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Plasma clearance and biodistribution of oxidatively modified ^{99m}Tc-ß-VLDL in rabbits

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Abstract

The biodistribution and removal from plasma (measured as fractional clearance rate, FCR, per hour) of native and oxidatively modified ^{99m}technetium-labeled ß-very low density lipoprotein (^{99m}Tc-ß-VLDL) were investigated in hypercholesterolemic (HC) and control (C) three-month old New Zealand rabbits. The intracellular accumulation of ß-VLDL labeled with ^{99m}Tc was studied *in vitro* in THP-1 cells and monocyte-derived macrophages isolated from rabbits. After intravenous injection into C rabbits, copper-oxidized ß-VLDL (^{99m}Tc-ox-ß-VLDL) was cleared from the circulation faster (0.362 ± 0.070/h) than native ß-VLDL (^{99m}Tc-nat-ß-VLDL, 0.241 ± 0.070/h). In contrast, the FCR of ^{99m}Tc-ox-ß-VLDL in HC rabbits was lower (0.100 ± 0.048/h) than that of ^{99m}Tc-nat-ß-VLDL (0.163 ± 0.043/h). The hepatic uptake of radiolabeled lipoproteins was lower in HC rabbits (0.114 ± 0.071% injected dose/g tissue for ^{99m}Tc-nat-ß-VLDL and 0.116 ± 0.057% injected dose/g tissue for ^{99m}Tc-ox-ß-VLDL) than in C rabbits (0.301 ± 0.113% injected dose/g tissue for ^{99m}Tc-nat-ß-VLDL and 0.305 ± 0.149% injected dose/g tissue for ^{99m}Tc-ox-ß-VLDL). The uptake of ^{99m}Tc-nat-ß-VLDL and ^{99m}Tc-ox-ß-VLDL by atherosclerotic aorta lesions isolated from HC rabbits (^{99m}Tc-nat-ß-VLDL: 0.033 ± 0.012% injected dose/g tissue and ^{99m}Tc-ox-ß-VLDL: 0.039 ± 0.017% injected dose/g tissue) was higher in comparison to that of non-atherosclerotic aortas from C rabbits (^{99m}Tc-nat-ß-VLDL: 0.023 ± 0.010% injected dose/g tissue and ^{99m}Tc-ox-ß-VLDL: 0.019 ± 0.010% injected dose/g tissue). However, ^{99m}Tc-nat-ß-VLDL and ^{99m}Tc-ox-ß-VLDL were taken up by atherosclerotic lesions at similar rates. *In vitro* studies showed that both monocyte-derived macrophages isolated from rabbits and THP-1 macrophages significantly internalized more ^{99m}Tc-ox-ß-VLDL than ^{99m}Tc-nat-ß-VLDL. These results indicate that in cholesterol-fed rabbits ^{99m}Tc-ox-ß-VLDL is slowly cleared from plasma and accumulates in atherosclerotic lesions. However, although the extent of *in vitro* uptake of ^{99m}Tc-ox-ß-VLDL by macrophages was high, the *in vivo* accumulation of this radiolabeled lipoprotein by atherosclerotic lesions did not differ from that of ^{99m}Tc-nat-ß-VLDL.

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Key words
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- Copper-mediated oxidation
- Biodistribution
- Fractional clearance rate
- Atherosclerosis
- Hypercholesterolemia
- Cholesterol feeding
- Rabbit

Introduction

ß-Very low density lipoprotein (ß-VLDL) is an atherogenic lipoprotein which induces the transformation of macrophages into foam cells and accumulates in plasma of hypercholesterolemic (HC) rabbits and in patients with type-III hyperlipoproteinemia (1). The oxidative modification of this lipoprotein may be induced by reactive oxygen species released by diverse cell types and increases the atherogenicity of this lipoprotein (2-6) in the presence of transition metals such as copper and iron. These oxidant species may induce changes in low density lipoprotein (LDL) and ß-VLDL (4,7) and they also act as mediators in the atherosclerotic process and other vascular diseases (8).

In hypercholesterolemia, the uptake of lipoproteins by the LDL receptor is reduced due to a down-regulation (9). However, native and oxidized ß-VLDL can be taken up by cells by other receptors whose expression is not regulated by cell sterol content (10). Therefore, the uptake of ß-VLDL by these receptors may be increased even during hypercholesterolemia, favoring the formation of foam cells. Increasing evidence demonstrates the presence of oxidatively modified lipoproteins in blood plasma from patients with heart disease and in cholesterol-fed rabbits (11-13). However, the pathophysiological role of oxidized lipoproteins present in blood plasma concerning the atherogenic process is not fully understood. Therefore, the objective of the present study was to compare the plasma clearance rate and the biodistribution of native and oxidized-ß-VLDL in cholesterol-fed (HC) and control rabbits (C) using ^{99m}technetium (^{99m}Tc)-radiolabeled lipoproteins (14).

Material and Methods

Cholesterol, benzamidine, phenylmethylsulfonyl fluoride (PMSF), aprotinin, butylated hydroxytoluene (BHT) and EDTA were purchased from Sigma Chemical Co. (St. Louis, MO). Sephadex G25 was from Pharmacia (Uppsala, Sweden). The enzymatic reagents for the cholesterol and triglyceride determinations were kindly donated by CELM (São Paulo, Brazil). All other chemicals were of the purest analytical grade available. Rompun® was kindly donated by Bayer Co. (São Paulo, Brazil).

Animals

Male New Zealand white rabbits weighing 2.5-3.5 kg were kept in an animal room on a 12-h light/12-h dark cycle, with free access to water and to the indicated diet. The 1% cholesterol diet was prepared by dissolving cholesterol (Sigma) in diethylether and then mixing it with Purina Rabbit Laboratory Chow (Purina, Campinas, SP, Brazil). The control diet consisted of Purina Rabbit Laboratory Chow without added cholesterol. The plasma cholesterol and triacylglycerols of control rabbits (C) and cholesterol-fed rabbits (HC) were determined by enzymatic methods.

ß-VLDL isolation and copper-mediated oxidation

Blood from HC rabbits (4-8 weeks of a cholesterol-rich diet) was collected into tubes containing EDTA (1 mg/ml) and centrifuged at 1000 *g* for 10 min at 4°C to isolate plasma. Immediately after isolation, 2 mM benzamidine, 1 mM PMSF, 1 µg/ml aprotinin and 20 µM BHT were added to plasma to inhibit proteolytic degradation of apolipoproteins and lipid peroxidation. ß-VLDL (density <1.019 g/ml) was isolated from plasma by sequential ultracentrifugation as described previously (15). ß-VLDL was dialyzed against 6 liters of 150 mM NaCl, 1 mM EDTA, and 3 mM NaN_3 in Tris buffer, pH 7.4. Before oxidation, the lipoprotein was dialyzed against the same buffer as described above but without EDTA. ß-VLDL (1 mg

protein/ml) was incubated with 200 µM copper sulfate for 18 h at 37°C in a shaking water bath.

Lipoprotein labeling with ^{99m}Tc

Native and copper-oxidized ß-VLDL were labeled with ^{99m}Tc according to Lees et al. (16). Briefly, ß-VLDL (4-8 mg protein) was mixed with 40-60 mCi ^{99m}Tc-pertechnetate (TcO_4^-) and 10 mg sodium dithionite, which was dissolved just before use in 0.5 M glycine buffer, pH 9.8, and the mixture was incubated for 30 min. ^{99m}Tc-ß-VLDL was separated from free ^{99m}Tc by Sephadex G 25 chromatography. The purified ^{99m}Tc-ß-VLDL was dialyzed against 150 mM NaCl, 1 mM EDTA, and 3 mM NaN_3 in Tris buffer, pH 7.4, at 4°C for 2 h, sterilized through a 0.22-µm filter to eliminate aggregates and immediately used in the experiments. Labeling efficiency was 75 to 85% for native ß-VLDL and 83-93% for copper-oxidized ß-VLDL. Upon precipitation with 20% (w/v) trichloroacetic acid (TCA), an average of 86% and 80% of the radioactivity of native and oxidized ß-VLDL, respectively, was recovered in the precipitate.

Biodistribution and plasma clearance studies

Overnight fasted rabbits were injected with native (^{99m}Tc-nat-ß-VLDL) or oxidized (^{99m}Tc-ox-ß-VLDL) ^{99m}Tc-ß-VLDL in a bolus (1-3 ml, *iv*) corresponding to 0.5-1.5 mg protein (5-10 mCi) into the marginal ear vein. Blood samples were drawn from the central ear artery into heparinized tubes 5, 15, 30, 60, 120, 160 and 240 min after injection. Plasma was isolated by centrifugation at 1000 *g* for 10 min and radioactivity was measured with a gamma-counter (Logic, Abbott Corp., Chicago, IL). The fractional clearance rate (FCR), defined as the fraction of the intravascular pool cleared per hour, was calculated from biexponential curves obtained from the radioactivity found in plasma after injection fitted by the least square procedure. The percentage of plasma radioactivity was calculated considering the value obtained at 5 min as 100% and the FCR of the ^{99m}Tc-lipoproteins from the intravascular compartment was estimated according to Matthews (17). After 6 h, the rabbits were sacrificed by exsanguination under ketamine/Rompun® anesthesia and the organs were removed. After rinsing the tissues with 150 mM NaCl, radioactivity per gram of wet tissue was counted. The radioactivity, expressed as percentage of the injected dose, was calculated for the liver, kidney, heart, ileo-psoas muscle, adrenal gland, lung, spleen, bone marrow and aorta.

Imaging studies

The biodistributions of native ^{99m}Tc-ß-VLDL and copper-oxidized ^{99m}Tc-ß-VLDL were imaged with a gamma camera (model ON 100, Ohio Nuclear Inc., Solon, OH) equipped with a high resolution collimator and stored in 128 x 128 matrices in a computer system. Planar images were acquired for the anterior view of the full body 6 h after the injection of ^{99m}Tc-labeled lipoproteins.

Autoradiography

Cholesterol-fed and control rabbits were sacrificed 6 h after injection of ^{99m}Tc-labeled lipoproteins and the aortas were removed, cleaned and washed with saline. The arteries were then opened, covered with plastic wrap, placed on X-ray film (Kodak X-OMAT XRP-5) and stored in a cassette for 48 h to perform autoradiography.

Rabbit monocyte-derived macrophages

Mononuclear cells were isolated from peripheral blood of control and cholesterol-fed rabbits using the Ficoll-Hypaque density gradient method as described previously (18). The mononuclear leukocytes (1 x 10^6 cells/

ml) were spread onto culture dishes in RPMI-1640 medium supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. After 1-h incubation at 37°C in a humidified incubator in a 5% CO_2 atmosphere, the contaminant lymphocytes and nonadherent cells were removed by gentle washing with PBS. To differentiate monocytes from macrophages, the adherent cells were cultured in fresh RPMI-1640 medium containing 10% fetal calf serum and antibiotics for 7 days. The medium was changed twice weekly. The adherent macrophages were then incubated with the medium described above supplemented with 1 mg/ml rabbit lipoprotein-deficient serum (LPDS) for 24 h at 37°C in a 5% CO_2 atmosphere. After incubation the cells were used for ß-VLDL uptake and degradation measurements.

THP-1 macrophages

Human THP-1 monocytic leukemia cells were maintained in RPMI-1640 containing 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. Cells cultured in the presence of 40 nM phorbol myristoyl acetate (PMA) were seeded onto multiwell dishes at a cell density of 1 x 10^6 cells/ml and incubated for 3 days. Induction of macrophage differentiation in THP-1 cells was characterized by increased adherence to culture dishes and typical changes in cell morphology. Thereafter, the cells were incubated for 24 h in medium containing 1 mg protein/ml LPDS and used for ß-VLDL uptake and degradation measurements.

Uptake of ^{99m}Tc-ß-VLDL by macrophages

After preincubation in RPMI-1640 containing LPDS for 24 h, the THP-1 macrophages or rabbit monocyte-derived macrophages were incubated with fresh medium containing increasing concentrations of native or copper-oxidized ^{99m}Tc-ß-VLDL for 4 h at 37°C in a 5% CO_2 atmosphere. The cells were then washed twice for 10 min with PBS containing 0.2% bovine serum albumin (BSA), pH 7.4, and once with PBS without BSA. The incorporation of ^{99m}Tc-ß-VLDL into the cells was determined after solubilizing the cells in 0.1 N NaOH and measuring the radioactivity with a gamma-counter. Aliquots of digested cells were assayed for protein content (19). Cell degradation of ^{99m}Tc-ß-VLDL was determined by measuring the 20% TCA-soluble radioactivity with a gamma-counter. Blank values were obtained with parallel cell-free incubations with ^{99m}Tc-β-VLDL and subtracted to calculate total cell-mediated lipoprotein degradation.

Other assays

The concentration of thiobarbituric acid-reactive substances (TBARS) was measured as described elsewhere (20). Hydroperoxides of cholesteryl esters, triacylglycerols and phospholipids were monitored by HPLC as described elsewhere (21,22). Formation of carbonyls was monitored according to Levine et al. (23) and thiol (-SH) content was determined according to Elman (24). Electrophoretic mobility of native and oxidized lipoproteins was evaluated in 1% agarose gel.

Statistical analysis

Data are reported as mean ± SD. All comparisons were made using ANOVA and the Scheffé test. Statistical significance was defined as $P<0.05$.

Results

Biochemical analysis of native and oxidized ß-VLDL

Incubation of ß-VLDL (1 mg/ml) with 200 µM copper sulfate for 18 h at 37°C resulted in increased electrophoretic mobil-

ity on agarose gel as compared to native ß-VLDL (3.4 cm *vs* 2.9 cm, respectively) (Table 1). Higher amounts of TBARS and lipid hydroperoxides were found in ß-VLDL after copper-dependent oxidation and the most significant increase was observed in phospholipid hydroperoxides (Table 1). The incubation of ß-VLDL with copper sulfate decreased the amount of apolipoprotein thiol (-SH) by 26% (Table 1). In addition, there was a significant increase of carbonyl content in oxidized ß-VLDL (Table 1).

Table 1 - Biochemical determinations of native and copper-oxidized ß-VLDL.

ß-VLDL (1 mg/ml) was incubated with 400 µM peroxynitrite at 37ºC for 18 h. Results are the mean of duplicates from two determinations. TBARS = Thiobarbituric acid-reactive substances.

	ß-VLDL	
	Native	Copper
Electrophoretic mobility (cm)	2.9	3.4
TBARS (nmol/mg protein)	0.23	22.60
Cholesteryl ester hydroperoxides (µM)	2.8	72.7
Triacylglycerol hydroperoxides (µM)	1.8	23.2
Phospholipid hydroperoxides (µM)	0.6	114.3
Carbonyl content (nmol/mg protein)	10.4	73.3
Thiol (-SH) content (µmol/mg protein)	6.8	5.0

Concentration of lipids in blood plasma

Two months after starting cholesterol feeding, the total cholesterol concentration in blood plasma increased approximately 40-fold in comparison to the basal cholesterol content of HC rabbits (1182.4 ± 172.0 mg/dl *vs* 31.9 ± 9.5 mg/dl, respectively, N = 20, P<0.001). Triacylglycerol concentration also increased on account of the cholesterol-rich diet (57.5 ± 21.8 mg/dl for basal *vs* 260.3 ± 72.3 mg/dl after 60 days, P<0.01). In HC rabbits, more than 85% of blood plasma cholesterol was recovered in the lipoprotein fraction with a density less than 1.019 g/ml.

Fractional clearance rate

After injection of the native and copper-oxidized ^{99m}Tc-labeled ß-VLDL into C and HC rabbits, the radioactivity in plasma was measured and the FCR (per hour) was calculated. Plasma radioactivity was also determined after 20% TCA precipitation. The FCR values calculated after TCA precipitation were similar to those obtained with total plasma (data not shown). In addition, the radioactivity was measured in the lipoprotein fraction with a density <1.019 g/ml after ultracentrifugation of plasma samples obtained from 2 rabbits of each group (C and HC) 5 min and 6 h after injection of the ^{99m}Tc-labeled lipoproteins. Approximately 85% radioactivity was observed in the lipoprotein fraction with a density <1.019 g/ml 6 h after injection of ^{99m}Tc-nat-ß-VLDL into C and HC rabbits. In contrast, when animals of both groups were injected with ^{99m}Tc-ox-ß-VLDL, 95-98% of the radioactivity was present in the lipoprotein fraction with a density <1.019 g/ml. In both C and HC groups the plasma clearance of ^{99m}Tc-nat-ß-VLDL and ^{99m}Tc-ox-ß-VLDL reflects a biexponential decay mode (Figure 1). Decay curves showed that plasma clearance of ^{99m}Tc-ox-ß-VLDL was faster than plasma clearance of ^{99m}Tc-nat-ß-VLDL in C rabbits (P<0.05). However, both ^{99m}Tc-labeled lipoproteins were removed from HC rabbit plasma at a slower rate than from C rabbits (P<0.05). Moreover, ^{99m}Tc-ox-ß-VLDL remained in HC rabbit plasma for a longer period of time in comparison to ^{99m}Tc-nat-ß-VLDL. FCR data are shown in Table 2.

Biodistribution studies

Biodistribution data for injected native and oxidized ^{99m}Tc-ß-VLDL were obtained for male New Zealand white control rabbits fasted overnight (N = 8) and cholesterol-fed rabbits (N = 9) 6 h after injection. Table 3 shows the percent of the injected dose of

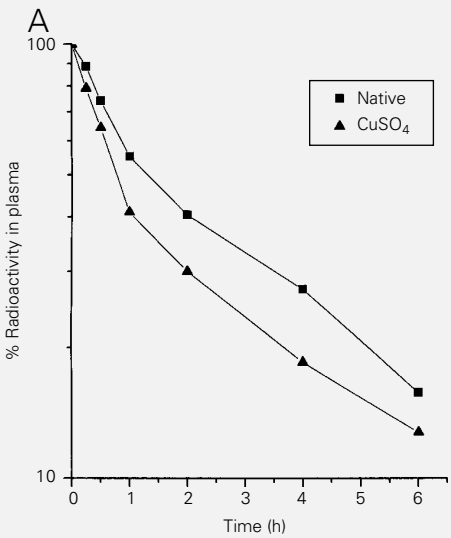

Figure 1 - Plasma decay curves of native ^{99m}Tc-ß-VLDL and copper-oxidized ^{99m}Tc-ß-VLDL in control (A) and hypercholesterolemic rabbits (B). Data are reported as means for 8-9 rabbits.

Table 2 - Removal of native and copper-modified ^{99m}Tc-labeled ß-VLDL from plasma of cholesterol-fed and control rabbits.

Data are reported as means ± SD of results obtained from (N) rabbits. *P<0.05 compared to the respective control rabbits; **P<0.05 compared to native ß-VLDL from control rabbits (ANOVA and Scheffé test).

	Fractional clearance rate/h	
^{99m}Tc-ß-VLDL	Cholesterol-fed	Control
Native	0.163 ± 0.043* (N = 9)	0.241 ± 0.020 (N = 8)
Copper-modified	0.100 ± 0.048* (N = 8)	0.362 ± 0.070** (N = 8)

^{99m}Tc-lipoproteins per gram tissue. No difference in ^{99m}Tc-ox-ß-VLDL or ^{99m}Tc-nat-ß-VLDL uptake was observed among the tissues studied (liver, kidney, spleen, adrenal glands, aorta, heart, muscle and adipose tissues, lung and bone marrow) in C rabbits.

In HC rabbits, both ^{99m}Tc-nat-ß-VLDL and ^{99m}Tc-ox-ß-VLDL were taken up to a lesser extent by the liver than in C rabbits. In contrast, the aorta and other tissues showed a similar accumulation of ^{99m}Tc-nat-ß-VLDL and ^{99m}Tc-ox-ß-VLDL in both C and HC rabbits (Table 3). This suggests that internalization of these lipoproteins by these tissues may be mediated by receptors that are not down-regulated by cell cholesterol content. A 1.5- and 2-fold higher uptake of ^{99m}Tc-nat-ß-VLDL and ^{99m}Tc-ox-ß-VLDL, respectively, was noted in the whole aorta and in the aortic arch containing atherosclerotic lesions when compared to normal aorta. However, there was no significant difference between ^{99m}Tc-ox-ß-VLDL and ^{99m}Tc-nat-ß-VLDL accumulation in the atherosclerotic aorta.

The cholesterol-rich diet increased the weight of liver, adrenals and spleen but did not affect the weight of the aorta (data not shown). Moreover, the uptake of both ^{99m}Tc-ox-ß-VLDL and ^{99m}Tc-nat-ß-VLDL calculated as % of injected dose per whole organ (Table 4) was similar to the uptake calculated as % of injected dose per gram tissue. The effect of tissue weight enhancement due to the cholesterol-rich diet was observed in the spleen where an increased ^{99m}Tc-nat-ß-VLDL uptake was found in HC rabbits. Since the presence of atherosclerotic lesions did not affect the weight of the aorta, the ^{99m}Tc-labeled lipoprotein uptake by the whole aorta was similar to that obtained by % of injected dose per gram tissue.

Imaging studies

The gamma camera images of the biodistribution of ^{99m}Tc-labeled lipoproteins in one

control rabbit and one cholesterol-fed rabbit obtained 6 h after injection are shown in Figure 2. The liver was the predominant uptake site for ^{99m}Tc-nat-ß-VLDL (Figure 2A) and ^{99m}Tc-ox-ß-VLDL (Figure 2C) in the control rabbit. The uptake of all ^{99m}Tc-labeled lipoproteins by the liver was higher in the control rabbit than in the cholesterol-fed rabbit. In the latter, there was a high uptake of both ^{99m}Tc-nat-ß-VLDL and ^{99m}Tc-ox-ß-VLDL by the kidneys (Figure 2B and 2D).

Table 3 - Biodistribution of ^{99m}Tc-ß-VLDL in rabbits.

Data are reported as means ± SD of results obtained from control (C) and hypercholesterolemic (HC) rabbits and are expressed as percentage of the injected radioactivity dose/g tissue. *P<0.05 compared to the respective C group (ANOVA and Scheffé test).

Tissue	Native ß-VLDL		Copper-oxidized ß-VLDL	
	C (N = 8)	HC (N = 9)	C (N = 8)	HC (N = 9)
Liver	0.301 ± 0.113	0.114 ± 0.071*	0.305 ± 0.149	0.116 ± 0.057*
Kidney	0.575 ± 0.106	0.536 ± 0.183	0.523 ± 0.201	0.517 ± 0.169
Heart	0.028 ± 0.014	0.030 ± 0.009	0.026 ± 0.012	0.026 ± 0.009
Muscle (m. psoas)	0.002 ± 0.0007	0.003 ± 0.002	0.002 ± 0.001	0.004 ± 0.002
Adrenal gland	0.086 ± 0.023	0.052 ± 0.021	0.103 ± 0.055	0.092 ± 0.057
Lung	0.049 ± 0.007	0.066 ± 0.041	0.057 ± 0.025	0.112 ± 0.090
Spleen	0.113 ± 0.034	0.073 ± 0.034	0.162 ± 0.128	0.207 ± 0.200
Aorta (whole)	0.021 ± 0.009	0.030 ± 0.010*	0.016 ± 0.008	0.032 ± 0.015*
Aortic arch	0.023 ± 0.010	0.033 ± 0.012*	0.019 ± 0.010	0.039 ± 0.017*
Aorta (thoracic)	0.019 ± 0.009	0.028 ± 0.012	0.015 ± 0.008	0.029 ± 0.016
Aorta (abdominal)	0.020 ± 0.010	0.028 ± 0.009	0.015 ± 0.009	0.027 ± 0.013
Bone marrow	0.097 ± 0.021	0.079 ± 0.004	0.068 ± 0.027	0.053 ± 0.020
Adipose tissue	0.0025 ± 0.002	0.006 ± 0.001*	0.003 ± 0.0006	0.004 ± 0.002

Table 4 - Biodistribution of ^{99m}Tc-ß-VLDL in rabbits (% injected dose/organ).

Data are reported as means ± SD of results obtained from (N) rabbits. C = Control rabbits; HC = hypercholesterolemic rabbits. *P<0.05 compared to the respective control group; **P<0.05 compared to native ß-VLDL of the HC group (ANOVA and Scheffé test).

Tissue	Native ß-VLDL		Copper-oxidized ß-VLDL	
	C (N = 8)	HC (N = 9)	C (N = 8)	HC (N = 9)
Liver	25.50 ± 3.97	15.35 ± 1.38*	28.25 ± 4.47	8.31 ± 2.40*,**
Kidney	6.11 ± 1.19	5.82 ± 2.11	5.85 ± 1.66	4.83 ± 1.32
Heart	0.123 ± 0.057	0.121 ± 0.056	0.104 ± 0.052	0.113 ± 0.046
Adrenal	0.039 ± 0.022	0.051 ± 0.033	0.046 ± 0.032	0.073 ± 0.031
Spleen	0.085 ± 0.040	0.157 ± 0.030*	0.111 ± 0.072	0.312 ± 0.215
Aorta	0.012 ± 0.004	0.019 ± 0.006	0.010 ± 0.005	0.021 ± 0.010*

Accumulation of ^{99m}Tc-labeled ß-VLDL by the atherosclerotic lesion

Figure 3 shows the uptake of ^{99m}Tc-ox-ß-VLDL by rabbit aorta. For comparison, we analyzed the aortas of one control (Figure 3A) and one cholesterol-fed rabbit (Figure 3B). The hypercholesterolemic rabbit aorta accumulated ^{99m}Tc-ox-ß-VLDL mainly in the aortic arch where numerous atherosclerotic lesions occurred (Figure 3B). In control rabbits, the accumulation of ^{99m}Tc-ox-ß-VLDL was barely observed (Figure 3A). Autoradiographs showing the same pattern were observed for ^{99m}Tc-nat-ß-VLDL.

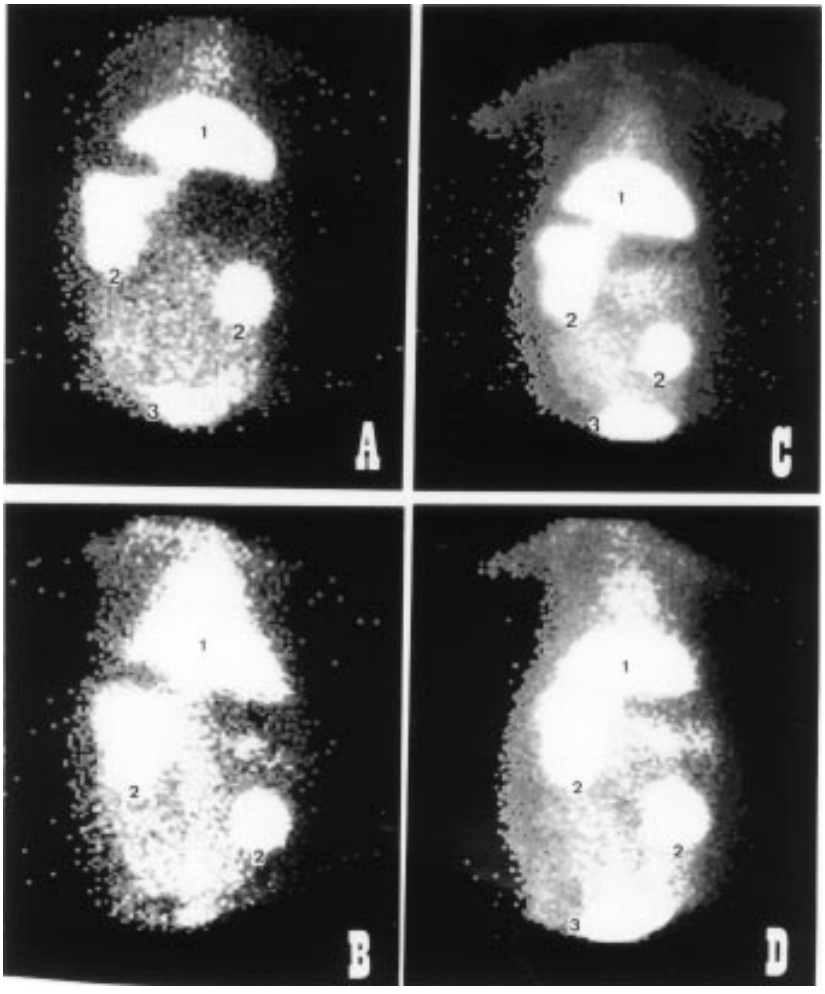

Figure 2 - Biodistribution of ^{99m}Tc-labeled ß-VLDL 6 h after tracer injection. *A*, ^{99m}Tc-nat-ß-VLDL in a control rabbit; *B*, ^{99m}Tc-nat-ß-VLDL in a cholesterol-fed rabbit; *C*, copper-oxidized ^{99m}Tc-ß-VLDL in a control rabbit; *D*, copper-oxidized ^{99m}Tc-ß-VLDL in a cholesterol-fed rabbit. 1, Liver; 2, kidney; 3, urinary bladder.

Uptake of ^{99m}Tc-ß-VLDL by macrophages

To determine whether ^{99m}Tc-labeling affects the binding of lipoproteins by cell membrane receptors, as well as to validate our *in vivo* findings, the uptake of ^{99m}Tc-labeled lipoproteins was tested in THP-1 macrophages and rabbit monocyte-derived macrophages. For uptake rate measurements these cells were initially preincubated with LPDS and further incubated with increasing amounts of ^{99m}Tc-nat-ß-VLDL and ^{99m}Tc-ox-ß-VLDL (Figure 4). Data obtained after 4-h incubation showed that both THP-1 macrophages and rabbit monocyte-derived macrophages internalized ^{99m}Tc-ox-ß-VLDL to a greater extent than ^{99m}Tc-nat-ß-VLDL. ^{99m}Tc-ox-ß-VLDL uptake was approximately 5-fold higher in THP-1 macrophages (Figure 4A) and 2-fold higher in monocyte-derived macrophages (Figure 4B) than ^{99m}Tc-nat-ß-VLDL uptake.

Discussion

Hypercholesterolemia is an important risk factor for the development of the atherosclerotic process, particularly in terms of the cholesterol transported in the LDL and in ß-VLDL of rabbits fed a cholesterol-rich diet. *In vitro* studies have shown that oxidative modification of ß-VLDL can contribute to atherogenesis (25). Oxidized ß-VLDL increases foam cell formation through binding and internalization into macrophages via macrophage scavenger receptors (25). The presence of oxidatively modified lipoproteins in the plasma of animals and atherosclerotic patients has been demonstrated (11-13). However, the pathophysiological role of these circulating oxidized lipoproteins remains a matter of speculation. The oxidized lipoproteins present in blood plasma may participate in atherogenesis by different

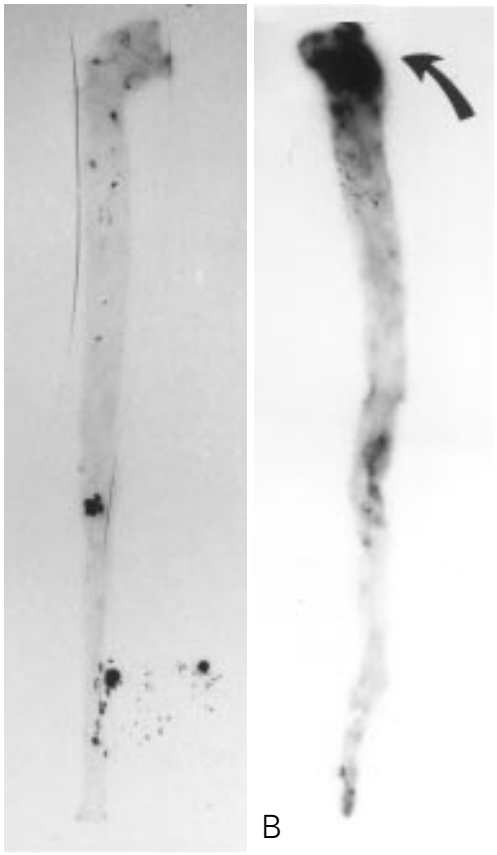

Figure 3 - Autoradiography for ^{99m}Tc-ox-ß-VLDL uptake in the aorta. *A*, Aorta from a control rabbit; *B*, aorta from a hypercholesterolemic rabbit. The arrow indicates the accumulation of copper-oxidized ^{99m}Tc-ß-VLDL in the atherosclerotic lesions of the aortic arch from cholesterol-fed rabbits.

mechanisms. The present study shows data of biodistribution, fractional clearance rate and atherosclerotic lesion uptake of oxidized ^{99m}Tc-ß-VLDL in cholesterol-fed rabbits. This radiotracer was chosen because ^{99m}Tc-labeled lipoproteins can be used for noninvasive quantitative biodistribution studies by scintigraphic imaging and for quantitatively determining the uptake and degradation of lipoproteins (14).

The biodistribution and fractional clearance rate differ between native and oxidized ß-VLDL in cholesterol-fed and control rabbits. A relevant and new finding observed here was the significant reduction of plasma ^{99m}Tc-ox-ß-VLDL removal in hypercholesterolemic rabbits (Table 2 and Figure 1). Our data show that the exogenously oxidized ß-VLDL remains in plasma for as long a time as the native one. This contrasts with previous findings showing that exogenously oxidized lipoproteins are rapidly removed from blood plasma by cells of the monocytic phagocyte system (26). This result suggests that in these animals there is a significant pool of oxidatively modified ß-VLDL in blood plasma responsible for the competitive inhibition or saturation of common cell receptors, leading to a slower removal of ^{99m}Tc-ox-ß-VLDL from plasma. Indeed, we re-

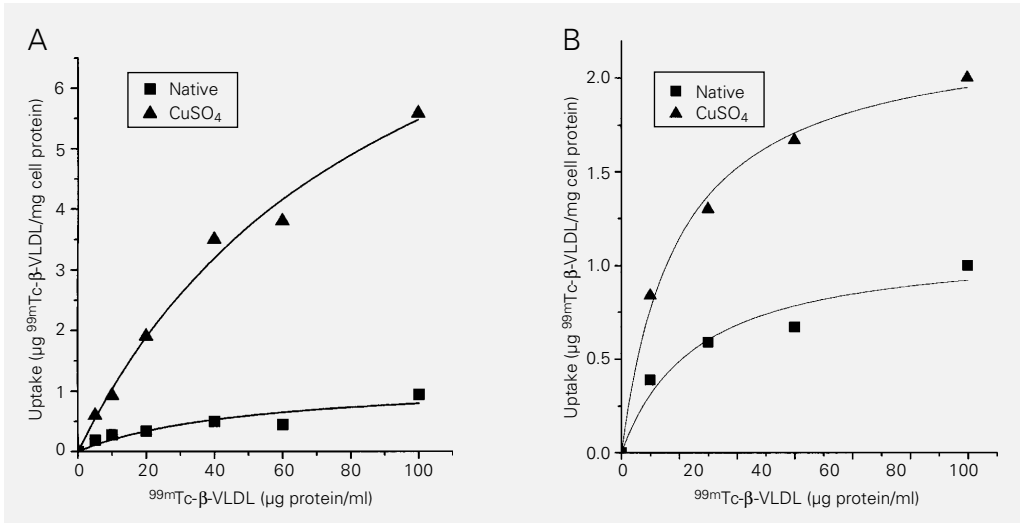

Figure 4 - Uptake of ^{99m}Tc-nat-ß-VLDL and ^{99m}Tc-ox-ß-VLDL by THP-1 macrophages (A) and rabbit monocyte-derived macrophages (B). Uptake was determined after incubating the cells with medium containing increasing concentrations of labeled lipoproteins for 4 h at 37°C. The internalization of the ^{99m}Tc-labeled ß-VLDL into cells was determined after solubilizing cells in 0.1 N NaOH and measuring the radioactivity with a gamma-counter. Each point is the mean of triplicate determinations made in 2-3 experiments.

cently demonstrated that ß-VLDL isolated from blood of cholesterol-fed rabbits has an increased content of lipid hydroperoxides (13).

Consistent with the low fractional clearance rate of ^{99m}Tc-ß-VLDL is the decreased uptake of ^{99m}Tc-nat-ß-VLDL and ^{99m}Tc-ox-ß-VLDL by the liver of hypercholesterolemic rabbits. Down-regulation of B/E receptors in cholesterol-fed rabbits has been previously shown (27-29). Accordingly, our results indicate that the binding and internalization of oxidatively modified ß-VLDL by hepatic cells can be partially mediated by B/E or other down-regulated receptors. Native ß-VLDL is recognized by remnant and B/E receptors of liver parenchymal cells from rats and rabbits (30). In contrast, copper-oxidized ß-VLDL is taken up mainly by Kupffer cells of rat liver (31). *In vitro* studies have shown that acetylated LDL competes only 10-20% with oxidized ß-VLDL for cell association and degradation by both liver endothelial cells and Kupffer cells (31). Thus, distinct receptors responsible for the recognition of oxidized ß-VLDL appear to occur in rabbit liver, similarly to mouse peritoneal macrophages which recognize modified lipoproteins by different receptors (32,33).

In contrast to the phenomenon observed in the liver, the other organs from hypercholesterolemic rabbits incorporated the native and oxidized ß-VLDL possibly through receptors which are not down-regulated by cell cholesterol content, since both control and cholesterol-fed animals showed a similar accumulation of ^{99m}Tc-labeled lipoproteins in these organs (Tables 3 and 4). The accumulation of native ^{99m}Tc-ß-VLDL by tissues with low LDL receptor activity, such as spleen and bone marrow, suggests that the uptake of these radiolabeled particles occurring in the reticulo-endothelial system is probably mediated by the scavenger receptor, LDL receptor-related protein (LRP) or by phagocytosis.

The high uptake of ^{99m}Tc-labeled ß-VLDL by the kidney observed in our study may be related to the diverse lipoprotein receptors present in the cells of this organ. The presence of apolipoprotein E on the surface of ß-VLDL allows this lipoprotein to be internalized by receptors other than the LDL receptor such as the LRP, the VLDL receptor and glycoprotein-330 (Gp-330) (10). In the kidney, the epithelial cells of the plasma membrane in proximal tubules and glomeruli show a high expression of Gp-330, a recently identified receptor that mediates the endocytosis of ß-VLDL (10). Gp-330 is also present in type-2 pneumocytes and in the epithelium lining the epididymis and yolk sac (10). Accumulation of ^{99m}Tc-ß-VLDL in kidneys was not decreased in cholesterol-fed rabbits, indicating that the receptor mediating its internalization is not down-regulated by the sterol content of the cells (Tables 3 and 4). Although lipoproteins are not expected to be filtered by the kidneys due to their size, they may bind to Gp-330 present on the glomerular plasma membrane. Another possibility for the appearance of radioactivity in kidneys is the presence of free ^{99m}technetium (^{99m}Tc-pertechnetate) released from labeled lipoproteins in the body. However, it has been reported that in both animals and humans only 5% to 12% of injected ^{99m}Tc activity was excreted through the urine within a 24-h period following an injection of ^{99m}Tc-LDL, indicating that ^{99m}Tc-labeled lipoprotein acts as an intracellular ligand (27). In fact, ^{99m}technetium is frequently used as a radiotracer because it acts as an intracellularly trapped ligand providing an accurate measurement of lipoprotein uptake by tissues (14).

The presence of oxidized LDL has been previously shown in atheromas of hypercholesterolemic rabbits and subjects with atherosclerosis (34). However, the *in vivo* uptake of oxidized ß-VLDL by the arteries of cholesterol-fed rabbits has not been reported previously. The atheroma foam cells express scavenger receptors that recognize negatively

charged oxidized lipoproteins (35). In fact, the copper-dependent oxidation of ß-VLDL originated particles with increased negative charge (Table 1) due to derivation of ε-amino groups of apolipoprotein lysine residues by aldehydes released from peroxidized lipids, allowing the recognition of oxidized lipoproteins by the scavenger receptor (25,36). Although the atherosclerotic lesions of aorta arteries from cholesterol-fed rabbits, mainly in the aortic arch, accumulated more ^{99m}Tc-ox-ß-VLDL than aortas from control rabbits (Table 3 and Figure 3), the atherosclerotic lesions of cholesterol-fed rabbits did not accumulate higher amounts of ^{99m}Tc-ox-ß-VLDL than of ^{99m}Tc-nat-ß-VLDL. Therefore, both ^{99m}Tc-labeled lipoproteins when present in the blood circulation could cross the endothelium to a similar extent. In fact, Simionescu et al. (37) and Vasile et al. (38) showed that in rats and in hypercholesterolemic rabbits, lipid deposition on the vessel wall occurs by transcytosis not mediated by specific receptors.

Previous data have shown (14,26,39) that ^{99m}Tc-LDL is intracellularly trapped, thus representing an adequate radiotracer for non-invasive imaging of LDL metabolism. Moreover, ^{99m}Tc-LDL is recognized by the high affinity LDL receptor (39) and is also taken up by the scavenger pathways (14). A possible effect of ß-VLDL ^{99m}Tc-labeling on lipoprotein uptake in our study was ruled out by *in vitro* cell culture experiments. Our results with THP-1 macrophages and rabbit monocyte-derived macrophages (Figure 4) showed that ^{99m}technetium did not affect the binding or internalization of either native or oxidized ß-VLDL. Accordingly, oxidatively modified ß-VLDL was taken up in high amounts by macrophages which express scavenger receptors (40). However, our biodistribution data indicate that when oxidized ß-VLDL is present in blood plasma its accumulation on the artery wall is not significantly higher than that of native ß-VLDL. These data agree with those previously reported for ^{99m}Tc-labeled oxidized LDL whose uptake was not different from that of ^{99m}Tc-native LDL by aorta of cholesterol-fed rabbits (41). Besides macrophages, the atherosclerotic lesion contains other cells such as smooth muscle cells that can take up oxidized and non-oxidized ß-VLDL (42). Therefore, the *in vivo* removal of both ß-VLDL particles from plasma reflects the total uptake by the different cells present in atheroma. The slow removal of oxidized ß-VLDL from plasma of cholesterol-fed rabbits suggests that this oxidatively modified lipoprotein may also participate in the atherogenic process in other ways, including the induction of increased leukocyte adherence to endothelium and its cytotoxicity to endothelial cells (43). Finally, our *in vitro* experiments support previous data showing that oxidized ß-VLDL is taken up more than the native lipoprotein by the macrophage scavenger receptors (25). However, this does not correspond to the *in vivo* process, where other cells and receptors may also participate in ß-VLDL uptake.

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