non-invasive method for detecting magnetically active nuclei *in vivo*. Given these advantages, we have developed the ^{19}F-containing residualizing label *NN*-dilactitol-3,5-bis(trifluoromethyl)benzylamine (DLBA), which can be used to study protein metabolism *in vivo* by ^{19}F n.m.r. spectroscopy (Daugherty *et al.*, 1989). The n.m.r. active nucleus, ^{19}F, was chosen for incorporation into the protein label because it is 100% naturally abundant, possesses a high relative sensitivity compared with protons (83.3%), and problems with background contamination of ^{19}F *in vivo* are minimal. Previous studies *in vivo* with rats using DLBA attached to asialofetuin (ASF) demonstrated the utility of this n.m.r. spectroscopic approach. These studies confirmed the quantitative nature of the n.m.r. experiment and demonstrated the ability of the technique to monitor protein metabolism non-invasively.

Our long-range goals are to non-invasively characterize the hepatic accumulation and degradation of lipoproteins in rabbits, a species that is commonly used to define lipoprotein metabolism and to explore the links between lipoprotein metabolism and the

Abbreviations used: DLBA, *NN*-dilactitol-3,5-bis(trifluoromethyl)benzylamine; β-VLDL, β-very-low-density lipoprotein; FT, Fourier transform; T_2, spin–spin relaxation time constant; TFA, trifluoroacetic acid; ASF, asialofetuin; DLT, dilactitol tyramine; TC, cellibiitol tyramine.

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development of atherosclerosis. Initial results are presented from the study of DLBA covalently attached to the potentially atherogenic lipoprotein fraction, β-very-low-density lipoproteins (β-VLDL). This approach demonstrated the possibility of non-invasively quantifying the accumulation of lipoproteins within the hepatic tissue of rabbits. In addition, analysis of the n.m.r. spectra revealed the feasibility of evaluating the degradation state of the apoprotein moiety *in vivo* by measuring spin–spin (transverse) relaxation times which serve to distinguish molecular species based on motional correlation times, and thus on state of degradation.

This work was presented in preliminary form at the 9th Annual Meeting of the Society of Magnetic Resonance in Medicine, New York, August 1990, and at the American Heart Association, Dallas, November 1990.

MATERIALS AND METHODS

Materials

Lactose, galactose oxidase, Pronase, human plasma thrombin, and $NaBH_3CN$ were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bovine pancreatic trypsin was purchased from Worthington (Freehold, NJ, U.S.A.). New Zealand white rabbits were purchased locally (Boswells, Pacific, MO, U.S.A.). $Na^{125}I$ and $Na^{131}I$ were obtained from Amersham Corp. (Arlington, IL, U.S.A.) and DLBA was prepared as described by Daugherty *et al.* (1989), with the following modification. Preparative isolation of DLBA was carried out by ion-exchange chromatography (Strobel *et al.*, 1985). Acidified reaction mixture (pH 4.0), diluted with water to a conductivity of no more than twice that of the equilibrating buffer (10 mM-acetic acid), was applied to a 30 cm × 2 cm DOWEX 50-X2 column. The column was washed with 10 mM-acetic acid to remove unreacted sugar, and then eluted with a 500 ml gradient of 10–500 mM-ammonium acetate (pH 7.0). Fractions of DLBA eluted with approx. 250 mM-ammonium acetate and were pooled and concentrated by rotary evaporation.

Preparation and radioiodination of β-VLDL

β-VLDL was harvested from hypercholesterolaemic rabbits fed a 2% (w/w) cholesterol-enriched diet (Daugherty & Schonfeld, 1985). The mass of lipoprotein protein was determined as described by Lowry *et al.* (1951), with BSA as a standard and ether extraction of lipids for β-VLDL samples. β-VLDL was directly radioiodinated with ^{125}I and ^{131}I using the solid-phase reactant Iodogen. Radiolabelled lipoprotein was separated from unbound radioiodide by gel filtration through Sephadex G-25 2.2 ml spin columns (Isolab, Akron, OH, U.S.A.), followed by dialysis against NaCl (0.15 M)/EDTA (1 mM, pH 8.2). The radioactive analogue of DLBA, DLT, was radioiodinated and coupled to β-VLDL as described by Daugherty *et al.* (1985).

Coupling of DLBA to β-VLDL

DLBA (4 μmol) in potassium phosphate buffer (0.2 M, pH 7.7) was oxidized at 37 °C by incubation with galactose oxidase in phosphate buffer (0.5 M, pH 7.7; total volume 0.4 ml). Galactose oxidase (14 units) was added hourly until the reaction was judged to be approximately 60% completed, as determined by the bicinchoninic acid assay for reducing sugars (McFeeters, 1980) with galactose as a standard. The period of incubation was 5–6 h. Galactose oxidase was removed from the mixture by passage through a Centricon-10 microconcentrator (Amicon, Danvers, MA, U.S.A.), and DLBA aldehyde was recovered in the filtrate after centrifugation at 4500 *g* for 30–60 min. β-VLDL (3 mg/μmol of DLBA aldehyde) was added to the incubation mixture along with $NaBH_3CN$ (40 mM) and the coupling reaction was incubated at 37 °C for 2 h. Labelled protein was separated from unbound label by dialysis against NaCl/EDTA.

Clearance of radioiodinated DLBA–β-VLDL from plasma

Normocholesterolaemic rabbits (1.0–1.5 kg) were fasted for 12 h prior to clearance studies. DLBA–^{131}I-β-VLDL and ^{125}I-β-VLDL (both approx. 400 μg of protein in 2 ml of EDTA/NaCl) were injected simultaneously via a marginal ear vein into four recipient animals. Blood was drawn into EDTA-containing tubes at the indicated intervals and plasma was separated by centrifugation; radioactivity was determined in 100 μl aliquots of plasma.

Enzymic digestion and characterization of β-VLDL

DLBA–β-VLDL was dialysed against a buffer (0.15 M-NaCl, 0.01 M-$CaCl_2$, 0.02 M-Tris, pH 7.4) at a ratio of 1:1000 (v/v) with three changes of dialysis over 4 h. Enzymic digestions of β-VLDL (1 mg) were performed by incubation of lipoprotein at 37 °C with pronase (0.02 unit), trypsin (10 μg) or thrombin (40 units). All samples, including a control of β-VLDL, were incubated for 18 h, except for the trypsin sample (3 h incubation). Samples were immediately analysed by high-resolution ^{19}F n.m.r. or delipidated according to the procedure of Herbert *et al.* (1978) in preparation for SDS/PAGE.

Delipidated samples of β-VLDL were dissolved in sample buffer containing SDS (5%, w/v), glycerol (10%, v/v), β-mercaptoethanol (5%, v/v), Bromophenol Blue (5%, v/v) and Tris (5%, v/v; 0.5 M, pH 6.8) by incubating in boiling water for 5 min. Vertical gel electrophoresis was performed using a gradient (3–20%, w/v) polyacrylamide gel. Samples subjected to SDS/polyacrylamide included β-VLDL, β-VLDL incubated with either $NaBH_3CN$ or galactose oxidase, and enzymic digestions of β-VLDL. Gels were stained with Coomassie Blue, and protein migrations were compared with those of known molecular mass standards. The electrophoretic mobilities of β-VLDL and DLBA–β-VLDL were assessed with agarose gels (1%, w/v) stained with Fat Red O as described by Papadopaulos & Kintzios (1969).

Characterization of DLBA–β-VLDL by high-resolution ^{19}F n.m.r. spectroscopy

^{19}F n.m.r. spectroscopy was performed with a Varian VXR-500 spectrometer operating at 470.3 MHz and a SIS 200/400 spectrometer operating at 188.2 MHz. Measurements of spin–spin relaxation (T_2) times were performed on intact protein and enzyme-digested samples using either the Carr–Purcell–Meiboom–Gill (CPMG; Carr & Purcell, 1954) or Hahn spin-echo (Hahn, 1950) pulse sequences with echo times (2τ) ranging from 0.3 ms to 400 ms. Measurement of T_2 times were performed twice on each sample. Chemical shifts of lipoprotein were externally referenced to trifluoroacetic acid (TFA), which was assigned a chemical shift of 0 p.p.m. More-positive shifts are shifts to higher frequency. Quantification of ^{19}F (μmol of DLBA/mg of protein) was determined under fully relaxed (magnetization) conditions by comparing peak areas of DLBA–β-VLDL with an external TFA reference of known concentration.

Fourier transform (FT) and Bayesian spectral analysis of n.m.r. data

Two methods were employed to determine the signal amplitude, or equivalently the peak area, of the ^{19}F-protein resonance from the array of spin-echoes. The first method employed the discrete FT of the time–domain signal to yield the frequency–domain spectrum. Regions of ^{19}F resonances were selected manually and correction of baseline was achieved by fitting to a fourth-order polynomial. Digitally integrated areas were

obtained using the Varian NMR software program employing Gaussian quadrature. The amplitude for each echo was plotted as a function of echo time and the resulting decay curve was analysed to obtain the exponential T_2 time constants via a non-linear least-squares curve-fitting program using a mono- or bi-exponential decay model. Comparison of the residuals, the difference between the model and the experimental data, was used as a criteria for the quality of fit to the model.

The second method for determining the amplitudes used Bayesian spectral analysis (Bretthorst, 1990). The time–domain signal obtained from a solution-state n.m.r. experiment is described by exponential decaying sinusoids that can be modelled according to eqns. (1) and (2), where f_R and f_i represent the signal functions from the real and imaginary channels, n is the number of frequencies, B_j and B_{j+n} contain the amplitude and phase of the jth resonance, ω is the frequency, and α is the exponential decay rate constant:

$$f_R(t) = \sum_{j-1}^{n} [B_j \sin(\omega_j t) - B_{j+n} \cos(\omega_j t)] \exp(-\alpha_j t) \quad (1)$$

$$f_i(t) = \sum_{j-1}^{n} [B_j \cos(\omega_j t) - B_{j+n} \sin(\omega_j t)] \exp(-\alpha_j t) \quad (2)$$

These equations represent the Bayesian quadrature n.m.r. model and were used to calculate the posterior probability of the frequencies and decay rate constants for each echo-free induction decay. The decay rate, α_j, (not the decay of the echo train envelope) includes contributions from both T_2 relaxation and magnetic field inhomogeneities. The amplitude of each free induction decay was determined after optimal estimation of the frequencies and decay rate constants and was plotted as a function of echo time. The resulting echo train envelope decay curve was analysed in the same manner as the FT method.

Non-invasive ^{19}F n.m.r. spectroscopy of liver *in vivo*

New Zealand rabbits (0.5–1.5 kg) were injected with 10 mg of DLBA–β-VLDL (approx. 1.5 μmol of DLBA) via a marginal ear vein and anaesthetized with a mixture of ketamine (100 mg/kg, intramuscular) and xylazine (60 mg/kg, intramuscular). Detection of hepatic ^{19}F content was achieved with a 4 cm-diam. surface coil used in the single-coil mode placed over the ventral surface of animals. Body temperature was maintained constant with the use of a circulating water jacket. ^{19}F n.m.r. spectroscopy was performed non-invasively on an SIS 200/400 spectrometer operating at 188.2 MHz. Signal-to-noise ratio was optimized using a pulse width of 50–60 μs (110 W) and a pulse repetition period of 0.1 s. A spectral width of 30 kHz and acquisition time of 0.068 s were used. Optimization of the static-field homogeneity was obtained by shimming on the free induction decay of tissue water protons with the ^{19}F surface-coil receiver. Spectra acquired at 5 and 30 h after injection were processed identically with a signal-to-noise enhancing time domain apodization that resulted in a 200 Hz line broadening. Estimation of signal contamination from blood was obtained using incremented intravenous doses of the vascular imaging agent perfluorotributylamine, utilizing the same n.m.r. experimental conditions above.

All procedures performed on animals had the prior approval of the Washington University Animal Studies Committee.

^{19}F n.m.r. spectroscopy of excised liver *in vitro*

New Zealand rabbits (0.5–1.0 kg; $n = 4$) were co-injected with DLBA–β-VLDL (10 mg) and ^{125}I-DLT–β-VLDL via a marginal ear vein. Animals were killed with an overdose of sodium pentobarbital 20 h after injection. Livers were perfused via the portal vein with NH_4Cl (10 mM) for inhibition of further proteolysis (Diment & Stahl, 1985), removed from the animal, and sealed in plastic wrap to retain moisture. ^{19}F n.m.r. experiments were performed on an SIS 200/400 horizontal magnet system operating at 188.2 MHz. An antenna producing a homogeneous radiofrequency magnetic field consisting of either a 2.5 cm-diam. one-turn-in-parallel saddle-shaped coil or a 2.5 cm-diam. Alderman–Grant (Alderman & Grant, 1979) was employed in the single-coil mode for spectroscopy of excised liver *ex vivo*. T_2 times were measured using either the CPMG or the Hahn spin-echo pulse sequence. Typical data acquisition parameters are as follows: spectral width, 20 KHz; acquisition time, 0.050 s; 180° pulse width, 60 μs at 110 W; range of echo times 0.3–20 ms; repetition time, 3.5 s; number of scans, 600–1200. The T_2 relaxation times were determined by the Bayesian spectral analysis method. A small section of the liver was homogenized and the percentage of degraded protein was determined by measuring the radioactivity in the soluble fraction after trichloroacetic acid precipitation.

Statistical analyses

Values reported from relaxation studies and estimates of protein degradation in excised livers are means ± S.E.M. A Wilcoxon non-parametric test was used to determine statistical significance for values reported in relaxation studies of livers.

RESULTS

Chemical and metabolic characterization of DBLA-labelled β-VLDL

To confirm that the derivatization procedure did not significantly modify the physical and chemical forms of the lipoprotein, SDS/PAGE was performed on samples of β-VLDL subjected to incubation at 37 °C with the reducing agent, $NaBH_3CN$, and galactose oxidase. There was no noticeable degradation of β-VLDL which had been incubated with or without $NaBH_3CN$ at 37 °C for 2 h. However, there was noticeable degradation of lipoprotein on treatment with large amounts of galactose oxidase (50 units/mg of protein), necessitating its removal from the labelling procedure before reductive amination of protein to DLBA. This was readily achieved by filtration through an anisotropic membrane of a microconcentrator with a molecular mass cut-off of 30 kDa.

To ensure that the DLBA-labelled particle did not have gross changes in charge brought about by derivatization of lysine residues, agarose gel electrophoresis was performed. The electro-

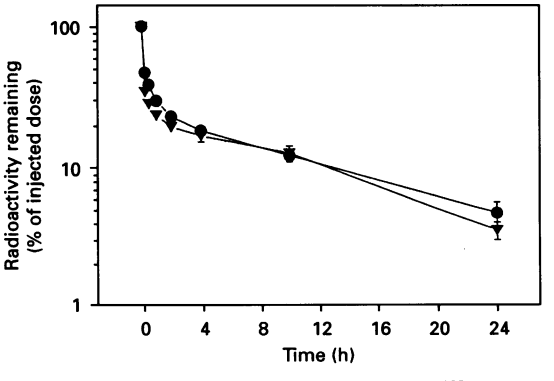

Fig. 1. Clearance of DLBA–^{131}I-β-VLDL (●) and ^{125}I-β-VLDL (▼) from plasma of normocholesterolaemic rabbits following simultaneous injection

Radioactivity was calculated at 0 min from a knowledge of injected counts and an estimate of the plasma volume. Points represent the means of four observations.

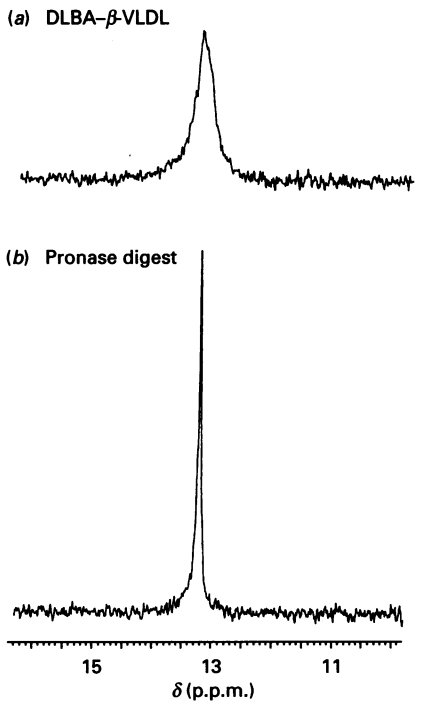

Fig. 2. High-resolution n.m.r. spectroscopy of intact and Pronase-digested DLBA-β-VLDL

Spectra were acquired under rapid-pulse-repetition conditions and referenced to an external capillary of trifluoroacetic acid. (*a*) ^{19}F n.m.r. spectrum of a solution containing DLBA conjugated to β-VLDL; (*b*) ^{19}F n.m.r. spectrum of Pronase-digested DLBA-β-VLDL.

phoretic mobility on agarose gels of intact particles of native β-VLDL and DLBA-β-VLDL were similar with substitutions of up to 0.15 μmol of DLBA/mg of protein, indicating that the surface charge density of native and labelled lipoprotein are identical (results not shown).

To determine whether the attachment of DLBA influenced the metabolic behaviour of β-VLDL, the kinetics of clearance of radioactivity from plasma after the simultaneous injection of ^{125}I-β-VLDL and DLBA-^{131}I-β-VLDL into four rabbits were measured (Fig. 1). Clearances of both the native and DLBA-labelled material were rapid and bi-exponential, as demonstrated previously (Daugherty *et al.*, 1986), and not significantly different. These data indicated that derivatization with the ^{19}F-containing residualizing label did not influence the cellular interaction of this lipoprotein.

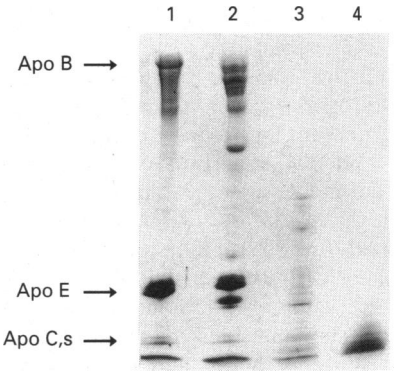

Fig. 3. SDS/PAGE of intact and digested β-VLDL

Samples were: control (lane 1), β-VLDL digested with thrombin (lane 2), β-VLDL digested with trypsin (lane 3) and β-VLDL digested with pronase (lane 4). Lanes 2 and 3 show both high- and low-molecular-mass fragments. Lane 4 (Pronase-digested sample) does not show any high-molecular-mass species. Apo, apolipoprotein.

Characteristics of high-resolution n.m.r. spectra of DLBA-β-VLDL

The high-resolution n.m.r. spectrum of DLBA-β-VLDL demonstrated a broad resonance ~ 13 p.p.m. to lower shielding (higher frequency) from an external reference of TFA (Fig. 2*a*). Quantification of ^{19}F/mg of protein by n.m.r. spectroscopy methods yielded a mean of 0.1–0.15 μmol of DLBA/mg of β-VLDL. Shown in Fig. 2(*b*) is the n.m.r. spectrum of DLBA-β-VLDL that was extensively digested during incubation with Pronase. The linewidths at half-height of intact and digested protein are ~ 200 and 40 Hz respectively. An important measurable n.m.r. parameter that influences the linewidth of an n.m.r. resonance is the T_2 time constant. This constant is inversely proportional to the linewidth of an n.m.r. resonance in the absence of dominating magnetic field inhomogeneities. Also, under most solution state conditions the T_2 time constant is inversely proportional to the molecular correlation time, τ_c, which is an indication of the time scale for reorientation and thus size. In general, shorter T_2 times generally reflect slower molecular motions, and thus longer τ_c times. Consequently, DLBA-β-VLDL was digested with thrombin, trypsin or Pronase to yield fragments of protein with selected molecular sizes as described by Cardin *et al.* (1984). Incubation of β-VLDL with thrombin, trypsin or Pronase resulted in progressive increases in the extent of degradation (Fig. 3). T_2 times of the digested fractions were analysed by both FT and Bayesian methods, which yielded similar results for all samples. The T_2 times obtained by either FT or Bayesian analysis of data from the low-molecular-mass

Table 1. T_2 times of intact and digested DLBA-β-VLDL

Measurements of T_2 times and the procedure for digestions are described in the Materials and methods section. T_2 times reported are the ranges of two measurements on each sample. Results are given for the Bayesian method of analysis. N.A., not applicable.

Sample	Relaxation decay	Short T_2 (ms)	Long T_2 (ms)	Amplitude of short T_2 (%)	Amplitude of long T_2 (%)
Intact DLBA-β-VLDL	Mono-	1.36–1.56	N.A.	100	N.A.
Intact DLBA-β-VLDL*	Mono-	3.34–5.05	N.A.	100	N.A.
Thrombin-digested DLBA-β-VLDL	Bi-	1.10–2.16	121–166	16.7–27.5	72.5–83.3
Trypsin-digested DLBA-β-VLDL	Bi-	4.16–5.22	82–100	6.9–24.5	75.5–93.1
Pronase-digested DLBA-β-VLDL*	Mono-	N.A.	279–288	N.A.	100
DLBA	Mono-	N.A.	355–384	N.A.	100

* Relaxation measurements were made on intact and Pronase-digested samples from a separate lot of labelled β-VLDL.

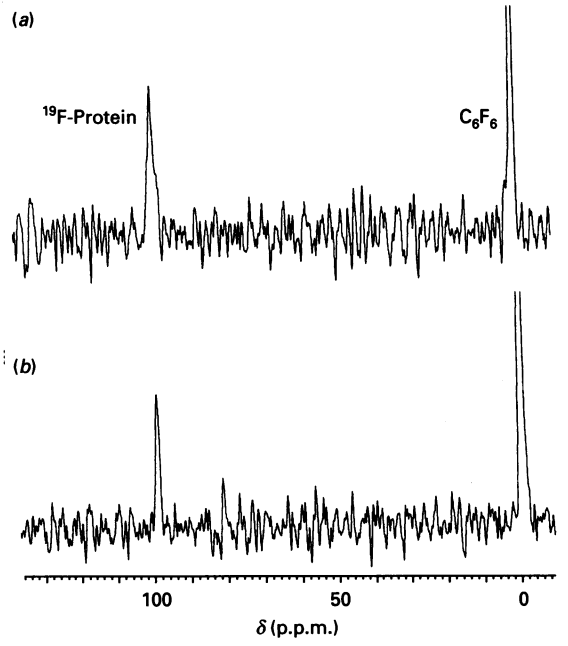

Fig. 4. N.m.r. spectra of ^{19}F-protein, obtained non-invasively from the liver by surface-coil detection following injection of DLBA–β-VLDL (10 mg) into a rabbit

Spectra represent 30 min acquisition time (two successive 15 min time domain signals added together using VNMR software). The ^{19}F-apoprotein resonance is referenced to an external capillary of hexafluorobenzene. Spectra were processed identically. (*a*) Spectrum acquired 5 h after injection of DLBA–β-VLDL; (*b*) spectrum acquired 30 h after injection of DLBA–β-VLDL.

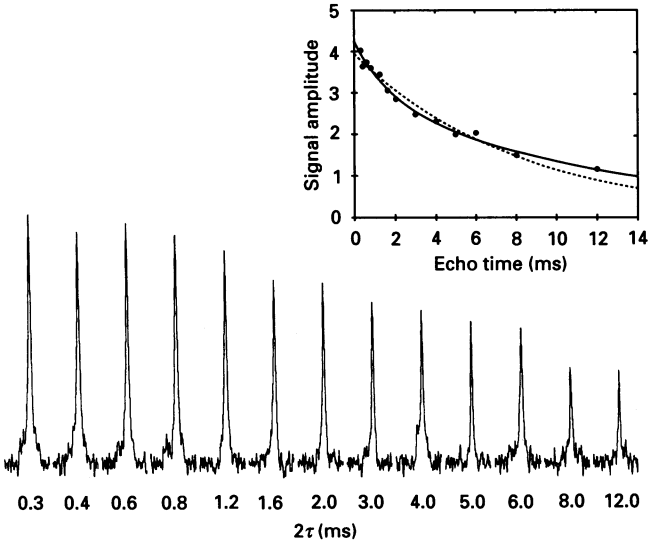

Fig. 5. N.m.r. spectra from a Hahn spin-echo experiment of ^{19}F in livers excised 20 h after injection of ^{19}F-DLBA–β-VLDL

Spectra are plotted as a function of echo time (2τ). The inset is a plot of the signal amplitude determined by Bayesian analysis of the time domain signal versus the echo time (●). The solid line represents data fitted to a biexponential decay, and the broken line indicates a monoexponential fit.

unbound DLBA label and the Pronase-digested sample best fit the monoexponential model, indicating the presence of one molecular species or a population of similarly sized molecules with a long T_2 time (~ 300 ms). Bayesian analysis of data from intact samples also revealed that intact labelled protein consisted of one dominant population of molecules with a short T_2 time (~ 2 ms). T_2 measurements were performed on two different sample preparations of β-VLDL (i.e. isolation of β-VLDL from different rabbits followed by labelling with DLBA), of which both demonstrated monoexponential behaviour upon either FT or Bayesian analysis. Both methods of analysis of data from thrombin and trypsin samples gave a biexponential model, with two distinct T_2 times in the ranges of 1–5 ms and 80–170 ms. The detection of two T_2 times is indicative of the presence of two distinct size clusters of ^{19}F-labelled protein fragments distinguished by a long and a short T_2 time (Table 1).

Measurements of T_2 times for intact and Pronase-digested samples were also performed at the lower field strength (188.2 Mz) used for *in vivo*/*in vitro* studies, to ensure that the increase in T_2 times upon digestion observed at 470.3 MHz would also be present at 188.2 MHz. T_2 times for the intact lipoprotein and the Pronase-digested moiety were 1.5–1.7 ms and 105–126 ms respectively at 470.3 MHz; the same samples exhibited T_2 relaxation times of 2.0–2.3 ms (intact) and 185–204 ms (digested) at 188.2 MHz.

Detection of DLBA–β-VLDL metabolism in tissues

The monitoring of the accumulation and degradation of DLBA–β-VLDL in rabbit liver *in vivo* was achieved non-invasively using surface-coil ^{19}F n.m.r. spectroscopy. After intravenous administration of DLBA–β-VLDL (10 mg of protein; ~ 1.5 μmol of DLBA). ^{19}F n.m.r. spectra were acquired at ~ 5 and 30 h, with data accumulation times of 15 min (Fig. 4) and a fluorine–protein signal observable within 10 min. Fluorine resonances from the DLBA-labelled lipoprotein were 98 p.p.m. less shielded (higher frequency) from an external reference of hexafluorobenzene (or 13 p.p.m. less shielded from TFA). Fluorine–protein resonances at 5 and 30 h had linewidths of 220 and 130 Hz respectively, representing an almost 50% decrease in linewidth (increase in T_2 time; decrease in molecular size). Perfluorotributylamine is an intravascular imaging agent with a long plasma residence time (Berkowitz, 1987) and is useful for determining the limits of detection of ^{19}F from the plasma. Analysis of data from the studies of intravenous administration of perfluorotributylamine provided evidence that the ^{19}F-lipoprotein signals arose from intracellular accumulation of ^{19}F in the liver, as opposed to contamination from the blood pool (results not shown). The fluorine concentration in plasma at which a ^{19}F n.m.r. signal could be observed with 30 min time averaging using a surface coil localized on the liver was almost 30 mM. This concentration of ^{19}F represents an ~ 200-fold excess above that used in the lipoprotein studies.

To evaluate whether the changes in T_2 times observed with selectively degraded β-VLDL *in vitro* also occurs *in vivo*, n.m.r. spectroscopy was performed on intact perfused livers removed after intravenous administration of DLBA–β-VLDL. Results of measurements of livers removed at 20 h after injection indicated two major dominant size clusters of molecular species having T_2 relaxation times of 1.8 ± 0.4 ms and 16 ± 3 ms ($P = 0.029$) respectively. The relative percentage of each component obtained from the signal amplitude versus echo-time curve indicated that, at 20 h, 69 ± 4% of the ^{19}F-protein fragments possessed the longer T_2 time. A representative plot of signal amplitude (as determined by Bayesian analysis) versus echo time and the FT spectra plotted as a function of echo time are shown in Fig. 5. This result was comparable with the percentage of trichloroacetic acids precipitable radioactivity (59 ± 4%) determined in homogenates of a small section from the above livers after the co-injection of the radioiodinated residualizing label, DLT, attached to β-VLDL.

DISCUSSION

The present study has demonstrated that it is feasible to detect the accumulation of lipoproteins in hepatic tissue non-invasively through ^{19}F n.m.r. spectroscopy. The n.m.r. studies demonstrated non-invasive hepatic detection of micromolar quantities of ^{19}F-protein in the rabbit model, a preferred animal model for the study of lipoprotein metabolism and atherosclerosis. The successful detection of n.m.r. active nuclei is dependent on the concentration of nuclei; thus detection of such small quantities of ^{19}F in the large rabbit liver is significant. In addition to detection of lipoprotein accumulation, the present study also demonstrated the feasibility of discriminating the state or extent of degradation of the apoprotein moiety.

DLBA conjugates to β-VLDL via lysine residues of apolipoprotein-B and/or -E. Because lysine groups have been established as critical amino acid residues for binding to cell-surface receptors (Weisgraber *et al.*, 1978; Lund-Katz *et al.*, 1988), it was important to establish that the chemical modification of β-VLDL did not affect its receptor-mediated cellular recognition or structural integrity. The clearance of DLBA–^{131}I-β-VLDL and ^{125}I-β-VLDL from plasma were not significantly different, indicating that the reductive amination procedure used for conjugation of DLBA to lysine residues does not alter recognition of the lipoprotein by the receptor. Data from SDS/polyacrylamide and agarose gel electrophoresis were consistent with a lack of any significant modification of β-VLDL resulting from the labelling procedure. SDS/PAGE performed on native β-VLDL, incubated with or without $NaBH_3CN$ at 37 °C for 2 h, confirmed that apoproteins remained intact on exposure to the reducing agent and/or heat. Because $NaBH_3CN$ is a mild reducing agent, it does not readily reduce disulphide bonds or cause cleavage of other peptide bonds (Borch *et al.*, 1971; Jentoft & Dearborn, 1983). Treatment of lipoprotein with large amounts of galactose oxidase (50 units/mg of protein) resulted in notable degradation of both apolipoprotein-B and apolipoprotein-E probably due to a contaminant in the enzyme. Since significant amounts of galactose oxidase were used in the preparation of DLBA aldehyde, removal of enzyme was necessary and easily accomplished with a centrifugal filter.

The correlation between the T_2 time and molecular size is dependent upon the more primary, functional relationship of T_2 time and the molecular correlation time, τ_c (s). The correlation time is generally interpreted as the mean time a molecule takes to tumble one radian for global re-orientational motion, although localized large amplitude motions affecting the ^{19}F moiety can also be important. The discussion assumes correlation times indicative of global molecular re-orientation, and the increase in T_2 relaxation times upon digestion of β-VLDL are consistent with this interpretation. The correlation time of a molecule is dependent upon several factors, including molecular size, symmetry and viscosity. In general, a short τ_c (10^{-12} s) is indicative of low-molecular-mass molecules with high motional frequencies, and a longer τ_c (10^{-9}–10^{-10} s) correlates with high-molecular-mass molecules with low motional frequencies. As the molecular motion of a molecule decreases (longer τ_c), the corresponding T_2 time also decreases. In addition, the width of an n.m.r. resonance at half height is inversely proportional to the T_2 time ($\Delta\nu_{\frac{1}{2}} \approx 1/\pi T_2$), but factors such as magnetic field inhomogeneities or chemical shift dispersion from slightly dissimilar structures also contribute to the width of the resonance. If inhomogeneities are not limiting, large molecules reflect broad resonances while small molecules have narrow resonances. Using CPMG or Hahn spin-echo studies, T_2 times can be evaluated rigorously by excluding the effects of magnetic field and chemical shift inhomogeneties. A resonance reflecting a single molecular species or a population of species with similar molecular sizes in the same environment will yield a monoexponential decay curve (plot of signal amplitude versus echo time) and therefore one relaxation time constant. A resonance reflecting two differently sized molecular species or two populations of species with an overall average of two distinct sizes/molecular motions gives rise to a biexponential curve with two T_2 times.

Intact DLBA–β-VLDL has a broad resonance ($\Delta\nu_{\frac{1}{2}} \approx 200$ Hz, Fig. 1), corresponding to a single T_2 relaxation time of ~ 2 ms (Table 1). The consistency of obtaining a single T_2 time was demonstrated by performing relaxation measurements on β-VLDL isolated from several hypercholesterolaemic rabbits followed by labelling with DLBA. Samples of intact protein from both preparations exhibited monoexponential behaviour upon analysis of spin-echo data. In addition, observation of rather similar T_2 relaxation times at 470.3 MHz and 188.2 MHz indicate that chemical shift anisotropy, a relaxation mechanism that is more efficient at higher magnetic field strengths (being proportional to the square of the field strength), is not the dominant mechanism for DLBA–β-VLDL. If it were dominant, T_2 relaxation times at the lower field strength would be six times longer than those at the higher field strength. Following digestion of apoproteins with specific enzymes to yield protein fragments of decreasing molecular size, the mean T_2 time from all fragments increased and was associated with a corresponding decrease in linewidth. The extent of this degradation was dependent upon the enzyme used. Pronase cleaves at non-specific sites and digests almost any protein to free amino acids, as demonstrated by SDS/PAGE (Fig. 3). Therefore it was expected to produce the smallest fragments of ^{19}F-labelled β-VLDL and a T_2 time similar to that of free DLBA. Thrombin is a proteolytic enzyme with a specificity for arginine and glycine bonds, while trypsin has a specificity for a substrate with lysine or arginine bonds. However, thrombin is much more specific than trypsin, cleaving only at selected arginine–glycine bonds. Because of this specificity, it was not surprising that different sizes of protein fragments are present, as demonstrated by SDS/PAGE (Fig. 3). The n.m.r. T_2 times computed from the decay curves from both FT and Bayesian methods revealed the presence of at least two dominant ^{19}F apoprotein fragments or size clusters for the thrombin and trypsin digests. It should be emphasized that the ^{19}F label was covalently attached to exposed lysine residues on apoproteins B and E (Daugherty *et al.*, 1985), and thus not all of the bands shown on the SDS/polyacrylamide gel reflect ^{19}F-labelled fragments. As expected, these methods detected only a single T_2 component for intact DLBA–β-VLDL, Pronase-digested DLBA–β-VLDL and free DLBA because of the presence of only one molecular species or of one population of species with similar molecular mass fragments. Also, the percentage of protein digested was greater for trypsin than for thrombin because of the differences in substrate specificity, although this percentage is variable for each enzyme depending on the amount of enzyme used and the length of incubation with protein.

Most importantly, analyses by either FT or Bayesian methods verify the n.m.r. technique for discriminating between molecular size based on relaxation behaviour. The accuracy in the determination of signal amplitudes is critical when attempting to discriminate between mono- and bi-exponential decay curves (signal amplitude versus echo time). Difficulties in FT analysis in accurately defining these amplitudes arose not from the process of integration itself but in the contamination from systematic errors such as the defining of the baseline level of spectra. Sources of baseline distortion include pulse breakthrough, probe or filter ringing and errors in defining the timing for the echo maximum. These problems resulted in large errors for discriminating the relative amount of each decaying component

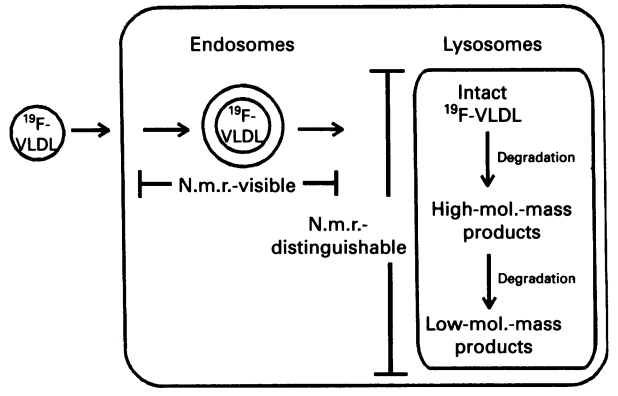

Fig. 6. Intracellular processing of ^{19}F-β-VLDL

The labelled lipoprotein is recognized by receptors on the cell surface and is cleared from the plasma. The intact lipoprotein is delivered to the endosome. At this stage, the lipoprotein accumulates and an ^{19}F signal can be detected. The labelled protein is then delivered to the lysosome, the site of enzymic degradation of the lipoprotein. Because DLBA is a residualizing label, it remains trapped within the lysosome and is not excreted from the cell. The rate and/or extent of degradation of protein in the lysosome can be determined, since n.m.r. methods can distinguish molecular species of different sizes.

using the frequency domain method. As a result, the effect of baseline roll and offset on the accuracy of the integral in the frequency domain are minimal with narrow lines, but lines whose widths are large have substantial errors in defining their areas. Because Bayesian is a time domain analysis method, frequency domain baseline distortion does not corrupt parameter estimation. Therefore the amplitudes estimated from this method are more precisely defined than those from the FT method (Bretthorst, 1992). We found that the Bayesian method led to smaller residuals in the echo decay curve analysis compared with the FT method (although for the higher signal-to-noise narrow line spectra, results from both analyses were similar). Thus Bayesian analysis was used for characterizing the ^{19}F spectra in excised livers.

N.m.r. spectroscopy was performed *in vivo* to determine if the dramatic changes in T_2 times observed by high-resolution methods could be detected. Comparison of the linewidths at 5 and 30 h after injection of labelled lipoprotein indicates an almost 2-fold decrease in linewidth. The 5 and 30 h time points were chosen to represent protein in a semi-intact/intact or degraded state respectively. It is highly probable that this linewidth change represents an increase in T_2 time; however, caution must be taken when interpreting these linewidth changes, since magnetic field inhomogeneities contribute to the width of an n.m.r. resonance. Because the homogeneity of the field was shimmed on the water proton to less than 100 Hz, it is reasonable to conclude that the decrease in linewidth is primarily due to an increase in T_2 time. Unfortunately, because of the limited ^{19}F sensitivity in the *in vivo* experiment, direct measurement of the T_2 time could not be performed with the present residualizing label (six ^{19}F atoms per molecule of DLBA), because of the length of time required to perform the experiment.

Spin–spin relaxation time constants were measured in livers *in vitro* to verify results of detection of degradation *in vivo*. The time point chosen (20 h) for perfused liver experiments represents a stage at which lipoprotein is extensively cleared from the plasma and degraded in the liver. The data from T_2 studies were biexponential, indicating the presence of two populations of ^{19}F-labelled proteins with differing molecular sizes. The n.m.r. determination of the percentage of the more slowly decaying component (i.e. smaller or motionally unrestrained molecule) was in agreement with the percentage of protein degraded calculated by the trichloroacetic acid method. It should be noted that the two distinct T_2 times could reflect either protein degradation or two differing environments of β-VLDL, one of which is more motionally restricted than the other. Since trichloroacetic acid precipitation is a commonly used index of protein degradation and the n.m.r. and acid precipitation results concur, it is likely that the two distinct T_2 times reflect a change in molecular size, i.e. degradation of protein.

An intracellular scheme can be devised based on data acquired *in vivo* and *in vitro* (Fig. 6). β-VLDL is cleared from the plasma through a receptor-mediated process and delivered intracellularly to endosomes. Once delivered to the endosome, the intact particle is transported to lysosomes and enzymically degraded into various fragments. Here most of the protein is degraded, but some remains in an intact/semi-intact form. In this case, the T_2 times are taken to be a reflection of degraded and intact/semi-intact states of the lipoprotein. An alternative interpretation of the presence of different T_2 times may be the existence of compartments within the cell that have differing environments, such that the reflection on the molecular correlation time is dissimilar depending on the intracellular location of the protein.

The results of this study demonstrate the feasibility of monitoring lipoprotein metabolism *in vivo* using ^{19}F n.m.r. spectroscopy. No other non-invasive monitoring method offers the potential for such powerful chemical specificity. At present the limited sensitivity of ^{19}F detection with DLBA prevented the non-invasive measuring of T_2 times *in vivo*. However, use of a label containing a greater number of equivalent fluorines would permit measurement of T_2 times *in vivo* and absolute quantification of ^{19}F from hepatic tissue. Thus future studies should be aimed at the development of a more highly fluorine-enriched residualizing label and to accurately characterize the intracellular state of the protein as reflected in two distinct T_2 times.

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