MDL 29311, a phenolic antioxidant, interferes with the interaction of apoC with VLDL: a possible explanation for its triglyceride-lowering effect

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Abstract MDL 29311 is an antioxidant that lowers plasma triglycerides and raises high density lipoprotein (HDL) cholesterol in rats. It lowers triglycerides in rats by enhancing the clearance of very low density lipoprotein (VLDL) by the liver (Sheetz, M. J., et al. 1994. *Metabolism.* **43:** 232–240). In this paper, the possibility that MDL 29311 enhances VLDL clearance by altering the apolipoprotein (apo) content of lipoproteins is examined. Treatment of rats with 1% MDL 29311 in the diet for 7 days lowered plasma triglycerides and markedly increased total lipoprotein-associated apoE. The increase in apoE was confined to the HDL fraction; no increase in VLDL-associated apoE was detected. No apparent alterations in the amount of total lipoprotein-associated apoC were observed, although there was a decrease in VLDL-associated apoC-II and C-III-0. Consistent with this finding, the amount of ^{125}I-labeled apoC transferred from HDL to VLDL in plasma from MDL 29311-treated rats was only 40% of the amount transferred in control plasma. Sepharose 6B gel filtration of mixtures of ^{125}I-labeled apoC with increasing concentrations of MDL 29311 in the absence of plasma or lipid revealed that proportionally increasing amounts of the ^{125}I-labeled apoC eluted in a high molecular weight (HMW) complex with MDL 29311. An HMW complex was not formed when MDL 29311 was mixed with ^{125}I-labeled soybean trypsin inhibitor. The ^{125}I-labeled apoC in the HMW complex bound to VLDL only 20% as well as uncomplexed ^{125}I-labeled apoC. MDL 29311 also caused the dissociation of ^{125}I-labeled apoC from VLDL at concentrations of MDL 29311 similar to those obtained in vivo. Other phenolic antioxidants related to MDL 29311 caused the formation of HMW ^{125}I-labeled apoC-containing complexes to an extent proportional to their abilities to lower triglycerides in rats. These studies support the hypothesis that MDL 29311 lowers triglycerides in rats by interfering with apoC association with VLDL, thereby relieving the apoC-mediated inhibition of hepatic VLDL uptake.—**Sheetz, M. J.** MDL 29311, a phenolic antioxidant, interferes with the interaction of apoC with VLDL: a possible explanation for its triglyceride-lowering effect. *J. Lipid Res.* 1995. **36:** 2609-2621.

Supplementary key words HDL • gel filtration • apoE • probucol analog

The clinical findings of high plasma triglycerides (TG) and low HDL cholesterol are often associated with a broader syndrome of hypertension, insulin resistance, and central obesity known as Syndrome X or Reaven's Syndrome (1–3). It is likely that the dyslipidemia found in Syndrome X contributes to the elevated risk of coronary heart disease (4–7). New drugs that lower TG and raise HDL could represent useful therapies for this syndrome.

MDL 29311 (**Fig. 1**) is a lipophilic antioxidant structurally similar to probucol. It has been evaluated as both a lipid-modulating and a glucose-lowering agent (8, 9). In addition to these activities, it has also been shown to inhibit the onset of insulin-dependent diabetes mellitus in genetically susceptible non-obese diabetic mice (10). The TG-lowering effect of MDL 29311 was studied in considerable detail (8). MDL 29311 lowers TG in rats by facilitating the clearance of TG-carrying VLDL (and probably chylomicrons as well) by the liver. It does not inhibit the secretion of VLDL by the liver, nor does it stimulate the uptake of VLDL-TG by peripheral tissues. It also does not up-regulate the liver's ability to clear VLDL (8). Our working hypothesis for how MDL 29311 facilitated VLDL clearance in rats was that it either enhanced the association of apoE or inhibited the association of apoC with VLDL, thereby stimulating the uptake of VLDL by the liver.

ApoE stimulates VLDL clearance by the liver by associating with VLDL and serving as a ligand for the LDL receptor (11, 12). ApoC represents a family of small apolipoproteins (apoC-I, C-II, and C-III) that bind to VLDL and HDL. Experiments with isolated apoC and apoE in the perfused rat liver (13, 14), and studies with transgenic mice overexpressing apoC-I, C-II, C-III, or E

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; PDB, plasma density buffer; TG, triglycerides; apo, apolipoprotein; HMW, high molecular weight; BSA, bovine serum albumin.

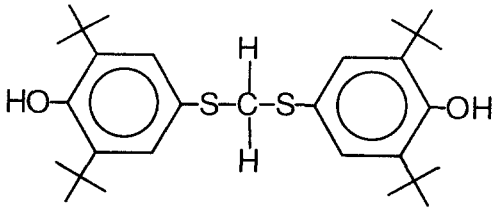


Fig. 1. Structure of MDL 29311.

(15–18) have demonstrated convincingly that these apolipoproteins are critical for controlling the residence time of VLDL in the circulation. Specifically, apoE enhances VLDL clearance while apoC inhibits it. Although it is well known that apoC-II is an activator of lipoprotein lipase and is considered to be an apolipoprotein that enhances TG clearance (19), transgenic mice overexpressing apoC-II were actually hypertriglyceridemic (16) indicating that, at least in rodents, its activities may be multiple and complex.

This paper shows how MDL 29311 treatment affects the relative levels of C and E apolipoproteins and how MDL 29311, along with related compounds, can interact with apoC to inhibit its interaction with VLDL. These findings suggest a novel potential mechanism whereby compounds such as MDL 29311 can lower TG.

EXPERIMENTAL PROCEDURES

Animals

Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 200–250 g were housed four per cage with food and water provided ad libitum, and maintained on a 14-h/10-h light/dark cycle. Rats were randomly divided into groups receiving control chow or chow containing 1% MDL 29311 as a dietary admixture. The admixture was prepared by mixing MDL 29311 (Dow Chemical, Midland, MI) with powdered feed and then reforming the feed into pellets (5001 rodent chow, Purina, Richmond, IN). Heparinized blood samples collected from the tail veins of conscious rats were analyzed for plasma triglycerides, total cholesterol (both from Roche Diagnostic Systems, Nutley, NJ), and HDL cholesterol (phosphotungstic acid/magnesium chloride precipitation method, Sigma, St. Louis, MO) on a Cobas Mira S automated analyzer (Roche Diagnostic Systems).

Lipoprotein isolation and characterization

For total lipoprotein isolation, 300 μl of blood was obtained from the tail vein of each conscious rat and combined with EDTA (1 mg/ml final concentration) and aprotinin (0.03 trypsin inhibitory units/ml final concentration), chilled, and centrifuged (20 min at 2500 *g*, 4°C, in a Beckman [Palo Alto, CA] GS-6KR centrifuge). The plasma was removed and 100 μl was mixed with 100 μl of plasma density buffer (PDB, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA) and adjusted to a density of 1.21 g/ml with solid KBr (Sigma). The mixture was centrifuged in a 42.2 Ti rotor (Beckman) for 24 h at 200,000 *g*, 8°C in a Beckman L8-80M ultracentrifuge. The upper 100 μl containing the lipoproteins was removed and diluted 1:10 with PDB. Aliquots of the diluted lipoproteins were then precipitated with 10 volumes of acetone–ethanol 1:1 overnight at -20°C in preparation for SDS-PAGE.

For isolation of VLDL, LDL, and HDL, blood was collected from the abdominal aorta of metofane (Pitman-Moore, Mundelein, IL)-anesthetized rats and was anticoagulated and plasma was obtained as described above. Lipoproteins were isolated from 4 ml of plasma by sequential ultracentrifugation (18–45 h, 200,000 *g*, 8°C in a Beckman 50.3 Ti rotor) at the following densities (obtained by adding solid KBr): VLDL, $d < 1.006$ g/ml; LDL, $1.006 < d < 1.063$ g/ml; and HDL, $1.063 < d < 1.21$ g/ml. Lipoprotein samples were dialyzed against PDB; total cholesterol and triglycerides were measured as described above, and protein was determined using the bicinchoninic acid (BCA) reagent (Pierce, Rockford, IL). Aliquots of the lipoprotein fractions were then precipitated using 10 volumes of acetone–ethanol 1:1 overnight at -20°C in preparation for SDS-PAGE.

SDS-PAGE

Precipitated apolipoproteins were dissolved in 20 μl of SDS sample buffer (2% SDS, 2% 2-mercaptoethanol, 0.01% bromophenol blue) and heated at 60°C for 15 min. The entire sample was then loaded onto a 4–20% MiniPlus SepraGel polyacrylamide gel (Integrated Separation Systems, Natick, MA) and proteins were separated by electrophoresis in SDS gel running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS, pH 8.3–8.6) at a constant voltage of 150 volts for 2 h in a Daiichi mini 2-gel device (Integrated Separation Systems). The proteins were then visualized by staining with 0.1% Coomassie R-250 (Bio-Rad, Richmond, CA) in 40% ethanol, 10% methanol and destaining with Novex (San Diego, CA) Gel-Clear destain solution.

Isoelectric focusing

Isoelectric focusing was carried out essentially as described (20). Precipitated apolipoproteins (approximately 10 μg protein) were focused on 0.5 × 12 cm gels over a pH range of 3.5–7.0 (Ampholine, Sigma) and stained with 0.04% Brilliant Blue G in 3.5% perchloric acid (Sigma).

Gel filtration

All gel filtration experiments were performed using a

1.5 × 20 cm column of Sepharose 6B (Pharmacia, Uppsala, Sweden) equilibrated with PDB containing 0.5% BSA (Fraction V, Sigma). Fractions of 25 drops each (approximately 1 ml) were collected and analyzed as described below.

HPLC analysis of MDL 29311

An aliquot of 200 µl of each gel filtration fraction was added to 2 ml of ether–ethanol 3:1 while vortexing. The organic layer was separated by centrifugation and dried under nitrogen. The residue was dissolved in 200 µl of acetonitrile–hexane–0.1 M ammonium acetate 180:13:7 and 160 µl was injected onto a Waters (Milford, MA) deltapak C18 reverse phase column (3.9 × 150 mm) in 85% acetonitrile at a flow rate of 1.5 ml/min. The elution of peaks was monitored on a Waters 991 photodiode array detector at 240 and 255 nm and the mass of MDL 29311 was calculated by comparison with a reference standard.

Purification and iodination of apoC

Rat apoC was purified from pooled rat VLDL by the following procedure. Approximately 1 mg of VLDL protein was precipitated using 10 volumes of acetone–ethanol 1:1 at -20°C overnight. The precipitated apolipoproteins were dissolved in 2 ml of PDB containing 6 M urea. This mixture was then gel-filtered on a 1.5 × 20 cm column of Sephadex G-75 (Pharmacia) in the same buffer and 1.5-ml fractions were collected. The absorbance of each fraction at 280 nm was measured and the second peak (see Fig. 6) was pooled and dialyzed against water. The protein concentration was determined and aliquots of 100 µg were lyophilized and stored at -20°C. SDS-PAGE analysis of the gel filtration fractions confirmed that the second peak contained primarily apoC. The final preparation contained a small amount (< 5%) of apoE but was otherwise indistinguishable from apoC by SDS-PAGE or isoelectric focusing. The purified apoC was iodinated using a modification (21) of the ICl method (22). An aliquot of 50 µg of apoC protein in 500 µl of PDB was added to a mixture of 200 µCi (2 µl) of $Na^{125}I$ (100 mCi/ml, Amersham, Arlington Heights, IL), 12 µl of 200 µg/ml ICl (Sigma) in 0.01 N HCl, and 100 µl of 0.1 M glycine, pH 9.5. The solution was mixed and allowed to stand at room temperature for 2 min before the free $^{125}I^-$ was separated by gel filtration over a Sephadex G-15 (Pharmacia) column in PDB. Fractions containing protein-bound radioactivity were pooled and dialyzed against PDB. The ^{125}I-labeled apoC thus obtained typically had a specific activity of 2×10^6 cpm/µg apoC and was > 97% precipitable by trichloroacetic acid. Greater than 95% of the ^{125}I-labeled apoC radioactivity co-migrated with apoC on SDS-PAGE. Less than 1% co-migrated with apoE. Similar procedures were used for the iodination of both apoE (purified from rat VLDL) and soybean trypsin inhibitor (Sigma) and similar results were obtained.

RESULTS

Rat plasma TG, total cholesterol, and HDL cholesterol levels, as well as the relative amounts of lipoprotein-associated apolipoproteins, were determined after feeding rats a 1% dietary admixture of MDL 29311 (Fig. 1) for 2, 4, or 7 days. This experiment was designed to determine whether increases in plasma apoE or decreases in plasma apoC could account for the observed TG-lowering effect of MDL 29311. Plasma total cholesterol levels were unchanged after 2 or 4 days of MDL 29311 treatment but were increased by 27% ($P < 0.05$) after 7 days (**Fig. 2A**). Correspondingly, HDL cholesterol levels were unchanged after 2 or 4 days, but were increased 27% ($P < 0.05$) after 7 days (Fig. 2B). In fact, the MDL 29311-induced elevation in total cholesterol (89 - 70 = 19 mg/dl) could be accounted for almost entirely by its effect on HDL cholesterol (79 - 62 = 17 mg/dl). Plasma TG began to fall after 4 days of 1% MDL 29311 feeding, but levels were not significantly lowered compared to controls ($P = 0.07$, although in other experiments with more animals per group the TG were significantly lowered by 4 days of MDL 29311 treatment; M. J. Sheetz, unpublished observations). However, by 7 days of treatment TG were reduced by 54% ($P < 0.05$, Fig. 2C). At each time point, total plasma apolipoproteins were obtained and analyzed by SDS-PAGE. Although densitometry was not performed, it is apparent that apoE was decreased relative to control after 2 days of MDL 29311 treatment (**Fig. 3a**). By 4 days, there may have been slightly more apoE relative to control in the lipoproteins from treated rats (Fig. 3b). By 7 days, the apoE level in the lipoproteins derived from treated rats was markedly increased relative to control (Fig. 3c). Although it is difficult to quantitate apoC levels on 4–20% SDS-PAGE gels as the apoC runs near the dye front, there were no obvious changes in total lipoprotein-associated apoC after 7 days of MDL 29311 treatment. Non-lipoprotein-associated apoC was not measured, but it has been shown to represent less than 10% of the total rat apoC pool (23). The partial TG lowering seen by day 4 and the significant lowering seen by day 7 seem to be accompanied by a slight increase in apoE by day 4 and a marked increase by day 7.

As described in the introduction, elevations in apoE should lead to enhanced clearance of lipoproteins that bind apoE (including VLDL). The apolipoprotein composition of isolated lipoproteins was investigated in MDL 29311-treated rats. After 7 days of treatment with

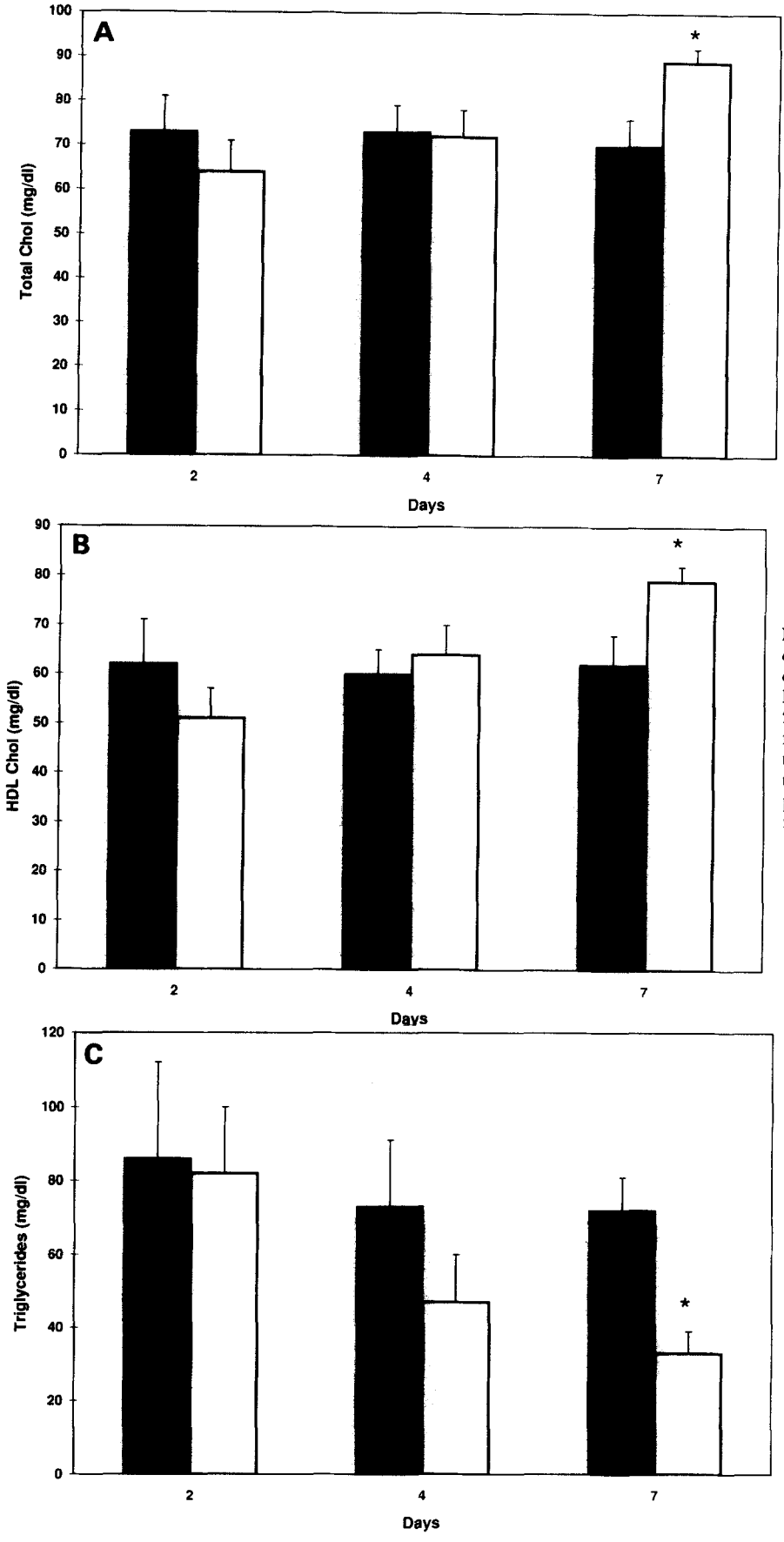


Fig. 2. Rat plasma total cholesterol (A), HDL cholesterol (B), and triglycerides (C) after 2, 4, or 7 days of treatment with 1% MDL 29311. Rats were fed a dietary admixture of 1% MDL 29311 for 2, 4, or 7 days. Plasma was obtained from a tail vein blood sample and total cholesterol (A), HDL cholesterol (B), and triglycerides (C) were measured. Each data point represents the mean ± SD for 4 rats/group (*, $P < 0.05$ vs. control group).

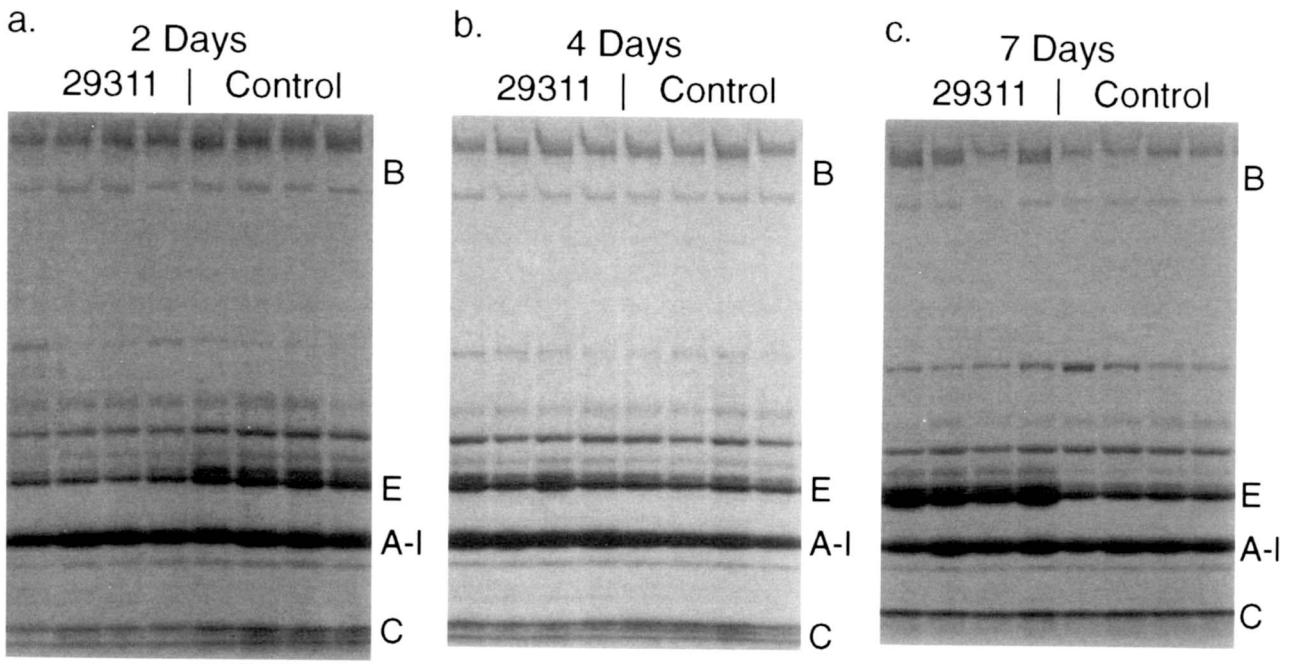


Fig. 3. SDS-PAGE analysis of apolipoproteins from total lipoproteins of control rats or rats treated with 1% MDL 29311 for 2 (a), 4 (b), or 7 (c) days. Total lipoproteins (d < 1.21 g/ml) were obtained from 100 µl plasma samples from control rats or from rats fed with 1% MDL 29311 for 2 (a), 4 (b), or 7 (c) days. The lipoproteins (20 µl out of 100 µl total) were diluted 1:10 with PDB and precipitated. Each sample was dissolved in SDS loading buffer and electrophoresed on a 4–20% polyacrylamide gel as described in Methods. Protein bands were visualized by Coomassie R-250 staining. Each lane contains apolipoproteins from one rat.

1% MDL 29311, rats were killed and their lipoproteins (VLDL, LDL, HDL) were separated by differential density ultracentrifugation. Equivalent amounts of apolipoproteins were analyzed by SDS-PAGE. Surprisingly, although the HDL-apoE was markedly elevated in the treated rats (**Fig. 4c**), the VLDL-apoE did not appear to be increased (Fig. 4a). An important question to consider at this point is: does an elevation of apoE sufficient

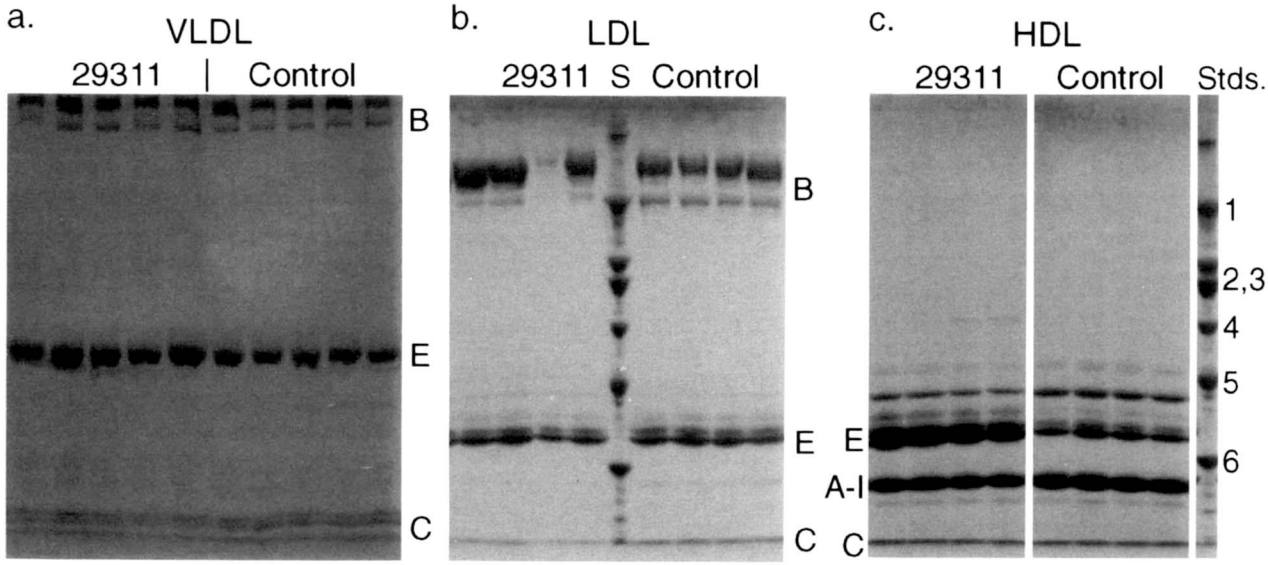


Fig. 4. SDS-PAGE analysis of VLDL (a), LDL (b), and HDL (c) apolipoproteins from control rats or rats fed 1% MDL 29311 for 7 days. VLDL (a), LDL (b), and HDL (c) lipoproteins were obtained by differential ultracentrifugation of plasma from control rats or rats treated with 1% MDL 29311 for 7 days. Equivalent amounts of protein (5 µg) were precipitated and dissolved in SDS sample buffer and electrophoresed on a 4–20% polyacrylamide gel as described in Methods. Protein bands were visualized by Coomassie R-250 staining. Each lane contains apolipoproteins from one rat. B, apo B; S, standards, etc. Molecular weights of standards labeled 1–6 in *c* are: 1 = 200,000, 2 = 116,000, 3 = 97,400, 4 = 66,000, 5 = 45,000, 6 = 29,000. The same standards are also shown in b but are not labeled for the sake of simplicity.

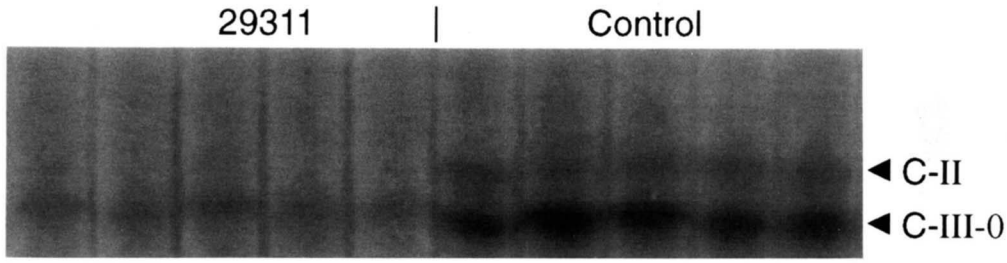


Fig. 5. Isoelectric focusing of VLDL apolipoproteins from control and MDL 29311-treated rats. VLDL were obtained by ultracentrifugation of plasma from control rats or rats treated with 1% MDL 29311 for 7 days. Equivalent amounts of protein (10 μg) were precipitated and focused on 7.5% acrylamide tube gels over a pH range of 3.5–7.0 as described in Methods. The identities of the bands were determined by slicing pieces from a gel run in parallel and incubating them in water for 2 h at room temperature and measuring the pH of the resulting solution. The isoelectric points of apoC-III-0 and C-II were obtained from the literature (29). ApoC-III-3 represents about 1/4 to 1/3 of the total apoC-III mass in these rats (M. J. Sheetz, unpublished observations) but it was not visualized well in this experiment due to interference from non-proteinaceous material. In other experiments involving isoelectric focusing of VLDL apolipoproteins from MDL 29311-treated rats, changes in the amounts of apoC-III-3 paralleled changes in the amounts of apoC-III-0.

to lower TG indeed cause an increase in the VLDL-apoE level? Fortunately, this question has been answered in the literature. Shimano et al. (18) made rat apoE-overexpressing transgenic mice which, when heterozygous, had plasma apoE levels of 9.3 mg/dl, compared to 4.7 mg/dl for non-transgenics. This elevation of total apoE resulted in a 2.4-fold increase in VLDL-apoE and decreased plasma TG by about 50%.

Although VLDL-apoE did not appear to be affected by MDL 29311 treatment, it did appear that VLDL- and LDL-apoC were decreased in the MDL 29311-treated rats (Figs. 4a,b). Isoelectric focusing of the VLDL apolipoproteins from MDL 29311-treated rats showed that both apoC-II and apoC-III-0 (the predominant apoC-III isoform in these rats; M. J. Sheetz, unpublished observations) were decreased (**Fig. 5**). To further investigate whether VLDL-apoC was indeed decreased by MDL 29311 treatment, VLDL were obtained from pooled plasma from control or 1% MDL 29311-treated (7 days) rats. The VLDL apolipoproteins were precipated and redissolved in 6 M urea. Under these conditions, all of the apoC and apoE is redissolved but only a small amount (< 20% for samples from both control and treated rats) of the apoB is redissolved (M. J. Sheetz, unpublished observations). When the VLDL apolipoproteins were separated by gel filtration on a Sephadex G-75 column (**Fig. 6**), the ratio of the second peak (apoC) to the first peak (mainly apoE) was 1.89 (control) compared to 1.11 (MDL 29311 treated). Equivalent results were obtained from two other experiments in which

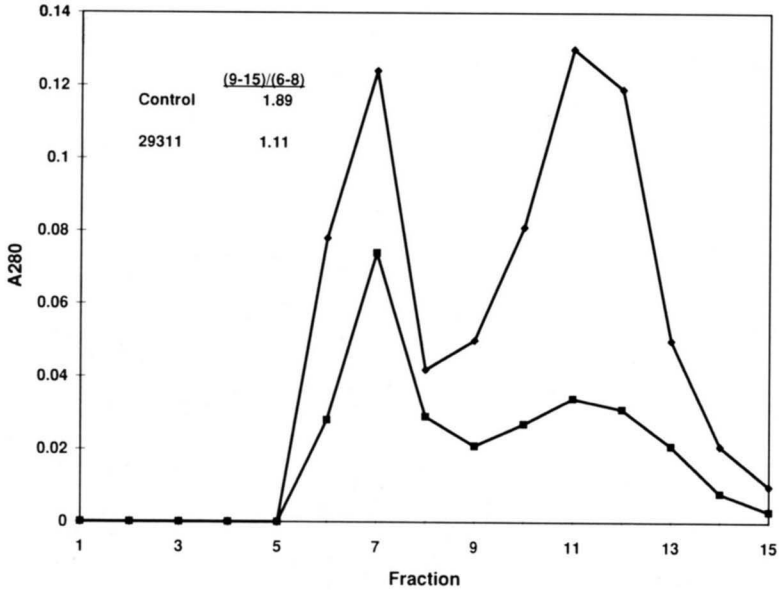


Fig. 6. Gel filtration on Sephadex G-75 of VLDL apolipoproteins from control and MDL 29311-treated rats. VLDL were isolated from pooled plasma of five rats fed either control chow (solid diamonds) or chow containing 1% MDL 29311 (solid squares) for 7 days. All of the VLDL (1.3 mg protein from controls and 0.7 mg protein from MDL 29311-treated) were precipitated overnight at -20°C with 10 volumes of acetone–ethanol 1:1. The pellet was dissolved in 2 ml of PDB containing 6 M urea. The solubilized apolipoproteins were then gel-filtered over a 40-ml Sephadex G-75 column equilibrated with the same buffer. Two-ml fractions were collected and their absorbance at 280 nm was determined. The ratio of the total absorbance in fractions 9–15 (apoC) to the total absorbance in fractions 6–8 (mainly apoE) is shown for each preparation.

TABLE 1. Transfer of ^{125}I-labeled apoC from HDL to VLDL in plasma from control rats or rats treated with 1% MDL 29311 for 7 days

	Control		MDL 29311	
Rat Number	TG	C Transfer	TG	C Transfer
	mg/dl	*%*	*mg/dl*	*%*
1	27	6.3	25	2.8
2	24	3.7	19	1.5
3	27	6.2	24	2.0
4	25	4.4	23	1.7
5	24	5.7	25	1.8
6	23	2.8	26	2.3
Mean ± SD	25 ± 2	4.9 ± 1.4	24 ± 3	2.0 ± 0.5[a]

Plasmas were obtained after a 24-h fast from control rats or from rats treated with 1% MDL 29311 for 7 days. TG were measured, and an aliquot of ^{125}I-labeled apoC-HDL was added and allowed to incubate with the plasma for 20 min at 37°C. The VLDL-associated ^{125}I-labeled apoC was then determined by gel filtering the mixture on a Sepharose 6B column and measuring the amount of ^{125}I-radioactivity that co-eluted with VLDL.

[a] $P < 0.05$ vs. control group.

different amounts of total protein were gel-filtered. Taken together, the data from Figs. 4a, 5, and 6 suggest that VLDL-apoC is decreased in MDL 29311-treated rats. A decrease in VLDL-apoC should make the VLDL more rapidly cleared by the liver (13, 14).

As VLDL-apoC appears to be decreased in MDL 29311-treated rats (Figs. 4a, 5, and 6) without a detectable decrease in total lipoprotein-associated apoC (Fig. 3c), the possibility that MDL 29311 treatment somehow interferes with apoC-VLDL association was considered. To test whether the association of apoC with VLDL was impaired in MDL 29311-treated rats, the transfer of ^{125}I-labeled apoC from HDL to VLDL in plasma from control rats, or in plasma from MDL 29311-treated rats, was measured. In order to minimize differences between treated and control VLDL levels, rats were fasted for 24 h so that the plasma TG (which are primarily carried in VLDL) were 25 ± 2 mg/dl in the controls and 24 ± 3 mg/dl in the treated animals (**Table 1**). One ml of plasma from fasted control or MDL 29311-treated rats was mixed with ^{125}I-labeled apoC-HDL (9 μg HDL protein) and the VLDL-associated counts were separated by gel filtration on a Sepharose 6B column. The percentage of applied radioactivity which co-eluted with VLDL was significantly higher in plasma from control rats than from MDL 29311-treated rats (4.9 ± 1.4% vs. 2.0 ± 0.5%, $P < 0.05$, Table 1).

As MDL 29311 treatment altered apoC distribution among different lipoproteins but did not appear to alter total apoC levels, the possibility that the MDL 29311 molecule might somehow interact directly with apoC in the absence of plasma or lipid was investigated. MDL 29311 is very poorly soluble in aqueous solutions: 20 μl of a DMSO solution containing 600 μg/ml MDL 29311 added to 1 ml of Tris-buffered saline will cause a visible precipitate (final MDL 29311 concentration = 12 μg/ml). However, when 200 μl of the same DMSO solution of MDL 29311 is added to 1 ml of Tris-buffered saline containing 0.5% BSA, a clear solution is obtained (final MDL 29311 concentration = 120 μg/ml). Sepharose 6B gel filtration of MDL 29311 in 0.5% BSA revealed that most of the compound eluted in the void volume with an apparent molecular weight of > 10^6 (**Fig. 7**). When a 12, 36, or 120 μg/ml solution of MDL 29311 in 0.5% BSA was mixed with ^{125}I-labeled apoC in the absence of

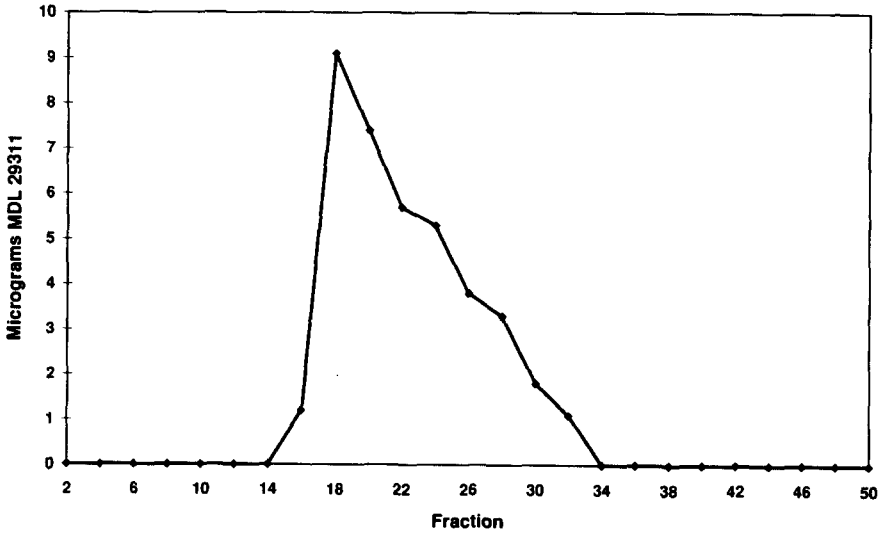


Fig. 7. Gel filtration on Sepharose 6B of MDL 29311. MDL 29311 (200 μg) in 2 ml of PDB containing 0.5% BSA was applied to a 1.5 × 20 cm Sepharose 6B column equilibrated with the same buffer. The column was run at a flow rate of 1 ml/min and 1-ml fractions were collected. Even numbered fractions were analyzed for MDL 29311 content by HPLC as described in Methods.

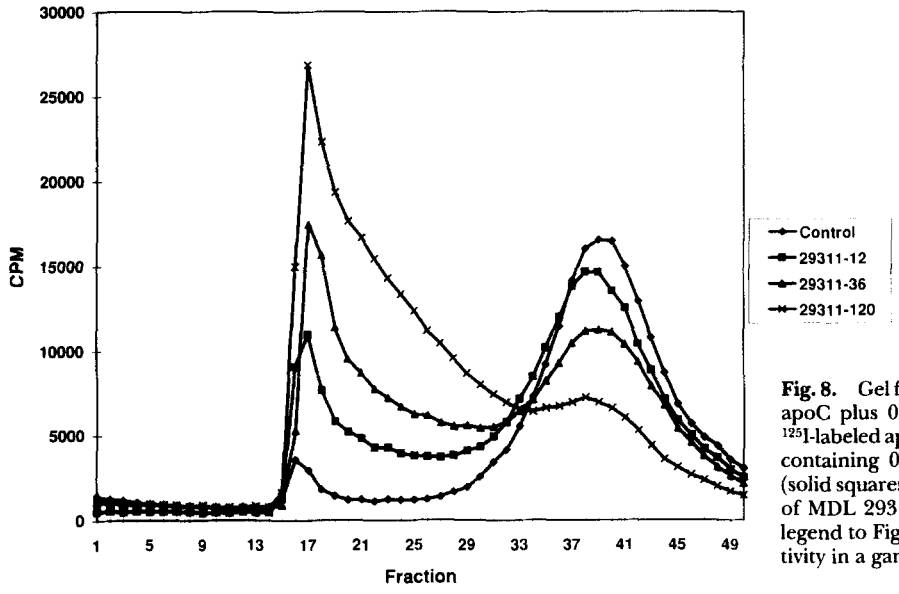


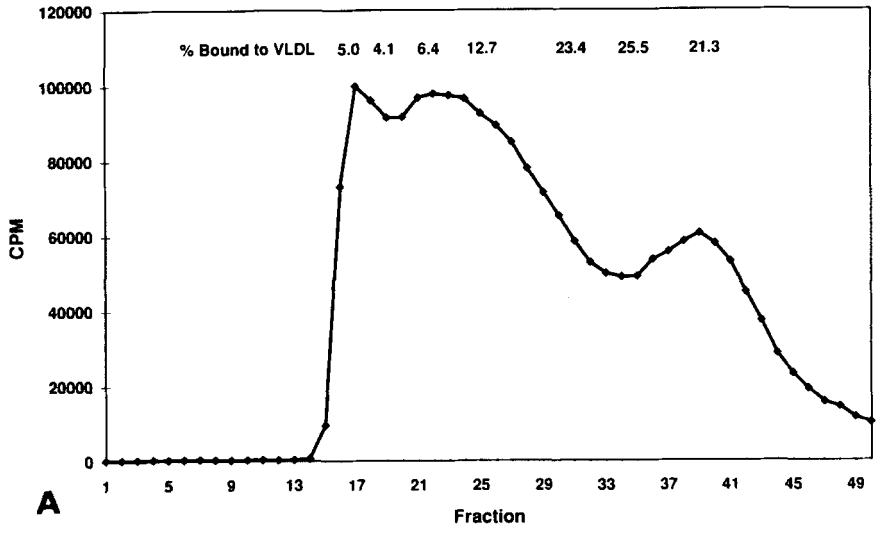
Fig. 8. Gel filtration on Sepharose 6B of ^{125}I-labeled apoC plus 0, 12, 36, or 120 mg/ml MDL 29311. ^{125}I-labeled apoC (400,000 cpm) was mixed with PDB containing 0.5% BSA and 0 (solid diamonds), 12 (solid squares), 36 (solid triangles), or 120 (X) μg/ml of MDL 29311 and gel-filtered as described in the legend to Fig. 7. Fractions were assayed for radioactivity in a gamma scintillation counter.

plasma or lipid and gel-filtered on Sepharose 6B, from 18% (12 μg/ml MDL 29311) to 39% (120 μg/ml MDL 29311) of the ^{125}I-labeled apoC eluted in the void volume. Only 5% of the ^{125}I-labeled apoC eluted in the void volume when no MDL 29311 was present (**Fig. 8**). Therefore, the amount of apparently high molecular weight (HMW) ^{125}I-labeled apoC formed by MDL 29311 was proportional to the amount of MDL 29311 in solution. The HMW material did not change in apparent size upon repeat gel filtration of the HMW peak (not shown). MDL 29311 also increased the apparent size of ^{125}I-labeled apoE (a structural relative of apoC) but not of ^{125}I-labeled soybean trypsin inhibitor (an unrelated protein, not shown).

The ability of the HMW material to bind to VLDL was measured. ^{125}I-labeled apoC was mixed with 120 μg/ml MDL 29311 in 0.5% BSA and gel-filtered on Sepharose 6B (**Fig. 9A**). Pooled fractions from different points on the elution profile were then allowed to bind to VLDL. The percent of total radioactivity bound to VLDL in each set of pooled fractions decreased as the apparent size of the ^{125}I-labeled apoC material increased (Fig. 9B). The percent of the ^{125}I-labeled apoC bound to VLDL observed for each pool was; 16–17 (5.0%), 18–19 (4.1%), 21–22 (6.4%), 24–25 (12.7%), 30–32 (23.4%), 36–38 (25.5%) and 39–41 (21.3%). Therefore, it appears that MDL 29311 can interact with ^{125}I-labeled apoC and impair its binding to VLDL.

MDL 29311 was also tested for its ability to displace apoC from VLDL. ^{125}I-labeled apoC-VLDL were incubated with increasing concentrations of MDL 29311. The VLDL were then separated by ultracentrifugation and the amount of ^{125}I-labeled apoC remaining VLDL-bound was determined. MDL 29311 displaced ^{125}I-labeled apoC from VLDL with a half-maximally active concentration between 20 and 60 μg/ml (**Fig. 10**). The total concentration of MDL 29311 and its metabolite (which also lowers TG) in plasma from treated rats is 20–30 μg/ml (M. J. Sheetz, unpublished observations).

The ability of structural relatives of MDL 29311 to interact with ^{125}I-labeled apoC was then tested. We have tested the TG-lowering ability of many phenolic antioxidants by feeding them to rats at 0.3% in the diet for 4 days (R. L. Barnhart and B. L. Rhinehart, unpublished observations). Many parameters were measured at the end of each experiment in addition to plasma TG levels. From these data, compounds were picked that showed more than 30% TG lowering without changing food consumption (MDL 29311, compounds A, B, and C), and compared to compounds that lowered TG less than 10% but were present in plasma at concentrations comparable to those observed with compounds that did lower TG (compounds D and E). ^{125}I-labeled apoC was added to a 36 μg/ml solution of each compound in 0.5% BSA in the absence of plasma or lipid and the mixture was gel-filtered on a Sepharose 6B column. The results are shown in **Fig. 11.** The compounds that lowered TG in rats all were as effective as, or more effective than, MDL 29311 at causing the appearance of HMW ^{125}I-labeled apoC complexes (Fig. 11A). The compounds that did not lower TG caused the formation of much less HMW ^{125}I-labeled apoC (Fig. 11B).



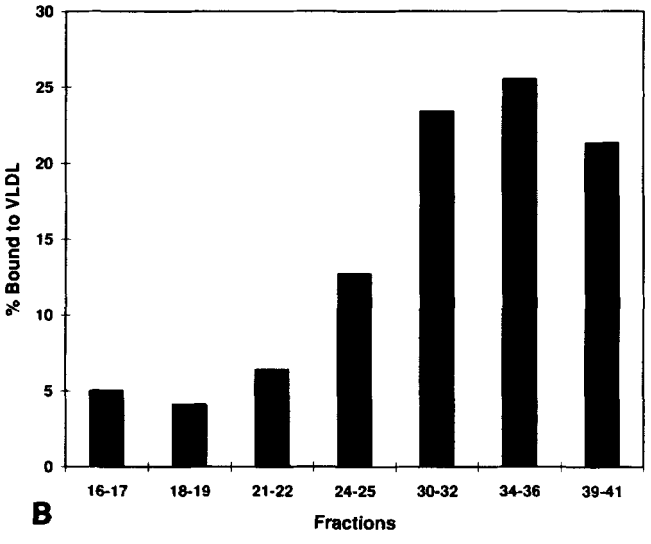


Fig. 9. Binding to VLDL of ^{125}I-labeled apoC-MDL 29311 complexes obtained by Sepharose 6B gel filtration. (A) ^{125}I-labeled apoC (3,000,000 cpm) was mixed with PDB containing 0.5% BSA and 120 μg/ml of MDL 29311. The mixture was gel-filtered as described in the legend to Fig. 7. Fractions were assayed for radioactivity in a gamma scintillation counter. (B) Consecutive fractions were pooled from different points on the elution profile (i.e., different apparent sizes). Fractions 16–17 and 18–19 were from the void volume; 21–22, 24–25, and 30–32 were from intermediate size ranges; and fractions 34–36 and 39–41 contained material of the same apparent size as uncomplexed ^{125}I-labeled apoC. The total radioactivity in each pool was between 180,000 and 200,000 cpm. Each pool was divided into two equal parts and 0.5 ml of a preparation of control VLDL (TG concentration = 200 mg/dl) was added to one part while buffer alone was added to the other. The volumes were adjusted to 4.8 ml with PDB and the mixtures were allowed to equilibrate for 15 min at room temperature before the VLDL were separated out by ultracentrifugation. The percent of total radioactivity associated with VLDL was determined by subtracting the very low density radioactivity (i.e., the radioactivity in the top 0.5–1.0 ml) in the buffer-only tubes from the very low density radioactivity in the VLDL-containing tubes (the amount of very low density radioactivity in the buffer-only tubes was between 5 and 10% of the amount of very low density radioactivity in the VLDL-containing tubes).

DISCUSSION

The data presented in this report provide support for the hypothesis that MDL 29311 lowers TG in rats by interfering with the association of apoC with the VLDL particle, thereby relaxing the apoC-mediated inhibition of VLDL uptake by the liver and allowing more rapid clearance. The pertinent findings presented in this report include: *a)* no detectable change in plasma total lipoprotein-associated apoC after MDL 29311 treatment (Fig. 3); *b)* reduced VLDL-associated apoC (both C-II and C-III-0) after MDL 29311 treatment (Figs. 4–6); *c)* impaired transfer of ^{125}I-labeled apoC to VLDL in plasma from MDL 29311-treated rats (Table 1); *d)* Interaction of MDL 29311 with ^{125}I-labeled apoC in the absence of plasma or lipid to form HMW complexes (Figs. 7–9); *e)* HMW ^{125}I-labeled apoC complexes show impaired VLDL binding activity (Fig. 9); *f)* MDL 29311 induces the dissociation of ^{125}I-labeled apoC from VLDL (Fig. 10); *g)* phenolic antioxidants that lower TG in rats strongly induce HMW ^{125}I-labeled apoC complex formation (Fig. 11A); and *h)* phenolic antioxidants that do not lower TG in rats only very weakly induce HMW ^{125}I-labeled apoC complex formation (Fig. 11B).

The possibility that MDL 29311 might stimulate VLDL clearance by enhancing apoE binding to VLDL either directly or by elevating plasma apoE levels was also considered. Consistent with this possibility is the finding of elevated plasma total lipoprotein apoE (Fig. 3). However, the finding that VLDL-associated apoE was not increased by MDL 29311 treatment (Fig. 4) argued against apoE elevation being responsible for MDL

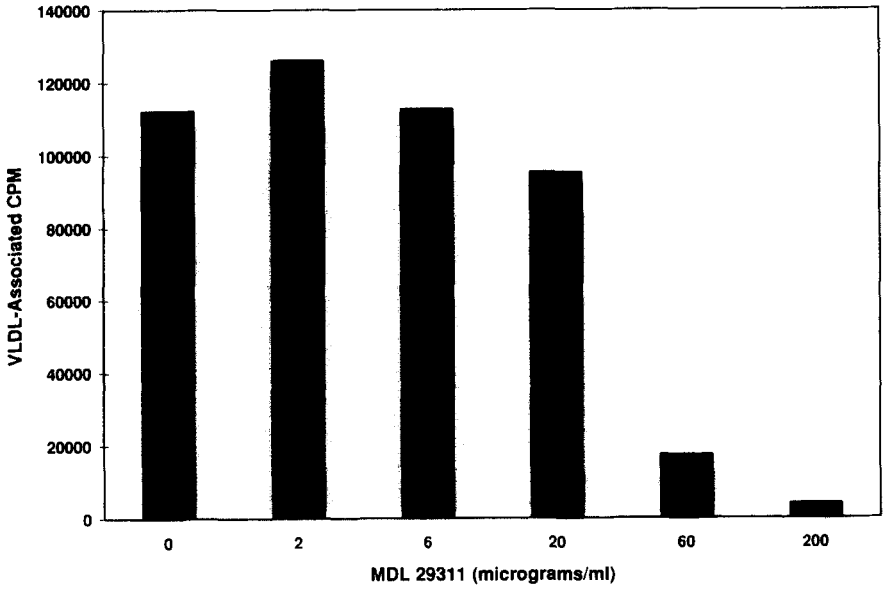


Fig. 10. Displacement of ^{125}I-labeled apoC from VLDL by MDL 29311. ^{125}I-labeled apoC was allowed to bind to VLDL from control rats and the resulting ^{125}I-labeled apoC-VLDL were obtained by ultracentrifugation. ^{125}I-labeled apoC-VLDL (180,000 cpm) were then incubated in 3 ml of PDB containing 0.5% BSA plus 600 µl DMSO containing MDL 29311 to make final concentrations of 0, 2, 6, 20, 60, and 200 µg/ml MDL 29311. After 1 h at room temperature, the VLDL were separated by ultracentrifugation and the amount of VLDL-associated radioactivity was determined by gamma scintillation counting. The results are expressed as cpm remaining VLDL-associated. In other experiments measuring the recovery of lipoprotein lipids, only about 75% of the lipids were recovered after ultracentrifugation (M. J. Sheetz, unpublished observations). Therefore, no more than 135,000 cpm would be expected to be recovered in the experiment shown.

29311's TG-lowering activity. These two findings, taken together with data from Shimano et al. (18) from rat apoE-overexpressing transgenic mice that have 50% lower TG with 140% higher VLDL-apoE levels, argue that MDL 29311 is more likely to lower TG by an apoC-dependent mechanism than by an apoE-dependent mechanism. On the other hand, one could argue that MDL 29311 treatment might enhance low affinity binding of apoE to VLDL. This interaction might not be detected when isolating the VLDL by ultracentrifugation because the apoE could possibly redistribute to HDL during the isolation. This possibility requires further investigation.

Although it is evident that MDL 29311 interacts directly with ^{125}I-labeled apoC (and presumably with unmodified apoC as well) in the absence of plasma or lipid, the nature of the HMW complex formed remains unclear. It is possible that ^{125}I-labeled apoC simply binds to MDL 29311 in the HMW structure thereby changing the apparent size of the ^{125}I-labeled apoC (Figs. 7 and 8). The fact that the HMW complexes containing ^{125}I-labeled apoC (Fig. 8) co-elute with MDL 29311 (Fig. 7) suggests that ^{125}I-labeled apoC may in fact be binding to MDL 29311. Consistent with this possibility is the fact that both apoC and apoE (as well as other apolipoproteins) contain numbers of amphipathic lipid-binding helices (24) whereas soybean trypsin inhibitor (which does not form complexes with MDL 29311) does not.

It has been shown that liposomes containing probucol (a close structural relative of MDL 29311) bind apoC-III less well than control liposomes (25). In order to study the apoC-MDL 29311 (or analog) interaction independent of the influence of lipid, the system shown in Figs. 7–9 was used. In the absence of lipid and other plasma components (except albumin), MDL 29311 appears to interact directly with apoC. Analogs of MDL 29311 that lower TG in rats also interact with apoC, but analogs that do not interact with apoC are not effective TG-lowering agents in rats (Fig. 11). In vivo, the HMW complex described in this paper is probably modified by the presence of lipoproteins. Nevertheless, for the small sample of compounds evaluated in the experiments set forth in Fig. 11, it serves as an interesting surrogate marker for TG-lowering activity. In addition, the fact that ^{125}I-labeled apoC in the HMW complex binds VLDL less well than uncomplexed ^{125}I-labeled apoC suggests a mechanism whereby MDL 29311 might be interfering with apoC binding to VLDL (Fig. 9).

As one might expect, the interaction of MDL 29311 with ^{125}I-labeled apoC is not very specific. This is borne out by the fact that both apoC-II and apoC-III-0 are reduced on the VLDL particles in treated rats (Fig. 5) and by the fact that MDL 29311 appears also to form complexes with apoE. ApoC-III-3 is another isoform of

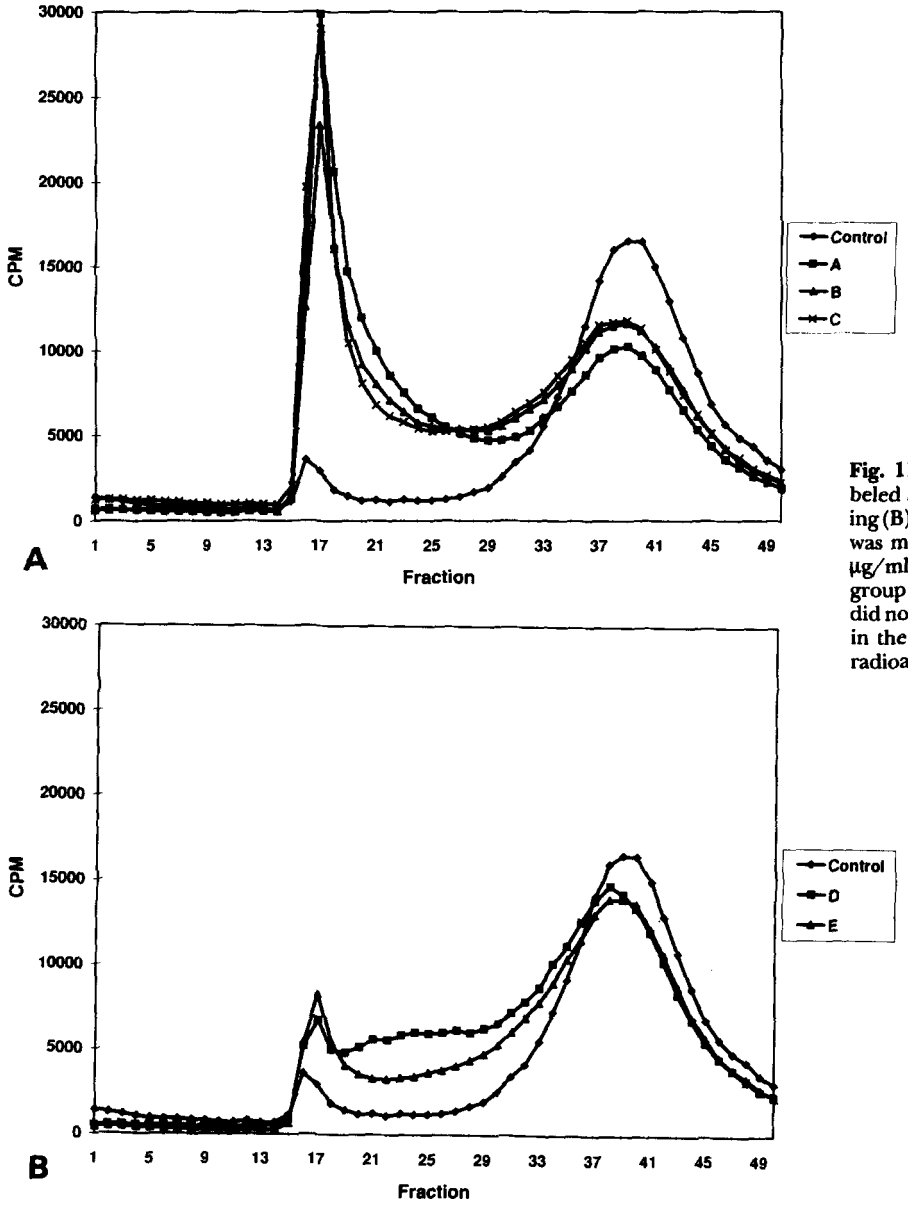


Fig. 11. Gel filtration on Sepharose 6B of ^{125}I-labeled apoC plus TG-lowering (A) or non-TG-lowering (B) antioxidants. ^{125}I-labeled apoC (300,000 cpm) was mixed with PDB containing 0.5% BSA and 30 μg/ml of the indicated antioxidant from either a group that lowered TG in rats (A) or a group that did not (B). The mixture was gel filtered as described in the legend to Fig. 7. Fractions were assayed for radioactivity in a gamma scintillation counter.

apoC-III that represents about 1/4 of the total apoC-III mass in the rats used in this study in the fasted state. In the experiment shown in Fig. 5, it is not shown because it was not well visualized on the isoelectric focusing gels due to interference from non-proteinaceous material. In other unrelated experiments, MDL 29311 treatment has been shown to reduce VLDL-apoC-III-3 in parallel with VLDL-apoC-III-0 (M. J. Sheetz, unpublished observations). The fact that VLDL-associated apoE is not reduced, whereas VLDL-associated apoC is, might be because the lipoprotein-associating properties for apoE-MDL 29311 complexes differ from those of apoC-MDL 29311 complexes. In support of this explanation is the fact that HDL-associated apoE is markedly increased in MDL 29311-treated rats whereas HDL-associated apoC does not appear to be altered by MDL 29311 treatment (Fig. 4c). Alternatively, there may be different dose dependencies of association for apoC and E. It is possible that the concentration of MDL 29311 and its metabolite in vivo is too low to interact with apoE but high enough to interact with apoC. The investigation of these and other possibilities provides avenues for future research.

Recent studies with transgenic mice have confirmed the crucial role apoC plays in TG metabolism (15–17). Although apoC-II and apoC-III are thought to have opposite effects upon TG clearance, recent studies with transgenic mice overexpressing human apoC-II (16)

have shown that apoC-II can have an inhibitory effect on TG clearance in addition to its stimulatory effect. Given these findings, the observation that MDL 29311 reduces VLDL-apoC-II and VLDL-apoC-III is not inconsistent with this effect contributing to its TG lowering activity in rats. Clinical epidemiology and genetic studies (26–28) point to apoC-III as an important contributor to abnormal TG levels. The studies described in this paper outline a novel mechanism whereby apoC distribution might be pharmacologically altered. This alteration could conceivably affect TG levels in a beneficial manner.

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