Diabetes Mellitus Is Associated With Reduced High-Density Lipoprotein Sphingosine-1-Phosphate Content and Impaired High-Density Lipoprotein Cardiac Cell Protection


Objective—The dyslipidemia of type 2 diabetes mellitus has multiple etiologies and impairs lipoprotein functionality, thereby increasing risk for cardiovascular disease. High-density lipoproteins (HDLs) have several beneficial effects, notably protecting the heart from myocardial ischemia. We hypothesized that glycation of HDL could compromise this cardioprotective effect.

Approach and Results—We used in vitro (cardiomyocytes) and ex vivo (whole heart) models subjected to oxidative stress together with HDL isolated from diabetic patients and nondiabetic HDL glycated in vitro (methylglyoxal). Diabetic and in vitro glycated HDL were less effective (P<0.05) than control HDL in protecting from oxidative stress. Protection was significantly, inversely correlated with the degree of in vitro glycation (P<0.001) and the levels of hemoglobin A1c in diabetic patients (P<0.007). The ability to activate protective, intracellular survival pathways involving Akt, Stat3, and Erk1/2 was significantly reduced (P<0.05) using glycated HDL. Glycation reduced the sphingosine-1-phosphate (S1P) content of HDL, whereas the S1P concentrations of diabetic HDL were inversely correlated with hemoglobin A1c (P<0.005). The S1P contents of in vitro glycated and diabetic HDL were significantly, positively correlated (both <0.01) with cardiomyocyte survival during oxidative stress. Adding S1P to diabetic HDL increased its S1P content and restored its cardioprotective function.

Conclusions—Our data demonstrate that glycation can reduce the S1P content of HDL, leading to increased cardiomyocyte cell death because of less effective activation of intracellular survival pathways. It has important implications for the functionality of HDL in diabetes mellitus because HDL-S1P has several beneficial effects on the vasculature.

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Key Words: AGE ■ cardiomyocytes ■ glycation ■ HDL ■ ischemia reperfusion injury ■ sphingosine-1-phosphate ■ type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) is one of the most important risk factors for developing cardiovascular disease. The dyslipidemia found in T2DM has multiple etiologies and affects all lipoprotein subclasses. Unfavorable composition and quantities of lipoproteins are paralleled by deterioration of their function under the pressure of insulin resistance and processes of oxidation and glycation. The complex process of protein glycation leads ultimately to accumulation of advanced glycation end products that have been correlated with the severity of coronary heart disease. Low plasma high-density lipoproteins (HDL) cholesterol is a strong risk factor for cardiovascular disease. HDL cholesterol levels are decreased in T2DM with alterations of both the HDL proteome and lipidome. These modifications can be related to early markers of arterial disease. HDL from T2DM patients also exhibit impaired anti-inflammatory and antioxidant properties. The protective effect of HDL has more recently been shown to extend into the field of cardiac hypoxic stress and ischemia. Oxidative stress, which is also induced by...
the anthracycline drug doxorubicin, leads to cardiotoxicity.\(^{10,11}\) We and others have shown that HDL protects the cardiomyocyte against both doxorubicin-\(^{12}\) and hypoxia-\(^{13}\)-induced oxidative stress in vitro. We have also shown that HDL protects the whole heart against ischemia–reperfusion (IR) injury by preserving mitochondrial integrity.\(^{14}\) On the molecular level, the protective effects of HDL are mediated by inducing phosphorylation of intracellular prosurvival proteins.\(^{15}\)

Sphingosine-1-phosphate (S1P) is a potent messenger molecule operating both intra- and intercellularly.\(^{16}\) In plasma, it is mainly associated with HDL via apolipoprotein M (apoM).\(^ {17}\) S1P is enriched in small dense HDL3 with a positive correlation between HDL S1P concentration and apoptotic protection.\(^{18}\) Theilmeier et al\(^ {19}\) have also shown that HDL and its constituent S1P protect the heart during IR injury via an S1P receptor.

Little is known about how diabetes mellitus and the pathophysiological process of glycation affect HDL function in the setting of ischemia-induced oxidative stress. In the present work, we investigated the functionality of glycated HDL (glyHDL) during oxidative stress and ischemia. We hypothesized that glycation could compromise this function, which would contribute to an increased risk of vascular disease in diabetes mellitus. The study has identified a novel, physiopathological mechanism specific to diabetes mellitus. It also has wider implications for the functionality of HDL in diabetes mellitus.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

#### Diabetic HDL Is Less Protective Than Control HDL

Previous studies have shown that HDL protects cells against oxidative stress induced by doxorubicin.\(^ {12}\) The effect of HDL on cell viability was quantified in primary cultures of cardiomyocytes after doxorubicin exposure, considering doxorubicin-only treated cells as 100% (Figure 1A). Cells exposed to doxorubicin+control HDL (cHDL) and doxorubicin+diabetic HDL (dmHDL) had mean viabilities of, respectively, 194.1±18.3% and 137.9±9.5% (\(P=0.011\) for comparison). Thus, dmHDL was less effective in protecting against doxorubicin-induced cell death than cHDL. The characteristics of T2DM patients are given in the Table.

#### Figure 1. High-density lipoprotein (HDL) from type 2 diabetes mellitus (T2DM) patients and in vitro glycated HDL (glyHDL) are less protective than healthy HDL.

**A**. Cell viability in primary cultures of cardiomyocytes after a 20-h incubation (20 h) with doxorubicin (DOX; 0.5 \(\mu\)M) measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cardiomyocytes were preincubated with HDL (200 \(\mu\)g/mL) from diabetic patients (dmHDL) and from healthy controls (cHDL) for 30 min before addition of DOX. Results were normalized to DOX-treated cells. \(n=6\). **B**, HDLs with increasing level of glycation were prepared in vitro by increasing methylglyoxal (MG) incubation times (2–40 h; black bars) in parallel with corresponding control HDLs (cHDL; hatched bars). The level of HDL glycation measured by the TNBS (2,4,6-trinitrobenzene sulfonic acid) assay with glycation being inversely correlated to absorbance. Mean absorbance for chDL 0 h (open bar) was set to 100%. **C**, Cell viability in cardiomyocytes after a 20-h incubation with DOX and various HDL (200 \(\mu\)g/mL) preparations measured by the MTT assay. (\(n=3\) for all groups). *\(P<0.001\), analysis of variance (ANOVA) for trend across glyHDLs. **D**, Infarct sizes in mouse hearts after ex vivo ischemia–reperfusion (IR) injury. Isolated hearts were treated with 400 \(\mu\)g/ mL cHDL (\(n=7\)) or glyHDL (\(n=8\)); control mice without treatment (IR, \(n=8\)). Quantification of infarct size is expressed as percentage of total heart area. \(P\) value was calculated by 1-way ANOVA, Bonferroni post hoc test. Student t test (unilateral, unpaired) was used to calculated difference between cHDL and glyHDL. N.T. indicates nontreated.
### Table. Study Cohort

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*Calculated by Friedwald’s formula.

### HDL Glycated In Vitro Is Less Protective Than Control HDL

To examine specifically the effects of glycation, we used a model based on methylglyoxal (MG) modification of HDL. Using the TNBS (2,4,6-trinitrobenzene sulfonic acid) assay to monitor glycation, we observed that MG increased HDL glycation (P<0.001), whereas chHDLs showed no increase (P=0.73; Figure 1B). Increased glycation was associated with a significant decrease in HDL-mediated protection from doxorubicin (P<0.001; P=0.84 for cHDLs; Figure 1C). HDL glycation was inversely correlated with cell viability (r=-0.674, P=0.002, n=18). Immunoblots of apoM and apoAI after glycation of HDL are shown in Figure I in the online-only Data Supplement.

The protective effect of chHDL and glyHDL against IR injury was also evaluated in the ex vivo isolated heart perfusion model (Figure 1D). Control mice subjected to ischemia but without HDL treatment had an infarct size of 12.8±1.2%. Treatment with chHDL during reperfusion significantly decreased average infarct size by 39% compared with controls (P=0.01), whereas infarct size did not differ significantly between glyHDL and control mice.

### Glycated HDL Does Not Induce Phosphorylation of Prosurvival Proteins as Effectively as Control HDL

The ability of HDL to activate prosurvival intracellular signaling pathways by inducing phosphorylation is important for cardiomyocytes during oxidative stress. We examined phosphorylation of Akt, Stat3, and Erk1/2 in relation to HDL glycation by Western blotting (Figure 2). chHDL more efficiently induced phosphorylation of each respective pathway in comparison to glyHDL after both 5 and 30 minutes of stimulation (see also Figure II in the online-only Data Supplement for phosphorylation levels for Stat3 and Erk1/2 at 60 minutes).

### SR-BI Participates in the Binding of HDL to Cardiomyocytes But Does Not Influence Its Protective Effects

To study more specifically the role of SR-BI in cardiomyocytes under oxidative stress, we manipulated its expression by transfecting cardiomyocytes with siRNA-targeting SR-BI. SR-BI siRNA downregulated mRNA and protein levels to 14.0±7.0% and 32.7±9.7%, respectively, compared with Scr-siRNA (120.0±16.3% and 90.6±2.4%; the expression of lipofectamine-only transfected cells was arbitrarily set to 100%; Figure 3B and 3C). Specific binding of HDL was reduced 36% (Scr-siRNA cardiomyocytes, 510±56 ng/mg versus SR-BI-siRNA cells, 327±41 ng/mg; P=0.005; Figure 3D).

The impact of SR-BI silencing on cell viability was examined after HDL+doxorubicin exposure (Figure 3E). SR-BI–downregulated cardiomyocytes had similar viability to Scr-siRNA–treated cells, 119.4±7.0% and 121.6±8.3%, respectively (P=0.86), in the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay with cHDL. Cardiomyocyte viability was also evaluated by DNA fragmentation after 8 hours of HDL+doxorubicin exposure. SR-BI-siRNA– and Scr-siRNA–transfected cells displayed similar fragmentation levels of 81.6±4.4% and 83.0±3.0%, respectively (P=0.81). A final evaluation of the role of SR-BI exploited SR-BI knockout mice using the ex vivo heart exposed to IR injury (Figure 3F). Without HDL treatment during reperfusion, wild-type and SR-BI knockout mouse did not differ in infarct size (P=0.23). HDL treatment reduced the infarct size by 37% (P=0.002) and 46% (P=0.044) in wild-type and SR-BI knockout mice respectively.

In addition, we investigated the involvement of SR-BI in activating HDL prosurvival pathways by comparing the phosphorylation of proteins in Scr-siRNA– and SR-BI-siRNA–transfected cardiomyocytes (Figure III in the online-only Data Supplement). There were no significant differences in stimulation responses for any of the phosphorylated proteins.

### In Vitro Glycation of HDL Decreases Its SIP Content Which Inversely Correlates to Its Protective Capacity

Given the reported importance of SIP, we next studied the consequences of glycation on HDL SIP content. We could find no evidence for the presence of derivatives of the SIP molecule by liquid chromatography mass spectrometry (data not shown). However, SIP content in the glyHDLs decreased with increasing incubation time (P<0.001; Figure IV in the online-only Data Supplement). SIP content in the cHDL preparations did not differ between groups (P=0.49).

Glycation Reduces HDL Binding to Cardiomyocytes

Given the role attributed to the scavenger receptor class B type I (SR-BI) receptor in certain HDL functions, we compared binding of cHDL and glyHDL to cardiomyocytes. Using 125I-labeled HDL in the concentration range 1 to 40 μg/mL, saturable, specific binding of cHDL and glyHDL was observed (Figure 3A) with (at 40 μg/mL) 245±44 ng bound HDL/mg and 104±41 ng bound HDL/mg of cell lysate proteins, respectively, corresponding to a 58% decrease for glyHDL (P=0.023).

Glycation increases the binding of HDL to cardiomyocytes but does not influence its protective effects.

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S1P concentration in HDL was significantly and inversely correlated to its glycation level ($r=-0.810; P<0.001$) and significantly and positively correlated to its protective capacity ($r=0.715; P<0.001$; Figure 4A and 4B). Similar results were achieved using albumin (BSA), instead of HDL, as a vehicle for S1P. S1P was resuspended with BSA into BSA:S1P complexes and then glycated with MG, which led to a significant glycation compared with control (Figure VA in the online-only Data Supplement). Glycation also led to a decrease in S1P content by 22.7±6.3% compared with control ($P=0.03$), and the protection capacity decreased from 141.1±5.7% to 121.9±4.6% ($P=0.036$; Figure VB in the online-only Data Supplement).

HDL S1P Content in T2DM Patients Show Correlations to Both Their Hemoglobin A1c and Their Protective Capacities
HDL was individually prepared from a second cohort of T2DM patients (n=26) with a range of hemoglobin A1c (HbA1c) levels (Figure 5). Using HbA1c as a measure of in vivo glycation, we observed that patient HDL S1P content correlated inversely with their HbA1c levels (Figure 5A; $r=-0.551; P=0.005$). The S1P content of dmHDL was also correlated to cell viability (Figure 5B; $r=-0.557, P=0.007$).

Adding S1P to Glycated HDL Improves Its Protective Capacity
In vitro glycated HDL and HDL from T2DM patients were resuspended with S1P and the HDL S1P content, and their protective capacities were evaluated. S1P content increased similarly in glyHDL (86.7±1.7 to 150.5±7.1 pmol/mg; $P<0.001$) and dmHDL (123.2±12.1 to 189.9±12.3 pmol/mg; $P=0.006$; Figure 6A and 6C). Concomitantly with the S1P increase, the protective capacity increased for both glyHDL (122.6±3.7% to 135.6±4.3%; $P=0.034$) and dmHDL (135.2±13.3% to 158.8±15.6%; $P=0.028$; Figure 6B and 6D).

Discussion
The main findings of this study are that the cardioprotective function of HDL manifested during acute oxidative stress and IR injury is impaired by glycation in vitro and inversely correlates in vivo with measures of glycation. On a molecular level, we demonstrate that glycation of HDL impairs its capacity to stimulate previously identified, prosurvival signaling pathways. Finally, we demonstrate that impaired function is directly related to loss of S1P from HDL, which correlates with measures of glycation in vitro and in vivo.

HDL isolated from diabetic patients was defective in protecting cardiomyocytes from stress. As diabetes mellitus can modify HDL in several ways, we used in vitro modification with MG to study specifically the consequences of glycation. It is a widely accepted methodology provoking protein glycation comparable to that observed in diabetic patients. Indeed, we observed similar levels of HDL glycation of our in vitro preparations and the diabetic patient group. Moreover, recent studies have reported that in vivo glycated products derived from MG best differentiate diabetic and nondiabetic HDL. In our studies, the consequence of such in vitro glycation was to reduce by 20% to 30% the protective capacity of HDL in both in vitro and in vivo.
ex vivo models of acute oxidative stress. It was linked to compromised activation of Akt, Stat3, and Erk1/2, protein kinases that have key roles in the SAFE (survivor activating factor enhancement) and RISK (reperfusion injury salvage kinase) myocardial prosurvival pathways and that we previously identified as important in HDL-mediated protection of cardiomyocytes.20

One potential explanation for the reduced protective capacity of HDL could be variations in apoM concentrations. The role of apoM in the association and function of S1P in HDL has been demonstrated in several models, even beyond the vascular field.25,26 However, we could observe no variations in apoM concentrations (Figure VI in the online-only Data Supplement), in accordance with previous reports that apoM is not decreased in non-MODY3 diabetic patients.27–29

An alternative explanation may be defective interaction with cell receptors. Glycation did reduce binding of HDL to cardiomyocytes. SR-BI, the major HDL receptor, is expressed in the heart.30 Complementary studies showed that siRNA-mediated reduction of SR-BI expression in cardiomyocytes did not affect the efficacy of HDL. This was confirmed in SR-BI knockout mice where absence of the receptor did not affect the protective function of HDL. Thus, we cannot demonstrate a major role for SR-BI in our model, although its central importance to the impact of HDL elsewhere in the vasculature, notably endothelium function, is clear.31 We cannot exclude, however, a possible, secondary role of anchoring HDL to the membrane to facilitate interaction of S1P with its receptor.31 This data suggest that the impact of S1P is independent of

![Figure 3](image-url)

**Figure 3.** The role of scavenger receptor class B type I (SR-BI) in cardiomyocyte high-density lipoprotein (HDL) binding and HDL protective effect. A, Cardiomyocytes bind in vitro glycated HDL (methylglyoxal [MG], 24 h) less efficiently than control HDL. White circles=specific 125I-cHDL binding; black circles=specific 125I-glyHDL binding (n=4). *P=0.023 (repeated measurement ANOVA interaction concentration×group). Mean expression levels of SR-BI in cardiomyocytes of mRNA (B) and protein (C) after transient transfection with scramble (Scr) siRNA, SR-BI-siRNA, or lipofectamine only (Lipofect; n=3). D, Specific 125I-HDL binding to cardiomyocytes transfected with Scr-siRNA or SR-BI-siRNA cells (n=4). *P=0.005 (repeated measurement ANOVA for interaction concentration×group). E, Cell survival after oxidative stress in cardiomyocytes knocked down for SR-BI. Cells were exposed to doxorubicin (DOX) and HDL for 20 h (n=4) or 8 h (n=3), and cell viability and DNA fragmentation were quantified, respectively. F, Infarct sizes in wild-type (WT) and SR-BI knockout (KO) mice hearts after ischemia-reperfusion (IR) injury ex vivo. n=7 for WT IR+HDL, n=5 for other groups. Hatched bars/circles=Scr-siRNA treated cells; black bars/circles=SR-BI-siRNA treated cells; Student t test. cHDL indicates control high-density lipoprotein; glyHDL, glycated high-density lipoprotein; and n.s., not significant.

![Figure 4](image-url)

**Figure 4.** Correlations between high-density lipoprotein (HDL) sphingosine-1-phosphate (S1P) content and glycation level (**A**) and protective capacity (**B**) of in vitro glycated HDL (n=18). Content of S1P was analyzed from in vitro glycated HDL and correlated to its level of glycation measured by TNBS (2,4,6-trinitrobenzene sulfonic acid; **A**) and its protective capacity measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (**B**), as described in Figure 1 (n=18).
Sphingosine-1-phosphate (S1P) content in high-density lipoprotein (HDL) from type 2 diabetes mellitus (T2DM) patients shows correlations to the (A) hemoglobin A1c (HbA1c) levels and (B) HDL protective capacity (viability). HDL was isolated from a group of diabetes mellitus patients (n=26) with a range of HbA1c between 5.7% and 9.5%. Viability was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as described in Figure 1.

We also considered whether there could be direct glycation of S1P. The molecule has one amine group potentially susceptible to modification by MG, thus creating S1P-MG derivatives. In collaboration with the Sciences Mass Spectrometry platform (core facility of the University of Geneva), we developed a mass spectrometry procedure to try to detect glycated derivatives of S1P. Several attempts were made to identify derivates in S1P–albumin complexes subjected to glycation. No derivatives suggestive of direct glycation of S1P were detected. Neither were derivatives observed in HDL preparations. On the other hand, our in vitro data clearly demonstrate an inverse relationship between the level of HDL glycation, its S1P content, and its cardioprotective capacity. The clinical relevance of these findings is underlined by the inverse correlation we observed between S1P concentrations of HDL from diabetic patients and their HbA1c levels. This may seem surprising as HbA1c integrates glycemic control over a longer period than the plasma residence time of HDL (estimated to 4 days). However, several studies have shown a strong correlation between HbA1c and HDL-apoA1 glycation products measured by the mass spectrometry procedure.23,33 Moreover, a recent study reported an inverse correlation between HbA1c levels and the concentration of S1P in HDL.34 Overall, these observations, together with data underlining the importance of S1P for HDL-mediated protection of cardiomyocytes, clearly identify loss of S1P from HDL as one explanation for impaired HDL function. Glycation itself would seem to be an important factor because (1) in vitro, it was sufficient to compromise HDL function and (2) it had the same effects in our simplified BSA:S1P model in the absence of HDL (loss of S1P which is associated with the degree of glycation and impaired protection).

The pathophysiological relevance of loss of S1P is underlined by the observation that HDL function can be recovered by restoring S1P to HDL. It highlights the need for a better understanding of the metabolic factors that influence the S1P content of HDL. The potential clinical consequences are illustrated by recent studies demonstrating increased risk of restenosis in patients with reduced HDL-S1P.35

The question arises as to how glycation may provoke loss of S1P from HDL. The major determinant of S1P binding to HDL is apoM. HDL-apoM could be susceptible to glycation-induced changes in HDL, which can destabilize HDL structure, modifying particle size and protein composition.36 Glycation also reduces the lipid-binding capacity of HDL,21 perhaps arising from an increase in hydrophilicity. The latter may influence binding of apoM to HDL, which is achieved via its hydrophobic signal sequence. However, we could not demonstrate a correlation between HDL–apoM concentrations and HbA1c levels in diabetic patients (Figure VI in the online-only Data Supplement), and as mentioned previously, apoM concentrations did not differ between diabetic patients and controls. Alternatively, apoM could be vulnerable to direct glycation. A model37 of its association with HDL shows the apolipoprotein protruding from the lipoprotein surface with the S1P-binding pocket facing outwards. In vitro glycation of apoAF3 and HDL38 strongly modifies tryptophan residues inducing conformational changes. Tryptophan residues of apoA have been implicated in binding S1P.37 We were able to confirm glycation of apoA in our dmHDL samples. When
challenged with an antibody to advanced glycation end products, advanced glycation end products were detected in apoM from diabetic patients, whereas no advanced glycation end products were evident in nondiabetic HDL (Figure VII in the online-only Data Supplement).

The failure in recent years to show clinical benefit from raising HDL cholesterol has prompted intense re-evaluation of the role of the lipoprotein in vascular disease. Although previous studies had established a protective role for HDL-S1P in ischemia, the present study is the first to show the potential pathological impact of a diabetes mellitus–associated modification of HDL on this function. Moreover, we identify a mechanism, loss of S1P, that could have wider implications for the role of HDL in vascular disease. The S1P component of HDL has been associated with several beneficial effects, including endothelial function, endothelial barrier integrity, and anti-inflammatory reactions. More recent studies have extended the potential impact of HDL-S1P to other cells, including pancreatic beta cells, where it was shown to improve insulin secretion, notably under conditions of endothelial stress.

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Disclosures
None.

References
4. Ikucchi K, Nejima J, Takano T, Ohta M, Hashimoto H. Increased serum advanced glycation end products were evident in nondiabetic HDL (Figure VII in the online-only Data Supplement) were evident in apoM from diabetic patients, whereas no advanced glycation end products were evident in nondiabetic HDL (Figure VII in the online-only Data Supplement).


The present study is the first to show the potential pathological impact of a diabetes mellitus–associated modification of high-density lipoprotein (HDL) on the protective role for HDL sphingosine-1-phosphate (S1P) in ischemia. Moreover, we identify a mechanism, loss of S1P, that could have wider implications for the role of HDL in vascular disease. The S1P component of HDL has been associated with several beneficial effects, including endothelial function, endothelial barrier integrity, and anti-inflammatory reactions. Recent data demonstrate a direct correlation between low S1P-HDL and cardiac disease. Our data underline the diagnostic potential of measuring the S1P content of HDL as a marker of HDL functionality. They also have therapeutic implications with respect to the addition of S1P to reconstituted HDL currently used in clinical trials.
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Material and Methods

Type 2 diabetic patients and controls

T2DM patients were recruited from the hospital outpatient diabetic clinic and healthy volunteers were recruited from university personnel. Their characteristics are given in Table 1. Four patients were treated with insulin and oral anti-diabetic drugs and two with oral anti-diabetic drugs. Five patients were asymptomatic for cardiac disease, and one had a previous myocardial infarction. Three patients were treated with statins. No patient had indications of renal insufficiency.

Preparation of HDL

For preliminary studies HDL (d=1.063-1.21 g/mL) was prepared by a standard double ultracentrifugation protocol from pooled human plasma of healthy donors as described \(^1\). This preparation was used in experiments if not otherwise indicated. Subsequently, and notably when individual preparations from diabetic patients (dmHDL) were required, a more rapid isolation procedure (7h) was developed. It entailed precipitation of apolipoprotein B containing lipoproteins by addition of sodium phosphotungstate (12mM, pH 6.15) and MgCl\(_2\) (50mM) then centrifuging (3500g, 1h, 4\(^\circ\)C). The supernatant was recovered and the density adjusted to 1.21g/L with NaBr before ultracentrifugation (5h, 430,000g). HDL was recovered and dialyzed against PBS-EDTA (100µM) or by several washes with PBS-EDTA in microspin columns. HDL protein concentration was determined by Lowry \(^2\). HDL concentration is expressed as its protein concentration.

Ethical permission (Ethics Commission, University Hospital, Geneva) was accorded for the study and informed consent was obtained from all donors. All human investigation was conducted according to the principles expressed in the Declaration of Helsinki.

In vitro glycation of HDL

HDL (3mg/ml) was incubated in sterile PBS-EDTA, 0.01% NaAzide and 6mM methylglyoxal (MG; Sigma, Germany) for various times (24h unless otherwise stated, 37\(^\circ\)C) under 5% CO\(_2\). Glycated HDL (glyHDL) was then dialyzed (24h, 4\(^\circ\)C) against sterile PBS-EDTA and the protein concentration determined by Lowry. Control HDL (cHDL) was incubated the same way except for the exclusion of MG. Immunoblots of apoM and apoAI after glycation of HDL are shown in supplementary figure S1.

Quantification of glycation of HDL in vitro

The degree of glycation of HDL was quantified by determining available amine groups using the colorimetric TNBS-assay (2,4,6-Trinitrobenzene Sulfonic Acid). The glycation level is inversely correlated to absorbance \(^3\).

Resuspension of S1P with HDL
S1P (S1P d18:1; Tocris Bioscience, Bristol, UK) was incubated with HDL (60pmol/mg protein, 30min, 37°C) with gentle shaking. The resuspension was washed three times with sterile PBS-EDTA with microspin columns before HDL protein measurement.

Animals

Experiments involving animals were approved by the local Animal Ethics Committee (Geneva). Animals were housed and treated in accordance with the Guide for Care and Use of Laboratory Animals Eighth Edition (US National Institute of Health Publication). Neonatal (2 days old) Wistar rats and male mice aged from 8-14 weeks were used.

Cell culture of primary cardiomyocytes and evaluation of cell survival

Primary cultures of cardiomyocytes were established from neonatal rats as described. Cell viability was measured by the MTT-assay. Cell death was measured by DNA-fragmentation using the cell death detection ELISA kit (Roche, Germany) according to the manufacturer’s instructions.

Perfusion of isolated mouse hearts

Male SR-BI knockout (SR-BI KO) and wild type (WT, C57BL/6 background) were anaesthetised via an intraperitoneal injection of sodium pentobarbital (60mg/kg). The heart was excised and the aorta was cannulated for retrograde perfusion in a Langendorff apparatus. Hearts were perfused with a modified Krebs–Henseleit buffer (NaCl 108mM; NaHCO₃ 25mM; KCl 5.9mM; MgSO₄ 1.19mM; CaCl₂ 2.5mM; Mannitol 1.1mM; di-sodium EDTA 0.5mM; glucose 10mM; sodium-pyruvate 5mM; gassed with 95% O₂/5% CO₂ at 37°C). A balloon was introduced into the left ventricle and left ventricular end-diastole pressure (LVEDP) fixed to 8-10mmHg. Hearts were subjected to 30min stabilisation followed by 35min global no-flow ischemia and 60min reperfusion. HDL (400µg/ml) was perfused during the first 7min of reperfusion. At the end of the protocol, the heart was immediately frozen. Frozen hearts were cut into 5-6 slices and incubated with triphenyltetrazolium chloride (TTC, 1%) to stain viable myocardium. Total myocardial and infarcted areas were measured from computed images using NIH Image software. Infarct areas were normalized to total cardiac area and averaged for individual hearts.

Knock-down of SR-BI by siRNA

Primary rat cardiomyocytes were transiently transfected using small interfering RNA (siRNA) according the manufacturers’ instructions (Lipofectamine RNAiMAX, Invitrogen, Switzerland) using siRNAs Silencer Select Negative Control 1 siRNA (#4390843) and anti-rat SR-BI (Scarb1) (#4390771) from LifeTechnologies, Switzerland.

Immunoblots and real-time PCR

Immunoblots according to standard procedures were performed using primary antibodies from Cell Signaling Technology (#9275 Phospho-AktSer⁴⁷³; #9134
Phospho-Stat3Ser\textsuperscript{727}; #9131 Phospho-Stat3Tyr\textsuperscript{705}; #9101 Phospho-Erk1/2\textsubscript{Thr202/Tyr204}, (#9272 total Akt), Abcam (ab37231 αSR-B1, ab154712 apoM polyclonal, ab47711 apoM monoclonal, # ab23722 anti-AGE antibody), Santa Cruz Biotechnology (#sc-94 Total Erk1/2), Calbiochem (#178422, apoAl polyclonal) and Millipore (#06-596 Total Stat3; #MAB374 α-GAPDH, glyceraldehyde 3-phosphate dehydrogenase). Total RNA was extracted (NucleoSpin RNA, Macherey-Nagel) and reverse transcribed using the manufacturers’ protocols. Real-time PCR was performed on a Light-Cycler480II (Roche). Primers to detect SR-BI were (sense) gtgctgctagctgttcttg and (anti-sense) acagactcagctgcatcttcac and for house-keeping gene 9S were (sense) ctccgaacaaacggtgaggt and (anti-sense) tccagcttcatcttgccctc.

\textbf{125I-iodination of HDL and binding experiments}

HDL was iodinated using iodination beads from Pierce Biotechnology (Rockford, IL) as described \textsuperscript{7}. Binding experiments performed as outlined \textsuperscript{7}. Briefly, primary rat cardiomyocytes were incubated with increasing concentrations of \textsuperscript{125I}-HDL (2h, 37°C) in the absence or presence of cold HDL (1mg/ml) after which cells were lysed and protein concentration and radioactivity were determined. Specific binding was considered as the difference between total and unspecific binding.

\textbf{Measurement of HDL S1P concentration}

S1P concentrations in HDL were determined by liquid-chromatography tandem mass spectrometry system (LC-MS/MS). Isolated HDL was diluted 1:9 in MeOH containing 10ng/mL internal standard (S1P-d7, Avanti Polar Lipids Inc. Alabaster, AL, USA) prior to injection into the LC-MS/MS (Ultimate 3000 LC Series, Thermo Fisher Scientific Inc., Whaltham, MA, USA and 5500 QTrap \textsuperscript{®} triple quadrupole linear ion trap system equipped with a Turbolon Spray\textsuperscript{™} interface (AB Sciex, Framingham, MA, USA)). Data acquisition and analysis were performed using Analyst\textsuperscript{™} software (version 1.6.2; AB Sciex, Framingham, MA, USA). For further details, see detailed measurement of S1P concentration by LC-MS/MS below.

\textbf{Detailed measurement of S1P concentration by LC-MS/MS}

Standard (S1P) and internal standard (S1P-d7) solutions were prepared from a stock solution at a concentration of 1 mg/mL (1 mg of substance in 1 mL DMSO/MeOH 1:4 + 4% HCl 30%). Working solutions were prepared by dilution in MeOH of the stock solutions to reach concentrations of interest ranging from 1 to 100 ng/mL. Stock and working solutions were stored at -20°C. Ultra-pure water (for chromatography) was produced using a milli-Qplus 185 from Millipore (Volketswil, Switzerland). MeOH (HPLC grade) was obtained from Merck (Darmstadt, Germany) and formic acid (FA, MS grade) was obtained from Sigma-Aldrich (Buchs, Switzerland).

\textbf{MS detection}

The Turbolon Spray interface was operated in the negative ionization mode. The parameters of the source were used with the following settings using nitrogen as curtain and nebulizer gas: capillary voltage -4.0 kV, temperature 625°C, curtain gas 20 psi, collision gas -8, GS1 45 psi, and GS2 45 psi. For the MRM parameters, a
dwell time of 50 ms, a declustering potential of -100 eV, and a collision energy of -40 eV were used for both S1P and S1P-d7. The precursor ions are 378.2 m/z and 385.2 m/z for S1P and S1P-d7, respectively. The same product ion of 78.8 m/z was selected for both compounds. In this mode, Q1 and Q3 resolutions were placed to give a 0.7 u at full width at half maximum (FWHM) for both precursor and product ions. The MS/MS experiments were based on collision-induced dissociation (CID) occurring in the collision cell (quadrupole 2), with nitrogen as collision gas set at 10.

**Validation of LC-MS/MS method for measurement of S1P concentrations**

For each day of the validation procedure (n=3), 5 repetitions of the basal level were carried out to establish the response corresponding to the endogenous level of our pool of HDL. This measured basal concentration was then removed in all calculations for the validation procedure. Based on the guidelines of the “Société Française des Sciences et des Techniques Pharmaceutiques” (SFSTP)\(^8\), validation was carried out over three non-consecutive days (p=3). For each day, calibration samples (cal) were prepared in duplicate (n=2), at six concentrations levels (k=6; cal = 1, 2, 5, 10, 50, 100 ng/mL) for the determination of the response function of S1P for each day. In the same way, quality control samples (QC) were prepared independently in quadruplicates (n=4), at four concentrations levels representing the calibration range (k=4; QC = 1, 5, 25, 100). Based on analysis of variance, specific criteria such as trueness, precision, accuracy, linearity and limit of quantification were determined. Matrix effect was also investigated\(^9\).

**Validation.** A linear regression model gave us the most suitable response function, with a weighting factor of 1/x².

**Trueness and precision.** A linear regression model gave us the most suitable response function, with a weighting factor of 1/x². Trueness represented the systematic error of the method and was calculated as the ratio between the absolute and the mean measured concentration. The trueness varied from 88 to 104%. On the other hand, precision represents the random error of the method that is expressed by the repeatability (R\(_{\text{RSD}}\)) and the intermediate precision (IP\(_{\text{RSD}}\)). The intra- and inter-day precision was calculated by computing the relative standard deviation (RSD) at each concentration level of the QC. RSDs were found to be lower than 10%, for every tested concentration levels that is in agreement with the limits fixed by the validation guideline.

**Linearity.** The linearity was expressed as the capacity inside a concentration range to provide results directly proportional to the concentration contained within the sample. It was determined by fitting the back-calculated concentrations of the QCs as a function of the introduced concentrations and by applying the linear regression model based on the least square method. The correlation coefficient for S1P over the concentration range was of 0.99992.

**Accuracy profile and limit of quantification (LOQ).** The sum of the trueness (bias) and the precision (standard deviation), called also accuracy, corresponds to the total error
of the method. The LOQ of S1P was set at 1 ng/mL, since the limit of quantification is defined to be the concentration showing a total error lower than 20%, as presented in Figure A.

**Matrix effect and recovery.** The matrix effect was determined by post-infusion of the deuterated S1P-d7 after injection of the diluted HDL sample (n=6) on the LC-MS system. Neither suppression nor enhancement of the signal was observed at the specific S1P retention time.

Accuracy profile for the determination of S1P using a linear regression with a weighted factor of 1 / \(x^2\). The continuous line represents the trueness, the dashed lines are the upper and lower accuracy limits in relative values and the dotted lines are the upper and lower 20% tolerance limits.

**Statistical analysis**

Values are presented as mean±SEM if not otherwise stated. Differences between data sets were assessed by Student’s t-test (paired or unilateral where applicable), Pearson product-moment correlation coefficient (Pearson’s \(r\)) or ANOVA (repeated measurement and post-hoc tests Bonferroni, Dunnett’s and for trend) (Statistica 7.1, StatSoft). A value of \(p<0.05\) was considered significant.
References


Fig. SI. Immunoblots with anti apoM or anti apoAl of SDS-PAGE profiles of HDL fractions ± glycation.

cHDL and *in vitro* glycation HDL (20µg) were separated by SDS-PAGE and then immunobotted with polyclonal anti-apoM (Abcam, ref 154712). The same membrane was stripped and incubated with polyclonal anti-apoAl (Calbiochem, ref 178422).
Glycated HDL is less efficient than control HDL in inducing phosphorylation of signal pathway proteins.

Primary cultures of cardiomyocytes were stimulated with cHDL or glyHDL 60min before cells were lysed and 30μg of protein was analysed by SDS-PAGE. A representative gel is shown with all bands from same gel (lane removed). Specific phospho-protein expression was quantified and normalized to GAPDH. Non-stimulated cells were set to 100%. n=7 for all groups.
Fig. SIII

HDL is equally efficient in inducing phosphorylation of intracellular signaling proteins in SR-BI knock-downed cardiomyocytes as normal cells.

Cardiomyocytes transfected with Scr-siRNA or SR-BI-siRNA were stimulated with HDL (200 μg/ml) for 5 min (AktSer⁴⁷³) or 60 min (Stat3Ser⁷²⁷, Erk1/2Thr²⁰²/Tyr²⁰⁴ and Stat3Tyr⁷⁰⁵) before cells were lysed. Thirty μg of proteins were analysed by SDS-PAGE. Representative gel is shown with all bands from same gel. Specific phospho-proteins expression were quantified and normalized to GAPDH expression. Results were expressed as percentage of the corresponding non-stimulated cells. n=6 for AktS, Stat3Ser, Erk 1/2; n=4 for Stat3Tyr.
Fig. SIV. *In vitro* glycation of HDL decreases its S1P content.

HDLs with increasing level of glycation were prepared *in vitro* by increasing MG incubation times (2-40h) in parallel with corresponding HDLs without MG (cHDL). S1P concentrations per mg of HDL protein were measured by LC-MS/MS. (n=3 for all groups). cHDL=hatched bars, glyHDL=black bars. *p<0.001, ANOVA for trend across glyHDLs.
Fig. SV. Glycation of BSA:S1P complexes reduces their protective effect against oxidative stress.

A. The level of glycation of BSA:S1P complexes measured in the TNBS-assay. S1P was prepared by resuspension into BSA by repeated vortexing during 30 min at 30°C (3 mg/ml:100 µM) before glycation with MG (6 mM, 24h at 37°C)(glyBSA:S1P) or not (cBSA:S1P) and then dialyzed against water. BSA without S1P was used as control and the mean absorbance for cBSA was set to 100% (n=5 for all groups). B. Cell viability in cardiomyocytes after incubation with DOX measured with the MTT-assay. Cardiomyocytes were pre-incubated for 30 min with BSA or BSA:S1P complexes corresponding to a concentration of 24 µg/ml:800 nM followed by 20h of DOX (0.5 µM) incubation. Results were normalized to DOX-treated cells (100%)(n=6 for all groups). n.s.=not significant. P-values calculated with ANOVA, post-hoc test Dunnett’s (A) and Bonferroni (B).
Fig. SVI.

**Fig. SVI. HDL apoM concentrations as a function of HbA1c levels.**

HDL (20µg) from non-diabetic subjects (cHDL) and diabetic patients (dmHDL) was fractionated by SDS-PAGE then immunoblotted with an anti-apoM monoclonal antibody (Abcam, ref ab47711). A) An example of apoM immunoblots for cHDL and dmHDL (1-6). B) Correlation of apoM (measured as staining intensity of immunoblot) with plasma HbA1c concentrations of diabetic patients.
Fig. SVII. Immunoblots of HDL using anti-apoM and anti-AGE (advanced-glycation end-products) antibodies.

HDL (20µg) from non-diabetic subjects (HDL) and diabetic patients (dmHDL) was fractionated by SDS-PAGE then immunoblotted with an anti-apoM monoclonal antibody (ref ab47711) and polyclonal anti-AGE antibody (ref ab23722) from Abcam, Cambridge, UK. HDL; HDL from non-diabetic control; dmHDL, HDL from diabetic patients.