Altered hepatic catabolism of low-density lipoprotein subjected to lipid peroxidation *in vitro*

William L. STONE,*§ Murray HEIMBERG,† Robert L. SCOTT,‡ Irene LeCLAIR* and Henry G. WILCOX†

*Department of Pediatrics, East Tennessee State University, James H. Quillen College of Medicine, Johnson City, TN 37614–0578, U.S.A.,

†Department of Pharmacology, University of Tennessee–Memphis, The Health Sciences Center, Memphis, TN 38163, U.S.A.,

and ‡Department of Internal Medicine, Barnes Hospital, Washington University, St. Louis, MO 63110, U.S.A.

Recent evidence suggests that oxidatively modified forms of low-density lipoprotein (LDL) may be particularly atherogenic. In this investigation, the catabolism of human LDL modified by lipid peroxidation *in vitro* was studied with a recirculating rat liver perfusion system. A dual-labelling technique was used that permitted native LDL and modified LDL to be studied simultaneously in the liver perfusion system. Native human LDL was found to have a fractional catabolic rate (FCR) of $1.00 \pm 0.21\%$ /h, in agreement with other investigators. Subjecting LDL to oxidation for 12 h in the presence of $30\ \mu\text{M}$ FeEDTA did not significantly affect its FCR. LDL treated with a superoxide-generating system (xanthine oxidase, hypoxanthine, O_2) in the presence of $30\ \mu\text{M}$ FeEDTA did, however, show a significant increase in FCR ($3.23 \pm 0.19\%$ /h). The hepatic up-

takes of native LDL and LDL oxidized with FeEDTA + O_2 were similar, but both were significantly lower than the hepatic uptake of LDL treated with the superoxide-radical-generating system. The proteolysis of LDL with pancreatin did not influence either its susceptibility to oxidation or its FCR. LDL oxidation resulted in the preferential loss of α -tocopherol rather than γ -tocopherol. These data indicate that the rat liver effectively catabolizes LDL oxidatively modified by treatment with the superoxide-generating system. Furthermore, our results suggest that only very low plasma levels of highly oxidized LDL could be found under conditions *in vivo*. The liver may therefore play a major role in protecting the arterial vasculature from highly atherogenic forms of LDL.

INTRODUCTION

Considerable evidence suggests that oxidative modifications to low-density lipoprotein (LDL) may be a contributing factor in atherosclerosis [1–4] and that antioxidants can inhibit these damaging oxidative alterations [5–9]. A major line of supporting evidence arises from the observation that macrophages incubated with oxidatively modified LDL assume many characteristics typical of the ‘foam cells’ found in the atherosclerotic lesions [10–14]. Foam cells are thought to arise from blood-borne monocytes that have invaded the endothelial lining of arteries and become cholesteryl ester-laden macrophages.

Foam-cell formation *in vitro* is well characterized in macrophages incubated with LDL chemically modified by acetylation (acetyl-LDL), which modifies lysine residues on apolipoprotein B (apo-B), resulting in an increased negative charge on LDL [15–17]. Oxidized LDL and acetyl-LDL both compete for one class of ‘scavenger receptors’ found on macrophages [13,14]. A second class of macrophage scavenger receptors recognizes oxidized LDL but not acetyl-LDL [18]. In contrast, macrophages incubated with native LDL are not transformed into foam cells [15,16].

The potential physiological significance of oxidatively modified LDL *in vivo* is not well defined. Oxidized LDL is cytotoxic to both endothelial cells and smooth-muscle cells in tissue culture [19]. Recent immunological data suggest that oxidatively modified LDL exists in atherosclerotic lesions [20,21]. It might, however, be very difficult to demonstrate significant amounts of oxidatively modified LDL in human plasma, due to its potentially

rapid plasma clearance by macrophages. Kupffer cells are liver-resident macrophages, and they are the largest population of phagocytic cells in an organism. About 30% of all non-parenchymal cells in the liver are Kupffer cells [22]. It is therefore likely that oxidatively modified LDL generated *in vivo* would be rapidly catabolized by the Kupffer cells. Acetyl-LDL (from humans or rats) injected into rats is rapidly taken up by the liver non-parenchymal cells [22,23]. Similarly, freshly isolated non-parenchymal liver cells catabolize acetyl-LDL 50-fold more than do freshly isolated parenchymal liver cells [22].

In this investigation, a recirculating rat liver perfusion system was used to compare the hepatic catabolism of: (1) human native LDL; (2) human LDL oxidized with O_2 and $30\ \mu\text{M}$ FeEDTA; and (3) human LDL oxidized with $30\ \mu\text{M}$ FeEDTA, xanthine oxidase, hypoxanthine and O_2 . Xanthine oxidase, hypoxanthine, O_2 and transition-metal ions generate superoxide and other oxyradicals that oxidatively modify LDL [12,24]. The commercially available xanthine oxidase used in our initial studies was purified from cow’s milk treated with pancreatin. We found, however, that pancreatin was not completely removed from the xanthine oxidase, as evidenced by a proteolytic activity that degraded the apoB100 protein of LDL. The proteolytic activity of this commercial xanthine oxidase towards apoB100 was, however, easily removed by column chromatography. Since the influence of pancreatic protease on the uptake of LDL by the liver has never been measured, we decided to use protease-containing xanthine oxidase (PXOase) as well as protease-free xanthine oxidase (PFXOase) in our experiments. Moreover, the combined influence of oxidation and proteolysis on the hepatic

Abbreviations used: LDL, low-density lipoprotein; apo, apolipoprotein; PMSF, phenylmethanesulphonyl fluoride; FCR, fractional catabolic rate; PXOase, protease-containing xanthine oxidase; PFXOase, protease-free xanthine oxidase; TCA, trichloroacetic acid; TBARS, thiobarbituric acid-reactive substances.

§ To whom requests for reprints should be addressed.

catabolism of LDL has not been studied. Although controversial [25–27], in acute pancreatitis it is possible that LDL would be subjected to both pancreatic proteases as well as free radicals generated from xanthine oxidase [28,29].

METHODS

Chemicals and enzymes

All chemicals were reagent grade, and all organic solvents were h.p.l.c. grade. Xanthine oxidase (product no. x4500, grade III from buttermilk) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). This xanthine oxidase was tested for protease activity by using Bio-Rad (Melville, NY, U.S.A.) protease-substrate gel tablets. The grade III xanthine oxidase was further purified by chromatography on a column (2.5 cm × 52 cm) of Sephacryl S-200 (Pharmacia, Piscataway, NJ, U.S.A.). Phenylmethanesulphonyl fluoride (PMSF) obtained from Sigma Chemical Co. was also used to inhibit the protease activity of xanthine oxidase. ^{125}I and ^{131}I in pH 7–11 NaOH for LDL iodination were purchased from Amersham (Arlington Heights, IL, U.S.A.). RRR- α -Tocopheryl acetate was purchased from Kodak Laboratory and Research Products (Rochester, NY, U.S.A.) and required no further purification.

Overall experimental design

We used a recirculating rat liver perfusion system to measure the catabolism of human radioiodinated LDL subjected to a variety of experimental treatments. Our overall methodology was very similar to that used by Sigurdsson et al. [30]. We did, however, use a dual-isotope labelling procedure, in which the catabolism of ^{125}I -LDL and ^{131}I -LDL subjected to different treatments could be simultaneously measured in the same rat liver. LDL catabolism was monitored by measuring the appearance of trichloroacetic acid (TCA)-soluble radioiodine radioactivity in the liver perfusate. In our experiments, we compared the hepatic catabolism of: (1) ^{125}I -labelled native LDL versus ^{131}I -labelled LDL oxidized by using only FeEDTA and O_2 (i.e. no enzymic oxidation); (2) ^{125}I -labelled native LDL versus ^{131}I -labelled LDL treated only with PXOase (i.e. proteolysis alone but no oxidation); (3) ^{125}I -LDL treated only with PFXOase (i.e. no oxidation and no proteolysis) versus ^{131}I -LDL treated with PFXOase, hypoxanthine, O_2 and FeEDTA (i.e. enzymic oxidation but no proteolysis); (4) ^{125}I -LDL oxidized with hypoxanthine, O_2 , FeEDTA (i.e. no enzymic oxidation) versus ^{131}I -LDL treated with PXOase, hypoxanthine, O_2 and FeEDTA (i.e. enzymic oxidation as well as proteolysis). Each of these four perfusion experiments was done in triplicate. A control 'liverless' perfusion was also performed for each experiment, and the TCA-soluble radioactivity obtained was subtracted from that measured in the presence of a rat liver.

Lipoprotein isolation

Venous blood was drawn from a single fasting healthy human male donor and mixed with NaN_3 (final concn. 1 mM) and EDTA (1 mg/ml of blood). LDL (density 1.02–1.063 g/cm³) was obtained by sequential ultracentrifugation [31] in a Sorvall Ti865.1 rotor (Dupont Co., Newton, CT, U.S.A.) at 60000 rev./min for 18 h in a Sorvall OTD-65B ultracentrifuge. The LDL obtained was washed by re-centrifugation at a density of 1.063 g/cm³ for 18 h and dialysed against 0.15 M NaCl/0.25 mM EDTA, pH 7.4 (no NaN_3). A sample of the dialysed LDL was further purified, after radioiodination (see below), by Sepharose CL-4B chroma-

tography to remove a small amount of contaminating albumin. SDS/PAGE (5.0% gel) [32] indicated a single apolipoprotein polypeptide, with an electrophoretic mobility identical with that of apo-B.

Radioiodination of LDL

Native LDL was radioiodinated by a modification [33] of the ICI method of McFarlane [34]. Most of the unbound iodine was rapidly removed (and the LDL further purified) by eluting the iodination reaction mixture from a column (1.2 cm × 8 cm) of Sepharose CL-4B (Pharmacia) with 0.15 M NaCl/0.1 mM EDTA, pH 7.4. Protein was monitored at 280 nm and radioiodine (^{125}I or ^{131}I) monitored with a model 4000 Beckman γ -radiation counter. The radiolabelled LDL fractions were pooled and exhaustively dialysed against 0.15 M NaCl/0.25 mM EDTA, pH 7.4, to remove the small amount of remaining unbound radioiodine. EDTA was not, however, present in the dialysis buffer if the LDL was to be oxidized. After dialysis, the LDL was assayed in duplicate for protein by a modification [35] of the Lowry method [36]. At least 97–98% of the LDL radiolabel was found to be precipitable in 10% TCA. We found 7–8% of the total LDL radiolabel to be extractable into chloroform/methanol (2:1, v/v).

Modified LDLs

LDL was oxidatively modified by both enzymic and non-enzymic methods. In both cases the LDL was radioiodinated before being subjected to oxidation, thereby ensuring that iodine did not add across any conjugated carbon-carbon double bonds created during lipid oxidation. In the non-enzymic method, radioiodinated LDL (1.0–1.5 ml) was placed in dialysis tubing that was then placed in a beaker containing 200 ml of 0.15 M NaCl/30 μM FeEDTA. Filtered air was continuously bubbled through a 60 μm -pore gas-diffusing stone (Fisher Scientific, Norcross, GA, U.S.A.) into the dialysate of the beaker. After 12 h of oxidation, the radioiodine on the modified LDL was 97–98% precipitable by 10% TCA, and 7–8% was found to be extractable in chloroform/methanol (2:1, v/v). In the enzymic method, radioiodinated LDL (1.0–1.5 ml) was placed in a dialysis membrane along with xanthine oxidase (10 or 20 m-units). Xanthine oxidase activity was measured by monitoring the superoxide reduction of ferricytochrome *c* at 550 nm [37] or by the appearance of uric acid absorbance at 290 nm [38]. The sealed dialysis membrane was placed in a beaker containing 200 ml of 0.15 M NaCl/30 μM FeEDTA/100 μM hypoxanthine. The dialysate in the beaker was maintained at maximum oxygen saturation by bubbling air through a gas-diffusing stone. The reaction mixture was incubated at 4 °C for 12 h, but the dialysate was replaced after 5 h with an additional 200 ml of 100 μM hypoxanthine. The oxidized LDL in the dialysis tubing was removed and used in the perfusion system within 1.5–2.0 h. After oxidation, 94% of radioactive label in the ^{131}I -LDL was precipitable in 10% TCA and 8–9% was extractable in chloroform/methanol (2:1, v/v). After being subjected to either of the oxidation procedures, the iodinated LDL was passed through a 0.45 μm -pore Acrodisc filter and assayed for vitamin E (α - and γ -tocopherol), thiobarbituric acid-reactive substances (TBARS) and protein.

Characterization of modified LDLs

The extent of LDL peroxidation was measured by a sensitive fluorescent thiobarbituric acid assay [39] in which a phospho-

tungstic acid/ H_2SO_4 system is used to precipitate LDL (protein and lipids). The TBARS in the washed LDL pellet was therefore a measure of TBARS bound to LDL particles. To estimate free TBARS in the aqueous phase, we also performed a thiobarbituric acid assay on the supernatant from the phosphotungstic acid/ H_2SO_4 precipitation of LDL. Excitation was at 515 nm, and fluorescence was measured at 553 nm in a Tuner model 430 spectrofluorimeter. Freshly diluted 1,1,3,3-tetramethoxypropane was used as a standard. The levels of α -tocopherol and γ -tocopherol in native and modified LDL were measured by reverse-phase h.p.l.c. with an Altex-Ultasphere ODS (Beckman) column (4.6 mm internal diam. $\times$ 25 cm) [40]. RRR- α -Tocopheryl acetate was used as an internal standard.

Liver perfusion, uptake of radiolabelled native LDL and modified LDLs and bile secretion

Livers from 9.5-month-old inbred Fischer-344 male rats weighing 415 ± 28 g were perfused as previously described [41]. The rats were fed on a standard laboratory ration (Rodent Laboratory Chow 5001; Ralston Purina Co., St. Louis, MO, U.S.A.) and water *ad libitum*. The livers were removed when the rats were in the fed state. After cannulation of the portal vein, the liver was perfused with oxygenated 0.9% NaCl at room temperature until positioned in the perfusion chamber, which was maintained at 37 °C. The liver was then perfused for 20 min with 100 ml of Krebs–Henseleit bicarbonate buffer (pH 7.4), containing 25% washed bovine erythrocytes, glucose (100 mg/ml) and 4% delipidated BSA. Non-radioactive moniodotyrosine was added to the perfusion medium (1 mg/ml) to inhibit non-specific deiodination due to any deiodinase activity [30] and to inhibit reutilization of radiolabelled iodotyrosine, released by LDL catabolism, for new protein synthesis by the liver. The medium was gassed continuously with O_2/CO_2 (19:1). During the 30 min period of equilibration, maximal rates of perfusate flow through the liver were attained, and these rates were maintained for the duration of the experiment. After the 30 min equilibration period, a portion (< 1.5 ml) of ^{125}I -LDL (native or modified), alone or in combination with a portion of modified (see above) ^{131}I -LDL, was injected into the perfusion apparatus. Duplicate 1.2 ml portions of the perfusate were removed at 30 min time intervals, with zero time being the point at which the radiolabelled LDLs were injected into the perfusion system. Each 1.2 ml sample of perfusate was centrifuged at 13000 *g* for 5 min to sediment the erythrocytes. A 700 μl portion of supernatant was placed in a 1.5 ml Eppendorf microfuge tube, followed by 700 μl of 20% TCA. After centrifugation for 5 min at 13000 *g* the supernatant was collected and the pellet washed by an additional centrifugation in 700 μl of 10% TCA. Both supernatants were combined and counted for radioactivity to determine the amount of radioiodotyrosine released by catabolism of the LDL. The radioactivity remaining in the pellet was also determined.

At the end of the experiment, the liver was perfused with 50 ml of ice-cold 0.9% NaCl to remove any medium from the hepatic sinuses, trimmed of extraneous tissue, blotted, weighed and homogenized in 10 vol. of 0.9% NaCl. In order to determine the uptake of radiolabelled LDLs by liver, a 500 μl sample of the liver homogenate was placed in a 1.5 ml Eppendorf microfuge tube, followed by 500 μl of 20% TCA. After centrifugation for 5 min at 13000 *g*, the radioactivity in the pellet and supernatant was measured. During the perfusion, the perfusate packed erythrocyte volume and the volume of bile produced by the liver were measured every 30 min. The bile produced was collected and the ^{125}I or ^{131}I radioactivity in it was measured.

Data analysis

When both ^{125}I -LDL and ^{131}I -labelled modified LDL were present in the perfusion media, standard adjustments were made for the appearance of ^{131}I radioactivity in the ^{125}I channel. The percentage of LDL degraded was calculated as the percentage of total radioiodine radioactivity (supernatant plus pellet) appearing as TCA-soluble radioactivity. A small correction was made to the TCA-soluble radioactivity since, after each withdrawal of perfusate, the newly catabolized LDL released radioiodotyrosine into a smaller volume of perfusate. Similarly, small corrections were made for minor fall in haematocrit that would have the effect of diluting both the TCA-soluble and TCA-precipitable radioactivity. Radioactivity appearing in bile was attributed to LDL catabolism, and was therefore included as 'TCA-soluble' radioactivity. It was assumed that the bile appeared as a linear function of time throughout the perfusion experiment.

Statistical analyses

For each perfusion experiment, we calculated the linear regression coefficients (and one-way ANOVA tables) for (1) time versus % of water-soluble ^{125}I radioactivity and (2) time versus % of water-soluble ^{131}I radioactivity (Sigma Plot Scientific Graph System; Jandel Scientific, Corte Madera, CA, U.S.A.). Standard statistical methods were employed to determine if the regression lines (and slopes) were significantly different [42].

RESULTS

Xanthine oxidase purification and LDL proteolysis

Figure 1 shows the elution profile of a commercial preparation of PXOase from a Sephacryl S-200 column. Two A_{280} peaks (1 and 2) were found, but enzymic activity was mostly localized in peak 1. SDS/PAGE indicated (results not shown) that both peak 1 and peak 2 primarily contained two polypeptides with molecular masses of 94000 and 38000 Da. These molecular-mass data agree very well with those obtained by Waud et al. [43] for xanthine oxidase purified from buttermilk treated with pancreatin. The Bio-Rad protease-detection assay indicated signifi-

Figure 1 Purification of xanthine oxidase

Grade III xanthine oxidase was eluted from a Sephacryl S-200 column (2.5 cm $\times$ 52 cm) in 50 mM phosphate buffer, pH 7.4, with 0.1 mM EDTA. Protein (●) was monitored at 280 nm and xanthine oxidase activity (▼) was measured at the indicated fraction numbers.

Figure 2 Proteolysis of apoB100 by xanthine oxidase preparations

Human LDL (200 μ g of protein) was incubated with 10 m-units of either PFXOase or PXOase. An equal sample of each reaction mixture (at 4 $^{\circ}$ C) was removed immediately (T_0) or after 8 h (T_8), and the fragmentation of apoB100 was analysed by SDS/PAGE (5% gels). In some experiments PMSF was added to the incubation mixture to give a final concentration of 0.1 mM. The label (at the bottom of the gel) and sample for each lane is as follows: (A) molecular-mass standards (from top to bottom, they were myosin, β -galactosidase, phosphorylase *b* and BSA); (B) PFXOase, (10 m-units), but no LDL; (C) PXOase, but no LDL; (D) apoB100, LDL but no xanthine oxidase; (E) LDL + PXOase at T_0 ; (F) LDL + PXOase at T_8 ; (G) LDL + PXOase + PMSF at T_0 , same as lane E but with PMSF; (H) LDL + PXOase + PMSF at T_8 , same as lane F but with PMSF; (I) LDL + PFXOase at T_0 ; (J) LDL + PFXOase at T_8 ; (K) LDL + PFXOase + PMSF at T_0 , same as lane I but with PMSF; (L) LDL + PFXOase + PMSF at T_8 , same as lane J but with PMSF; (M) as lane A.

cant protease activity (towards casein) with PXOase that could be suppressed, but not eliminated, by treatment with 0.1 mM PMSF. In contrast, xanthine oxidase peaks 1 and 2 from the column did not show digestion of casein. The pooled peak 1 fractions were designated as PFXOase.

Figure 2 shows the influence of both PXOase and PFXOase on the proteolytic fragmentation of apoB100 in LDL. When 10 m-units of PXOase was added to 200 μ g of LDL (see lane E, 'LDL + PXOase at T_0 '), in Figure 2), there was a very rapid proteolysis of apoB100. Moreover, after 8 h almost all the apoB100 was fragmented into smaller polypeptides (see lane F, 'LDL + PXOase at T_8 '). The addition of PMSF was able to suppress, but not to eliminate, the proteolysis of apoB100 caused by addition of PXOase (see lane G, 'LDL + PXOase + PMSF at T_0 '), and lane H, 'LDL + PXOase + PMSF at T_8 '). In marked contrast, the PFXOase, either treated or not treated with PMSF, did not result in any detectable proteolytic fragmentation of apoB100 (e.g., see lane J, 'LDL + PFXOase at T_8 ', and lane L, 'LDL + PFXOase + PMSF at T_8 ').

Hepatic catabolism and uptake of native and modified LDL

Figure 3(a) shows the simultaneous hepatic catabolism of both native LDL and LDL treated only with PXOase as a function of time. In this experiment, the modified LDL was treated with PXOase, but was not subjected to oxidative stress, since neither O_2 nor hypoxanthine was present. These data indicate that apoB100 proteolysis, in the absence of oxidative stress, had little influence on the catabolism of LDL by the rat liver. The fractional catabolic rate (FCR) (i.e. the slope of any line in Figure 3) of native LDL (1.00 ± 0.21 %/min) was not significantly different from that of PXOase (10 m-units)-treated LDL (1.22 ± 0.21 %/h).

Figure 3 Percentage of water-soluble radiiodine from the catabolism of native LDL or modified LDL as a function of time in the rat liver perfusion system

(a) Native 125 I-LDL (103.0 ± 2.8 μ g of protein) and 131 I-LDL (90.6 ± 7.5 μ g of protein) treated with 10 m-units of PXOase for 12 h (LDL + PXOase); (b) native 125 I-LDL (50.0 ± 2.2 μ g of protein) and 131 I-LDL (62.0 ± 4.9 μ g of protein) treated with O_2 and 30 μ M FeEDTA for 12 h (LDL + FeEDTA + O_2); (c) 125 I-LDL (53 ± 1.6 μ g of protein) treated with 10 m-units PFXOase for 12 h (LDL + PFXOase) and 131 I-LDL (55.0 ± 2.4 μ g of protein) treated with 10 m-units of PFXOase, O_2 , 100 μ M hypoxanthine and 30 μ M FeEDTA for 12 h (LDL + PFXOase + O_2 + HX + FeEDTA); (d) 125 I-LDL (92.2 ± 1.4 μ g of protein) treated with O_2 , 100 μ M hypoxanthine and 30 μ M FeEDTA for 12 h (LDL + O_2 + HX + FeEDTA) and 131 I-LDL (92.3 ± 3.4 μ g of protein) treated with 10 m-units of PXOase, O_2 , 100 μ M hypoxanthine and 30 μ M FeEDTA for 12 h (LDL + PXOase + HX + O_2 + FeEDTA). Each data point is the mean $\pm$ S.D. obtained at each time point for three independent liver-perfusion experiments.

Moreover, the FCR of native LDL measured in the absence of any modified LDL was very similar to that given in Figure 3(a) for native LDL (results not shown). The contribution to the FCR made by radioactivity appearing in bile was 0.36 ± 0.12 for native LDL (all conditions) and 0.41 ± 0.14 for LDL treated only with PXOase, i.e. about one-third of the total FCR in both cases.

Figure 3(b) indicates that the catabolism of LDL subjected to mild non-enzymic oxidation (12 h air in the presence of 30 μ M FeEDTA) was not markedly different from that of native LDL. In this experiment, native LDL and modified LDL had FCRs of 0.63 ± 0.10 %/h and 0.81 ± 0.09 %/h respectively. The contribution of bile radioactivity to the total FCR for the modified LDL was 0.20 ± 0.05 . In contrast, Figure 3(c) shows that LDL subjected to 12 h of oxidation in the enzymic superoxide-generating system using PFXOase (10 m-units, + hypoxanthine + O_2 + FeEDTA) was catabolized at a dramatically higher rate than that of native LDL treated only with PFXOase. The FCR for the enzymically oxidized LDL (3.23 ± 0.19 %/h, with bile radioactivity contributing 0.91 ± 0.10) was more than twice ($P < 0.01$) that observed for LDL treated with 10 m-units of PFXOase alone (1.46 ± 0.08 %/h, with bile radioactivity contributing 0.72 ± 0.09). Moreover, the FCR of LDL treated only with PFXOase (see Figure 3c) was not statistically different from that of native LDL treated only with PXOase (see Figure 3a).

Figure 4 Percentage of water-soluble radiolabelled iodine from the catabolism of native LDL or LDL treated with enzymically generated superoxide ions as a function of time in the rat liver perfusion system

Native ^{125}I -LDL ($92.2 \pm 1.4 \mu\text{g}$ of protein) and ^{131}I -LDL ($92.3 \pm 3.4 \mu\text{g}$ of protein) were treated with 20-munits of PXOase, O_2 , 100 μM hypoxanthine and 30 μM FeEDTA for 12 h (LDL + PXOase + O_2 + HX + FeEDTA). Each data point is the means $\pm$ S.D. obtained at each time point for three independent liver-perfusion experiments.

As shown in Figure 3(d), LDL subjected to both proteolysis and superoxide radicals was catabolized more rapidly (total FCR of $2.72 \pm 0.29 \text{ \%}/\text{h}$, $P < 0.05$, with bile radioactivity contributing 0.36 ± 0.09) than LDL treated with FeEDTA, O_2 and 100 μM hypoxanthine (FCR of $1.59 \pm 0.10 \text{ \%}/\text{h}$, with bile radioactivity contributing 0.37 ± 0.11). It should be noted that proteolysis combined with exposure to enzymically generated superoxide radicals (Figure 3d) did not increase the FCR above that observed when LDL was treated with superoxide radicals alone (Figure 3c).

In the experiments described above, the activity of xanthine oxidase was 10 m-units. Figure 4 indicates that subjecting LDL to a superoxide-generating system with 20 m-units of xanthine oxidase activity dramatically increased its hepatic catabolism. The FCR of LDL treated with the 20 m-units of xanthine oxidase system (total FCR of $10.32 \pm 1.5 \text{ \%}/\text{h}$, with bile radioactivity contributing 0.72 ± 0.25) was at least 3 times that observed for LDL oxidized with the 10 m-units of xanthine oxidase system.

For the data presented in Figure 3, the perfusate pool of native LDL protein varied from about 50 to 100 μg . Our data show that

the FCR for native LDL was not, however, markedly dependent on the pool size of LDL. These data are consistent with those of Sigurdsson et al. [30], who found only a small fall in FCR in the 50–500 μg range of LDL protein. At higher levels, i.e. greater than 1000 μg of LDL protein, these investigators did, however, find a marked fall in FCR [30].

The relative uptake (percentage of liver pellet c.p.m./total c.p.m. in perfusate per g of liver) of native and modified LDLs was also measured in each perfusion experiment. Slater et al. [44] have shown that tissue uptake of LDL reflects the immediate binding to membranes rather than the cumulative uptake of LDL, since internalization and degradation occurs very rapidly. We found that about 85% of the radioactivity associated with the liver was in the TCA pellet. It should also be noted that $97.3 \pm 3.6 \text{ \%}$ of the radiolabelled iodide counts initially added to the perfusate could be recovered in the perfusate plus bile radioactivity at the end of the perfusion. These data indicated that very little non-protein-bound radio-iodide accumulated in the rat livers during the perfusion. The relative uptake of native LDL was found to be similar ($0.27 \pm 0.07 \text{ \%}$ of liver pellet c.p.m./total perfusate c.p.m. per g of liver, with a range of 0.21–0.39) in each perfusion experiment and was independent of the presence of modified LDL. Modification of LDL by treatment with FeEDTA + O_2 (uptake of $0.27 \pm 0.03 \text{ \%}$ of liver) or by PXOase ($0.34 \pm 0.03 \text{ \%}$ of liver) did not dramatically alter the hepatic uptake compared with that of native LDL. However, oxidation of LDL, in the presence of either PXOase ($1.24 \pm 0.30 \text{ \%}$ of liver) or PFXOase ($1.54 \pm 0.55 \text{ \%}$ of liver), significantly increased hepatic uptake compared with that of native LDL. These data were qualitatively similar to those shown in Figure 3, i.e. the high FCRs rates of enzymically oxidized LDL were paralleled by higher relative hepatic uptakes.

LDL oxidation was monitored by measuring the appearance of TBARS and the consumption of tocopherols. As shown in Table 1, native LDL not subjected to any oxidation system showed $2.3 \pm 0.37 \text{ nmol}$ of TBARS/mg of protein, but only 35% of the TBARS was associated with the LDL precipitate and the rest was in the aqueous phase. LDL oxidized with FeEDTA + O_2 showed $9.1 \pm 1.8 \text{ nmol}$ of TBARS/mg of protein, with 66% now associated with the LDL precipitate. LDL oxidized with 10 m-units of PXOase showed $32 \pm 11 \text{ nmol}$ of TBARS/mg of protein, and 54% was associated with the LDL precipitate. LDL oxidized with PFXOase showed an oxidation pattern similar to that observed for the LDL oxidized with the PXOase. Table 1 also indicates that the α -tocopherol content of LDL was only slightly diminished after 12 h in 30 μM FeEDTA + O_2 . In contrast, LDL subjected to the superoxide-generating system (10 m-units of PXOase) showed a marked decrease in α -tocopherol after 12 h. LDL γ -tocopherol was also measured, but its consumption was

Table 1 Effect of oxidation on the formation of TBARS and tocopherol consumption in LDL

LDL was non-enzymically oxidized by incubation for 12 h in the presence of 30 μM FeEDTA and 100 μM hypoxanthine (HX) and O_2 at 4 $^\circ\text{C}$. For enzymic oxidation, LDL was incubated with 30 μM FeEDTA, 100 μM HX, O_2 and 10 m-units of either protease PXOase or PFXOase. TBARS associated with precipitated LDL (pellet) and in the aqueous phase (supernatant) were measured in triplicate. TBARS are given as means $\pm$ S.D. Means in each column not sharing a common letter are significantly different ($P < 0.05$).

Conditions	TBARS (nmol/mg protein)			α -Tocopherol (% consumed)
	Pellet	Supernatant	Total	
LDL (no oxidation)	0.8 ± 0.1^a	1.6 ± 0.3^a	2.3 ± 0.4^a	0
LDL + FeEDTA + O_2 + HX	6.0 ± 1.9^b	3.2 ± 0.1^b	9.1 ± 1.8^b	4
LDL + FeEDTA + O_2 + HX + PXOase	16.0 ± 6.0^c	15.0 ± 5.3^c	32.0 ± 11.0^c	100%
LDL + FeEDTA + O_2 + HX + PFXOase	12.0 ± 2.0^c	15.0 ± 7.1^c	28.0 ± 8.0^c	100%

negligible after either 12 or 24 h of oxidation in 30 μ M FeEDTA + O₂. LDL α -tocopherol was 100% consumed after 12 h of oxidation in the enzymic superoxide-generating systems, but γ -tocopherol consumption was only 50%.

DISCUSSION

High plasma levels of oxidatively modified LDL may be particularly atherogenic and may lead to the formation of 'foam cells', observed in the early stages of arterial plaque formation. In this investigation, it was demonstrated that the hepatic catabolism of human LDL is altered dramatically by being exposed to a superoxide-anion-radical-generating system in the presence of 30 μ M FeEDTA. In particular, we found that the FCR of LDL oxidized by the superoxide-generating system (20 m-units of PXOase) was about 9 times the FCR for native LDL in the same isolated-rat-liver perfusion system. The increased FCR rate of LDL exposed to superoxide radicals was also accompanied by (1) a significantly higher hepatic uptake compared with native LDL and (2) a loss of both α - and γ -tocopherol. α -Tocopherol was, however, oxidized more rapidly than γ -tocopherol. The α -/ γ -tocopherol ratio might, in some circumstances, prove a useful analytical parameter to estimate the extent of LDL oxidation *in vivo*. LDL subjected to the superoxide-generating system contained a significant amount of particle-associated TBARS. In contrast, LDL subjected to FeEDTA + O₂ showed significant loss of neither α - nor γ -tocopherol and only low amounts of LDL-associated TBARS. Moreover, the FCR rate of LDL subjected to FeEDTA + O₂ was not significantly different from that of native LDL.

There is some evidence suggesting that proteolytic enzymes, xanthine oxidase and oxyradicals play an important role in acute pancreatitis. The data obtained *in vivo* presented in this report do not, however, support the hypothesis that treatment of LDL with pancreatin either increases the susceptibility of LDL to oxidative modification or influences the catabolism of the oxidatively modified LDL. Figure 2 shows that treatment of LDL with only PXOase (but no O₂ or hypoxanthine) resulted in the extensive degradation of apoB100. Nevertheless, the hepatic catabolism of LDL treated with PXOase was not very different from that of native LDL, suggesting that enzymic proteolysis is not a critical factor influencing LDL degradation. Fong et al. [45] similarly found that the rate at which ¹²⁵I-LDL was degraded by macrophages in tissue culture was not affected by pretreating the LDL with purified proteolytic enzymes (trypsin, kallikrein, plasmin, thrombin, or elastase). Moreover, our results *in vitro* indicated that enzymic proteolysis of LDL combined with oxidative modification does not increase the FCR above that observed with just oxidative modification alone. Neither our results nor those of Fong et al. [45] addressed the issue of whether proteolysis and oxidation *in vivo* could interact to influence the rate of LDL degradation or its cytotoxicity.

Steinbrecher et al. [13] reported that catabolism of oxidized LDL injected intravenously into guinea pigs was rapid and that over 80% of the degradation occurred by hepatic catabolism. In those experiments, LDL was oxidized by exposure to 5 μ M Cu²⁺. Our isolated-liver-perfusion studies and the intact-animal studies of Steinbrecher et al. [13] suggest that the liver plays a critical role in preventing the accumulation of oxidatively modified LDL in plasma. The isolated-liver-perfusion experiments reported here do not, however, rule out the possibility that low levels of partially oxidized LDL could be detected *ex vivo* in fresh plasma. Avogaro et al. [46] have provided evidence for the presence of an oxidatively modified form of LDL in normolipidaemic humans. This modified LDL species was more electronegative than native

LDL, and had a low phospholipid content and a high content of conjugated dienes. These properties are all consistent with oxidative modification. Furthermore, the modified human LDL had a lower uptake by cultured macrophages and a diminished ability to bind to the apoB,E receptor. In a recent paper, Bowry et al. [47] reported that fresh human plasma contained very low levels of oxidatively modified LDL. This observation is certainly consistent with the results of the present study. Bowry et al. [47] also found that HDL was the major carrier of lipid hydroperoxides in plasma.

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