Radioiodination of low density lipoprotein initiates lipid peroxidation: protection by use of antioxidants

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Abstract It is now apparent that low density lipoprotein (LDL) is very susceptible to lipid peroxidation and that the resulting oxidized LDL has altered biological properties. Radiation, particularly of longer duration and lower intensities, initiates lipid peroxidation, yet radioiodination with ^{125}I and ^{131}I is a frequently used method to label LDL for biological studies. To test the possibility that this procedure alters the biological properties of LDL, native LDL was radioiodinated with $^{125}I/^{131}I$ using ICl to average specific activities of approximately 300 and approximately 100 cpm/ng protein, respectively. Lipid peroxidation was monitored by TBARS and conjugated diene formation. Biological properties were monitored by fibroblast and macrophage uptake of LDL as well as by rate of plasma clearance (FCR) in guinea pigs. ^{131}I-labeled LDL showed enhanced indices of lipid peroxidation compared to ^{125}I-labeled LDL and both were greater than native LDL. The FCR of ^{131}I-labeled LDL was greater than that of ^{125}I-labeled LDL (by 20–40%) and both increased progressively (by >250%) when measured at 2, 6, and 13 days after iodination. The radioiodinated LDL samples were also more susceptible to pro-oxidant conditions. Thus, after exposure to Cu^{2+}, ^{131}I-labeled LDL showed greatly enhanced lipid peroxidation, decreased uptake by fibroblasts, increased uptake by macrophages and greatly accelerated FCR in guinea pigs. Exposure of LDL to ^{131}I-labeled albumin produced similar changes. Protecting LDL with antioxidants such as BHT and ascorbate immediately after radioiodination generally ameliorated the adverse effects.—**Khouw, A. S., S. Parthasarathy, and J. L. Witztum.** Radioiodination of low density lipoprotein initiates lipid peroxidation: protection by use of antioxidants. *J. Lipid Res.* 1993. **34:** 1483–1496.

Supplementary key words oxidized LDL • antioxidants • metabolic studies • radiation

Many studies have shown that ionizing radiation such as X-rays, gamma rays, and high energy electrons will cause lipid peroxidation in aqueous solutions of fats and oils. Doses of X-rays and gamma rays as low as 1000 rads have been shown to produce lipid peroxidation in fat emulsions, fatty acid micelles, and phospholipid liposomes. These changes are manifested by increases in conjugated diene formation, lipid hydroperoxide formation, oxygen uptake, and decreases in polyunsaturated fatty acid contents (1–5). The extent of oxidation seems to be positively correlated with substrate concentration, and inversely correlated with dose rate. In other words, the extent of oxidation at a fixed total dose of radiation increases as longer durations of lower intensities are used (1). Furthermore, the presence of antioxidants seems to inhibit radiation-induced oxidation (5).

The mechanism of radiation-induced lipid peroxidation seems to involve free radicals, such as the hydroxyl radical ($HO^{\cdot}$) and the superoxide anion ($O_2^{\cdot -}$), which are produced through the radiolysis of water (6). The highly reactive hydroxyl radical is able to abstract a double allylic hydrogen from the acyl chain of a polyunsaturated fatty acid (1, 2, 6). This results in an alkyl radical that rearranges to form the conjugated diene, and reacts with molecular oxygen to produce the peroxyl radical. The peroxyl radical, in turn, is able to propagate the reaction by abstracting a hydrogen from another acyl chain to produce the lipid hydroperoxide and another alkyl radical. Hydroxyl radical scavengers such as ethanol and sodium formate seem to protect against radiation-induced formation of conjugated dienes (1). The superoxide anion is thought to initiate lipid peroxidation by either of two possible mechanisms (2, 6). It may be converted to the hydroxyl radical, via hydrogen peroxide, in the presence of a transition metal such as iron. Alternatively, it may promote the decomposition of trace amounts of lipid hydroperoxide, resulting in alkoxyl radicals that would be sufficiently reactive to initiate lipid peroxidation.

Iodine-125 (^{125}I) and iodine-131 (^{131}I) are two radioactive isotopes of iodine that emit ionizing radiation. ^{125}I

Abbreviations: LDL, low density lipoprotein; TBARS, thiobarbituric acid-reactive substances; FCR, fractional catabolic rate; BHT, butylated hydroxytoluene; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TCA, trichloroacetic acid; MDA, malondialdehyde; DME, Dulbecco's modified Eagle's medium; LPDS, lipoprotein-deficient serum.

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has a half-life of 60 days, and decays by electron capture to tellurium-125. In doing so, it emits a large variety of radiation in the form of gamma rays, X-rays, and electrons. These radiations generally have energies up to 35 keV. ^{131}I, on the other hand, has a shorter half-life of 8 days, but more energetic radiation. It decays by beta decay to xenon-131. ^{131}I also emits gamma radiation, but its predominant gamma radiation has a transition energy of 364 keV. In addition, ^{131}I also emits a variety of beta particles, the predominant one of which has a maximum energy of 606 keV (7, 8).

Both ^{125}I and ^{131}I are used extensively in lipoprotein research as radiolabels for various lipoproteins. Such radiolabeled lipoproteins are used in a wide variety of in vitro and in vivo systems designed to evaluate lipoprotein characteristics including receptor interaction, in vivo clearance and localization, composition, and recently the biological consequences of oxidation of lipoproteins. The value of these nuclides as labels is based on the assumption that they do not measurably alter the characteristics of the labeled particle. The fact that ionizing radiation can result in the peroxidation of lipids suggests the possibility that ^{125}I and ^{131}I may, in fact, alter the characteristics of the labeled lipoprotein through lipid peroxidation. For instance, in the case of low density lipoprotein (LDL), it has been well established that significant lipid peroxidation results in marked alterations in LDL's lipid composition, apoprotein integrity, electrophoretic mobility, cytotoxicity, interaction with LDL receptors as well as scavenger receptors, and in vivo clearance (9, 10). The following studies demonstrate that these radiolabels do have measurable effects on the characteristics of LDL, and that these effects are greater for ^{131}I than for ^{125}I. Furthermore, these effects can be minimized by the use of the antioxidants butylated hydroxytoluene (BHT) and ascorbic acid.

METHODS

LDL isolation and iodination

Fasting blood was collected by venipuncture from six to eight normal human subjects. Na_2EDTA was added at 1 mg/ml. The blood was pooled and plasma was recovered after centrifugation at 3000 rpm for 30 min at 10°C. In order to prevent proteolysis, benzamidine (Fisher), D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (Calbiochem), and phenylmethylsulfonyl fluoride (Calbiochem) were added at 2 mM, 1 μM, and 0.5 mM, respectively. Gentamicin and chloramphenicol were added at 100 μg/ml and 50 μg/ml, respectively (11, 12). The plasma was adjusted to a density of 1.020 g/ml by the addition of a NaBr solution containing 1 mM EDTA, and centrifuged at 50,000 rpm for approximately 18 h at 10°C in a Beckman ultracentrifuge equipped with a 60 Ti or 55 Ti rotor. The supernatant was discarded and the infranatant was recentrifuged for a "wash" under identical conditions after replenishing its volume with additional 1.020 density NaBr solution. The resulting infranatant was adjusted to a density of 1.063 g/ml and centrifuged as before for 24 h. LDL was recovered as the supernatant and "washed" through a second 1.063 g/ml ultracentrifugation for 24 hrs. The isolated LDL was extensively dialyzed at 5°C in phosphate-buffered saline (PBS) containing 0.27 mM EDTA (11, 12). LDL protein concentration was measured by the method of Lowry et al. (13) using a bovine serum albumin (BSA) standard.

Carrier-free ^{125}I and ^{131}I were obtained from Amersham (Chicago, IL). LDL was iodinated with either ^{125}I or ^{131}I using the iodine monochloride (ICl) method of McFarlane (14), as modified by Bilheimer, Eisenberg, and Levy (15) as previously reported by us (16). Labeled LDL was then extensively dialyzed in PBS containing 0.27 mM EDTA at 5°C, and recovered by filtering through a 0.45-μm filter. Specific activity for ^{125}I-labeled LDL ranged from 200 to 700 cpm/ng, and for ^{131}I-labeled LDL from 50 to 350 cpm/ng when measured 2 days after iodination in an LKB compugamma 1282 double-channel spectrometer (Turku, Finland) equipped with a 3" crystal with a rated efficiency of 81% for ^{125}I and 57% for ^{131}I. More than 98% of the radioactivity was precipitable by trichloroacetic acid (TCA), and less than 8% of the radioactivity was extractable by diethyl ether.

BSA (Sigma) was dissolved in PBS and iodinated using Iodobeads (Pierce). Samples were then extensively dialyzed in EDTA-free PBS, and recovered by filtering through a 0.45-μm filter. Specific activity for ^{125}I-labeled BSA was approximately 350 cpm/ng, and for ^{131}I-labeled BSA was approximately 130 cpm/ng.

Modification of LDL

Labeled and unlabeled LDL were diluted to 0.1–0.5 mg/ml in Ham's F-10 medium and oxidatively modified by the addition of copper sulfate (5–20 μM) (17, 18). For most of the oxidations the presence of EDTA in the saline buffer led to a small residual concentration of EDTA in the final oxidation mixture. Milder extents of oxidation were obtained in many of the experiments by decreasing the ratio of copper to EDTA. In one experiment (turnover #2), extensive oxidation was achieved by removing EDTA from the LDL solution prior to oxidation by dialysis against EDTA-free PBS. LDL was refiltered through a 0.45-μm filter after each dialysis. After incubation at 37°C for the prescribed duration, the reaction was stopped by the addition of EDTA to a concentration of $>5\times$ the copper concentration and refrigeration at 4°C.

Unlabeled LDL was also oxidatively modified by copper while in the presence of radioiodine labeled and unlabeled BSA. Native LDL was incubated with ^{125}I-labeled BSA, ^{131}I-labeled BSA, or unlabeled BSA at 4°C for 2 and

5 days. ^{125}I-labeled BSA was added to provide 300 cpm per ng of LDL protein. Similar incubations were performed with ^{131}I-labeled BSA and unlabeled BSA added to LDL aliquots in equal amounts by weight as the experiment in which ^{125}I-labeled BSA was added. After 2 days, an aliquot of each preincubated LDL/BSA sample was diluted to a final LDL concentration of 0.1 mg protein/ml of Ham's F-10 medium. Identical but freshly mixed non-preincubated samples of LDL and BSA were prepared in the same manner. Copper sulfate (5 μM) was added to all the diluted LDL/BSA samples, which were then incubated in cuvettes at room temperature while formation of conjugated dienes was followed.

After 5 days of preincubation, similar preincubated and non-preincubated samples were again prepared except each LDL/BSA sample was diluted to 0.2 mg/ml of LDL protein and copper was added to final concentration of 10 μM. Samples were then incubated in plastic cell culture plates at 37°C. At 0, 2, 4, 8, 14, and 24 h, aliquots in duplicate were frozen at −20°C for subsequent measurement of thiobarbituric acid reactive substances (TBARS).

Analysis of modified LDL

TBARS. Extent of LDL oxidation was measured by analysis of TBARS, using malondialdehyde (MDA) as the standard, as previously described (17, 18). Aliquots for TBARS analysis were immediately frozen at −20°C for subsequent simultaneous analysis after the indicated incubation duration.

Conjugated dienes. Extent of lipid peroxidation was also monitored by measuring the extent of conjugated diene formation as previously described (19). Samples of LDL/BSA were oxidized with copper in matched cuvettes while formation of conjugated dienes was continuously measured by absorbance at 234 nm using a Uvikon 810 spectrophotometer (Zurich, Switzerland).

LDL fatty acid composition. Lipids from native and modified LDL samples were extracted by a modification of the method of Folch, Lees, and Sloane Stanley (20). The fatty acids were transmethylated (21) and analyzed in a Varian gas chromatograph model 3700, equipped with a column of 10% Silar 5CP on 100/120 Gas Chrom Q2 (19). Fatty acid contents were expressed as a percentage of total fatty acids.

Fibroblast degradation. Cultured human fibroblasts were plated in 24-well cluster dishes. Prior to use, cells were incubated overnight in DME medium containing 0.05 mg/ml gentamicin and 5 mg/ml human lipoprotein-deficient serum (LPDS). After washing the cells with DME/gentamicin, pre- and post-oxidized labeled LDL were added to duplicate wells in the presence and absence of a 10-fold excess concentration of native LDL. After incubation at 37°C for 5 h, the media were recovered and the amount of LDL receptor specific degradation of LDL was determined as previously described (16). Cell protein in each well was solubilized by overnight incubation in 500 μl of 1 N NaOH and measured by a modification of the method of Lowry et al. (13).

Macrophage degradation. Resident mouse peritoneal macrophages were harvested by lavage as previously described (17, 18). Cells were plated in 24-well cluster dishes at a density of approximately 1.2 million cells per well in RPMI medium containing 10% (vol/vol) fetal calf serum. Pre- and post-oxidized labeled LDL in DME/LPDS were added to the cells in the presence and absence of polyinosinic acid at 250 μg/well. All other steps are as above. Scavenger receptor-mediated degradation was calculated as the extent of LDL degraded that was suppressible by polyinosinic acid.

Agarose electrophoresis. Agarose electrophoresis gels were obtained from Ciba Corning Diagnostic Corp. (Palo Alto, CA). LDL Samples were loaded at 0.4–8.0 μg protein per well and run at 86 volts for 35 min. After drying, gels were stained with Oil Red O to reveal lipoprotein bands.

In vivo clearance. Male Hartley guinea pigs weighing 500–600 g were purchased from Charles River Breeding Labs, Inc. (Wilmington, MA). Radioiodinated LDL samples prior to oxidation or after varying amounts of copper oxidation were diluted in PBS or Ham's F-10 medium, respectively. The LDL samples were then injected intravenously into guinea pigs via the jugular vein cut down as previously described (16). Blood samples were collected over the ensuing 24 h via cardiac puncture. Whole plasma radioactivity was determined in a double-channel gamma spectrometer. Plasma decay kinetics were analyzed using an interactive curve-peeling program as previously described (16, 22). Fractional catabolic rates (FCR) in pools/h were calculated with the assumption that labeled LDL preparations represent kinetically homogenous populations of particles. For samples with very rapid FCRs (greater than 0.5) only TCA-precipitable radioactivity in plasma measured at each time point was used for calculations in order to exclude free radioiodine present in plasma, which presumably was generated from the rapid degradation of the radioiodinated LDL.

Protection with antioxidants

Ascorbic acid (Sigma Chemical Co., St. Louis, MO) was dissolved in PBS at 500 mM and pH was adjusted to 7.4 with 10 N NaOH. Butylated hydroxytoluene (BHT) (J. T. Baker Chemical Co., Phillipsburg, NJ) was dissolved in absolute ethanol (Quantum Chemical Corp., Tuscola, IL) at 20 mM. Stock ascorbic acid and BHT were added to PBS containing 0.27 mM EDTA to final concentrations of 500 μM and 20 μM, respectively. BHT was noted to at least partially precipitate out of solution when the PBS was cooled to 4°C. In some experiments, LDL was iodinated as described above and then immediately dialyzed extensively in PBS containing ascorbic acid

and BHT. The antioxidants did not significantly affect the specific activity of the labeled LDL or the TCA precipitability of the radioactivity.

At 2 and 9 days after iodination, guinea pig turnovers were performed using the ^{125}I-labeled LDL and ^{131}I-labeled LDL tracers that had been dialyzed with PBS containing ascorbic acid and BHT, as well as ^{125}I-labeled LDL and ^{131}I-labeled LDL samples that had been simultaneously iodinated but dialyzed only in PBS with EDTA.

At 37 days after iodination, fatty acid compositions were measured in the four samples listed above, as well as in an aliquot of the stock native LDL.

RESULTS

Effects of radioiodination on parameters of lipid peroxidation

TBARS formation. When ^{125}I-LDL, ^{131}I-LDL, and unlabeled LDL were exposed to 10 μM copper to initiate oxidation, they showed significant differences in the rate of progression of lipid peroxidation, as assayed by TBARS formation (**Fig. 1**). Unlabeled LDL showed a slow rise in TBARS over the first 14 h before rapidly accelerating to 36 nmol MDA/mg LDL by 23 h. ^{125}I-LDL had a reduced lag phase of 4 h on its way to 33 nmol MDA/mg LDL. ^{131}I-LDL had a lag phase of only 2 h and showed the most rapid rise of TBARS, finally reaching 34 nmol MDA/mg LDL by 23 h.

In order to determine whether the radiation itself, or the covalent attachment of the iodine, or even the iodination procedure (which is an oxidation process) accounted for the increased susceptibility to oxidation of the labeled samples, we exposed LDL to ^{125}I- or ^{131}I-labeled BSA. Unlabeled native LDL was oxidized with copper exactly as in the previous experiment, but this time the LDL had been preincubated for 5 days with either ^{125}I-BSA, ^{131}I-BSA, or unlabeled BSA. As controls, native LDL was also oxidized in the presence of freshly added ^{125}I-BSA or ^{131}I-BSA (without preincubation). All the controls showed a similar pattern of TBARS formation, reaching approximately 9 and 33 nmol MDA/mg LDL by 8 and 14 h, respectively (**Fig. 2**). LDL that had been preincubated with ^{125}I-BSA for 5 days had a similar pattern, but slightly higher TBARS values at 8 and 14 h. In contrast, LDL which had been preincubated with ^{131}I-BSA showed a significantly earlier rise of TBARS, though again it reached similar levels by 24 h.

Conjugated diene formation. The formation of conjugated dienes, which can be measured by absorbance at 234 nm, is another measure of lipid oxidation (23). To corroborate the previous BSA experiment, it was repeated while measuring the formation of conjugated dienes. This time, the preincubation time for the preincubated samples was decreased to 2 days, and copper and LDL concentrations were halved. All the controls showed a similar starting absorbance, at approximately 0.700 units, and a similar pattern of increase in conjugated dienes (**Fig. 3A**). In contrast, the LDL samples that had been preincubated with ^{125}I-BSA and ^{131}I-BSA showed an increased absorbance even at the beginning of the oxidation process, at 0.815 and 0.901, respectively. When the curve origins were normalized (Fig. 3B), it can also be seen that the LDL samples preincubated with ^{125}I-BSA and ^{131}I-BSA had shorter lag phases and faster early slopes in conjugated diene formation, with ^{131}I-BSA having the greatest effect.

The previous two experiments suggest that the radioiodine-induced change in LDL oxidation does not require

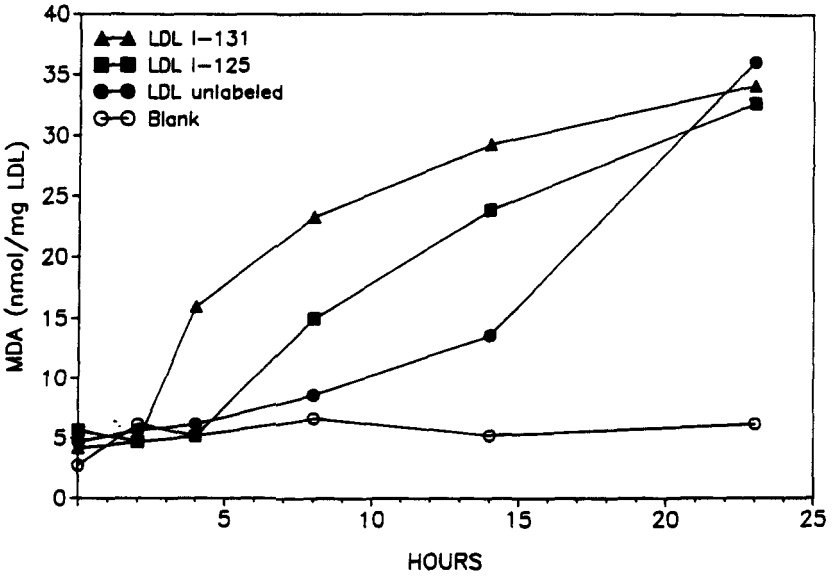

Fig. 1. Time course of TBARS formation of various LDL preparations during exposure to mild copper oxidation. Aliquots of the same LDL preparation were iodinated. Samples of ^{125}I-LDL, ^{131}I-LDL, and unlabeled LDL were diluted to 0.2 mg/ml in Ham's F-10 medium and then oxidized with 10 μM copper at 37°C for 23 h in the presence of 15 μM EDTA. Aliquots were taken in duplicate and TBARS were measured at indicated times. The blank sample contained only Ham's F-10 medium and copper.

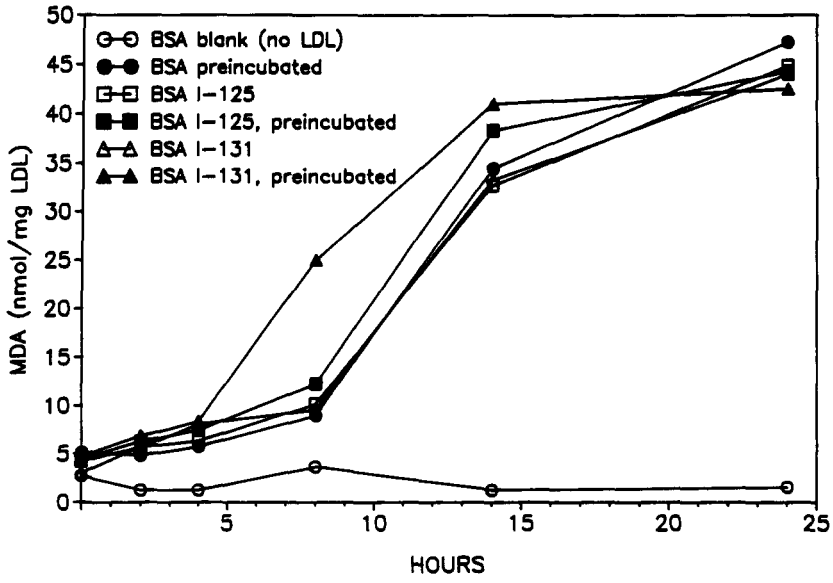

Fig. 2. Time course of TBARS formation of LDL exposed to iodinated BSA. Native unlabeled LDL was preincubated with ^{125}I-BSA, ^{131}I-BSA, or unlabeled BSA for 5 days at 4°C. ^{125}I-BSA had a specific activity of approximately 350 cpm/ng, and was added to provide 300 cpm per ng LDL protein. ^{131}I-BSA (130 cpm/ng) and unlabeled BSA were added to LDL in identical amounts by weight as ^{125}I-BSA. After 5 days of preincubation, identical but nonpreincubated samples of LDL/^{125}I-BSA and LDL/^{131}I-BSA were prepared. All five samples were then diluted in Ham's F-10 medium to 0.2 mg/ml and oxidized with 10 μM copper at 37°C in the presence of 7 μM EDTA. Aliquots were taken in duplicate and TBARS measured at indicated times.

covalent alteration of the LDL particle, nor is it an artifact of the iodination process itself. Therefore, it is more likely that it is radiated energy from the radiolabeled BSA which is affecting the LDL. Moreover, this alteration of LDL's characteristics seems to require a finite duration of exposure.

Fatty acid content. During lipid peroxidation there is consumption of polyunsaturated fatty acids. Therefore, differences in the rate of lipid peroxidation should be associated with differences in the rate of polyunsaturated fatty acid consumption. ^{125}I-LDL, ^{131}I-LDL, and LDL that had been taken through the iodination process but without the addition of iodine (sham-iodinated LDL), were oxidized for 8 and 24 h by the addition of 15 μM copper. The samples were used 37 days after the iodination procedure. Subsequent to the oxidation, they were extracted and fatty acid levels were measured. Before oxidation, sham-iodinated LDL and ^{125}I-LDL had similar linoleic acid (18:2) contents at 42.5% and 41.7%, respectively (**Table 1**). In contrast, ^{131}I-LDL already showed

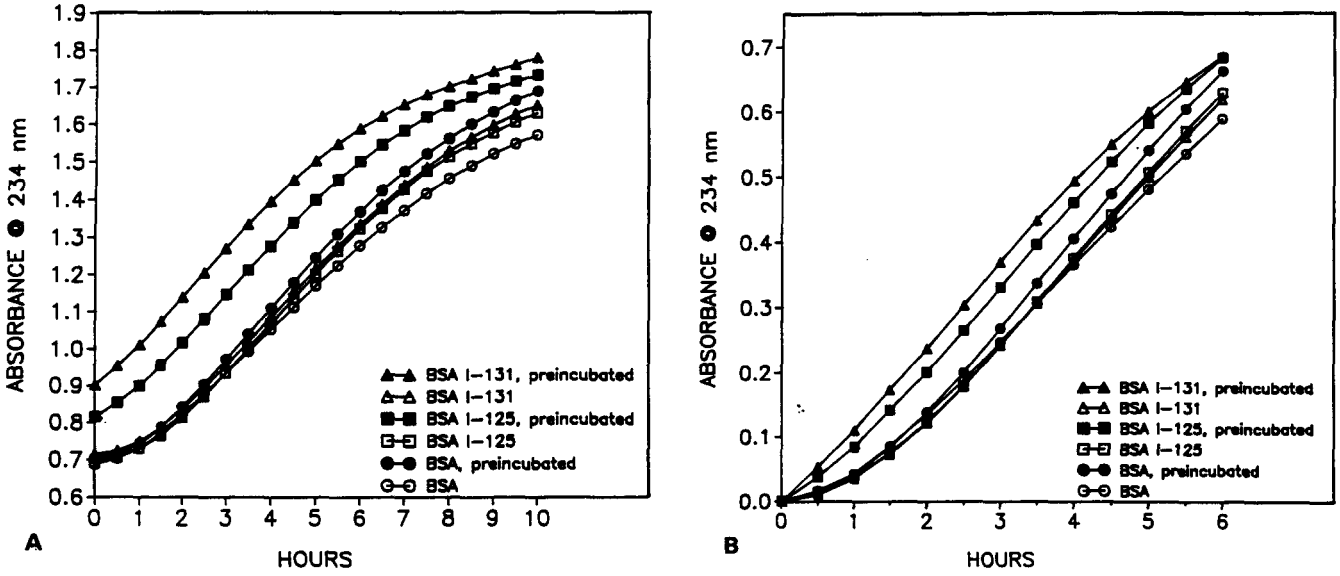

Fig. 3. Conjugated diene formation of LDL preparations exposed to iodinated BSA. Panel A: Samples were prepared as described in legend to Fig. 2 except that the preincubation time was reduced to 2 days, and LDL protein concentrations, EDTA concentrations, and copper concentrations were halved to 0.1 mg/ml, 3.4 μM, and 5 μM, respectively. Oxidations were done in matched cuvettes at room temperature while conjugated diene formation was measured every 30 min by absolute absorbance at 234 nm. Panel B: The same conjugated diene formation curves as Fig. 3A, but the origins were normalized and only the changes in absorbance at 234 nm were plotted.

TABLE 1. LDL fatty acid contents pre- and post-oxidation

Preparation	Percent Fatty Acid Distribution								
	14:0	16:0	16:1	18:0	18:1	18:2	20:3	20:4	20:5
Sham LDL									
Pre-ox	0.7	20.9	2.0	7.0	18.4	42.5	1.3	5.6	1.7
8 h Post-ox		23.4	2.2	7.3	20.7	39.3	1.1	3.8	2.3
24 h Post-ox	1.3	41.0	3.1	12.9	30.5	8.0			3.1
^{125}I-LDL									
Pre-ox		22.3	1.9	7.1	20.3	41.7	1.1	3.9	1.7
8 h Post-ox		36.7	3.1	12.4	28.3	16.3		0.4	2.9
24 h Post-ox		44.9	3.4	14.2	29.4	4.1			4.0
^{131}I-LDL									
Pre-ox		26.7	2.6	8.8	22.9	34.2	0.9	2.0	1.8
8 h Post-ox		39.2	3.0	12.3	30.2	11.8			3.4
24 h Post-ox		45.6	3.1	14.2	29.1	4.2			3.9

Aliquots of the same LDL preparation were iodinated. Sham LDL (LDL that had been taken through the iodination process but without use of radioiodine), ^{125}I-LDL, and ^{131}I-LDL were diluted to 0.2 mg/ml and oxidized at 37°C with 15 μM copper in the presence of 20 μM EDTA. Aliquots were taken prior to oxidation, as well as 8 and 24 h after oxidation, and stored at 4°C in the presence of added excess EDTA. Samples were extracted and percent fatty acid distribution was measured by high performance liquid chromatography. (The samples used were 37 days after iodination.) Fatty acid content identified as 20:5 probably also includes 22:1 and perhaps other minor fatty acids.

significant depletion of linoleic acid even prior to oxidation at 34.2%. After 8 h of oxidation, ^{125}I-LDL and ^{131}I-LDL showed significant depletion of linoleic acid contents at 16.3% and 11.8%, respectively, but the sham-iodinated LDL had changed only minimally at 39.3%. The sham-iodinated LDL showed depletion of linoleic acid to 8.0% only after 24 h of oxidation. Similar tendencies showing increasing potential for oxidation of sham-iodinated LDL, ^{125}I-LDL, and ^{131}I-LDL can also be seen in the baseline levels and subsequent oxidative consumption of arachidonic acid (20:4). Fatty acid measurements done on nonoxidized samples up to 21 days after iodination showed no significant differences.

Effects of radioiodination on biological properties of LDL

Cell culture experiments. Degradation of LDL before and after oxidation was measured using cultured human fibroblasts and mouse peritoneal macrophages. In order to achieve relatively mild levels of oxidation, ^{125}I-LDL and ^{131}I-LDL were exposed to 10 μM copper for 8 h in the presence of 15 μM EDTA. TBARS levels after oxidation were 15 and 23 nmol MDA/mg LDL for ^{125}I-LDL and ^{131}I-LDL, respectively. Even prior to copper oxidation, LDL receptor specific degradation was significantly reduced for ^{131}I-LDL compared to ^{125}I-LDL (**Fig. 4**). Levels of LDL degradation for ^{131}I-LDL were approxi-

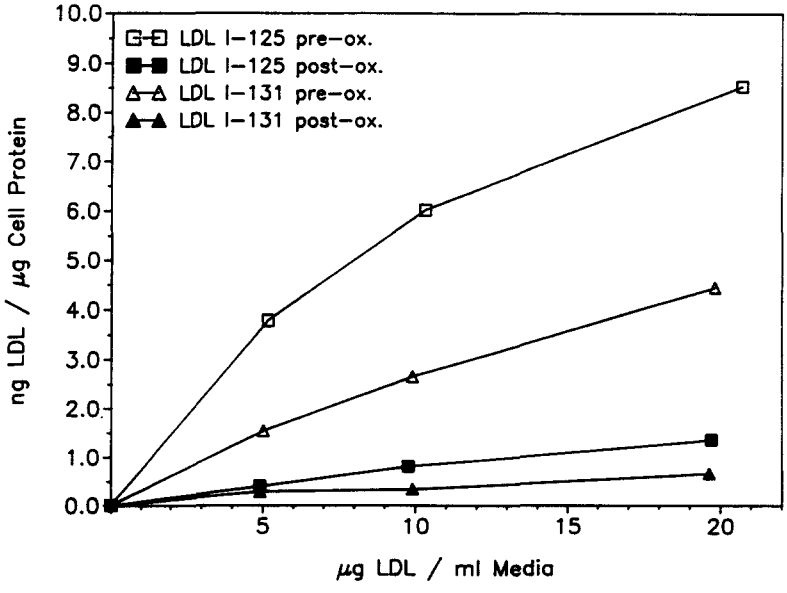

Fig. 4. Fibroblast degradation of variously iodinated and oxidized LDL samples. Aliquots of the same LDL preparation were iodinated and aliquots of ^{125}I-LDL and ^{131}I-LDL were diluted to 0.2 mg/ml and oxidized with 10 μM copper for 8 h in the presence of 15 μM EDTA. Aliquots of pre- and post-oxidized samples were then incubated with cultured human fibroblasts at 37°C for 5 h, in the presence and absence of 200 μg/ml of excess unlabeled LDL. The amount of LDL receptor specific degradation was measured as the amount of labeled LDL degraded that was suppressible by an excess of unlabeled LDL.

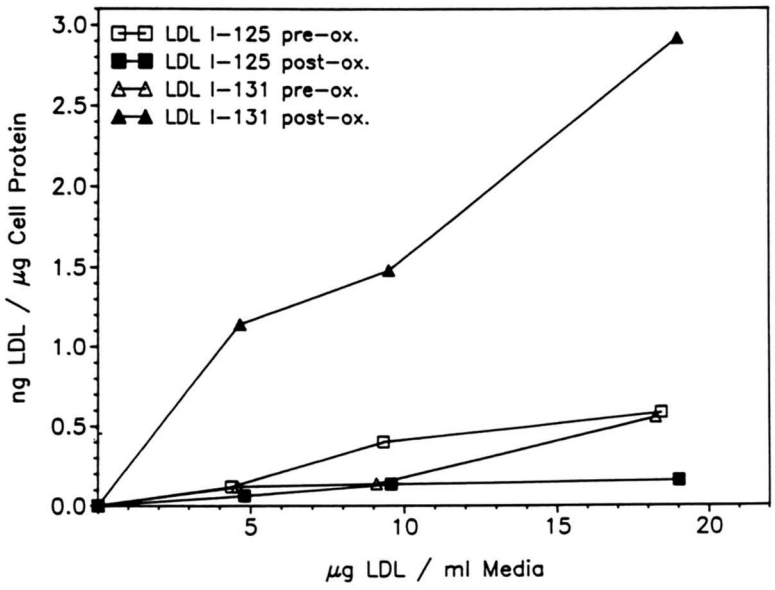

Fig. 5. Macrophage degradation of variously iodinated and oxidized LDL samples. Preparations of LDL used in Fig. 4 were used in this experiment, except that the LDL preparations were incubated with mouse peritoneal macrophages instead of cultured human fibroblasts, and polyinosinic acid (PI) at 250 μg/well (500 μg/ml) was used instead of excess unlabeled LDL. The amount of LDL degraded via the scavenger receptor pathway was calculated as that amount suppressible by the excess PI.

mately one-half of that for ^{125}I-LDL at the concentrations tested. With exposure to only mild oxidative conditions, both iodinated samples displayed very low levels of LDL receptor specific degradation with values approximately one-tenth of that for peroxidized ^{125}I-LDL. Again, the oxidized ^{131}I-LDL showed the greatest loss of ability to bind to the LDL receptor.

An experiment with mouse peritoneal macrophages was done to measure scavenger receptor-mediated degradation for the LDL samples used above. Prior to copper oxidation, both labeled LDLs showed very little scavenger receptor-mediated degradation (**Fig. 5**). At the mild levels of oxidation used, only ^{131}I-LDL showed a significant increase in scavenger receptor-mediated degradation after exposure to oxidizing conditions.

Agarose gel electrophoresis of these samples confirmed that the extent of lipoprotein modification of the samples used in the cell culture experiments, as manifested by electrophoretic mobility, are in the order of: native LDL < ^{125}I-LDL < ^{131}I-LDL < oxidized ^{125}I-LDL < oxidized ^{131}I-LDL (**Fig. 6**).

In vivo clearance of radiolabeled LDL preparations. Effect of oxidation (turnover experiment #1): ^{125}I-LDL and ^{131}I-LDL were exposed to a moderate level of oxidation (**Table 2**). TBARS levels for post-oxidized ^{125}I-LDL and ^{131}I-LDL were 18.9 and 29.4 nmol MDA/mg LDL, respectively. Each of the iodinated LDL samples, before and after oxidation, was injected into guinea pigs in pairs, and the FCRs were calculated. Even prior to oxidation, there was a significant difference between the clearance of ^{125}I-LDL and ^{131}I-LDL (Table 2). Mean FCRs were 0.080 pool/h for ^{125}I-LDL and 0.112 pool/h for ^{131}I-LDL. After oxidation, the FCR for ^{125}I-LDL increased 2-fold to 0.154 pool/h, but the FCR for ^{131}I-LDL increased to 1.915 pool/h, an increase of nearly 20-fold. An example of actual plasma decay curves obtained in individual guinea pigs for ^{131}I-LDL before oxidation and after 4 and 18 h of oxidation is shown in **Fig. 7.**

Effect of co-incubation (turnover experiment #2): In order to achieve more extensive levels of oxidation, ^{125}I-LDL and ^{131}I-LDL were dialyzed in EDTA-free PBS. After recovery and filtration, an aliquot of each sample was placed in a common container and incubated together overnight at 4°C. The next day, aliquots of all three samples (^{125}I-LDL, ^{131}I-LDL, and co-incubated ^{125}I-LDL/^{131}I-LDL) were oxidized with copper at 20 μM for 11 h (**Table 3**). After oxidation, the resulting TBARS were 30.7, 28.9, and 30.7 nmol MDA/mg LDL for ^{125}I-LDL, ^{131}I-LDL, and co-incubated ^{125}I-LDL/^{131}I-LDL, respectively. TBARS for these samples prior to oxidation were no greater than 1.6 nmol MDA/mg LDL.

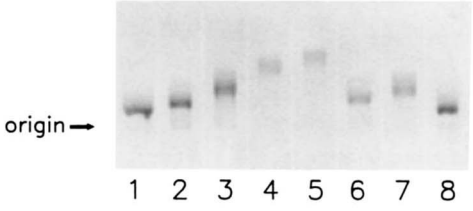

Fig. 6. Agarose gel electrophoresis of variously iodinated and oxidized LDL samples. Unlabeled LDL samples, stock labeled LDL samples, and the pre- and post-oxidized labeled LDL samples used in Figs. 4 and 5 were loaded on an agarose gel at 1–8 μg/well and run at 86 volts for 35 min. The gel was subsequently dried and stained with Oil Red O. The experiment was done 20 days after iodination. Lanes 1 and 8, unlabeled LDL in PBS; lane 2, stock ^{125}I-LDL in PBS; lane 3, stock ^{131}I-LDL in PBS; lane 4, post-oxidized ^{125}I-LDL in Ham's F-10; lane 5, post-oxidized ^{131}I-LDL in Ham's F-10; lane 6, pre-oxidized ^{125}I-LDL in Ham's F-10; lane 7, pre-oxidized ^{131}I-LDL in Ham's F-10.

TABLE 2. Effect of radioiodination on susceptibility of LDL to oxidative modification: turnover study #1—effect of oxidative stress

Guinea Pig #	^{125}I-LDL		^{131}I-LDL	
	FCR	TBARS	FCR	TBARS
	pools/h	*nmol/mg protein*	*pools/h*	*nmol/mg protein*
Pre-ox				
1	0.077		0.109	
2	0.083		0.114	
Mean	0.080 ± 0.004		0.112 ± 0.004	
Post-ox				
3	0.154	18.9	2.060	29.4
4	0.154	18.9	1.770	29.4
Mean	0.154 ± 0.0		1.915 ± 0.205	

Aliquots of the same LDL preparation were iodinated with ^{125}I and ^{131}I and diluted to 0.3 mg/ml in Ham's F-10 medium. To generate moderate levels of oxidation, each LDL was oxidized by exposure to 10 μM copper for 8 h and then to 20 μM copper for an additional 10 h in the presence of EDTA at 42 μM. TBARS were measured before and after oxidation and are expressed as nmol of MDA formed per mg protein. Pre- and post-oxidized LDL samples were then injected intravenously into guinea pigs and plasma clearance rates were calculated as fractional catabolic rates (FCR) expressed as pools/h.

Prior to oxidation, the mean FCR for ^{125}I-LDL was 0.087 pool/h, while that for ^{131}I-LDL was again greater at 0.123 pool/h (Table 3). With co-incubation of the two tracers, the mean FCR for ^{131}I-LDL remained essentially unchanged at 0.124 pool/h, but that for ^{125}I-LDL had now increased to 0.103 pool/h. After oxidation, all the samples markedly increased their clearance, but the LDL samples exposed to ^{131}I again showed the greatest increase. The mean FCR for ^{125}I-LDL was 3.185 pool/h, while that for ^{131}I-LDL was 5.970 pool/h. For the iodinated samples that were subjected to co-incubation, the mean FCR for ^{131}I-LDL was mildly increased to 6.690 pool/h, while that for ^{125}I-LDL was doubled to 6.285 pool/h, i.e., the ^{131}I radiation clearly had increased the susceptibility to oxidation of the LDL particles labeled with ^{125}I.

Effect of storage (turnover experiment #3): Because the earlier data indicated an increasing effect of radiation-induced oxidation over time, we examined the effects of storage on identical aliquots of LDL iodinated with ^{125}I and ^{131}I (**Table 4**). After iodination, the tracers were dialyzed extensively for 2 days in PBS with EDTA. Samples were then promptly recovered and turnover experiments were performed in guinea pigs. Turnovers were then repeated 4 and 11 days later (6 and 13 days after iodination) using the same labeled LDL samples and matched guinea pigs. Between experiments, samples were stored in the dark at 4°C.

When injected promptly (2 days) after iodination, the mean FCRs for both samples were relatively slow at 0.034 and 0.042 pool/h for ^{125}I-LDL and ^{131}I-LDL, respectively (Table 4). As the duration of storage increased, both samples showed a remarkable acceleration of their FCR. As little as 6 days after iodination, the mean FCR had increased to 0.063 pool/h for ^{125}I-LDL and 0.089 pool/h for ^{131}I-LDL. By 13 days, both samples had increased by over 250%.

Protection with antioxidants

The data above indicated that the radiation from the radioiodine had directly initiated lipid peroxidation and rendered the LDL samples more susceptible to oxidative modification when exposed to an oxidant challenge. If this is correct, then antioxidants should be able to inhibit lipid peroxidation and hence prevent the enhanced FCR after exposure to radioiodine. To test this hypothesis, identical aliquots of LDL were iodinated with ^{125}I and ^{131}I in duplicate. One pair of ^{125}I-LDL and ^{131}I-LDL samples was immediately dialyzed in PBS with EDTA as all previously

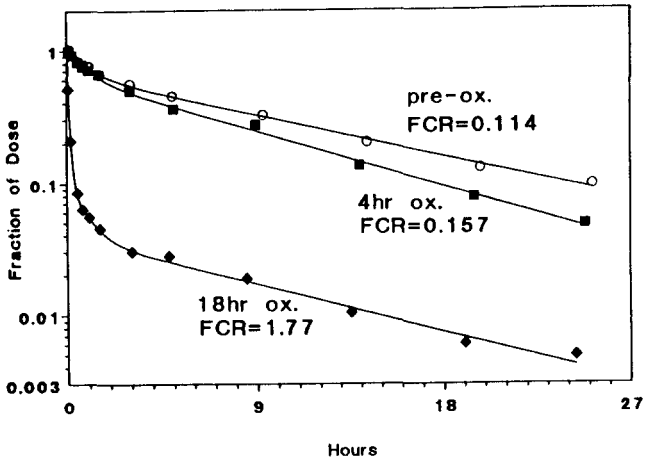

Fig. 7. Plasma decay curves in individual guinea pigs of ^{131}I-LDL before and after 4 and 18 h of copper-mediated oxidation as described in legend to Table 2.

TABLE 3. Effect of radioiodination on susceptibility of LDL to oxidative modification: turnover study #2—effect of co-incubation with labeled BSA

Guinea Pig #	^{125}I-LDL		^{131}I-LDL	
	FCR	TBARS	FCR	TBARS
	pools/h	*nmol/mg protein*	*pools/h*	*nmol/mg protein*
Pre-ox				
5	0.071		0.106	1.6
6	0.103		0.139	1.6
Mean	0.087 ± 0.023		0.123 ± 0.023	
Pre-ox/co-inc				
7	0.092	0.9	0.112	0.9
8	0.113	0.9	0.135	0.9
Mean	0.103 ± 0.015		0.124 ± 0.016	
Post-ox				
9	2.890	30.7	4.950	28.9
10	3.480	30.7	6.990	28.9
Mean	3.185 ± 0.417		5.970 ± 1.443	
Post-ox/co-inc				
11	5.720	30.7	5.990	30.7
12	6.850	30.7	7.390	30.7
Mean	6.285 ± 0.799		6.690 ± 0.990	

Aliquots of the same LDL preparation were iodinated with ^{125}I and ^{131}I. To generate extensive levels of oxidation, EDTA was removed from ^{125}I-LDL and ^{131}I-LDL by EDTA-free dialysis. Subsequently, an aliquot of each sample was incubated together in a common container at 4°C. One day later, aliquots of all three samples (^{125}I-LDL, ^{131}I-LDL, and co-incubated ^{125}I-LDL/^{131}I-LDL) were diluted in Ham's F-10 medium to 0.5 mg/ml and oxidized for 11 h with 20 μM copper. Paired tracers (individually incubated ^{125}I-LDL and ^{131}I-LDL, co-incubated ^{125}I-LDL/^{131}I-LDL) were then injected into guinea pigs and FCRs were determined for all pre- and post-oxidized samples.

described samples. The other pair of labeled LDL samples was dialyzed in PBS containing 500 μM ascorbic acid, 20 μM BHT, as well as EDTA (**Table 5**).

A guinea pig turnover was then performed 2 days after iodination. The mean FCR for the routinely prepared ^{125}I-LDL was 0.047 pool/h, while that for the corresponding ^{131}I-LDL was slightly faster at 0.052 pool/h (values in accord with previous experiments). In contrast, the LDL samples protected with the antioxidants ascorbic acid and BHT showed identical mean FCRs of 0.047 pool/h for both ^{125}I-LDL and ^{131}I-LDL.

Nine days after the iodination procedure the turnover was repeated using the same pairs of samples. By this time, the unprotected samples again showed marked acceleration of their mean FCRs to 0.090 and 0.099 pool/h for ^{125}I-LDL and ^{131}I-LDL, respectively. By comparison, the samples protected with antioxidants showed only minor changes in their mean FCRs to 0.052 pools/h and 0.055 pools/h for ^{125}I-LDL and ^{131}I-LDL, respectively.

At 37 days after iodination fatty acid distribution was measured in the four labeled LDL samples used above as well as in an aliquot of the original unlabeled LDL sample (**Table 6**). The unlabeled LDL had a linoleic acid content of 43% and an arachidonic acid content of 8.0% of total

TABLE 4. Effect of radioiodination on susceptibility of LDL to oxidative modification: turnover study #3—effect of storage

Guinea Pig #	Fractional Catabolic Rate	
	^{125}I-LDL	^{131}I-LDL
	pools/h	
2 Days		
13	0.036	0.042
14	0.038	0.046
15	0.029	0.037
Mean	0.034 ± 0.005	0.042 ± 0.005
6 Days		
16	0.057	0.083
17	0.075	0.101
18	0.056	0.083
Mean	0.063 ± 0.011	0.089 ± 0.010
13 Days		
19	0.084	0.100
20	0.093	0.110
21	0.093	0.113
Mean	0.090 ± 0.005	0.108 ± 0.007

Aliquots of the same LDL preparation were iodinated with ^{125}I and ^{131}I and then dialyzed at 4°C in PBS containing 0.27 mM EDTA. Two days after iodination, paired samples were injected into guinea pigs and FCRs were calculated. Aliquots of these samples were also stored in the dark at 4°C and turnovers were repeated at 6 and 13 days after iodination.

TABLE 5. Effect of radioiodination on susceptibility of LDL to oxidative modification: turnover study #4—effect of antioxidant protection

Guinea Pig #	Fractional Catabolic Rate			
	^{125}I-LDL		^{131}I-LDL	
	Control	Anti-ox	Control	Anti-ox
	pools/h			
2 Days				
22	0.052		0.057	
23	0.042		0.047	
24		0.050		0.051
25		0.045		0.046
26		0.045		0.045
Mean	0.047 ± 0.007	0.047 ± 0.003	0.052 ± 0.008	0.047 ± 0.003
9 Days				
27	0.084		0.092	
28	0.100		0.110	
29	0.086		0.096	
30		0.046		0.049
31		0.056		0.059
32		0.055		0.057
Mean	0.090 ± 0.009	0.052 ± 0.005	0.099 ± 0.010	0.055 ± 0.005

Using aliquots of the same LDL preparation, two pairs of ^{125}I-LDL and ^{131}I-LDL were iodinated. Immediately after iodination, one pair (^{125}I-LDL and ^{131}I-LDL) was dialyzed in PBS containing 0.27 mM EDTA. The other pair was dialyzed in PBS containing antioxidants BHT (20 μM) and ascorbic acid (500 μM) in addition to EDTA. Turnovers were performed at 2 and 9 days after iodination, with one pair of samples injected into each guinea pig. Between studies, all samples were stored in the dark at 4°C.

fatty acids. As before, the radiolabeled LDLs showed decreases in the content of polyunsaturated fatty acids. ^{125}I-LDL had a linoleic acid content of 41% and an arachidonic acid content of 5.2%. ^{131}I-LDL had a linoleic acid content of 36% and an arachidonic acid content of 3.6%. No significant decreases in saturated or monounsaturated fatty acid contents were noted. In contrast, the radiolabeled LDLs that were protected with antioxidants showed relative preservation of their fatty acid distribution. Linoleic acid contents for both ^{125}I-LDL and ^{131}I-LDL were 42% while arachidonic acid contents were 8% (Table 6).

DISCUSSION

The preceding studies demonstrate that exposure of LDL to ^{125}I and ^{131}I under commonly used experimental conditions resulted in lipid peroxidation and alterations in biological properties. The presence of the radiolabels

TABLE 6. LDL fatty acid contents of radioiodinated LDL preparations with and without antioxidant protection

Preparation	14:0	16:0	16:1	18:0	18:1	18:2	20:4
				%			
Without antioxidant							
Native LDL	0.6	21.4	2.0	6.4	18.8	42.7	8.0
^{125}I-LDL	0.7	23.4	2.4	7.2	20.5	40.8	5.2
^{131}I-LDL	0.7	26.3	2.5	7.8	22.7	36.4	3.6
With antioxidant							
^{125}I-LDL	0.9	22.5	2.4	6.5	18.6	41.6	7.6
^{131}I-LDL	0.7	21.9	2.0	6.7	18.8	42.0	7.8

Aliquots of the LDL preparation were iodinated in pairs with ^{125}I-LDL or ^{131}I-LDL. After iodination, one pair (^{125}I-LDL and ^{131}I-LDL) was dialyzed as usual in PBS containing 0.27 mM EDTA. The other pair was dialyzed in PBS containing the antioxidants BHT (20 μM) and ascorbic acid (500 μM) as well as EDTA. All samples were stored in the dark at 4°C. At 37 days after iodination, samples were extracted and percent fatty acid distributions were measured by high performance liquid chromatography.

induced oxygen-free radicals in the aqueous solutions and increased the content of hydroperoxides in LDL, even as the LDL was being dialyzed or stored in the presence of EDTA. Thus, the radiolabeled LDL had much greater susceptibility to oxidation mediated by pro-oxidants such as copper ions, presumably due to prior initiation of lipid peroxidation. This effect was significantly greater for ^{131}I than for ^{125}I.

Evidence of radioiodine-induced lipid peroxidation was manifested by accelerated formation of peroxidation products, as measured by TBARS and conjugated dienes. LDL labeled with radioactive iodine, as well as non-labeled LDL exposed for 5 days to radioiodine-labeled BSA, produced a more rapid rise in TBARS with a shortened lag phase when oxidized in the presence of copper. The same phenomenon was observed using the formation of conjugated dienes as the index of lipid peroxidation. LDL that had been exposed to radioiodine labeled BSA for only 2 days already showed increased formation of conjugated dienes, even before copper-induced peroxidation was performed. These same samples also showed a more rapid initial rise in conjugated diene formation upon exposure to copper, when compared to LDL that had not been exposed to radioiodine. Because each sample contained identical concentrations of a common LDL and BSA, and each was followed in matching cuvettes, the lag time required for each sample to reach a specific absorbance level, and therefore a specific level of conjugated diene formation, can be compared. Clearly the LDL exposed to ^{125}I and ^{131}I had enhanced susceptibility to lipid peroxidation as manifested by a decreased lag time. Experiments were also done in which copper-induced generation of conjugated dienes was measured in LDL that had been directly labeled with radioiodine (data not shown). Similar results were seen except that even at the start of the experiment, the absolute absorbance of ^{131}I-LDL was already so high that the additional increase occurring after the addition of copper was greatly diminished. This can probably be explained by the more prolonged and intense exposure to radioiodine brought on by direct radiolabeling of LDL. Studies by Esterbauer et al. (24), and Frei, Kim, and Ames (25) have shown that lipid peroxidation of LDL does not occur until endogenous antioxidants such as alpha tocopherol, carotenoids, and ubiquinone-10 have been consumed, and that this delay is manifested by a lag phase in the production of peroxidation products (23). Moreover, the addition of exogenous antioxidants to LDL results in an increase of the this lag phase (23, 24). Since exposure to radioiodine decreased the lag phase of copper-induced oxidation, it is likely that radioiodine exposure initiated lipid peroxidation and depletion of endogenous antioxidants.

Evidence that radiation-induced lipid peroxidation of LDL can also be seen in the consumption of the substrate of lipid peroxidation, namely polyunsaturated fatty acids. The two predominant polyunsaturated fatty acids of human LDL are linoleic acid and arachidonic acid. Prolonged exposure of LDL to a radioiodine label resulted in depletion of arachidonic acid and linoleic acid contents. With exposure to copper, these differences were all exaggerated due to the acceleration of lipid peroxidation. Changes in fatty acid contents occurring in the absence of overt pro-oxidant conditions required a significant duration of radiation exposure to become detectable, since measurements done at less than 2 weeks were not significantly different.

The radiation effects of radioiodine were even more pronounced on a number of the biological properties of LDL. The iodinated LDL samples bound less well to the LDL receptor and at least for the ^{131}I-LDL sample had enhanced macrophage uptake and these effects were exaggerated by exposure to copper. Evidence for radiation effect was seen in the difference between a relatively weak radiation emitter, in ^{125}I, and a more powerful radiation emitter, in ^{131}I. One consequence of lipid peroxidation of LDL is the loss of lysine residues on apolipoprotein B-100 through interaction with malondialdehyde (MDA) and other products. This results in loss of recognition by the LDL receptor (26–28). This is a very sensitive indicator, as modification of as few as 3–5% of lysine residues of LDL can affect recognition by the fibroblast LDL receptor (18, 29). Simply exposing LDL to ^{131}I through radiolabeling decreased its LDL receptor specific degradation by approximately 50%, when compared to ^{125}I-LDL. Mild copper oxidation resulted in very low LDL receptor specific degradation for both ^{125}I and ^{131}I labeled samples. In contrast, scavenger receptor-mediated uptake by macrophages was the same for pre- and post-oxidized samples of ^{125}I-LDL but uptake of the mildly oxidized ^{131}I-LDL was significantly increased. Two conclusions can be reached from these two experiments. First, that ^{131}I increases LDL's tendency for biological modification when compared to ^{125}I. And second, that loss of LDL receptor recognition occurs prior to the development of scavenger receptor recognition (26). These results are consistent with those of Philippot, Verma, and Lin (30), who irradiated human LDL with gamma rays from a cesium-137 source at doses of 100–2000 rad. They report that this resulted in LDL modification as manifested by decreased binding and degradation by Chinese hamster V79 fibroblasts, as well as increased degradation by mouse J774G peritoneal macrophages (30).

Radiation-induced biological modification of LDL was best demonstrated in the in vivo experiments measuring turnover of LDL in guinea pigs. Studies in our laboratory have shown that accelerated in vivo clearance of oxidized LDL is a most sensitive biological marker of oxidation and that even mild levels of oxidative modification of human LDL, not reflected in enhanced macrophage uptake, are nevertheless very sensitively reflected in in-

creased clearance when injected intravenously into guinea pigs (18). This is due to uptake by liver sinusoidal cells (31, 32). Since aliquots of ^{125}I- and ^{131}I-labeled LDL were injected simultaneously in each animal, direct comparisons could be made for the two samples in each animal. Turnover studies #1 and #2 showed that ^{131}I-LDL is consistently cleared faster than ^{125}I-LDL. Turnover #1 showed that when copper was used to provide equivalent oxidative stress, ^{131}I-LDL generated higher TBARS and demonstrated a much greater acceleration of its clearance compared to ^{125}I-LDL. With more extensive oxidation, as in turnover study #2, ^{125}I-LDL generated comparable TBARS, but its clearance was still not as accelerated as ^{131}I-LDL. This experiment also showed that when ^{125}I-LDL was exposed to ^{131}I through co-incubation with ^{131}I-LDL, its clearance was closer to that of ^{131}I-LDL. Turnover study #3 demonstrated that even without copper, radioiodine labeling produced rapid and progressive modification of LDL during storage.

The mechanism of radioiodine-induced lipid peroxidation and LDL modification is likely to be identical to that of lipid peroxidation induced by ionizing radiation in general. Gamma and beta radiation produce radiolysis of the aqueous buffer, producing oxygen free radicals. Polyunsaturated fatty acids would then undergo hydrogen abstraction, conjugated diene formation, and lipid hydroperoxide formation. This mechanism is consistent with the experimental findings. Since it is the radiolysis of the aqueous environment that generates the free radicals, only the presence of the radionuclide in the solution is required. There is no requirement for the iodination process or any covalent interaction. This explains why the presence of radiolabeled BSA was able to alter native LDL, and why the presence of ^{131}I-LDL was able to alter the behavior of ^{125}I-LDL. This mechanism also explains why exposure to radioiodine resulted in a prompt increase of conjugated dienes and progressive loss of polyunsaturated fatty acids, even without exposure to copper. In contrast, TBARS formation requires further decomposition of the lipid hydroperoxide and increases only after exposure to copper. This is consistent with the work of O'Connell and Garner (3) who irradiated phospholipid liposomes with gamma rays from a cobalt-60 source at doses up to 7500 rad. They reported increases in conjugated dienes and lipid hydroperoxides, but only mild increases in TBARS. Significant increases in measurable TBARS occurred only after the post-irradiated samples were exposed to ferric chloride. They determined that the ferric chloride effect was independent of oxygen radicals and represented an iron-catalyzed decomposition of radiation-induced lipid hydroperoxides (3). Since ionizing radiation produces lipid hydroperoxides, it is predictable that radiolabeled LDL produces oxidation products such as TBARS at a higher rate and after a shorter lag phase upon exposure to copper. The possibility that direct attack of LDL components by ionizing radiation may be responsible for radiation-induced LDL modification has also been proposed (30), but it is less clear how effective antioxidants would be in preventing this type of interaction.

Since radioiodine-induced modification of LDL occurs by lipid peroxidation, it should be prevented by antioxidants. BHT is a well-known lipid-soluble antioxidant. When added to LDL, it is carried in the lipid core and functions as a free radical scavenger. It has been shown to effectively inhibit metal-catalyzed lipid peroxidation (33), monocyte-macrophage-mediated lipid peroxidation (34), UV-A-induced lipid peroxidation (35), as well as delaying the onset of ionizing radiation-induced lipid peroxidation (6). Ascorbic acid is a potent water-soluble chain-breaking antioxidant. It can function directly as a scavenger of hydroxyl radicals, superoxide anions, singlet oxygen, as well as aqueous peroxyl radicals (36). It can also function indirectly by regenerating alpha tocopherol from the alpha tocopheroxyl radical in the lipid core (37). It has been shown that physiologic levels of ascorbic acid are able to inhibit the oxidative modification of LDL (23, 37, 38), as well as preserve the endogenous lipid-soluble antioxidants of LDL (37). The current studies demonstrate that when BHT and ascorbic acid were introduced immediately after iodination, radioiodine-induced lipid peroxidation and LDL modification were greatly inhibited. LDL polyunsaturated fatty acid levels were preserved compared to those of unlabeled native LDL during prolonged (37 days) storage. The guinea pig turnover studies using the protected tracers demonstrate that not only were the initial differences between ^{125}I-LDL and ^{131}I-LDL essentially abolished, but the effects of progressive modification with time were greatly diminished. It is impossible to determine the relative efficacy of BHT versus ascorbic acid as antioxidants in this system. While the content of ascorbic acid in the aqueous medium is known, the actual content of BHT that partitioned into the lipid core of LDL is unknown.

Radiolabeled LDL samples have been extensively used in a large number of in vitro and in vivo studies of LDL metabolism (39–41). Undoubtedly, most of these studies are valid. This is particularly true in most human subjects in which LDL turnover studies have been performed, as the labeled LDL samples are usually rapidly injected very soon after iodination, and in plasma there are powerful antioxidant defenses present. For example, we and others have shown that a single LDL sample, iodinated with either ^{125}I or ^{131}I, gave nearly equal FCRs when injected into human subjects (i.e., Fig. 2 of ref. 42). However, inspection of even these carefully handled samples indicates that the FCR of the ^{131}I aliquot (0.428) was faster than the ^{125}I-LDL (0.417) (42). Similarly, in a recent study of LDL turnover in guinea pigs, we saw a more pronounced ^{131}I effect (43). In this paper, it is demonstrated that exposure

of LDL to radioiodine can result in radiation-induced changes in its biochemical composition as well as its metabolic behavior. For this reason, the use of radioiodine as a label for LDL and perhaps other lipoproteins has some inherent limitations, if one is not aware of this phenomenon. These limitations include a significantly limited effective "shelf life," potential difficulty in interpreting data that directly compare ^{125}I- and ^{131}-labeled material, and in particular, an exaggeration of differences when prooxidants are present. This may be particularly important when LDLs are being compared that have different inherent susceptibility to oxidation, such as dense LDL (44). The relevance of these limitations varies greatly depending on the type of studies being done and the types of LDL being studied. Fortunately, these limitations can be greatly diminished in the systems described through the use of antioxidants. Optimal regimens remain to be determined for the conditions under study in a given experiment, but our data demonstrate that BHT and ascorbic acid were very effective in protecting LDL for use in a very sensitive assay of LDL modification such as in vivo turnover studies.

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