Effects on Lipoprotein Subclasses of Combined Expression of Human Hepatic Lipase and Human apoB in Transgenic Rabbits

Manfredi Rizzo, John M. Taylor, Carlo M. Barbagallo, Kaspar Berneis, Patricia J. Blanche, Ronald M. Krauss

Objective—The effects of combined expression of human hepatic lipase (HL) and human apolipoprotein B (apoB) on low-density lipoprotein (LDL) subclasses were examined in rabbits, a species naturally deficient in HL activity.

Methods and Results—In apoB-transgenic rabbit plasma, >80% of the protein was found in the 1.006- to 1.050-g/mL fraction. Gradient gel electrophoresis (GGE) of this fraction revealed two distinct species, designated large and small LDL. A denser fraction (d=1.050 to 1.063 g/mL) contained small LDL as well as another discrete LDL subspecies, designated very small LDL. Expression of HL resulted in reductions in protein concentrations in the 1.006- to 1.050-g/mL density-gradient subfractions containing large (6.5±4.1 versus 32.6±12.0 mg/dL, P<0.005) and small LDL (59.6±17.4 versus 204.3±50.3 mg/dL, P<0.002). A concomitant small but not significant increase in protein concentration in the denser LDL fraction (48.0±28.2 versus 44.6±18.2 mg/dL) was due primarily to an increase in very small LDL (25.9±3.1 versus 9.6±5.4% of total LDL GGE densitometric area, P<0.002).

Conclusion—These findings support a direct role for HL in regulating total plasma LDL concentrations as well as in the production of smaller, denser LDL from large, more buoyant precursors. (Arterioscler Thromb Vasc Biol. 2004; 24:141-146.)

Key Words: hepatic lipase ■ apoB ■ LDL ■ transgene ■ rabbit ■ density gradient ultracentrifugation

Hepatic lipase (HL) is a 476AA-glycoprotein that is synthesized by hepatocytes and binds to the external surfaces of hepatic cells.1 Hepatic lipase functions both as triacylglycerol hydrolase and phospholipase, hydrolyzing triacylglycerides in chylomicron remnants, intermediate density lipoproteins (IDL), and HDL, as well as phospholipids in HDL.2,3 In addition, it can also enhance cell association and removal of lipoproteins by initiating their binding to cell surface heparan sulfate proteoglycans.4

Human HL deficiency is accompanied by increases in large, buoyant LDL and HDL enriched in triglycerides and phospholipids,6 as well as increased plasma levels of β-VLDL remnants.7 Metabolic experiments in human HL deficiency4 and in HL-deficient mice8 have demonstrated a defect in conversion of IDL to LDL. Mice lacking HL show higher concentrations of plasma phospholipids and apoE and increases in HDL particles.9

Because of the naturally occurring, relatively low concentrations of HL, the rabbit is an effective model for investigating the consequences of deficiency of this enzyme. Rabbits lack apoB100 mRNA editing capability in liver, which results in substantially higher levels of circulating apolipoprotein B100 (apoB)-containing IDL and LDL particles in plasma.10 This property is in contrast to mice in which liver apoB100 mRNA is edited to yield apoB48 mRNA, resulting in a reduced plasma content of IDL and LDL. Rabbits have substantial plasma cholesterol ester transfer protein (CETP) activity, which is lacking in mice.10

We previously documented that the overexpression of hepatic lipase in rabbits resulted in reduced plasma levels of VLDL, IDL, and HDL.11 We showed that cholesterol-fed hepatic lipase transgenic rabbits have a marked reduction of IDL and large LDL with an induction in LDL particles of smaller size and higher density.12 However, the cholesterol-fed rabbit is not a good model for studying the full range of LDL subclasses found in humans because rabbit LDL consists mainly of large, buoyant particles, with very low levels of particles corresponding to the smaller, dense LDL subclasses found in humans.12 In contrast, human apoB transgenic rabbits exhibit increased levels of several LDL subclasses, including high levels of small, dense LDL.13 This model is therefore useful for studies of metabolism of human-like apoB-containing LDL subclasses.
In this study, we used previously generated models of transgenic rabbits\textsuperscript{11,13} to evaluate the effects of increased HL activity on specific lipoprotein subclasses. In particular, we tested the hypothesis that expression of HL in human apoB transgenic rabbits can alter the distribution of specific LDL subclasses resulting in a shift to smaller, denser LDL particles.

**Materials and Methods**

**Protocol**

Pathogen-free New Zealand White rabbits (Charles River Breeding Laboratories) were used in this study. Human hepatic lipase and human apoB-100 transgenic rabbit models have been described previously.\textsuperscript{11,13} Animals expressing both human hepatic lipase and apoB were produced by crossbreeding the two transgenic strains. Studies were performed in 8 animals expressing human apoB, 6 expressing both human hepatic lipase and apoB, and 10 nontransgenics, all females of 4 to 6 months of age.

The concentration of human apoB in the rabbits carrying the transgene was 100±5 mg/dL (mean±SD), similar to that previously reported and ~5-fold higher than endogenous rabbit apoB. Human HL activity level as measured in postheparin plasma\textsuperscript{11} was 9±3 (mean±SD) μEq of fatty acid released per mL per hour from a triolein substrate, a level ~10-fold higher than the activity found in nontransgenic controls.

All animals were maintained on a chow diet. The experimental procedures were conducted in accordance with NIH Guidelines and with the approval of the Committee on Animal Research of the University of California, San Francisco. Before the study, rabbits were fasted overnight (14 to 16 hours). Plasma from individual animals was separated by centrifugation at 3000 rpm for 20 minutes at 4°C.

**Preparative and Density-Gradient Ultracentrifugation**

Briefly, preparative ultracentrifugation was performed to isolate lipoproteins under standard conditions.\textsuperscript{14} Sodium bromide was added to adjust densities and samples were centrifuged at 40,000 rpm for 18 hours (VLDL, d<1.006 g/mL), 24 hours (d=1.006 to 1.050 g/mL), 24 hours (d=1.050 to 1.063 g/mL), and 48 hours (HL, d=1.063 to 1.21 g/mL) at 10°C in a Beckman 40ti fixed angle rotor. We modified previously described procedures for density-gradient ultracentrifugation\textsuperscript{15–16} to separate apoB-containing particles having a density range between 1.006 and 1.050 g/mL. The d=1.006 to 1.050 g/mL fraction was dialyzed for 24 to 48 hours against a d=1.025-g/mL sodium bromide solution at 4°C. The dialyzed d=1.006- to 1.050-g/mL fraction was then layered (2.0 mL) above a solution of d=1.045 g/mL (2.5 mL) in a 1/2×3.5-inch Ultraclear Tube (Beckman Instruments, Mountain View, CA) and 2.5 mL of a solution of d=1.063 g/mL, layered above the fraction. The tubes were centrifuged for 40 hours at 17°C in a Beckman SW45 rotor in a Beckman L5–75 ultracentrifuge. The slowest setting for acceleration was used at the start of the run. After 40 hours at 40,000 rpm, the rotor was allowed to coast to a stop without braking. The contents of the tube then were withdrawn by pipetting 14 individual 0.5 mL fractions.

**Nondenaturing Polyacrylamide Gradient Gel Electrophoresis**

Nondenaturing polyacrylamide gradient gel electrophoresis (GGE) of whole plasma and lipoprotein fractions was performed as described,\textsuperscript{12,17,18} using gradient gels manufactured in our laboratory by established procedures.\textsuperscript{19} Concentrations of lipoproteins across the range of particle diameters (Å) were estimated from densitometric scans of lipid-stained gels using absorbance in OD.cm\textsuperscript{2} units. Lipoprotein calibrators were used to monitor reproducibility of absorbance measurements and for the calculation of a normalization factor used to average densitometric scans obtained from different gels. Mean particle size profiles were generated from individual profiles of transgenic rabbits and control rabbits by averaging absorbance units of equivalent particle size for each group. Particle size measurement of the lipoprotein controls was within ±2.6 Å in the size range of IDL and LDL, and variation of total LDL absorbance was within ±12%. HDL particle size measurements were within ±1.3 Å and absorbance variation was ±11%.

**Chemical Analysis**

Total cholesterol and triglycerides were measured using enzymatic methods on a Gilford System 3500 analyzer (Gilford Instruments, Oberlin, Oh).\textsuperscript{20–21} HDL cholesterol was measured after heparin-manganese precipitation of the remaining plasma lipoproteins.\textsuperscript{22} Protein concentrations were determined by the Lowry method modified to include sodium dodecyl sulfate.\textsuperscript{23}

**Statistical Analysis**

Statistical analyses were performed using the Statview Program (Abacus Concepts Inc). Means and standard deviations were calculated and the differences were analyzed using the Mann-Whitney nonparametric test. Statistical significance was considered to be achieved when differences between groups had probability values <0.05. For correlation analyses, the Spearman rank correlation method was applied.

**Results**

Consistent with our previously reported findings, expression of human apoB100 at ~100 mg/dL in transgenic rabbits resulted in a 6- to 10-fold increase in total plasma triglyceride and total cholesterol levels, accompanied by a marked reduction in plasma HDL cholesterol (Table 1). Expression of HL in apoB+HL double transgenic rabbits resulted in >60% reductions in plasma levels of triglycerides and cholesterol, as well as an increase in HDL cholesterol. However, the increased expression of HL in the double transgenic rabbits did not restore plasma lipid concentrations to the levels found in nontransgenic controls. LDL peak particle sizes in apoB and apoB+HL transgenic rabbits were similar, and were smaller than the major species of LDL particles in nontransgenic rabbits.

The mean peak particle diameters of IDL and LDL density subfractions from apoB- and apoB+HL-transgenic rabbits as determined by gradient gel electrophoresis were similar (Table 1, available online at http://atvb.ahajournals.org). Figure 1A (available online at http://atvb.ahajournals.org) shows the particle diameters of distinct GGE peaks identified within the density subfractions of d=1.023 to 1.063 g/mL from both groups. This reveals a trend for smaller LDL diameters with increasing density and for clustering of values for particle diameters in several size regions. A histogram of the peak diameters reveals three distinct size groups, designated as large, small and very small LDL (Figure 1B). The results did not differ significantly in apoB versus apoB+HL transgenic rabbits.

Protein concentrations were measured in VLDL and pooled IDL and LDL density subfractions from transgenic and nontransgenic rabbits (Table 2). VLDL and IDL protein levels were low and similar in both transgenic groups and nontransgenics, whereas protein levels in the fractions containing large, small, and small and very small LDL were significantly higher in apoB transgenics than in nontransgenic controls (all P<0.0005). Expression of hepatic lipase resulted in significant reductions in protein concentrations in fractions containing large LDL (P<0.005) and small LDL (P<0.002).
There was also a small but not statistically significant increase in protein in the densest LDL fractions (d 1.050 to 1.063 g/mL).

Analysis of the distribution of triglyceride and cholesterol in LDL subclasses in a subgroup of transgenic animals (4 apoB and 3 apoB + HL transgenic rabbits) revealed that the triglyceride/total cholesterol ratio (wt/wt) did not differ among the LDL subtypes in the apoB transgenic rabbits (1.1 ± 0.1, 0.9 ± 0.05, and 1.0 ± 0.1, respectively). In contrast, the additional expression of HL resulted in a depletion of triglyceride in all the LDL subtypes. There was a significantly greater reduction in smaller particles, with a total triglyceride/cholesterol ratio of 0.8 ± 0.1 in large LDL, 0.5 ± 0.03 in small LDL (P < 0.0005 versus apoB transgenics), and 0.3 ± 0.1 in very small LDL (P < 0.005 versus apoB transgenics).

Further analysis of apoB-containing lipoproteins was performed by densitometric analysis of GGE of unfractionated lipid-stained whole plasma (Figure 1). As previously described, overexpression of apoB resulted in increased levels of both large and small LDL compared with nontransgenic controls. Consistent with the measurements of protein concentrations in Table 2, the added expression of HL in double transgenic rabbits resulted in a reduction of large and small LDL accompanied by a small increase of very small LDL particles. Densitometric analysis was performed on lipid-stained areas within the particle size subclasses shown in Figure 1. Compared with apoB transgenic rabbits, the double transgenic rabbits had significantly lower levels of small LDL (51.6 ± 3.9 versus 62.4 ± 6.5, P < 0.005) and higher levels of very small LDL (25.9 ± 3.1 versus 9.6 ± 5.4, P < 0.002). These results are consistent with the measurements of LDL protein concentrations in these fractions (Table 2).

HDL particle size distributions were analyzed by non-denaturing 3/31% GGE with protein staining (Figure 2). Compared with nontransgenic controls, the apoB-transgenic rabbits showed a marked reduction of HDL particles, most notably in larger size classes. HL expression in the double transgenic animals resulted in higher levels of HDL than in apoB-transgenic rabbits, with less of an effect in larger-sized HDL, a class of particles that corresponds in size to human HDL2b. In comparison with nontransgenic controls, apoB + HL transgenics showed a reduction in the larger-sized HDL, with a concomitant increase in the smallest particles that correspond in size to human HDL3b and HDL3c.

Spearman correlation analyses (Table 3) supported the relationships of plasma lipid concentrations with levels of large, small, and very small LDL particles, as assessed by areas under the curve in Figure 1. Plasma triglyceride concentrations were significantly correlated with levels of large LDL in both groups of transgenics; however, the correlation was positive in the apoB (R = 0.02) and negative in the apoB + HL transgenic rabbits (P < 0.05, Figure II, available online at http://atvb.ahajournals.org). The correlations between plasma triglycerides and levels of small LDL also showed opposite results in the two groups of transgenic animals: the correlation was negative in the apoB (P < 0.005) and positive in the apoB + HL transgenic rabbits, but the latter relationship did not reach statistical significance. Plasma HDL cholesterol was positively correlated with very small LDL (P < 0.02) in the apoB transgenics and non-HDL cholesterol was inversely correlated with large LDL (P < 0.02) in the apoB + HL rabbits.

**Discussion**

Hepatic lipase has been shown to modulate multiple steps in lipoprotein metabolism through both lipolytic and nonlipolytic functions. Human HL deficiency is accompanied by increases in large, buoyant LDL, HDL enriched in triglycerides and phospholipids, and increased plasma levels of β-VLDL remnants. Metabolic experiments in patients with human HL deficiency demonstrated a defect in conver-

| TABLE 1. Plasma Lipid Levels and LDL Peak Particle Diameters |
|-----------------|----------|----------|----------|
|                  | Nontransgenics | ApoB Tg  | ApoB + HL Tg |
|                  | (n = 10)      | (n = 8)  | (n = 6)   |
| Triglycerides, mg/dL | 37.4 ± 12.8  | 261.2 ± 61.6 | 74.7 ± 24.6* |
| Total cholesterol, mg/dL | 41.1 ± 20.6  | 485.0 ± 185.5 | 133.4 ± 64.9* |
| HDL cholesterol, mg/dL | 23.5 ± 8.9   | 2.1 ± 1.1   | 13.0 ± 4.9†  |
| Plasma LDL peak particle diameter, Å | 274.0 ± 6.7  | 239.1 ± 3.1 | 236.4 ± 2.7* |

Nontransgenics indicate New Zealand White nontransgenic rabbits; ApoB Tg, rabbits overexpressing human apoB; apoB + HL Tg, rabbits overexpressing both human hepatic lipase and human apoB; and NS, not significant. Values are reported as mean ± SD.

* P < 0.01, † P = 0.05, ¶ P < 0.002 apoB + HL Tg vs nontransgenics.

| TABLE 2. Protein Concentrations of apoB-Containing Particles |
|-----------------|----------|----------|----------|
|                  | Nontransgenics | ApoB Tg  | ApoB + HL Tg |
|                  | (n = 10)      | (n = 8)  | (n = 6)   |
| Density, g/mL    |            |          |           |
| VLDL             | < 1.006     | 5.0 ± 1.3 | NS        | 4.0 ± 2.0 | 4.9 ± 1.5 |
| IDL              | 1.006–1.023 | 4.0 ± 1.1 | NS        | 3.7 ± 2.0 | 2.8 ± 1.0 |
| Large LDL        | 1.023–1.035 | 4.9 ± 1.0 | < 0.0005  | 32.6 ± 12.0 | < 0.005  | 6.5 ± 4.1 |
| Small LDL        | 1.035–1.050 | 0.5 ± 0.3 | < 0.0005  | 204.3 ± 50.3 | < 0.002  | 59.6 ± 17.4* |
| Small + Very Small LDL | 1.050–1.063 | 0.2 ± 0.1 | < 0.0005  | 44.6 ± 18.2 | NS       | 48.0 ± 28.2* |

Values are reported in mg/dL as the mean ± SD. * P < 0.002 apoB + HL Tg vs nontransgenics.
ization of injected IDL to LDL. Increased HL activity has been associated with small, dense LDL particles in humans. 26,27 To understand the mechanisms by which HL regulates lipoprotein metabolism, animal models have been developed with altered levels of expression of this enzyme.

Mouse models have been used to evaluate the effects of overexpression or deficiency of HL on lipoprotein distributions. 9,28–30 Mice lacking HL were not able to process chylomicron remnants or to produce lipoproteins of density exceeding 1.029 g/mL. 9 By contrast, transgenic mice expressing increased levels of HL showed a transformation of the lipoprotein profile from predominantly buoyant (VLDL and IDL) lipoproteins to more dense (LDL) fractions, whereas higher levels of HL led to decreased levels of these particles. 10 These results indicate multiple actions of HL in metabolism of apoB-containing lipoproteins. However, the mouse model is limited for studies of plasma LDL metabolism because plasma LDL levels are very low and CETP activity is virtually absent. 10 The low levels of circulating LDL may be a consequence of the editing of a majority of apoB100 mRNA in the liver to yield a mRNA that produces apoB48. This editing mechanism for apoB 100 mRNA is not found in human liver or in rabbit liver.

By contrast, rabbits have higher levels of LDL and substantial CETP activity. 10 In addition, rabbits have naturally low levels of hepatic lipase, making this species an appropriate model for studies of hepatic lipase deficiency. In the past, we documented that the expression of human hepatic lipase in apoB-transgenic rabbits resulted in a marked reduction of IDL and large LDL with the appearance of LDL of smaller size and density. 12 However, the cholesterol-fed rabbit has a preponderance of large LDL and very low levels of particles corresponding to smaller, denser LDL subclasses typically found in humans. By contrast, human apoB transgenic rabbits exhibit increased levels of several LDL subclasses, including small, dense LDL. 13 Thus, transgenic rabbits are likely to be useful models for studying the effects of HL on metabolism of human-like apoB-containing LDL subclasses.

Using a combination of density-gradient ultracentrifugation and gradient gel electrophoresis, we identified three discrete LDL subspecies, designated large, small, and very small LDL, in apoB- and apoB+HL-transgenic rabbits. The size ranges of these LDL subclasses correspond with those found in humans, 17 but with slightly lower density of small LDL particles (230 to 253 Å) in the transgenic rabbits. We found that the addition of hepatic lipase in apoB-transgenic rabbits led to a 50% reduction in total LDL levels. This was restricted to large and small LDL, with a small increase in the smallest, most dense LDL subclass.

We confirmed our previous observations 13 that the major LDL species in chow-fed apoB transgenic rabbits are triglyceride-enriched. The finding that total plasma triglyceride levels were positively correlated with large LDL and inversely with small LDL in the apoB transgenic rabbits is consistent with the possibility of a precursor-product relationship with relative impairment in the lipolytic transformation of large to small LDL (Figure 3). Concomitant expression of

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**Figure 1.** Densitometric analyses of lipid-stained 2% to 14% non-denaturing GGE of the VLDL, IDL, and LDL regions of unfractionated plasma. The data are mean ± SE absorbance units for the groups of apoB transgenic (n = 8) and apoB+HL transgenic (n = 6) rabbits. Intervals for LDL subclasses refer to size subgroupings derived from data in Figure 1.

**Figure 2.** Densitometric analyses of protein-stained 3% to 31% non-denaturing GGE of human HDL in the density range of 1.063 to 1.21 g/mL density fraction. The data are mean ± SE absorbance units for non-transgenic controls (n = 8), apoB transgenic (n = 7), and apoB+HL transgenic (n = 5) rabbits. HDL size intervals are those previously described for humans. 16

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### TABLE 3. Spearman Correlations Between Plasma Lipid Concentrations and Levels of Large, Small, and Very Small LDL Particles as Assessed by Gradient Gel Electrophoresis

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<thead>
<tr>
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<th>Plasma Triglycerides</th>
<th>Plasma HDL Cholesterol</th>
<th>Plasma Non-HDL Cholesterol</th>
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<tbody>
<tr>
<td>ApoB transgenics (n = 8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large LDL</td>
<td>0.80†</td>
<td>−0.57</td>
<td>0.28</td>
</tr>
<tr>
<td>Small LDL</td>
<td>−0.89†</td>
<td>0.14</td>
<td>−0.09</td>
</tr>
<tr>
<td>Very small LDL</td>
<td>−0.27</td>
<td>0.78†</td>
<td>−0.36</td>
</tr>
<tr>
<td>ApoB+HL transgenics (n = 6)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Large LDL</td>
<td>−0.90*</td>
<td>0.34</td>
<td>−0.90†</td>
</tr>
<tr>
<td>Small LDL</td>
<td>0.66</td>
<td>−0.68</td>
<td>0.71</td>
</tr>
<tr>
<td>Very small LDL</td>
<td>0.21</td>
<td>0.38</td>
<td>0.27</td>
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*P < 0.05, †P < 0.02, ¶P < 0.005
hepatic lipase may overcome this limitation, leading to an inverse relation of plasma triglyceride to levels of large LDL, and triglyceride depletion of smaller LDL. However, the marked reduction in plasma levels of both large and small LDL in the combined transgenic rabbits suggests the operation of other actions of hepatic lipase, perhaps noncatalytic, that promote direct plasma clearance of these particles or their precursors.

The apoB transgenic rabbits had very low levels of HDL consistent with our earlier report. The reduction in HDL might result from increased CETP-mediated transfer of cholesterol esters from HDL to apoB-containing lipoproteins. We found that the addition of HL led to higher HDL levels than in apoB transgenics. It is possible that this resulted from a reduction of CETP-acceptor apoB-containing particles (Figure 3). On the other hand, as with LDL, there was a shift toward smaller particles which may have resulted from both lipolytic and noncatalytic functions of this enzyme.

We found a significantly positive correlation of HDL cholesterol concentration with levels of very small LDL in the apoB-transgenic rabbits. This is consistent with the hypothesis that variation in CETP activity results in reciprocal changes in HDL cholesterol and cholesterol content of precursors of very small LDL. Hence, higher CETP activity would result in both lower HDL cholesterol and reduced formation of very small LDL by lipolysis because of cholesterol enrichment of their metabolic precursors (Figure 3). The lack of a significant correlation between HDL cholesterol and very small LDL in the apoB+HL rabbits could reflect the primary role of HL rather than CETP in determining levels of these smaller particles (Figure 3).

The role of hepatic lipase in atherogenesis is at present uncertain. Recent studies in humans have indicated that reductions in hepatic lipase by pharmacologic therapy are associated with reduced coronary atherosclerosis protection in association with an increase in LDL particle buoyancy, and hence reduced levels of small, dense LDL. In the present model, however, the potentially proatherogenic effect of hepatic lipase in shifting the LDL distribution of apoB-transgenic rabbits to smaller, denser particles is accompanied by the potentially antiatherogenic effects of reduced total LDL mass and increased HDL cholesterol. Reduced LDL and increased HDL cholesterol induced by overexpression of lecithin:cholesterol acyl transferase has been shown previously to attenuate diet-induced atherosclerosis in rabbits.

In conclusion, the expression of hepatic lipase in apoB-transgenic rabbits resulted in a reduction in total LDL levels and in a dramatic change in the LDL subclass distribution: a near complete disappearance of larger, more buoyant particles and a small increase in the smallest, most dense subclass. These results are consistent with a central role for hepatic lipase in regulating total plasma LDL concentrations, as well as in the production of small, dense LDL from larger, more buoyant precursors.

Acknowledgments
This work was supported by NIH grants HL18574 (R.M.K.) and HL51588 (J.M.T.) and by a grant from the Dairy Management, Inc., administered in collaboration with the National Dairy Council. Dr. Rizzo was the recipient of grants from Consiglio Nazionale delle Ricerche, Rome, Italy, and from International Atherosclerosis Society, “Visiting Fellowship Award”. Houston, Tex. Kaspar Berneis was the recipient of a grant from the Swiss National Foundation.

The authors wish to thank Laura Holl and Joe Orr for laboratory analysis and Professor Alberto Notarbartolo, Head of the Department of Clinical Medicine and Emerging Diseases, University of Palermo, Italy, for his support and advice.

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Arterioscler Thromb Vasc Biol. 2004;24:141-146; originally published online November 13, 2003;
doi: 10.1161/01.ATV.0000107027.73816.ce
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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