Type I diabetes mellitus decreases in vivo macrophage-to-feces reverse cholesterol transport despite increased biliary sterol secretion in mice

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Abstract  Type I diabetes mellitus (T1DM) increases atherosclerotic cardiovascular disease; however, the underlying pathophysiology is still incompletely understood. We investigated whether experimental T1DM impacts HDL-mediated reverse cholesterol transport (RCT). C57BL/6J mice with alloxan-induced T1DM had higher plasma cholesterol levels (P < 0.05), particularly within HDL, and increased hepatic cholesterol content (P < 0.001). T1DM resulted in increased bile flow (2.1-fold; P < 0.05) and biliary secretion of bile acids (BA, 10.5-fold; P < 0.001), phospholipids (4.5-fold; P < 0.001), and cholesterol (5.5-fold; P < 0.05). Hepatic cholesterol synthesis was unaltered, whereas BA synthesis was increased in T1DM (P < 0.001). Mass fecal BA output was significantly higher in T1DM mice (1.5-fold; P < 0.05), fecal neutral sterol excretion did not change due to increased intestinal cholesterol absorption (2.1-fold; P < 0.05). Overall in vivo macrophage-to-feces RCT, using [3H] cholesterol-loaded primary mouse macrophage foam cells, was 20% lower in T1DM (P < 0.05), mainly due to reduced tracer excretion within BA (P < 0.05). In vitro experiments revealed unchanged cholesterol efflux toward T1DM HDL, whereas scavenger receptor class B1-mediated selective uptake from T1DM HDL was lower in vitro and in vivo (HDL kinetic experiments) (P < 0.05), conceivably due to increased glycation of HDL-associated proteins (+65%, P < 0.01). In summary, despite higher mass biliary sterol secretion T1DM impairs macrophage-to-feces RCT, mainly by decreasing hepatic selective uptake, a mechanism conceivably contributing to increased cardiovascular disease in T1DM.—Freark de Boer, J. W. Annema, M. Schreurs, J. N. van der Veen, M. van der Giet, N. Nijstad, F. Kuipers, and U. J. F. Tietge

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Atherosclerotic cardiovascular disease (CVD) is a predominant cause of morbidity and mortality in type I diabetes mellitus (T1DM) patients (1, 2). Compared with subjects without diabetes, T1DM confers a 7-fold increase in the risk of fatal CVD (2). However, the mechanisms underlying accelerated atherosclerosis in T1DM are still incompletely understood.

Plasma HDL cholesterol levels are inversely related to the incidence of CVD (3, 4). The role of this lipoprotein in promoting reverse cholesterol transport (RCT) is currently regarded as the main established atheroprotective property of HDL (5, 6). The critical steps in RCT comprise initial efflux of excess cholesterol from lipid-laden macrophages within atherosclerotic lesions toward HDL for transport through the plasma compartment, followed by the subsequent uptake of cholesterol into the liver for excretion into bile and feces (7, 8).

Although T1DM has been associated with changes in sterol metabolism (9–13), no data are currently available addressing the impact of T1DM on RCT. Therefore, this study explored the pathophysiological consequences of experimental T1DM on overall RCT as well as the individual steps involved in this process. Our data demonstrate that macrophage-specific RCT is decreased in T1DM despite increased biliary sterol secretion as well as increased fecal excretion of bile acids (BAs). Mechanistically, we

Supplementary key words  high density lipoproteins • bile acids • cardiovascular disease • atherosclerosis • neutral sterols • glucose • efflux • selective uptake • liver • bile

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Abbreviations: BA, bile acid; BW, body weight; CE, cholesteryl ester; CVD, cardiovascular disease; RCT, reverse cholesterol transport; SR-B1, scavenger receptor class B1; T1DM, type 1 diabetes mellitus.

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identified decreased hepatic selective uptake of cholesterol from glycated HDL as a major underlying factor for reduced RCT in T1DM.

MATERIALS AND METHODS

Animals

C57BL/6j mice were obtained from Charles River (Sulzfeld, Germany). The animals were caged in animal rooms with alternating 12 h periods of light (from 7.00 AM to 7.00 PM) and dark (from 7.00 PM to 7.00 AM), with ad libitum access to water and mouse chow diet (Arie Blok, Woerden, The Netherlands). Animal experiments were performed in conformity with PHS policy and in accordance with the national laws. All protocols were approved by the responsible ethics committee of the University of Groningen.

Induction of type 1 diabetes mellitus

To induce experimental T1DM, wild-type C57BL/6j were injected intravenously with a single dose of alloxan (65 mg/kg body weight, Sigma, St. Louis, MO), while control mice received an equivalent volume of PBS. Blood glucose levels were assessed by tail bleeding using a Onetouch Ultra glucometer (LifeScan Benelux, Beerse, Belgium). Plasma insulin levels were determined using an ultrasensitive mouse insulin ELISA kit (Alpco Diagnostics, Salem, NH).

Plasma lipid and lipoprotein analysis

Plasma total cholesterol, triglycerides, free fatty acids, and phospholipids were measured enzymatically using commercially available reagents (Roche Diagnostics, Basel, Switzerland and Wako Pure Chemical Industries, Neuss, Germany). Pooled plasma samples from mice of the same experimental group were subjected to fast protein liquid chromatography (FPLC) gel filtration using a Superose 6 column (GE Healthcare, Uppsala, Sweden) as described (14). Samples were chromatographed at a flow rate of 0.5 ml/min, and fractions of 500 µl each were collected. Individual fractions were assayed for cholesterol concentrations as described above. Plasma plant sterol levels were measured by gas chromatography exactly as previously published (15).

Analysis of liver lipid composition

To determine hepatic cholesterol, phospholipid, and triglyceride content, liver tissue was homogenized, and lipids were extracted following the general procedure of Bligh and Dyer as described (16). Triglycerides and cholesterol were measured using commercial kits as detailed above. Phospholipid content of the liver was determined essentially as published previously (16).

Bile collection and assessment of biliary excretion of cholesterol, phospholipids, and BAs

Continuous bile cannulation was performed on day 10 after injection with either alloxan or saline injection following a previously described protocol (18). Briefly, wild-type C57BL/6j donor mice were injected intraperitoneally with 1.0 ml of 4% Brewer thioglycollate medium (Becton Dickinson, Le Point de Claire, France) on day 4 after thioglycollate injection, peritoneal macrophages were harvested as described (19). Macrophages were plated in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (HyClone, Logan, UT) and penicillin (100 U/ml)/streptomycin (100 µg/ml) (Invitrogen) and were allowed to adhere for 4 h at 37°C under 5% CO₂ humidified air. Then nonadherent cells were removed by washing twice with PBS followed by loading of the macrophages with 50 µg/ml acetylated LDL and 3 µCi/ml [3H]cholesterol (Perkin Elmer, Boston, MA) for 24 h. After washing twice with PBS, the macrophages were equilibrated for 18 h in RPMI 1640 medium containing penicillin (100 U/ml)/streptomycin (100 µg/ml) and 2% BSA (Sigma). Immediately before injection, cells were harvested and resuspended in RPMI 1640 medium. The suspended [3H]cholesterol-loaded macrophage foam cells (2 million per mouse) were injected intraperitoneally into individually housed recipient mice. Plasma was collected at the indicated time points after macrophage injection by retroorbital puncture and for the final blood draw by heart puncture. At the end of the experimental period, livers were harvested, snap-frozen in liquid nitrogen, and stored at −80°C. Feces were collected continuously up to 48 h. Counts in plasma were assessed directly by liquid scintillation counting (Packard 1600CA TriCarb, Packard, Meriden, CT). Counts from a respective piece of liver were determined following solubilization of the tissue (Solvable, Packard) exactly as previously reported (20) and were related to total liver mass. Fecal samples were dried, weighed, and thoroughly ground. Aliquots were separated into BA and neutral sterol fractions as previously published (17). Briefly, samples were first heated for 2 h at 80°C in alkaline methanol and then extracted three times with petroleum ether. In the top layer, counts within the neutral sterol fraction were determined by liquid scintillation counting, whereas counts incorporated into BAs were assessed from the bottom layer. Counts recovered from the respective aliquots were related to the total amount of feces produced over the whole experimental period. All obtained counts were expressed relative to the administered tracer dose.

Determination of fractional intestinal cholesterol absorption

Fractional cholesterol absorption was determined using a dual isotope method essentially as described (21). Briefly, mice received an intravenous (i.v.) dose of 0.3 mg cholesterol-D₇ dissolved in 20% Intralipid ( Fresenius Kabi, Den Bosch, The Netherlands) and an oral dose of 0.6 mg cholesterol-D₅ dissolved in medium-chain triglyceride oil. Blood spots were collected from the tail on filter paper at t = 0, 3, 6, 12, 24, 48 and 72 h. Cholesterol was extracted from blood spots using 95% ethanol-acetone (1:1). After an overnight derivatization with N,N-Bis-(trimethyl) trifluoroacetamide and trimethylchlorosilane, isotope enrichments were determined by GC/MS. Fractional cholesterol absorption was calculated as the ratio of the area under the enrichment curves derived from the oral (cholesterol-D₅) and i.v. (cholesterol-D₇) administration, corrected for the respective administered doses.
In vitro efflux assay

HDL for efflux and cellular cholesterol uptake studies described below was isolated from mouse plasma by density gradient ultracentrifugation as described previously (20).

THP-1 human monocytes (ATCC via LGC Promochem, Teddington, UK) were grown in suspension culture in RPMI 1640 medium supplemented with 10% FBS and penicillin (100 U/ml)/streptomycin (100 µg/ml) until differentiation into macrophages by the addition of 100 nM PMA (Sigma). Differentiated THP-1 macrophages were loaded with 50 µg/ml acetylated LDL and 1 µg/ml [125I]cholesterol for 24 h followed by equilibration for 18 h as previously published (18). Then cells were washed with PBS and 50 µg protein/ml of isolated mouse HDL was added. After 24 h, radioactivity within the medium was determined by liquid scintillation counting. The cell layer was washed twice with PBS, whereafter 0.1 M NaOH was added. Plates were incubated 30 min at room temperature, and the radioactivity remaining within the cells was assessed by liquid scintillation counting. Wells incubated with RPMI without added HDL were used as blanks to determine HDL-independent efflux, and these values were subtracted from the respective experimental values. Efflux is given as the percentage of counts recovered from the medium in relation to the total counts present on the plate (sum of medium and cells).

In vitro selective cholesterol uptake assay

HDL was labeled with the nonhydrolyzable trap label [3H]cholesterol ether (Perkin Elmer) essentially as described previously (22). Cholesterol ether behaves metabolically as cholesterol ester (14), however, because of the ether bond resecretion by the cells is prevented. To assess selective uptake, LDL[α,mSR-BI] cells (kindly provided by Dr. Monty Krieger, Dept. of Biology, Massachusetts Institute of Technology, Cambridge, MA) were used, a CHO-derived cell line lacking LDL receptor expression that was in addition stably transfected with the mouse scavenger receptor class BI (SR-BI) cDNA. Cells were cultured as described except that 24 h before adding the labeled HDL preparations (50 µg HDL cholesterol/ml) 10% FBS was substituted by 10% lipoprotein-depleted serum (16). Labeled HDL preparations were then added to the cells in serum-free DMEM, and incubations were continued for 6 h. Supernatants and cells were processed as detailed above for macrophages, and radioactivity within medium and cells was determined. Cellular uptake is expressed as the percentage of counts recovered from the cells in relation to the counts present on the plate (sum of medium and cells).

Determination of HDL glycation

The extent of HDL glycation was determined using an enzymatic fructosamine assay (Diazyme, Dresden, Germany) according to the manufacturer’s protocol. Mouse HDL was isolated by ultracentrifugation as described above, and 40 µg of total protein from respective HDL preparations was used in the assay.

In vivo HDL kinetics studies

HDL kinetics studies were performed essentially as published previously (16, 20, 22). Autologous HDL was isolated by ultracentrifugation from pooled plasma of either control or T1DM mice (density 1.063 < d < 1.21) and dialyzed extensively against sterile PBS containing 0.01% EDTA. HDL was then labeled with the respective trap labels [125I]-tyramine-cellobiose and cholesteryl hexadecyl ether (cholesteryl-1,2,3H; Perkin Elmer Life Sciences). Then 0.4 µCi of [125I] and 0.7 million dpm of the 3H tracer were injected into the tail veins of fasted control and T1DM mice. Blood samples were obtained by retroorbital bleeding at 5 min, 1 h, 3 h, 6 h, 11 h, and 24 h after injection. Plasma decay curves for both tracers were generated by dividing the plasma radioactivity at each time point by the radioactivity at the initial 5-min time point after tracer injection and used to calculate fractional catabolic rates after fitting to a bicompartamental model using the SAAM II program (16). Hepatic uptake of HDL apolipoproteins (125I) and HDL-CEs (3H-cholesterol ether) was calculated by expressing the counts recovered in liver as a percentage of the injected dose, which was calculated by multiplying the initial plasma counts (5 min time point) with the estimated plasma volume (3.5% of total body weight). Selective uptake into liver was determined by subtracting the percentage of the injected dose of 125I-HDL recovered in liver from the percentage of the injected dose of 3H-HDL-CE.

Western blotting

Western blots for SR-BI were carried out on total liver homogenates as well as on hepatic membrane fractions prepared essentially as described (16). Protein concentrations were determined with the bicinchoninic acid (BCA) assay (Pierce Biotechnology, Inc., Rockford, IL). Equal amounts of protein were separated by SDS-PAGE electrophoresis and blotted onto nitrocellulose. SR-BI was visualized using a commercially available goat anti-mouse SR-BI antibody (Novus Biologicals, Littleton, CO), followed by the appropriate HRP-conjugated secondary antibody. HRP was detected using chemiluminescence (ECL, GE Healthcare). Quantitation was carried out using the freely available ImageJ software, adjusting the background for the area size of each band and subtracting it from the respective bands. Results were normalized for the average of the control mice.

Analysis of gene expression by real-time quantitative PCR

Total RNA from mouse livers was isolated using Trizol (Invitrogen) and quantified with a NanoDrop ND-100 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE). cDNA synthesis was performed from 1 µg total RNA using reagents from Invitrogen. Real-time quantitative PCR was carried out using an ABI-Prism 7700 (Applied Biosystems, Foster City, CA) fast PCR system with the default settings (16). Multi-exon spanning PCR primers and fluorogenic probes were designed with the Primer Express Software (Applied Biosystems) and synthesized by Eurogentec (Seraing, Belgium). The mRNA expression levels presented were calculated relative to the average of the housekeeping gene cyclophilin and further normalized to the relative expression levels of the respective controls.

Statistical analysis

Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL). Values are expressed as means ± SEM. Unpaired Student’s t-test was used to assess statistical differences between groups. Statistical significance for all comparisons was assigned at P < 0.05.

RESULTS

Plasma cholesterol levels and liver cholesterol content are increased in type 1 diabetic mice

Mice treated with alloxan became severely hyperglycemic within 2 days after injection and remained diabetic throughout the 11 days of the experiment (P < 0.001, Table 1). Consistent with these results, blood HbA1c levels were significantly increased in alloxan-injected mice on day 10 (P < 0.001, Table 1). The striking decrease in plasma
insulin levels ($P < 0.001$, Table 1) confirmed alloxan-induced destruction of pancreatic β cells.

Plasma total cholesterol was increased in diabetic mice ($P = 0.01$, Table 1). FPLC profiles showed an overall increase in HDL cholesterol levels and also the appearance of larger HDL particles in the diabetic group (Fig. 1). Plasma triglycerides were higher in diabetic mice compared with controls ($P < 0.01$, Table 1), whereas phospholipid and free fatty acid levels remained unchanged (Table 1).

Body weight (BW) in diabetic mice was lower than in controls ($P < 0.001$, Table 1). Although absolute liver weight was not different between the groups, liver weight relative to body weight was increased in T1DM mice ($P < 0.01$, Table 1). Hepatic cholesterol content was 22% higher in the diabetic group ($P < 0.001$, Table 1), whereas liver triglycerides and phospholipids were not affected (Table 1).

**Biliary cholesterol and BA secretion are increased in type I diabetic mice**

Continuous bile cannulation was performed to assess the impact of T1DM on biliary sterol secretion. Bile flow was 2.1-fold increased ($2.67 \pm 0.36 \text{ vs. } 1.25 \pm 0.11 \mu\text{l/min}$, $P < 0.01$, Fig. 2A) in diabetic mice. Furthermore, biliary BA secretion ($2333 \pm 354 \text{ vs. } 223 \pm 13 \text{ nmol/min/100 g BW}$, $P < 0.001$) was 10.5-fold increased, whereas biliary phospholipid ($103.2 \pm 14.9 \text{ vs. } 23.0 \pm 1.6 \text{ nmol/min/100 g BW}$, $P < 0.001$) and cholesterol secretion ($11.73 \pm 2.36 \text{ vs. } 2.15 \pm 0.17 \text{ nmol/min/100 g BW}$, $P < 0.01$) were 4.5-fold and 5.5-fold higher in the T1DM group, respectively (Fig. 2B–D).

Fecal BA excretion was significantly higher in diabetic mice ($2.85 \pm 0.20 \text{ vs. } 2.01 \pm 0.12 \text{ µmol/day}$, $P < 0.01$, Fig. 3A). However, fecal neutral sterol excretion did not change significantly ($4.04 \pm 0.28 \text{ vs. } 3.46 \pm 0.23 \text{ µmol/day}$, $P = 0.13$, Fig. 3B), conceivably attributable to increased intestinal cholesterol absorption as indicated by higher plasma campesterol/cholesterol ratios in T1DM mice compared with controls ($12.5 \pm 1.1 \text{ vs. } 8.4 \pm 0.4 \times 10^{-3}$, $P < 0.01$) as well as by a 2.1-fold higher intestinal fractional cholesterol absorption in direct measurements ($74 \pm 12 \text{ vs. } 35 \pm 8\%$, $P < 0.05$).

**Hepatic gene expression analysis indicates increased BA synthesis in type I diabetic mice**

The hepatic expression of the major transporters for biliary cholesterol, ATP-binding cassette transporter G5 ($A_{bcg5}$) and $A_{bcg8}$ was increased by 34% ($P < 0.05$) and 33% ($P < 0.01$), respectively (Table 2). The expression of the biliary phospholipid transporter $A_{bcg4}$ was increased by 19% ($P < 0.05$), whereas the expression of the BA transporter $A_{bcg11}$ ($A_{bcg11}$) was not different (Table 2). The expression of HMG-CoA reductase ($H_{mgr}$) remained unchanged, indicating that cholesterol synthesis rates were not altered in T1DM mice. Identical plasma lathosterol/cholesterol ratios in T1DM mice and controls as measure of endogenous cholesterol synthesis rates further supported this conclusion ($1.6 \times 10^{-4} \pm 0.2 \times 10^{-4} \text{ vs. } 1.6 \times 10^{-4} \pm 0.2 \times 10^{-4}$). However, a 94-fold ($P < 0.01$) increase in the expression of $C_{yp7a1}$ and a 1.7-fold elevated expression of $C_{yp8b1}$ ($P < 0.05$) indicated increased BA synthesis in diabetic mice.

**Macrophage-to-feces RCT is decreased in type I diabetic mice**

Biliary sterol secretion, either as free cholesterol or after metabolic conversion to BAs, is a critical step in RCT (7, 8). Therefore, a macrophage-to-feces RCT experiment was performed to investigate whether increased biliary sterol secretion in diabetic mice would translate into increased RCT. Following intraperitoneal injection with [³H]cholesterol-loaded macrophage foam cells, counts recovered from plasma tended to be higher in diabetic mice compared with controls ($3.13 \pm 0.46 \text{ vs. } 2.21 \pm 0.19\%$ of injected dose, $P = 0.07$ at 48 h, Fig. 4A). The amount of label present within the liver at time of sacrifice did not differ between the groups (Fig. 4B). Surprisingly, despite the increased mass biliary sterol secretion, overall macrophage-to-feces RCT was reduced by 20% in diabetic mice compared with controls ($9.23 \pm 0.79 \text{ vs. } 11.54 \pm 0.61\%$ of injected dose).
do not represent the underlying mechanism for decreased RCT in T1DM mice.

**Uptake of HDL cholesterol by the liver is impaired in type 1 diabetic mice**

Hepatic uptake of HDL cholesterol is another key process in RCT and impaired uptake of HDL cholesterol by the liver would offer an alternative explanation for decreased macrophage-to-feces RCT in diabetic mice. Therefore, the hepatic expression of the selective uptake transporter for HDL cholesterol, SR-BI, was investigated. Although the mRNA expression of SR-BI was 22% higher in diabetic mice compared with controls ($P < 0.05$, Fig. 6A), neither total nor membrane-associated SR-BI protein expression was different between groups (Fig. 6B). Besides the expression of SR-BI, a reduced affinity of the ligand, namely the HDL particle, could also impair hepatic selective uptake of HDL cholesterol. Selective uptake from T1DM HDL into ldlA[mSR-BI] cells, an in vitro model system for SR-BI-mediated selective uptake of cholesterol from HDL, was reduced by 41% (18.3 ± 2.9 vs. 31.2 ± 2.8%, $P < 0.05$, Fig. 6C).

To investigate whether reduced hepatic selective uptake also occurs in vivo in T1DM mice, a series of HDL kinetic studies was performed. Whereas the HDL protein turnover dose, $P < 0.05$, Fig. 4C). This difference was mainly due to a decreased amount of label excreted within the fecal BA fraction of T1DM mice (7.08 ± 0.77 vs. 9.11 ± 0.46% of injected dose, $P < 0.05$, Fig. 4C), whereas label recovered within the fecal neutral sterol fraction was not different between groups (Fig. 4C).

**The efflux capacity of HDL is not affected in type 1 diabetic mice**

Glycation of apoA-I has been associated with reduced functionality in cholesterol efflux assays (23). Thus, we first determined whether alloxan-induced T1DM results in increased glycation of HDL proteins. HDL from diabetic mice was more glycated compared with control HDL as judged by a significant increase in fructosamine residues (83 ± 3 vs. 50 ± 6 nmol/mg protein, $P < 0.01$, Fig. 5A). Next, we investigated whether increased glycation of HDL particles would translate into altered cholesterol efflux from these particles as a potential mechanism explaining decreased macrophage-to-feces RCT in T1DM mice. However, the amount of labeled cholesterol effluxed in vitro from macrophage foam cells toward either control HDL or HDL isolated from diabetic mice did not differ (Fig. 5B), indicating that changes in the efflux capacity of HDL do not represent the underlying mechanism for decreased RCT in T1DM mice.

**Fecal bile acid excretion is increased in T1DM mice, while fecal neutral sterol excretion is unaltered.** Feces of individually housed mice ($n = 8$ per group) were collected over a period of 24 h, starting on day 8 after alloxan or PBS injection. Fecal samples were dried, weighed, and thoroughly ground. Aliquots were used for determination of bile acids (A) and neutral steroids (B) by gas liquid chromatography as detailed in Materials and Methods. Data are given as means ± SEM. ** $P < 0.01$ compared with PBS-injected control mice.
Type I diabetes mellitus decreases RCT

Although cholesterol efflux toward glycated HDL was not impaired, the functionality of glycated HDL in SR-BI-mediated selective uptake was significantly decreased, conceivably representing a major contributing factor to reduced RCT in T1DM mice.

The starting point of RCT is cholesterol efflux from macrophage foam cells within the vascular wall. Under conditions of hyperglycemia, HDL-associated proteins readily become glycated (24, 25), which might have important functional implications as glycated apoA-I has been reported to be defective in mediating cholesterol efflux (23). However, our data demonstrate that in the context of a whole HDL particle in vitro cholesterol efflux toward HDL from T1DM mice was unchanged compared with control HDL. This observation is supported by previous studies using isolated total HDL (26, 27). Combined, these data suggest that glycation of HDL proteins might inhibit ABCA1-mediated efflux, whereas ABCG1-mediated efflux is not affected.

Following transport of cholesterol by HDL through the plasma compartment, the next important step of RCT is selective uptake into the liver mediated by SR-BI. Hepatic SR-BI expression remained largely unchanged in T1DM mice. However, HDL kinetic studies revealed that hepatic selective uptake was significantly lower in T1DM mice in vivo, and also in vitro the properties of T1DM HDL to function in selective uptake were significantly impaired. Thereby, reduced selective uptake of cholesterol from diabetic HDL likely contributes significantly to the decrease in RCT observed in T1DM mice by decreasing the input of cholesterol originating from macrophages into the hepatic cholesterol pool.

Biliary sterol secretion is thought to be a major determinant for the completion of the RCT pathway (7, 8). Our data show enhanced biliary secretion of BAs as well as did not differ between control and T1DM mice (0.098 ± 0.009 vs. 0.089 ± 0.006 pools/h, n.s., Fig. 6D), the HDL-CE fractional catabolic rate was significantly lower in diabetic animals (0.169 ± 0.005 vs. 0.131 ± 0.006 pools/h, P < 0.01, Fig. 6D), indicating reduced whole body selective uptake. Hepatic uptake of HDL proteins was similar in controls and T1DM mice (26.4 ± 2.6 vs. 23.8 ± 2.3%, n.s., Fig. 6E), however, uptake of HDL-CE was lower in T1DM than controls (46.0 ± 2.4 vs 36.6 ± 2.3%, P < 0.05, Fig. 6E) translating into a significant reduction of hepatic selective uptake under diabetic conditions (19.6 ± 1.9 vs 12.8 ± 0.9%, P < 0.05, Fig. 6E). These combined data demonstrate that impaired hepatic selective uptake of cholesterol from T1DM HDL particles occurs also in vivo and indicate that this mechanism contributes to reduced in vivo RCT in T1DM mice.

DISCUSSION

Our data demonstrate that experimental T1DM results in a decrease in macrophage-to-feces RCT despite increased biliary sterol secretion rates. To delineate the underlying mechanism of this finding, our study explored the impact of T1DM on key steps relevant for RCT. Although cholesterol efflux toward glycated HDL was not impaired, the functionality of glycated HDL in SR-BI-mediated selective uptake was significantly decreased, conceivably representing a major contributing factor to reduced RCT in T1DM mice.

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### Table 2. Hepatic mRNA expression levels at day 10 after injection with alloxan or PBS

<table>
<thead>
<tr>
<th>Gene</th>
<th>PBS (n = 6)</th>
<th>Alloxan (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abcg5</td>
<td>1.00 ± 0.08</td>
<td>1.34 ± 0.09</td>
</tr>
<tr>
<td>Abcg8</td>
<td>1.00 ± 0.05</td>
<td>1.33 ± 0.08</td>
</tr>
<tr>
<td>Abcl1</td>
<td>1.00 ± 0.07</td>
<td>1.19 ± 0.06</td>
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<tr>
<td>Abcl11</td>
<td>1.00 ± 0.02</td>
<td>0.93 ± 0.07</td>
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<tr>
<td>Hmgcr</td>
<td>1.00 ± 0.08</td>
<td>1.08 ± 0.22</td>
</tr>
<tr>
<td>Cyp7a1</td>
<td>1.00 ± 0.29</td>
<td>8.71 ± 2.16</td>
</tr>
<tr>
<td>Cyp8b1</td>
<td>1.00 ± 0.11</td>
<td>1.69 ± 0.27</td>
</tr>
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Expression levels were normalized to the housekeeping gene cyclophilin and further normalized to the mean expression levels of the PBS control group. Data are given as means ± SEM. § P < 0.05, † P < 0.01 each compared with PBS controls.

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**Fig. 4.** Macrophage-to-feces reverse cholesterol transport is decreased in T1DM mice. On day 9 after injection with either PBS (n = 8) or alloxan (n = 7), individually housed mice were injected intraperitoneally with 2 million [3H]cholesterol-loaded macrophage foam cells per mouse. Plasma was collected at the indicated time points after macrophage injection, and counts were assessed by liquid scintillation counting at the indicated time points (A). At the end of the experimental period, livers were harvested, snap-frozen in liquid nitrogen, and stored at −80°C. Counts in a weighed liver sample were determined following solubilization of the tissue and related to total liver mass (B). Feces were collected continuously up to 48 h and were dried, weighed, and thoroughly ground. Aliquots were separated into bile acid and neutral sterol fractions as detailed in Materials and Methods, and counts recovered from the respective aliquots were related to the total amount of feces produced over the whole experimental period (C). All obtained counts were expressed relative to the administered tracer dose. Data are given as means ± SEM. * P < 0.05 compared with PBS-injected control mice.
of BAs and cholesterol in gallbladder bile were increased in T1DM mice injected with alloxan as well as in nonobese diabetic mice (11, 29). Because biliary BA secretion is a major driving force for biliary cholesterol secretion (30), these results are consistent with previously published data demonstrating that alloxan- or streptozotocin-induced diabetes increased biliary sterol secretion rates in rats (13, 28). In addition, concentrations of BAs and cholesterol in gallbladder bile were increased in T1DM mice injected with alloxan as well as in nonobese diabetic mice (11, 29). Because biliary BA secretion is a major driving force for biliary cholesterol secretion (30),

Fig. 5. HDL from T1DM mice is glycated but HDL-mediated efflux from macrophage foam cells remains unchanged. HDL was isolated from mice injected with either PBS or alloxan, and fructosamine residues were determined (n = 4 per group) as measure of HDL glycation (A). Differentiated THP-1 macrophages were loaded with 50 μg/ml acetylated LDL and 1 μCi/ml [3H]cholesterol as detailed in Materials and Methods. Subsequently, cells were washed with PBS and 50 μg protein/ml of isolated mouse HDL was added (n = 4 per group). After 24 h, radioactivity within the medium was determined by liquid scintillation counting. The cell layer was washed twice with PBS, whereafter 0.1 M NaOH was added. Plates were incubated for 30 min at room temperature, then radioactivity remaining within the cells was assessed. HDL-independent efflux was calculated from wells incubated with RPMI without added HDL and these values were subtracted from the respective experimental values. Efflux is given as the percentage of counts recovered from the medium in relation to the total counts present on the plate (sum of medium and cells) (B). Data are given as means ± SEM. **P < 0.01 compared with HDL isolated from PBS-injected control mice.

Fig. 6. T1DM mice have impaired selective uptake of HDL cholesterol by the liver. Hepatic mRNA expression of SR-BI in mice that were injected with either PBS (n = 8) or alloxan (n = 7) was determined by real-time quantitative PCR (A). Protein expression of SR-BI in liver homogenates (n = 6 in each group, left panel) and in the hepatic membrane fraction (n = 6 in each group, right panel) was determined by Western blot and representative images are shown (B). SR-BI-mediated selective uptake of HDL cholesterol from isolated HDL was determined in vitro using ldlA[mSR-BI] cells as detailed in Materials and Methods (C). Fractional catabolic rates of HDL protein as well as HDL-CE were determined in control (n = 6) and T1DM (n = 5) mice after injection of autologous HDL labeled with 125I-tyramine cellobiose and 3H-cholesteryl ether as described in Materials and Methods (D). Hepatic uptake rates of HDL protein, HDL-CE as well as selective uptake rates in control and T1DM mice determined at the 24 h-time point of the kinetic experiment shown in panel D (E). Experimental conditions and calculations are detailed in Materials and Methods. All data are given as means ± SEM. * P < 0.05, ** P < 0.01 compared with PBS-injected controls.
Type I diabetes mellitus decreases RCT

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Fig. 7. Working model summarizing the impact of T1DM on sterol metabolism and RCT. Compared with controls (A), biliary secretion of bile acids and cholesterol is significantly increased in T1DM (B). Whereas hepatic cholesterol synthesis remains unaltered, bile acid synthesis is upregulated. Hepatic cholesterol levels do not decrease, because the liver cholesterol pool in diabetic mice receives more input via increased intestinal cholesterol absorption due to both increased food uptake and higher fractional absorption rates. These events result in an enhanced cycling of cholesterol between the liver and the intestine without having a net effect on fecal neutral sterol excretion. In terms of RCT, less cholesterol originating from macrophages enters the hepatic pool due to impaired hepatic SR-BI-mediated selective uptake from diabetic HDL, and a lower fraction of this cholesterol is subsequently used for BA synthesis. These changes are reflected by decreased overall RCT, primarily attributable to reduced tracer excretion within the fecal bile acid fraction. Bold arrows represent increased fluxes in T1DM mice (B) compared with PBS-injected controls (A), while thinner arrows represent decreased fluxes.

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the primary point of dysregulation in T1DM is likely in the metabolism of BAs. Hepatic gene expression analysis indicated increased de novo BA synthesis in T1DM animals, for which cholesterol serves as the substrate (31). In contrast to fecal BA excretion, fecal neutral sterol excretion was similar between groups despite the 5.5-fold higher biliary cholesterol secretion in the diabetic mice. These data are conceivably explained by 2.1-fold increased cholesterol absorption rates observed in the T1DM mice in our study. In addition, food intake was almost 2-fold higher in T1DM mice (data not shown), overall resulting in a substantial increase in cholesterol supply from diet. Increased intestinal cholesterol absorption has previously been observed by others in insulin-deficient diabetic rodent models as well as in humans (9, 10, 12, 32). However, it is important to point out that our study specifically identified decreased SR-BI-mediated selective uptake, which represents the point of entry for macrophage-derived cholesterol into the entero-hepatic system, as the major block in RCT affected in T1DM.

Certain methodological issues have to be considered in the interpretation of our results. HDL kinetic studies using trap labels for HDL proteins as well as HDL cholesteryl ester clearly demonstrated reduced hepatic selective uptake rates in vivo in T1DM mice. The hepatic tracer data in the RCT experiment, however, were obtained 48 h after injection of labeled macrophages using a freely distributable label. The methodology of the in vivo RCT assay is therefore not suitable to allow any conclusion on selective uptake. In addition, the data from the macrophage RCT assay only indicate, whether the tracer deloaded from the macrophages enters better or less good the RCT system and is finally deposited in the feces. Overall cholesterol fluxes through the body cannot be derived from these data, because then different pool sizes of cholesterol are important and would have to be taken into account.

Besides mediating RCT, HDL particles have additional anti-atherosclerotic properties such as inhibiting endothelial inflammation (33), promoting vascular nitric oxide generation (34) as well as protecting LDL against oxidative modification (35, 36). Increasing evidence suggests that also these atheroprotective functions of HDL particles are impaired in T1DM. The anti-oxidative properties of HDL appear to be diminished in T1DM (37, 38) and the ability of HDL from T1DM patients to counteract the inhibitory effects of oxidized LDL on endothelium-dependent vasorelaxation is also reduced (39). In addition, glycation of apoA-I was recently shown to decrease the potency of HDL to inhibit neutrophil infiltration and adhesion molecule expression using the carotid artery collar model in rabbits (40). In addition to decreased RCT, these alterations are expected to contribute to an overall reduced capacity of diabetic HDL to protect against atherosclerotic CVD.
Figure 7 summarizes our current working model on the effects of T1DM on sterol metabolism and RCT. Biliary secretion of BAs and cholesterol is significantly increased, whereas hepatic cholesterol synthesis remains largely unaffected. Nevertheless, hepatic cholesterol levels do not decrease, because the liver cholesterol pool in diabetic mice receives more input via increased food intake and higher intestinal cholesterol absorption. These events result in an enhanced cycling of cholesterol between the liver and the intestine without having a net effect on fecal neutral sterol excretion. To compensate for increased fecal BA loss, also hepatic BA synthesis from cholesterol is up-regulated. With respect to RCT, less cholesterol originating from macrophages enters the hepatic cholesterol pool because SR-BI-mediated selective uptake from diabetic HDL is impaired. Thereby, a smaller fraction of this cholesterol is then used for BA synthesis. These changes are reflected by decreased overall RCT, primarily due to reduced tracer excretion within the fecal BA fraction.

In conclusion, insulin-deficient T1DM mice exhibit decreased RCT despite increased biliary sterol secretion. These unfavorable changes are conceivably due to decreased properties of glycated HDL to function in hepatic selective uptake. Impaired RCT is expected to contribute to the increased risk for atherosclerotic CVD morbidity and mortality in patients with T1DM.

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