Stimulation of Liver-Directed Cholesterol Flux in Mice by Novel N-Acetylglalactosamine–Terminated Glycolipids With High Affinity for the Asialoglycoprotein Receptor


Objective—Interventions that promote liver-directed cholesterol flux can suppress atherosclerosis, as demonstrated for scavenger receptor-BI overexpression in hypercholesterolemic mice. In analogy, we speculate that increasing lipoprotein flux to the liver via the asialoglycoprotein receptor (ASGPr) may be of therapeutic value in hypercholesterolemia. Methods and Results—A bifunctional glycolipid (LCO-Tyr-GalNAc₃) with a high-nanomolar affinity for the ASGPr (inhibition constant 2.1±0.2 nmol/L) was synthesized that showed rapid association with lipoproteins on incubation with serum. Prior incubation of LCO-Tyr-GalNAc₃ with radiolabeled low-density lipoprotein or high-density lipoprotein (0.5 µg/µg of protein) resulted in a dramatic induction of the liver uptake of these lipoproteins when injected intravenously into mice (70±3% and 78±1%, respectively, of the injected dose at 10 minutes of low-density lipoprotein and high-density lipoprotein), as mediated by the ASGPr on hepatocytes. Intravenously injected LCO-Tyr-GalNAc₃, quantitatively incorporated into serum lipoproteins and evoked a strong and persistent (≥48 hour) cholesterol-lowering effect in normolipidemic mice (37±2% at 6 hours) and hyperlipidemic apoE⁻/⁻ mice (32±2% at 6 hours). The glycolipid was also effective on subcutaneous administration. Conclusions—LCO-Tyr-GalNAc₃ is very effective in promoting cholesterol uptake by hepatocytes and, thus, may be a promising alternative for the treatment of those hyperlipidemic patients who do not respond sufficiently to conventional cholesterol-lowering therapies. (Arterioscler Thromb Vasc Biol. 2006;26:169-175.)

Key Words: cholesterol-lowering drugs ■ hyperlipoproteinemia ■ lipoproteins ■ receptors ■ transgenic models

Clinical data indicate a positive correlation between low-density lipoprotein (LDL) cholesterol levels and the occurrence of arteriosclerosis, whereas a strong inverse correlation has been demonstrated between high-density lipoprotein (HDL) cholesterol levels and cardiovascular disease. Current therapies for hypercholesterolemia are mainly focused on enhancing the hepatic clearance and catabolism of LDL and very low-density lipoprotein (VLDL) by the induction of LDL receptors (LDLRs) in the liver through inhibition of cholesterol synthesis by 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors ("statins") or stimulation of cholesterol synthesis by 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors ("statins") or stimulation of cholesterol synthesis by 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors ("statins") or stimulation of cholesterol synthesis by 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors ("statins"). Leaving HDL levels relatively unaffected. However, recent findings indicate that the antiatherogenic capacity of HDL is not determined by its steady-state level in the serum, per se, but rather by its kinetics and functionality, which governs the cholesterol flux from peripheral cells to the liver ("reverse cholesterol transport"). as reviewed by Von Eckardstein and Assmann. Permanently overexpression of the hepatic HDL receptor (scavenger receptor BI [SR-BI]) in heterozygous LDLr-deficient mice on a high-fat/ high-cholesterol diet, resulted in a strong reduction in plasma HDL cholesterol (90%) and atherosclerotic lesion area. Even temporary stimulation of reverse cholesterol transport by transient hepatic overexpression of SR-BI in these mice, which resulted in a transient lowering of plasma LDL cholesterol (55%) and apolipoprotein AI (40%) levels, led to a significant decrease in lesion formation. These studies clearly illustrate that stimulation of the hepatic uptake of LDL and HDL will be an effective entry to antiatherosclerotic therapy and incited us to explore the feasibility to induce effective clearance of these lipoproteins via the asialoglycoprotein receptor. This high-capacity receptor is uniquely expressed on the surface of hepatocytes to mediate the hepatic uptake and subsequent lysosomal processing of galactose (Gal) and N-acetylgalactosamine.
(GalNAc)-terminated substrates from the serum, at which the affinity for GalNAc is 50-fold higher than for Gal. Previous proof-of-concept studies already showed that bifunctional glycolipids [TG(4Å)C and TG(20Å)C], consisting of a cholesteryl moiety for anchorage to lipoproteins and a triantennary Gal-terminated glycoside with moderate affinity for the ASGPr, were able to associate with lipoproteins and induce their liver uptake in rodents. However, TG(4Å)C induced the hepatic uptake of LDL by Kupffer cells instead of hepatocytes, exerted a cholesterol-lowering effect only at high doses (≥35 mg/kg), and had to be administered by slow infusion because of its hemolytic properties. TG(20Å)C was somewhat more effective, but the hepatocyte-targeted delivery of lipoproteins was still suboptimal because of rapid redistribution of the glycolipid from lipoproteins in serum somewhat more effective, but the hepatocyte-targeted delivery of lipoproteins was still suboptimal because of rapid redistribution of the glycolipid from lipoproteins in serum and inability to induce the hepatic uptake of triglyceride-rich lipoproteins, such as VLDL. The aim of the present study was to improve the hepatocyte-directed lipoprotein targeting efficiency of the glycolipids by enhancing their affinity for both lipoproteins and the ASGPr. For this purpose, we synthesized a novel triantennary glycolipid (LCO-Tyr-GalNAc3) with a highly lipophilic lithocholic acid-Gly-TRIS(Gal)3 structure, and a high-nanomolar affinity for the ASGPr. These modifications indeed resulted in a highly improved induction of lipoprotein uptake (LDL, VLDL, and HDL) by hepatocytes and a concomitantly enhanced hypocholesterolemic potency. Strong and persistent cholesterol-lowering effects were observed in normolipidemic and hyperlipidemic mice and even after subcutaneous injection. We conclude from these data that LCO-Tyr-GalNAc3 is very effective in promoting cholesterol transport to hepatocytes and, thus, may be a promising alternative for current cholesterol-lowering therapies, such as familial hypercholesterolemic patients.

**Methods**

Please see an expanded version of the Methods section online, available at http://atvb.ahajournals.org.

**Animals**

Male mice (C57Bl/6 background), fed ad libitum with regular chow, were used. The study was approved by the ethics committee of the Leiden University.

**Glycolipids**

[3α(oleoylxylo)-5β-cholanoyl]-gamma-aminobutyric acid-Gly-TRIS(Gal), (LCO-Gal3), and [3β(oleoylamido)-5β-cholanoyl]-Tyr-Gly-TRIS(GalNAc3) (LCO-Tyr-GalNAc3; Figure 1, available online at http://atvb.ahajournals.org) were synthesized as described.

**Isolation and Characterization of Lipoproteins**

Human lipoproteins were isolated from healthy volunteers, and protein was determined according to Lowry et al.

**Radiolabeling of Lipoproteins and LCO-Tyr-GalNAc3**

LDL and AcLDL were radioiodinated as described. LCO-Tyr-GalNAc3 was radioiodinated using a iodogen (10 μg)-coated reaction tube. HDL and AcLDL were radiolabeled with [3H]cholesterol oleate (CO). Association of LCO-Tyr-GalNAc3 With Lipoproteins

Sera were incubated with LCO-[125I]Tyr-GalNAc3, and electrophoresed on agarose gels. Lipids were visualized by Sudan black and [125I] activity using a Packard Instant Imager.

**Kinetic Studies in Mice**

Mice were anesthetized, and kinetic studies with radiolabeled lipoproteins were performed as described. The contribution of hepatocytes to the hepatic uptake of lipoproteins was determined by the isolation of hepatocytes at 10 minutes after injection. Conscious mice were injected via the tail vein with LCO-[125I]Tyr-GalNAc3, and placed in metabolic cages. After 48 hours, the mice were killed, and [125I] activity was determined in their organs, feces, and urine.

**Macrophage Association Studies In Vivo**

Mice were injected intraperitoneally with Brewer’s thioglycollate medium. After 5 days, mice were injected with radiolabeled lipoproteins with or without glycolipid. After 1 hour, mice were euthanized by cervical dislocation, and macrophages were isolated by peritoneal lavage with 10 mL of ice-cold PBS and washed 3 times with PBS. Cellular protein was determined according to Lowry et al. and radioactivity was counted.

**Cholesterol-Lowering Effect of Glycolipids**

PBS or glycolipids were injected intravenously into the tail vein or subcutaneously into the neck region of conscious mice. Blood samples were taken from the tail vein, and the sera were analyzed for total cholesterol. The distribution of lipids over lipoproteins was determined by fast protein liquid chromatography (Superose 6).

**Statistical Analysis**

Statistical significance was determined by using the 2-tailed Student t test, repeated measures ANOVA, and Dunnett t test. Values are expressed as means, and errors represent the variation between data points (in case of n=2) or SD (n≥3).

**Results**

**Incorporation of LCO-Tyr-GalNAc3 Into Serum Lipoproteins**

Incubation of LCO-Tyr-GalNAc3 with human serum resulted in a concentration-dependent reduction of the electrophoretic mobility of the lipoproteins (LDL, VLDL, and HDL), whereas the mobility of albumin was not affected relatively to the front marker bromophenol blue (Figure 1A). Similar results were obtained using LCO-Gal3 (data not shown). Analysis of the distribution of LCO-[125I]Tyr-GalNAc3 over the serum components showed association of the glycolipid mainly with LDL and HDL and virtually no binding to albumin (Figure 1B). Case LCO-[125I]Tyr-GalNAc3 preferentially bound to LDL in human serum (Figure 1B), the glycolipid predominantly associated with HDL in wild-type mouse serum (Figure 1C) and to (β)-VLDL in LDL in apoE⁻/⁻ serum (Figure 1D).

**Effect of LCO-Tyr-GalNAc3 on the Hepatic and Splenic Uptake of Lipoproteins in Mice**

On intravenous injection into wild-type mice, the hepatic uptake of [125I]-LDL and [3H]CO-labeled HDL was negligible (<1% of the dose at 10 minutes after injection). Prior incubation of LCO-Tyr-GalNAc3 with LDL or HDL caused a dose-dependent acceleration of the serum clearance of both lipoproteins and enhanced their liver uptake reaching 70±3%
and 78±1% of the dose (10 minutes after injection), respectively, at 0.5 μg of glycolipid per microgram of particle protein (Figure 2A and 2C). At this glycolipid loading, the ASGPr-selective inhibitor asialoorosomucoid (ASOR)27 inhibited the glycolipid-induced hepatic clearance of LDL and HDL by 63% (Figure 2B) and 94% (Figure 2C), respectively, indicating that the induced hepatic clearance of both lipoproteins is mainly mediated by the ASGPr on hepatocytes. Liver cell distribution studies confirmed the prominent role of hepatocytes in the uptake of LDL (52.3±1.5%) and HDL (95.6±0.7%; n=2). LCO-Gal3 enhanced the hepatic clearance of both lipoproteins to a similar extent as LCO-Tyr-GalNAc3, but the enhanced liver uptake of LDL could not be inhibited by ASOR (data not shown). Also, whereas LCO-Gal3 enhanced the association of LDL with the spleen by 33.5-fold, LCO-Tyr-GalNAc3 did not affect the splenic uptake (and extrahepatic distribution in general) of both lipoproteins (data not shown).

Effect of LCO-Tyr-GalNAc3 on the Association of Lipoproteins With Macrophages in Mice
Preincubation of 125I-LDL with the highest dose of glycolipid used in the kinetic studies (0.5 μg per microgram of protein) did not enhance the association of 125I-LDL with thioglycolate-ellicited macrophages in the peritoneal cavity in vivo (+7%, not significant; Figure IIA, available online at http://atvb.ahajournals.org). Likewise, the glycolipid did not significantly affect the association of [3H]CO-HDL with macrophages (+31%, not significant; Figure IIB). In fact, the total accumulation of 125I-LDL and [3H]CO-HDL remained much lower than that of 125I-AcLDL and [3H]CO-AcLDL,
respectively. Other than the observation that LCO-Tyr-GalNAc3 did not increase the extrahepatic distribution of LDL and HDL, the glycolipid apparently does not increase the affinity of both lipoproteins with nonresident macrophages either.

**Kinetic Studies on Glycolipids in Mice**

We have shown recently that LCO-[125I]Tyr-GalNAc3 almost completely associates with HDL after intravenous administration to wild-type mice and that the clearance from serum (half time \approx 30 minutes) could mainly be attributed to uptake by the liver (44\% at 60 minutes after injection).22 Intra-venous injection of LCO-[125I]Tyr-GalNAc3 (1.0 mg/kg) into conscious mice now revealed that, in addition to uptake by the liver, glycolipid-derived radioactivity is also excreted in feces and urine, as shown at 48 hours after injection (Figure IIIA, available online at http://atvb.ahajournals.org). Interestingly, a similar pattern of hepatic uptake and excretion from the body was found after subcutaneous administration of the glycolipid (5.0 mg/kg; Figure IIIB). Assuming that the radioactivity recovered in the intestinal tract and feces result from biliary secretion of (metabolized) glycolipid by the liver, it can be calculated that 56.9\% (\approx 570 \mu g) and 16.3\% (\approx 815 \mu g) of the doses have reached the liver at 48 hours after intravenous and subcutaneous injection, respectively.

**Cholesterol-Lowering Effect of Glycolipids in Mice**

On intravenous injection of LCO-Gal3 into conscious wild-type mice, a significant and dose-dependent hypocholesterolemic effect was detected [repeated measures ANOVA; F(2,5)=14.3; P<0.01], at which the effects of both doses differed significantly from the control group (Dunnett t test: 1.0 mg/kg: P<0.05; 3.3 mg/kg: P<0.01; Figure 3A). At 3.3 mg/kg, the effect peaked at 3 hours (28.4%; P<0.01) and was still significant (P<0.05) up to 12 hours after injection. LCO-Tyr-GalNAc3 also evoked a significant dose-dependent hypocholesterolemic effect on intravenous injection [F(2,6)=72.4; P<0.001], with both effects significantly different from the control group (1.0 mg/kg: P<0.001; 3.3 mg/kg: P<0.001; Figure 3B). However, as compared with LCO-Gal3, the effects were even more pronounced (36.5\% at 6 hours after injection of 3.3 mg/kg; P<0.01) and were remarkably persistent. At 48 hours after injection, serum cholesterol levels had still not reached control values at both doses (1.0 mg/kg: P<0.05; 3.3 mg/kg: P<0.05). Subcutaneous administration of LCO-Tyr-GalNAc3 also gave a significant dose-dependent cholesterol-lowering effect [F(2,8)=13.0; P<0.01], and both effects differed significantly from the control group (5.0 mg/kg: P<0.05; 16.7 mg/kg: P<0.01; Figure 3C). The effects had a slower onset and did not reach a maximum before 24 hours after injection (20.7\% at 48 hours after administration of 16.7 mg/kg; P<0.01). The effect of intravenous injection of LCO-Tyr-GalNAc3 (10 mg/kg) was also examined in apoE \textsuperscript{-/-} mice that have \approx 10-fold higher total cholesterol (TC) levels than wild-type mice on a regular diet. Also, in these severely hyperlipidemic mice, LCO-Tyr-GalNAc3 appeared highly effective, because it caused a 32\% (P<0.001) decrease in serum TC at 6 hours after injection, and a statistically significant effect (24\%±5%; P<0.05) was still observed at 24 hours after injection (Figure 3D). Importantly, 3 consecutive injections of LCO-Tyr-GalNAc3 into apoE \textsuperscript{-/-} mice at 72-hour time intervals resulted in consistent effects on TC reduction (27\% to 32\% after 6 hours; Figure 3E).

**Effect of LCO-Tyr-GalNAc3 on Mouse Serum Lipoproteins In Vivo**

To investigate the effects of the glycolipids on the serum levels of the individual lipoprotein classes, serum was taken...
from wild-type mice, before and 3 hours after injection of LCO-Tyr-GalNAc$_3$ (10 mg/kg), and was subjected to fast protein liquid chromatography (Figure 4A and 4B). The eluted fractions containing VLDL/chylomicrons, intermediate-density lipoprotein/LDL, HDL, and the total bed volume were pooled, and the total cholesterol (Figure 4A) and glycerol (Figure 4B) contents were determined. The decrease in the total cholesterol content of the fractions (42.6 ± 1.6%; mean ± SEM; $P<0.001$) could be attributed to a decrease in cholesterol in VLDL (61.3 ± 5.6%; $P<0.05$), LDL (29.2 ± 1.4%; $P<0.01$), and HDL (43.8 ± 1.5%; $P<0.001$). The total glycerol content of the fractions, a measure of triglyceride content, tended to be decreased (10.8 ± 7.2%; not significant), but a significant reduction was observed for the glycerol content of VLDL ($P<0.05$). Likewise, injection of LCO-Tyr-GalNAc$_3$ (10 mg/kg) into apoE$^−/−$ mice resulted in a 25.5% reduction of TC ($P<0.05$), which was caused by a decreased cholesterol content of VLDL (24.5%; $P<0.05$), LDL (26.3%; $P<0.05$), and HDL (39.9%; $P<0.05$). Similar effects were observed on glycerol reduction, which only was significant for VLDL ($P<0.05$; Figure 4C and 4D).

**Toxicity of Glycolipids in Mice**

The absence of toxicity of LCO-Tyr-GalNAc$_3$ was verified in wild-type mice (after single injection) and in apoE$^−/−$ mice (after 3 consecutive injections at 72-hour time intervals) at the highest dose applied in vivo (Table I, available online at http://atvb.ahajournals.org). In accordance with the results shown above, a bolus injection of LCO-Tyr-GalNAc$_3$ (10 mg/kg) in wild-type mice led to a significant reduction of plasma TC after 3 hours (37.8%; $P<0.0001$), which persisted for >24 hours (16.8%; $P<0.05$) as compared with control animals, leaving total plasma glycerol and protein levels unchanged ($P>0.05$). The serum parameters for systemic (lactate dehydrogenase) and liver toxicity (ASAT, ALAT, and γGT) remained unaffected at 3 and 24 hours after injection. No changes were observed in liver, heart, kidney, and spleen weight at both time points. Similarly, repeated injections of LCO-Tyr-GalNAc$_3$ in apoE$^−/−$ mice did not affect body and organ weights, and no effects were observed on the serum levels of lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, and γ-glutamyl transferase, as determined at 48 hours after the third injection. In addition, no signs of hemolysis were observed after incubation of heparinized blood with LCO-Tyr-GalNAc$_3$ at concentrations equivalent to doses of 100 mg/kg (data not shown).

**Discussion**

It is becoming increasingly clear that net cholesterol fluxes, rather than steady-state serum cholesterol levels, are instrumental to the occurrence of atherosclerosis. In particular, it has been shown in mice that the flux of LDL cholesterol and HDL cholesterol to the liver dictates the cholesterol homeostasis in the periphery. As shown by Kozarsky et al and Arai et al, overexpression of the HDL receptor SR-BI, be it transient or permanent, strongly decreases serum HDL levels (cholesterol and apolipoprotein AI) and atherosclerosis. Likewise, strategies aimed at stimulating the hepatic LDL-mediated uptake of LDL and VLDL in humans also reduce serum cholesterol and atherosclerosis. As such, interventions that promote the transport of LDL, VLDL, and HDL to hepatocytes, the only cell type able to irreversibly excrete...
choleretic from the body, may be effective in suppressing the atherogenic process.

Our newly developed glycolipid LCO-Tyr-GalNAc3 is superior to the parent compounds, because it combines stable incorporation into lipidic particles and a high affinity and selectivity for the ASGPr. The highly hydrophobic character of the lithocholic oleate moiety indeed resulted in an avid and quantitative association of the glycolipids with lipoproteins in vitro, withstanding dissociation of the glycolipids in the blood to endogenous lipoproteins. Therefore, both LCO-Gal3 and LCO-Tyr-GalNAc3 evoked a dose-dependent monophasic serum decay of both LDL and HDL, with a concomitant highly induced uptake of both lipoproteins by the liver (reaching 70% to 80%). In contrast, at similar concentrations, TG(20:0)C induced the liver uptake of LDL and HDL to only 20% to 25% and 35%, respectively. The potential therapeutic value of the glycolipids could not only be appreciated from their enhanced ability to promote the hepatic uptake of lipoproteins but also from their ability to induce ASGPr-mediated hepatocyte-specific uptake. It appeared that LCO-Gal, induced the uptake of LDL by Kupffer cells and spleen, instead of hepatocytes. Probably, the conformational properties of the individual galactose clusters, which are critical for proper recognition by the ASGPr, may be overruled by high-local surface concentrations of the surface of LDL. In contrast, LCO-Tyr-GalNAc3 enhanced the uptake of LDL mainly by hepatocytes. This indicates that the disturbing effects of sugar density on receptor specificity can be overcome by strongly enhancing the affinity of the triantennary glycoside for the ASGPr, as we have also demonstrated for 30 nm-sized liposomes. Importantly, LCO-Tyr-GalNAc3 also did not increase the association of LDL and HDL with peritoneal macrophages in vivo, suggesting that the glycolipid will not promote foam cell formation in the arterial wall by enhancing lipid accumulation into subendothelial macrophages. Indeed, we have evaluated the uptake of glycolipid-laden radiolabeled 3H-LDL and 3HCO-HDL by the aorta in mice, and we were not able to detect the presence of radioactivity above the background level (data not shown).

Because these glycolipids are aimed to evoke a cholesterol-lowering effect on administration to hyperlipidemic patients, they should accumulate into endogenous lipoproteins before being cleared by the liver. From LCO-[125]Tyr-GalNAc3 turnover studies, it can indeed be concluded that rapid binding of glycolipids to lipoproteins does occur, leading to a progressive uptake of glycolipid by the liver. This resulted in remarkably persistent dose-dependent decreased serum cholesterol concentrations, at which LCO-Tyr-GalNAc3 showed an ∼3-fold higher hypocholesterolemic potency than LCO-Gal3. Because it is clear that an enhanced ASGPr-mediated uptake of lipoproteins results in an enhanced secretion of bile acids, this pathway will ultimately lead to an increased fecal secretion of cholesterol.

LCO-Tyr-GalNAc3 not only caused a reduction of LDL and HDL cholesterol, but also of VLDL cholesterol, which was not observed for either TC(20:0)C or LCO-Gal3 (data not shown). These observations are in agreement with our recent findings that GalNAc-terminated glycolipids can efficiently induce the uptake of particles with a size ≤70 nm by hepatocytes, whereas Gal-terminated glycolipids are ineffective. Clearly, replacement of Gal by GalNAc within the glycolipid not only dramatically enhances the affinity of glycolipids for the ASGPr (inhibition constant, 2 nM versus 190 nM), but also largely improves the specificity of HDL, LDL, and β-VLDL for the ASGPr on hepatocytes versus the GP on Kupffer cells. Therefore, despite the significantly different (LDL+VLDL)/HDL molar ratios in wild-type mice (=0.2) and apoE−/− mice (=30), the hypocholesterolemic effects of LCO-Tyr-GalNAc3 in both the normolipidemic and hyperlipidemic mice were similar. This is an important observation with respect to application in humans (=3.0), because LCO-[125]Tyr-GalNAc3 predominantly associates with LDL in human serum. In addition, repeated administrations of LCO-Tyr-GalNAc3 resulted in consistent TC-reducing effects in apoE−/− mice, which indicates its suitability for long-term therapeutic usage in hypercholesterolemia.

In conclusion, we have shown that LCO-Tyr-GalNAc3 is a very effective inducer of lipoprotein uptake by the hepatic ASGPr, even after relatively noninvasive subcutaneous administration, without any sign of adverse effects. The HDL lowering capacity is similar to that evoked by transient hepatic overexpression of SR-BI, which was found to retard atherogenesis in LDLr-deficient mice. Therefore, if such an effect on atherogenesis can also be observed in humans, LCO-Tyr-GalNAc3-induced hepatic uptake of lipoproteins that include HDL may be an effective entry to hyperlipidemia-induced atherosclerotic therapy, which can be of benefit for those patients who do not respond to conventional 3-hydroxy-3-methylglutaryl-coenzyme A reductase-inhibiting cholesterol-lowering therapies.

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References


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METHODS

Animals. Sixteen male apoE−/− mice (15-16 week old; 26-30 g) and fifty-five male wild-type mice (12-14 week old; 24-28 g) (both C57Bl/6 background) from Broekman Instituut BV, Someren, The Netherlands, fed ad libitum with regular chow were used. The study was approved by the Ethics Committee of the Leiden University.

Glycolipids. (3α(Oleoyloxy)-5β-cholanoyl)-γ-aminobutyric acid-Gly-TRIS(Gal)₃ [LCO-Gal₃, Fig. IA] and (3β(oleoylamido)-5β-cholanoyl)-Tyr-Gly-TRIS(GalNAc)₃ [LCO-Tyr-GalNAc₃; Fig. IB] were synthesized as described earlier.¹⁹,²² The purity and identity of both glycolipids have been fully confirmed by NMR and mass spectroscopy.

Isolation and Characterization of Lipoproteins. Human LDL and HDL were isolated from the blood of healthy volunteers,²³ and dialyzed at 4°C against PBS, 1 mmol/L EDTA, pH 7.4. Protein concentrations were determined according to Lowry et al.²⁴ using BSA as a standard.

Radiolabeling of Lipoproteins and LCO-Tyr-GalNAc₃. LDL and AcLDL were radioiodinated with Na¹²⁵I and purified as described.²⁵ LCO-Tyr-GalNAc₃ was radioiodinated with Na¹²⁵I using a iodogen (10 µg)-coated reaction tube. More than 97% and 99% of the radiolabel in ¹²⁵I-LDL and ¹²⁵I-HDL were 10% TCA precipitable, and the specific ¹²⁵I-activities were ~150 dpm/ng of protein. LCO-[¹²⁵I]Tyr-GalNAc₃ (1300 dpm/ng) migrated as a single band (Rf 0.64) on TLC (n-butanol: n-propanol: 25% NH₄OH:H₂O = 15: 40: 30: 15, v/v/ v/v). HDL and AcLDL were radiolabeled with [³H]cholesteryl oleate (CO) (~25-30 dpm/ng protein) as described.²⁵ We deliberately used [³H]CO-HDL for in vivo studies, since ¹²⁵I-labeling of HDL results labeling of many exchangeable apolipoproteins.
Association of LCO-Tyr-GalNAc₃ with Lipoproteins. Serum was incubated (30 min at 37°C) with LCO-[¹²⁵I]Tyr-GalNAc₃ in PBS pH 7.4. The mixtures were electrophoresed on 0.75% (w/w) agarose gels at pH 8.8 using bromophenol blue as front marker, and the gels were fixed and dried. Lipids were visualized by Sudan Black and ¹²⁵I-activity using a Packard Instant Imager (Hewlett-Packard Co., Palo Alto, CA).

Kinetic Studies in Mice. Mice were anesthetized and the abdomens were opened. Radiolabeled lipoproteins, previously incubated with PBS or LCO-Tyr-GalNAc₃ (30 min at 37°C), were injected via the inferior vena cava. Blood and liver samples were taken and processed as previously described. Alternatively, conscious mice were injected via the tail vein with LCO-[¹²⁵I]Tyr-GalNAc₃, and placed in metabolic cages. At the end of the experiment, the mice were killed and organs were excised, weighed, and counted for radioactivity. The contribution of hepatocytes to the LCO-Tyr-GalNAc₃-induced hepatic uptake of radiolabeled lipoproteins was assessed by collagenase perfusion of livers at 10 min after injection, and separation of hepatocytes from non-parenchymal cells by differential centrifugation (purity of hepatocytes >95%).

Macrophage Association Studies In Vivo. Mice were injected i.p. with 1 mL of 3% Brewer's thioglycollate medium to attract macrophages towards the peritoneal cavity. After 5 days, mice were injected i.p. with ¹²⁵I-LDL or [³H]CO-HDL (20 µg of protein), previously incubated with PBS or LCO-Tyr-GalNAc₃ (0.5 µg of glycolipid per µg of particle protein; 30 min at 37°C), ¹²⁵I-AcLDL, or [³H]CO-AcLDL (20 µg of protein). After 1 h, mice were sacrificed by cervical dislocation, macrophages were isolated by peritoneal lavage with 10 mL of ice-cold PBS, and washed 3 times with PBS. Cellular protein was determined according to Lowry et al. and radioactivity was counted.

Cholesterol-Lowering Effect of Glycolipids. PBS or glycolipids were injected i.v. into the tail vein or s.c. into the neck region of conscious mice. Blood samples (25 µL) were taken
from the tail vein, and the sera were analyzed for total cholesterol. To determine the effect of
glycolipid on the distribution of lipids over the individual lipoprotein fractions, sera were
subjected to FPLC (SMART system; Amersham Pharmacia Biotech), using a Superose® 6
column at a flow rate of 50 µL/min and with PBS, 1 mM EDTA, 0.02% NaN₃, pH 7.4 as
eluens. The lipoprotein fractions were pooled and assayed for total cholesterol and glycerol
using the Roche Molecular Biochemicals enzymatic kits for cholesterol and triglycerides,
respectively.

**Statistical Analysis.** Statistical significance was determined by using the two-tailed
Student's t-test, repeated measures ANOVA, and Dunnett's t-test. Values are expressed as
means, and errors represent the variation between data points (in case of n=2) or standard
deviation (n≥3).
Figure I. Chemical structures of the glycolipids LCO-Gal$_3$ (A) and LCO-Tyr-GalNAc$_3$ (B).
Figure II. Effect of LCO-Tyr-GalNAc$_3$ on the association of LDL and HDL with macrophages in vivo. $^{125}$I-LDL (A) or [$^3$H]CO-HDL (B) (20 µg of protein) were incubated with PBS or LCO-Tyr-GalNAc$_3$ (0.5 µg of glycolipid per µg of particle protein; 30 min at 37°C), and injected i.p. into mice at 5 days after they had received an i.p. injection of thioglycollate. $^{125}$I-AcLDL (A) or [$^3$H]CO-AcLDL (B) (20 µg of protein) were injected as controls. After 1 h, macrophages were isolated, washed, and the association of the lipoproteins with the macrophages was determined. Values are means±SD ($n$=3). n.s., not significant.
Figure III. Distribution of LCO-Tyr-GalNAc<sub>3</sub> in mice. LCO-[<sup>125</sup>I]Tyr-GalNAc<sub>3</sub> was injected i.v. (1.0 mg/kg; A) or s.c. (5.0 mg/kg; B) into conscious wild-type mice. At 48 h after injection, the mice were killed, and <sup>125</sup>I-activity was determined in their organs, feces, and urine. Values are means ± variation of two experiments.
<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Wild-type (I, 3 h)</th>
<th>Wild-type (I, 24 h)</th>
<th>ApoE⁻/⁻ (III, 48 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>TC (mM)</td>
<td>1.96 ± 0.06</td>
<td>1.22(37.8%)↓</td>
<td>2.44±0.04</td>
</tr>
<tr>
<td>TG (mM)</td>
<td>0.53±0.06</td>
<td>0.50±0.02 (n.s.)</td>
<td>1.00±0.04</td>
</tr>
<tr>
<td>Prot (g/l)</td>
<td>55.4±2.0</td>
<td>54.3±0.8 (n.s.)</td>
<td>62.7±1.1</td>
</tr>
<tr>
<td>∆M (g)</td>
<td>-0.40±0.42</td>
<td>-0.55±0.07</td>
<td>0.20±0.57</td>
</tr>
<tr>
<td>Liver (%M)</td>
<td>4.58±0.29</td>
<td>4.64±0.15</td>
<td>5.13±0.26</td>
</tr>
<tr>
<td>Heart (%M)</td>
<td>0.45±0.02</td>
<td>0.45±0.01</td>
<td>0.47±0.04</td>
</tr>
<tr>
<td>Kidney (%M)</td>
<td>1.31±0.02</td>
<td>1.32±0.01</td>
<td>1.41±0.05</td>
</tr>
<tr>
<td>Spleen (%M)</td>
<td>0.33±0.03</td>
<td>0.33±0.01</td>
<td>0.33±0.02</td>
</tr>
<tr>
<td>ASAT (U/L)</td>
<td>132±43</td>
<td>125±33</td>
<td>71±4</td>
</tr>
<tr>
<td>ALAT (U/L)</td>
<td>40±7</td>
<td>41±1</td>
<td>34±1</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>637±98</td>
<td>712±55</td>
<td>412±43</td>
</tr>
<tr>
<td>γGT (U/L)</td>
<td>≤2</td>
<td>≤2</td>
<td>≤2</td>
</tr>
</tbody>
</table>

LCO-Tyr-GalNAc₃ (10 mg/kg) or PBS was injected i.v. into wild-type mice (once) or ApoE⁻/⁻ mice (three times at 72 h-intervals). At 3 and 24 h after injection (wild-type) or at 48 h after the final injection (ApoE⁻/⁻), heparinized blood samples were taken, and animals were killed. The plasma samples were stored at -80°C. Plasma total cholesterol (TC), total glycerol (TG), and protein (prot) were measured as described in ‘Methods’, and ASAT, ALAT, LDH, and γGT were determined using SYS-3 BM/Hitachi 747 kits from Roche Molecular Biochemicals. Liver, heart, kidneys, and spleen were excised, and their mass was calculated as percentage of total body mass (%M). Values are means±SD (n=3-5). *P<0.05; ***P<0.0001. All other differences are not statistically significant. ∆M, difference in body mass; n.d., not determined; n.s., not significant.