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Defects of High-Density Lipoproteins in Coronary Artery Disease Caused by Low Sphingosine-1-Phosphate Content

Correction by Sphingosine-1-Phosphate—Loading

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ABSTRACT

BACKGROUND Sphingosine-1-phosphate (S1P) is a constituent of high-density lipoproteins (HDL) that contributes to their beneficial effects. We have shown decreased HDL-S1P in coronary artery disease (CAD) but its functional relevance remains unclear.

OBJECTIVES This study investigated the functional consequences of reduced HDL-S1P content in CAD and tested if increasing it may improve or restore HDL function.

METHODS Human HDL from healthy and CAD subjects, as well as mouse HDL, were isolated by ultracentrifugation. HDL-S1P-dependent activation of cell-signaling pathways and induction of vasodilation were examined in vitro and in isolated arteries using native and S1P-loaded HDL, S1P receptor antagonists, and S1P-blocking antibodies.

RESULTS HDL-S1P-dependent signaling was clearly impaired and S1P content reduced in CAD-HDL as compared to healthy HDL. Both healthy and CAD-HDL could be efficiently and equally well loaded with S1P from cellular donors and plasma. S1P-loading greatly improved HDL signaling and vasodilatory potential in pre-contracted arteries and completely corrected the defects inherent to CAD-HDL. HDL-S1P content and uptake was reduced by oxidation and was lower in HDL_3 than HDL_2. Loading with S1P in vitro and in vivo fully replenished the virtually absent S1P content of apolipoprotein M-deficient HDL and restored their defective signaling. Infusion of erythrocyte-associated C17-S1P in mice led to its rapid and complete uptake by HDL providing a means to directly S1P-load HDL in vivo.

CONCLUSIONS Reduced HDL-S1P content contributes to HDL dysfunction in CAD. It can be efficiently increased by S1P-loading in vitro and in vivo, providing a novel approach to correcting HDL dysfunction in CAD. (J Am Coll Cardiol 2015;66:1470–85) © 2015 by the American College of Cardiology Foundation.

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High-density lipoproteins (HDLs) confer protection against the development of atherosclerosis (1). In addition to their crucial role in reverse cholesterol transport, HDLs have several reverse cholesterol transport–independent properties that potentially contribute to atheroprotection (2,3). Patients with clinically manifest atherosclerosis not only have low plasma HDL cholesterol levels but their HDLs are dysfunctional and harbor harmful metabolites (4). However, the reasons for this dysfunction and the biochemical, structural, and molecular correlates of HDL driving it are largely unknown. Among the most frequently proposed causes are oxidative modifications of HDL proteins and lipids (5,6).

In previous work, we and others have shown that HDLs contain sphingosine-1-phosphate (S1P) that contributes to several beneficial HDL effects, such as nitric oxide-dependent vasodilation and cardioprotection (3,7–11). Recently, we have shown that HDLs from patients with coronary artery disease (CAD) contain considerably lower amounts of S1P than healthy HDLs (12). However, it remains unknown whether this reduced S1P content translates into HDL dysfunction or, conversely, if increasing it may improve or restore CAD-HDL function.

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In the present study, we investigated the functional consequences of low S1P in CAD-HDL. We compared activation of signaling pathways by healthy HDLs and CAD-HDLs and have designed strategies to increase HDL-S1P content in vitro and in vivo to improve HDL function. We also examined whether CAD-HDL signaling may be impaired due to low S1P content and if this can be corrected by S1P-loading.

METHODS

A detailed description of all experimental procedures is provided in the Online Appendix.

In brief, venous blood was drawn from healthy human subjects (n = 70) and patients with stable CAD (n = 64) recruited consecutively to expand the groups used in our previous studies of S1P in CAD-HDL (12). The study was approved by the ethics committee of the University Hospital Essen and complies with the Declaration of Helsinki. Written informed consent was obtained from each participant. Demographic and clinical data are provided in Online Table 1. HDLs were isolated by sequential density gradient ultracentrifugation as described (12) from pooled plasma (mice) or individual samples (humans).

S1P was measured in plasma and HDLs using liquid chromatography tandem mass spectrometry (LCMS) (12) by blinded investigators.

HDLs were loaded with S1P either by incubation with sphingosine-laden human erythrocytes (10 μmol/l sphingosine for 1 h) or by adding 6 nmol S1P (in vitro studies) or 3 pmol S1P (vasodilation studies) to 0.1 mg HDL.

C17-laden mouse erythrocytes (10 μmol/l C17-S1P for 1 h) were extensively washed, diluted to a hematocrit of 0.5, and injected intravenously (150 μl).

STATISTICAL ANALYSIS. Data are expressed by mean ± SD or median (range) for continuous variables and frequency count and percentage for qualitative variables, respectively. Groups were compared by unpaired or paired Student *t* test, Mann-Whitney *U* test, or chi-square test. Statistical significance was assumed for $p < 0.05$.

ABBREVIATIONS AND ACRONYMS

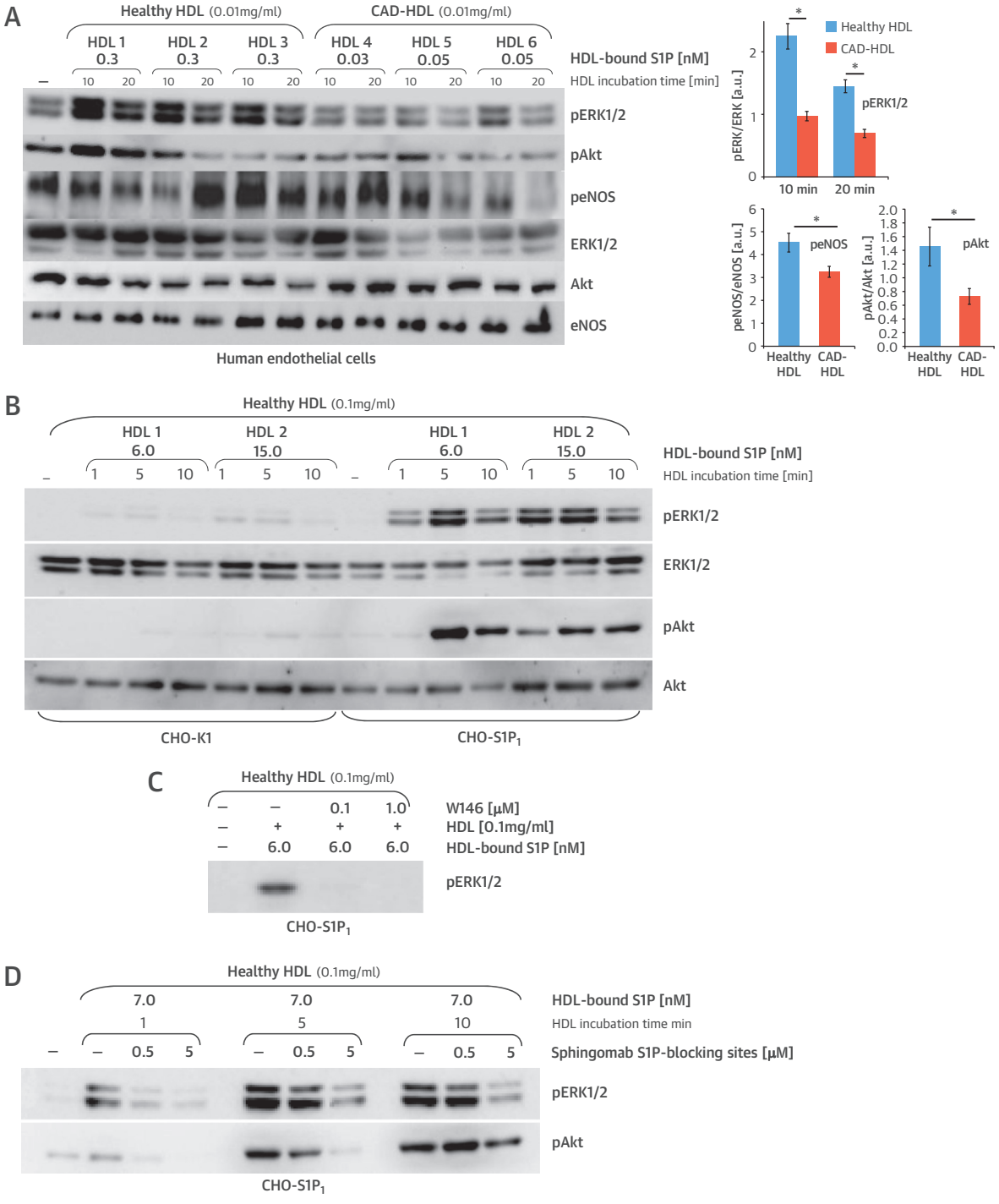
apo = apolipoprotein
CAD = coronary artery disease
CHO = Chinese hamster ovarian cells
DOP = 4-deoxypyridoxine
eNOS = endothelial nitric oxide synthase
ERK1/2 = extracellular signal-regulated kinases 1/2
HDL = high-density lipoprotein
HNF 1A = hepatocyte nuclear factor 1 alpha
HUVEC = human umbilical vein endothelial cell
S1P = sphingosine-1-phosphate

RESULTS

IMPAIRED CAD-HDL SIGNALING DUE TO LOWER S1P CONTENT. We have previously shown that CAD-HDLs contain lower S1P concentrations than HDLs from healthy individuals in a total of 95 healthy subjects and 85 patients with stable CAD (12). To test whether this translates into impaired S1P-dependent HDL signaling, we compared the effectiveness of healthy HDL and CAD-HDL to activate 3 important intracellular signaling cascades in human umbilical vein endothelial cells (HUVEC): the extracellular signal-regulated kinases 1 and 2 (ERK1/2) mitogen activated protein kinases, the Akt pathway, and the endothelial nitric oxide synthase (eNOS). We used Western blotting to detect the active phosphorylation sites of ERK1/2 (Thr202/Tyr204), Akt (Ser473), and eNOS (Ser1177). HDL preparations from 14 healthy subjects and 9 patients with stable CAD (Online Table 2) were chosen randomly and compared. As measured by LCMS, CAD-HDL contained 4 to 5 times less S1P than healthy HDL (45.06 ± 6.74 pmol/mg of HDL protein vs. 273.78 ± 15.01 pmol/mg; $p < 0.05$). Using this experimental setup, we observed that CAD-HDLs were much less efficient than healthy HDLs in activating all 3 kinases both in magnitude and duration of the evoked signaling response when used at the same protein concentration (**Figure 1A**).

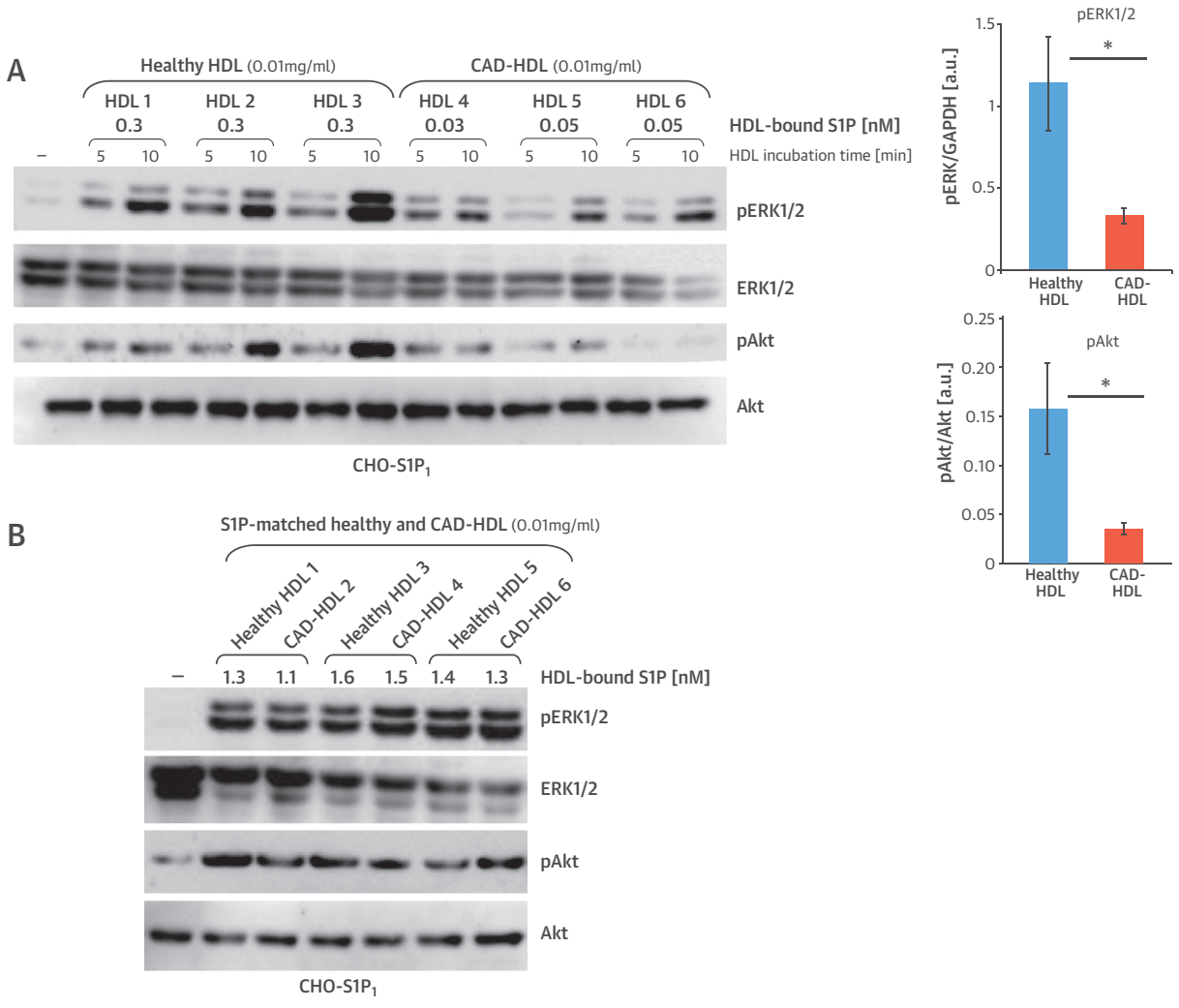
To test if the observed differences can be attributed to the differences in HDL-S1P content, we established a cellular system in which S1P-dependent

FIGURE 1 Compromised Signaling by CAD-HDL Due to Lower S1P Content



(A) Western blotting for activating phosphorylation of ERK1/2 (Thr202/Tyr204), Akt (Ser473), and eNOS (Ser1177) in HUVECs stimulated with healthy and CAD HDLs. The S1P concentration present in an HDL-associated form is expressed as nmol/l (nM) of HDL-bound S1P for each HDL. **(B)** Western blotting for pERK1/2 and pAkt in HDL-stimulated CHO-K1 and CHO-S1P$_1$ cells. **(C)** HDL-induced ERK1/2 phosphorylation in the presence of increasing concentrations of the S1P$_1$ antagonist W146. **(D)** ERK1/2 and Akt activation by HDL after pre-blocking of HDL with the S1P-neutralizing antibody Sphingomab for 30 min. *$p < 0.05$. CAD = coronary artery disease; CHO = Chinese hamster ovary; eNOS = endothelial nitric oxide synthase; ERK1/2 = extracellular signal-regulated kinases 1 and 2; HDL = high-density lipoprotein; HUVEC = human umbilical vein endothelial cell; p = phosphorylated; S1P = sphingosine-1-phosphate.

FIGURE 2 Disparities in HDL-S1P-Dependent Signaling Due to S1P Content Differences

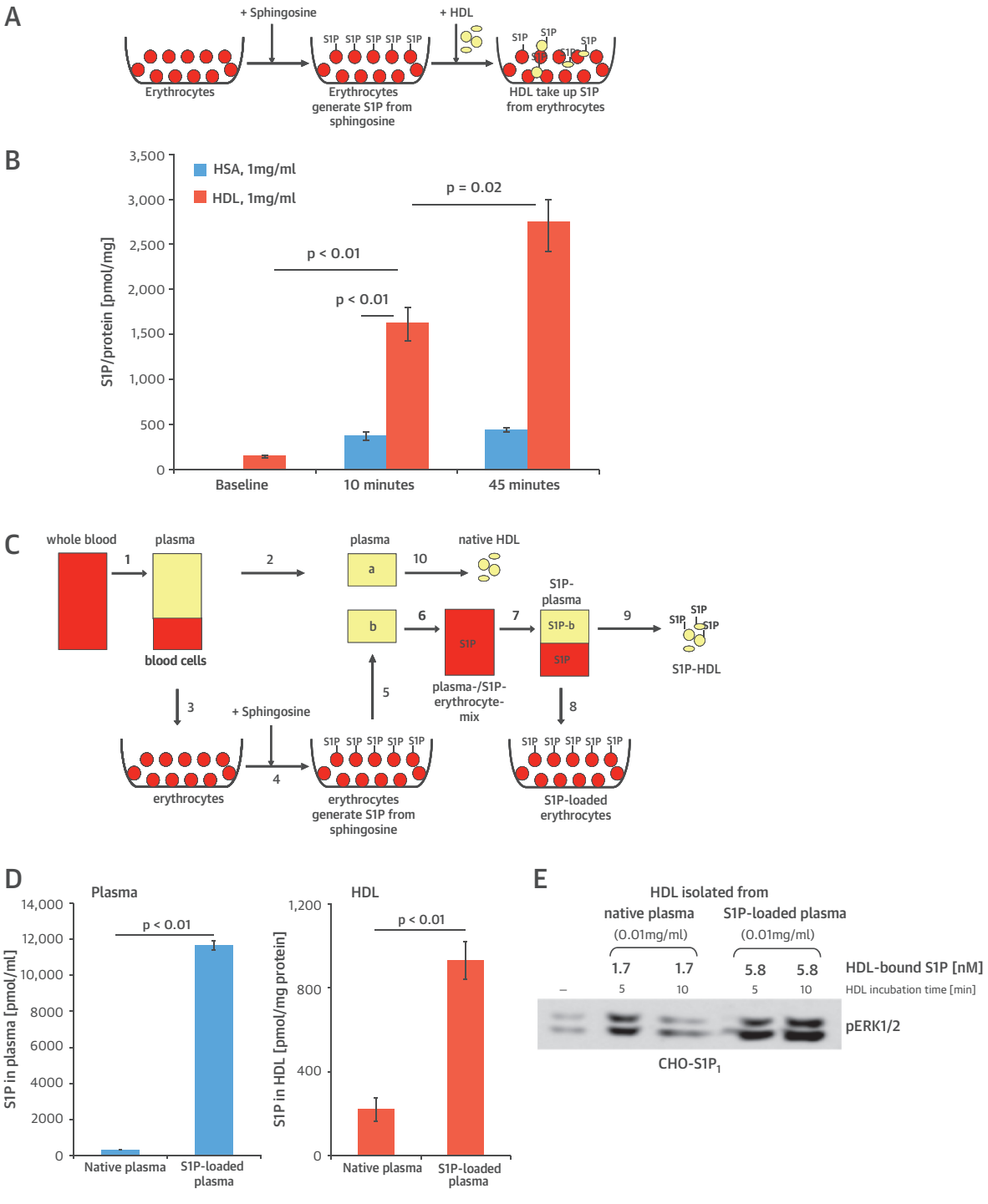


(A) Comparison of ERK1/2 and Akt activation by healthy and CAD-HDL. **(B)** No difference between ERK1/2 and Akt activation by individual pairs of healthy HDL and CAD-HDL matched for an identical S1P content. *p < 0.05. Abbreviations as in Figure 1.

HDL signaling can be reliably identified, quantified, and compared among different HDL preparations. For this, we used Chinese hamster ovarian (CHO) cells that were rendered highly sensitive to S1P by stable overexpression of the human $S1P_1$ receptor (Online Figure 1) and were thus expected to respond with prominent S1P-dependent signaling to any S1P associated with and presented by HDL. Indeed, stimulation with HDL induced potent ERK1/2 and Akt signaling responses in CHO-$S1P_1$ cells compared to a negligible one in control CHO-K1 cells (Figure 1B). We then examined whether HDL-induced ERK1/2 activation was, indeed, dependent on the overexpressed $S1P_1$ by adding the $S1P_1$-specific antagonist W146 and observed that it completely abolished ERK1/2 activation by HDL (Figure 1C). Finally, we directly showed that the HDL signaling response was dependent on their S1P content as pre-incubation of HDL with the S1P-neutralizing antibody Sphingomab (13) abolished the HDL signal in a concentration-dependent manner (Figure 1D).

Comparing healthy and CAD-HDL signaling in CHO-$S1P_1$ cells revealed that CAD-HDLs were much less efficient in inducing ERK1/2 and Akt signaling, similar to the results obtained in HUVEC (Figure 2A). To test whether this may be caused by differences

FIGURE 3 S1P Uptake by HDL From Erythrocytes and Plasma



(A) S1P-loading of HDL from erythrocytes. **(B)** Kinetic of S1P uptake by HDL (n = 12) from erythrocytes. HSA was used for comparison (n = 4). **(C)** S1P uptake by HDL contained naturally in plasma after exposure to S1P-loaded erythrocytes. **(1)** Plasma and erythrocytes were separated. **(2)** Plasma were divided into pools **a** and **b**. Erythrocytes **(3)** were incubated with sphingosine **(4)** to generate S1P, washed, and added **(5)** to plasma pool b for 30 min **(6)**. Plasma and erythrocytes were then separated **(7, 8)** and HDL isolated by ultracentrifugation **(9)**. HDL was also isolated **(10)** from the unexposed pool **b. (D)** S1P levels in native and S1P-loaded plasma **(left)**; S1P content in native HDL and HDL isolated from S1P-loaded plasma **(right)**. **(E)** ERK1/2 phosphorylation by native HDL and HDL isolated from S1P-loaded plasma. HSA = human serum albumin; other abbreviations as in Figure 1.

other than those in S1P content, we compared signaling by 6 pairs of healthy and CAD-HDL samples that were specifically matched for the same S1P content (129.67 ± 9.60 pmol/mg protein in healthy and 122.17 ± 8.24 pmol/mg in CAD-HDLs) despite the overall statistical difference in HDL-S1P between groups (12). ERK1/2 and Akt phosphorylation by these HDL-S1P–matched pairs was virtually identical (**Figure 2B**) suggesting that the poorer signaling observed generally for CAD-HDL was, indeed, due to their lower S1P content.

LOADING HDL WITH EXTERNAL S1P IMPROVES SIGNALING AND VASODILATION. Next we addressed whether and to what extent human HDL can take up additional S1P. To do this, we incubated healthy HDL with human erythrocytes, given that they are the main physiological S1P sources in plasma (14) and considering our observations that they release S1P efficiently only in the presence of HDL (15). To evoke rapid S1P release, we increased erythrocyte S1P content ~4-fold by short pre-incubation with sphingosine (**Figure 3A**). After exposure to the erythrocyte suspension, HDL rapidly and potently took up S1P: HDL-S1P increased ~10-fold after 10 min and ~20-fold after 45 min (**Figure 3B**). In comparison, human serum albumin acquired 10-fold less S1P on a protein basis (**Figure 3B**).

We then asked whether HDL contained naturally in plasma can take up S1P and, if so, how much would be taken up by the entire HDL plasma fraction. To answer this, we isolated erythrocytes and plasma from healthy individuals, S1P-loaded the erythrocytes, and incubated them with their own plasma (**Figure 3C**). HDLs were then isolated by ultracentrifugation and their S1P content compared with that of native HDLs from the same individual (**Figure 3C**). As a result, plasma S1P increased ~10-fold (**Table 1**, **Figure 3D**), HDL-S1P 4.18-fold (**Figure 3D**), and S1P associated with the entire HDL fraction 2.43-fold (**Table 1**). This indicated that HDLs in a normal plasma environment are highly efficient and potent acceptors of S1P. Interestingly, when tested for ERK1/2 activation in CHO-$S1P_1$ cells, plasma S1P-loaded HDLs were more efficient than native HDL (**Figure 3E**).

To pursue this finding systematically, we tested whether S1P-loading per se was able to improve HDL signaling. To do this, we loaded HDL with S1P from erythrocytes and stimulated CHO-$S1P_1$ cells side by side with native HDL. At all concentrations tested, S1P-loaded HDLs were much more efficient in activating ERK1/2 and Akt versus the corresponding native HDL preparations (**Figures 4A and 4B**). This was due to the increased S1P content as the S1P-neutralizing antibody Sphingomab was much less efficient in blocking signaling by S1P-loaded HDL than native HDL (**Figure 4B**). Subsequent experiments with human endothelial cells delivered similar results: S1P-loaded HDL was much more effective than native HDL in inducing ERK1/2 and eNOS phosphorylation (**Figure 4C**). We also confirmed that in human endothelial cells, the $S1P_3$ receptor is required for HDL signaling (9) as the $S1P_3$ inhibitor TY52156 effectively blocked kinase activation by HDL (**Figure 4D**).

We then transferred the in vitro observations to functional arterial vasodilation studies that we performed with healthy native and S1P-loaded HDLs in explanted norepinephrine-pre-contracted rat mesenteric arteries. There, we observed that S1P-loading clearly improved the vasodilator potency of HDL (**Figure 4E**).

HDL-S1P, OXIDATION, AND HDL SUBCLASSES. CAD-HDL is well known to differ from healthy HDL in many structural and metabolic characteristics associated with functional impairment (4,5). We thus hypothesized that the ability of CAD-HDL to contain and take up S1P may be compromised by CAD-associated alterations of the HDL particle. To pursue this experimentally, we examined if 2 such major alterations—CAD-associated oxidative modifications (4,5) and the prevalence of HDL_3 versus HDL_2 in CAD (16)—have an impact on S1P content and uptake by HDL. HDL oxidation resulted in a 44% loss of HDL-S1P compared to native HDL (82 ± 26 pmol/mg vs. 147 ± 77 pmol/mg) (**Figure 5A**). It also reduced S1P uptake by HDL by more than 50% (**Figure 5A**). Western blot analysis of apolipoprotein AI (apo AI) as the major HDL apolipoprotein and apo M as an important S1P binding partner in HDL (11) showed that both proteins were altered by oxidation: apo M was virtually absent in oxidized HDL, whereas apo AI appeared as several

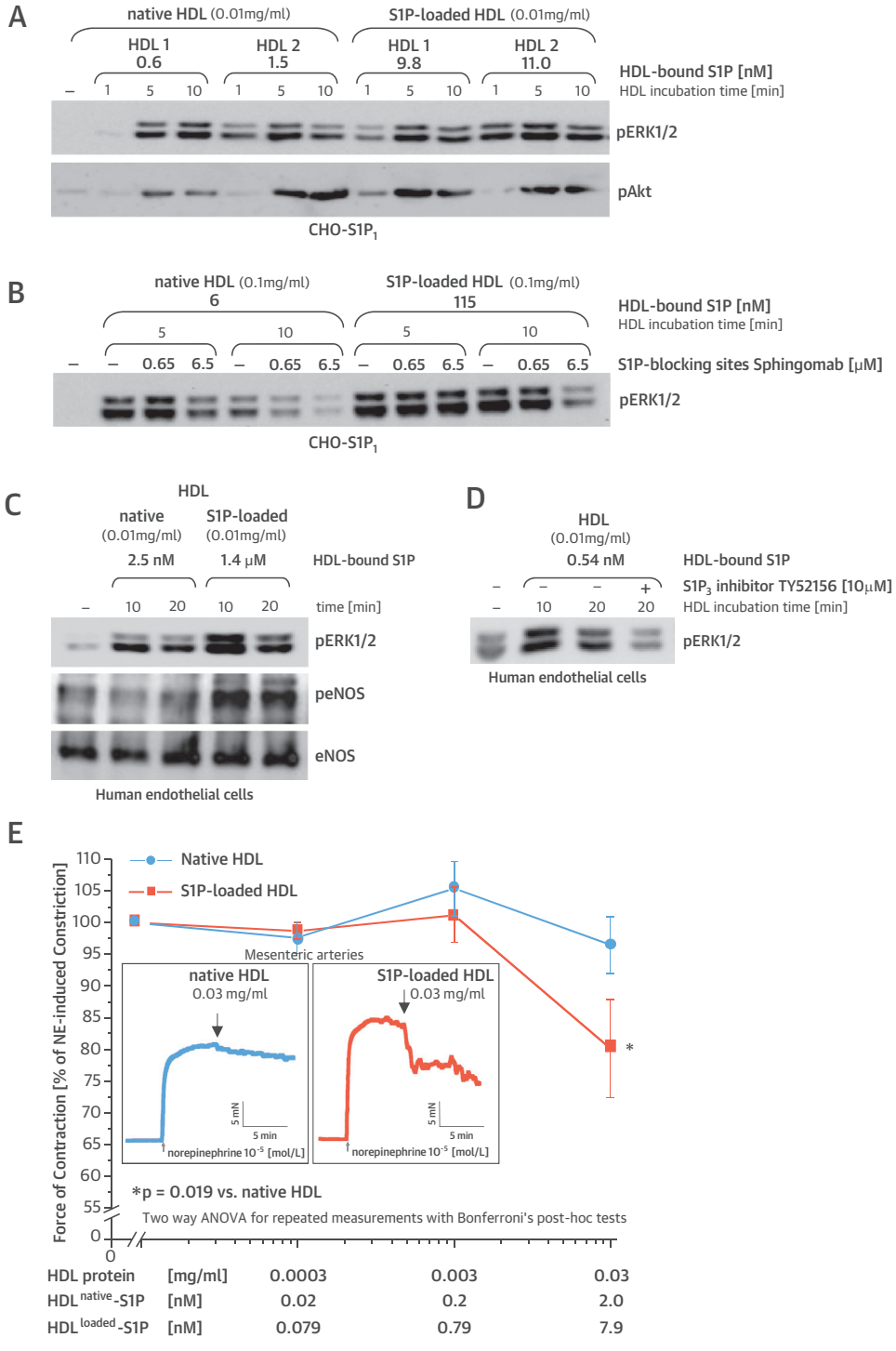
TABLE 1 Characteristics of HDL From Native and S1P-Loaded Human Plasma*

	Native Plasma	S1P-Loaded Plasma	HDL Isolated From Native Plasma	HDL Isolated From S1P-Loaded Plasma
S1P, nmol/l	442 ± 19	11,655 ± 340†	–	–
HDL-C, mmol/l	1.4 ± 0.2	1.0 ± 0.1	–	–
S1P/protein, pmol/mg	–	–	223 ± 56	929 ± 90‡
Cholesterol, mg/ml	–	–	0.56 ± 0.07	0.46 ± 0.05
S1P in the HDL fraction, nmol/l plasma	393 ± 74	954 ± 97†	–	–

Values are mean ± SEM. *Isolated from the same plasma at baseline and after incubation with S1P-loaded erythrocytes. †$p < 0.05$ vs. native plasma. ‡$p < 0.05$ vs. native HDL.

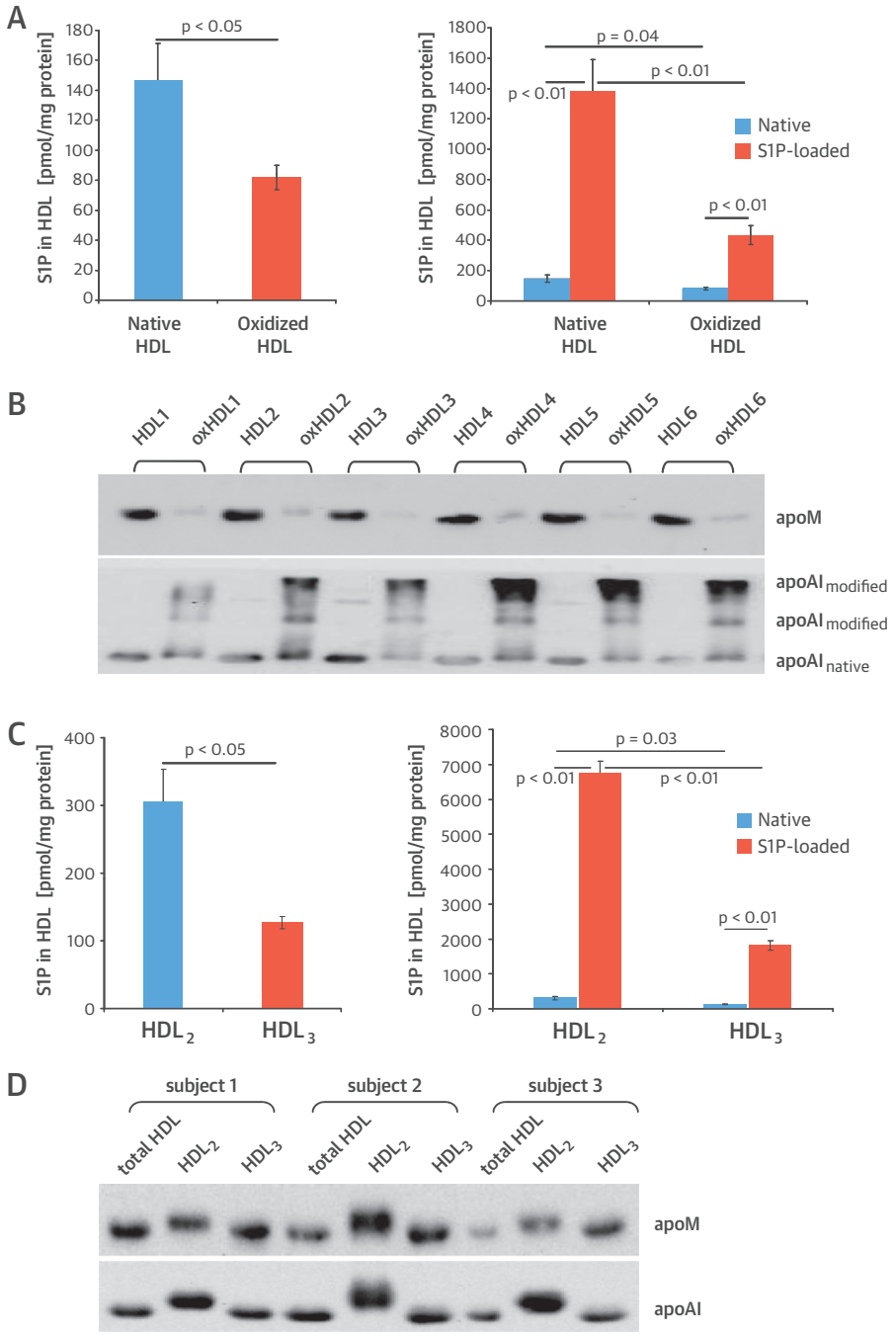
HDL = high-density lipoproteins; HDL-C = high-density lipoprotein cholesterol; S1P = sphingosine-1-phosphate.

FIGURE 4 S1P-Loading of Healthy HDL Augments Signaling and Vasodilator Potential



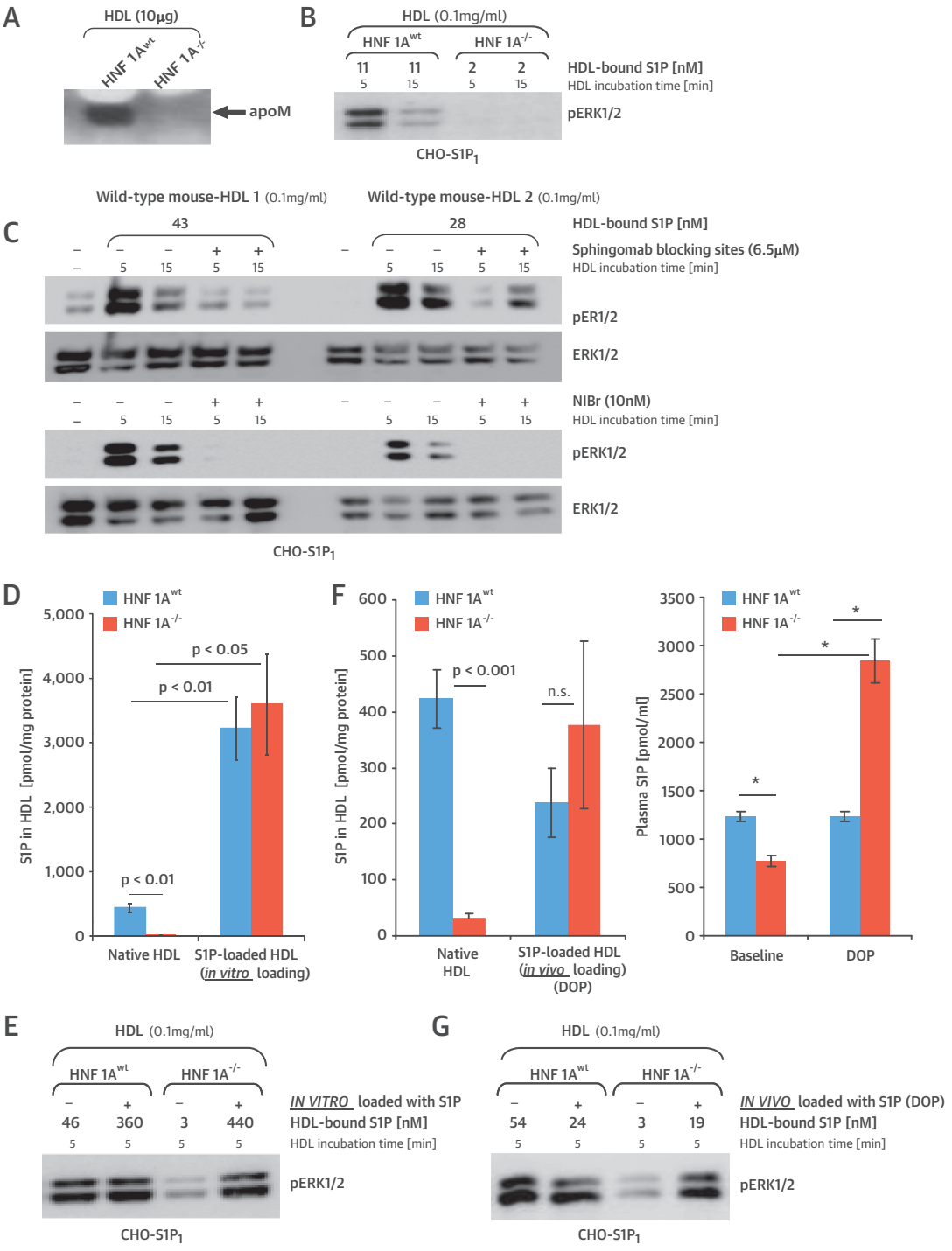
(A) ERK1/2 and Akt activation by native HDL and S1P-loaded HDL (erythrocytes) from the same individuals. **(B)** Sphingomab is less efficient in preventing ERK1/2 phosphorylation by S1P-loaded than native HDL. **(C)** ERK1/2 and eNOS phosphorylation by native and S1P-loaded HDL (direct loading) in HUVEC. **(D)** Inhibition of HDL-induced ERK1/2 phosphorylation by the $S1P_3$ inhibitor TY52156. **(E)** Vasodilation of NE–pre-contracted rat mesenteric arteries by native and S1P-loaded HDL (direct loading) from the same individuals. The corresponding HDL-S1P concentrations are depicted below the protein concentrations. Also shown are representative original tracings of the response to native and S1P-loaded HDL. NE = norepinephrine; other abbreviations as in Figure 1.

FIGURE 5 Effects of HDL Oxidation and HDL Subclasses on S1P Content and Uptake Capacity



(A) S1P content of native and oxidized HDL from the same individuals (n = 6) before and after S1P-loading with erythrocytes. **(B)** Western blotting for apo AI and apo M in the same HDL samples. **(C)** S1P content of human HDL_2 and HDL_3 (n = 5) before and after S1P-loading from erythrocytes. **(D)** Western blotting for apo M and apo AI in HDL_2, HDL_3, and total HDL from the same individuals (10 μg each). apo = apolipoprotein; other abbreviations as in Figure 1.

FIGURE 6 HDL Lacking S1P Due to apo M Deficiency: Correction of Signaling by S1P-Loading



(A) Absence of apo M in HNF 1A$^{-/-}$ HDL on Western blotting. **(B)** ERK1/2 activation by HNF 1A$^{-/-}$ and wild-type HDL (HNF 1A^{wt}). **(C)** Abrogation of ERK1/2 activation by mouse HDL with Sphingomab **(top)** and the S1P$_1$ inhibitor NIBr **(bottom)**. **(D)** S1P uptake by HNF 1A^{wt} and HNF 1A$^{-/-}$ HDL from erythrocytes (n = 4 and 6). **(E)** ERK1/2 activation by native and in vitro S1P-loaded HNF 1A$^{-/-}$ and HNF 1A^{wt} HDL. **(F)** HDL-S1P and plasma S1P in HNF 1A^{wt} and HNF 1A$^{-/-}$ mice treated with or without DOP (6 mg/l for 16 days with the drinking water). **(G)** ERK1/2 activation by HDL isolated from untreated or DOP-treated HNF 1A^{wt} and HNF 1A$^{-/-}$ mice. *p < 0.05. DOP = 4-deoxypyridoxine; HNF = hepatocyte nuclear factor; other abbreviations as in Figures 1 and 5.

immunoreactive bands (Figure 5B). To then address the issue of altered HDL subclass distribution in CAD as a cause for lower HDL-S1P, we determined the S1P content of HDL_2 and HDL_3 and measured their S1P uptake. Interestingly, S1P levels were ~2-fold lower in HDL_3 than HDL_2 on a pmol per protein basis (Figure 5C). Another difference became apparent after S1P-loading: although both HDL_2 and HDL_3 took up S1P efficiently, HDL_3 took up 3.7-fold less than HDL_2 (Figure 5C, right panel). No differences between apo AI and apo M content were evident between HDL_2 and HDL_3 (Figure 5D).

TABLE 2 Characterization of Plasma and HDL in Mice Lacking apo M

	HNF 1A^{wt}	HNF 1A $^{-/-}$	HNF 1A^{wt} After DOP	HNF 1A $^{-/-}$ After DOP
Plasma				
S1P, nmol/l	1,233 ± 56	772 ± 61*	1,236 ± 49	2,844 ± 228†‡
HDL-C, mmol/l	1.95 ± 0.5	4.2 ± 0.3*	2.3 ± 1.0	5.3 ± 2.0
HDL				
S1P/protein, pmol/mg	423 ± 52	32 ± 7*	238 ± 62	376 ± 149
Cholesterol/protein, mg/mg	0.5 ± 0.1	0.5 ± 0.03	0.4 ± 0.01	1.0 ± 0.5

Values are mean ± SEM. *$p < 0.05$ vs. wild type. †$p < 0.05$ vs. the respective genotype without DOP. ‡$p < 0.05$ vs. wild-type mice with DOP (6 mg/l for 16 days).

-/- = knockout mice; DOP = 4-deoxypyridoxine, HNF 1A = hepatocyte nuclear factor 1A; wt = wild type; other abbreviations as in Table 1.

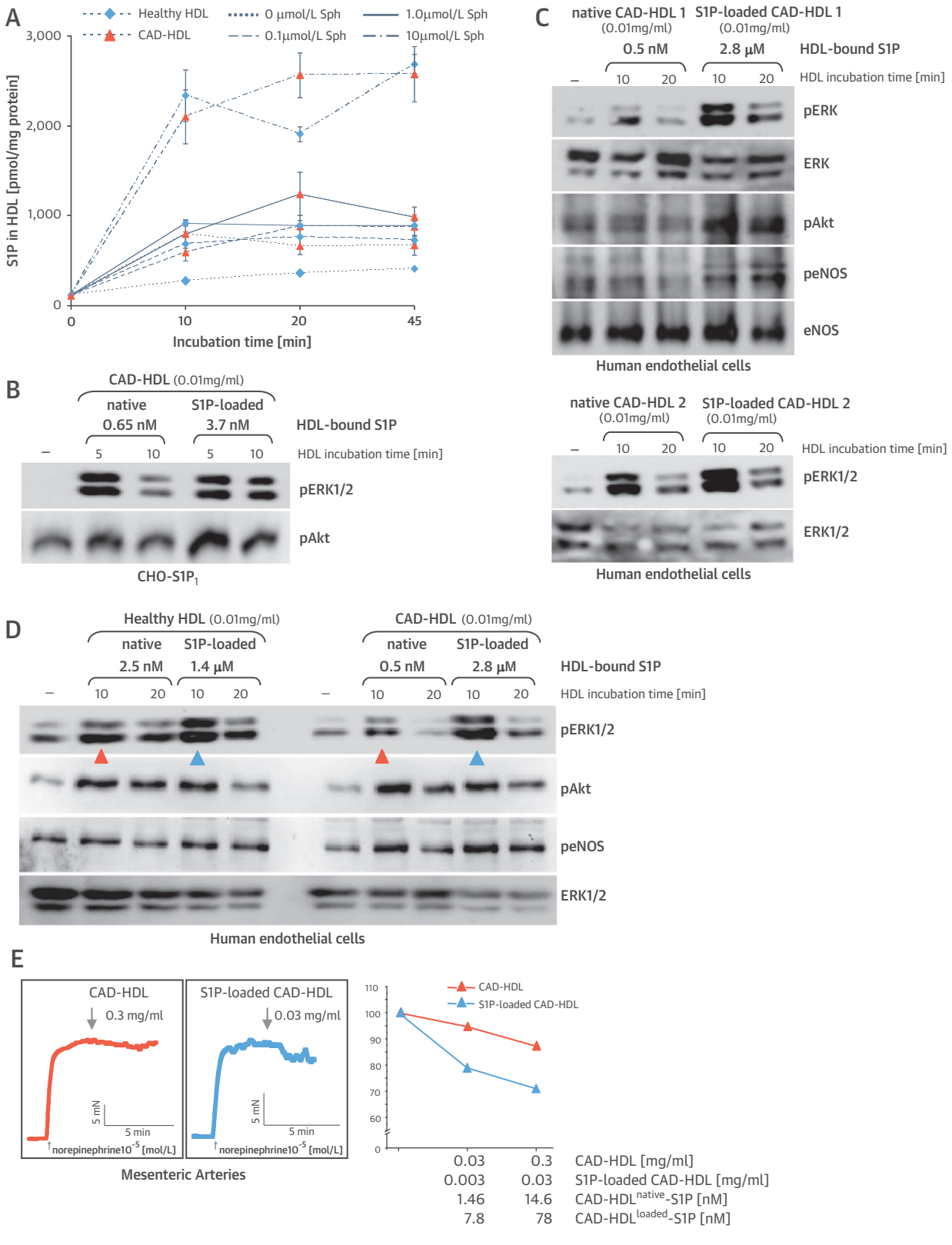
S1P-LOADING CORRECTS HDL DEFECTS IN apo M DEFICIENCY. apo M is the main HDL protein that binds S1P and apo M–deficient mice have severely reduced HDL-S1P, along with distinct HDL signaling defects (11). To test whether apo M may be a limiting factor for S1P-loading of HDL, we examined S1P uptake by apo M–deficient HDL (Figure 6A) isolated from mice deficient for hepatocyte nuclear factor 1 alpha (HNF 1A), the transcription factor indispensable for apo M gene expression (17,18). HNF $1A^{-/-}$ HDL contained virtually no S1P (10-fold less S1P per mg HDL protein than wild-type HDL) (Table 2, Figures 6D and 6F), identical to what has been published for genuine apo $M^{-/-}$ HDL (11), and featured a similar defect in ERK1/2 activation (Figure 6E). The signaling defect observed was due to the lack of HDL-S1P and not to that of apo M as both Sphingomab and $S1P_1$ inhibitors abrogated ERK1/2 signaling by wild-type HDL (Figure 6C). This excluded apo M properties (other than its S1P-binding) to be responsible for the signaling defects of apo M–deficient HDL. Surprisingly, despite the complete absence of apo M, HNF $1A^{-/-}$ HDL efficiently acquired S1P from erythrocytes in vitro and could be S1P-loaded to the same extent as wild-type HDL (Figure 6D). Of note, such S1P-loading completely corrected the signaling defect of apo M–deficient HDL (Figure 6E).

To test whether S1P-loading of HNF $1A^{-/-}$ HDL lacking apo M was also feasible in vivo, we treated mice with low-dose 4-deoxypyridoxine (DOP), a pharmacological inhibitor of the S1P-degrading enzyme S1P lyase. Indeed, HDL-S1P increased ~20-fold and plasma S1P ~3-fold, respectively, in HNF $1A^{-/-}$ mice (Figure 6F, Table 2), reaching levels comparable with or exceeding those in DOP-treated wild-type mice. Of note, the severe signaling defect of HNF $1A^{-/-}$ HDL was completely corrected by DOP as shown by the similar effectiveness of HDL from DOP-treated HNF $1A^{-/-}$ and wild-type at activating ERK1/2 (Figure 6G). These data suggest that even HDLs with severe and inherent S1P deficiency due to the lack of its main binding protein in HDL (apo M) can be efficiently S1P-loaded in vitro and in vivo, and that such S1P-loading will completely correct the signaling defects due to low HDL-S1P.

CONSEQUENCES OF S1P-LOADING FOR CAD-HDL FUNCTION. As S1P-loading was possible both in S1P-proficient human and S1P-deficient mouse HDLs, we examined whether CAD-HDLs can also be S1P-loaded. Using sphingosine-laden erythrocytes (0.1, 1 and 10 μmol/l), we observed that CAD-HDLs took up S1P extremely well and did so as efficiently as healthy HDLs (Figure 7A). Consequently, we tested if S1P-loading of CAD-HDLs improved signaling potential. Even the lowest S1P-loading dose of CAD-HDLs (593 ± 93 pmol S1P/mg taken up from 0.1 μM sphingosine-laden erythrocytes) clearly enhanced ERK1/2 phosphorylation in CHO-$S1P_1$ cells as compared with the native CAD-HDL (297 ± 101 pmol S1P/mg) (Figure 7B). Thus, even a modest 2-fold increase in S1P content was sufficient to improve the signaling potential of CAD-HDL. Also in human endothelial cells, S1P-loading clearly enhanced the ability of CAD-HDL to phosphorylate ERK1/2, Akt, and eNOS (Figure 7C). Interestingly, when comparing signaling by S1P-loaded healthy HDL and S1P-loaded CAD-HDLs, signaling effectiveness of both was increased to the same maximum extent (blue arrows in Figure 7D). Finally, we observed that in norepinephrine–precontracted arteries, S1P-loaded CAD-HDL achieved a similar or even better vasodilation at only one-tenth of the corresponding native CAD-HDL concentration (Figure 7E).

S1P-LOADING OF HDL BY ERYTHROCYTE TRANSFUSION. To directly elevate HDL-S1P in vivo, we loaded mouse erythrocytes with S1P ex vivo and injected them intravenously into C57Bl6 mice (0.91×10^6 per

FIGURE 7 S1P-Loading of CAD-HDL Improves Signaling and Vasodilator Potential



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mouse) (**Figure 8A**). This corresponded to a ~9.4% increase in erythrocyte number (based on 9.65×10^6 erythrocytes/ml blood) and equaled a dose of ~0.9-1.7 nmol erythrocyte-associated S1P per mouse.

To track the fate of injected S1P in vivo, we used the synthetic C17-S1P analogue for loading, as it is easily distinguished from its native C18-analogue by LCMS. Five minutes after intravenous administration, we collected plasma, isolated HDL, and measured C17-S1P content. We found virtually 100% of the injected C17-S1P in the HDL fraction (1.5 ± 0.09 nmol C17-S1P/mg HDL protein corresponding to 1,038 ± 0.06 pmol C17-S1P in the HDL fraction based on 0.76 mg/ml HDL cholesterol in plasma, 0.6 mg HDL-cholesterol/mg HDL protein, and 2 ml blood). Altogether, the total amount of S1P (C17- and C18-) contained in HDL per mg protein increased 4.5-fold compared to native HDL (**Figure 8B**). Thus, S1P administered systemically in vivo using S1P-loaded erythrocytes was acquired predominantly by HDLs and efficiently increased their S1P content.

DISCUSSION

HDLs from patients with manifest atherosclerotic disease display several functional defects, such as impairment of antioxidative and anti-inflammatory properties (4,19). This has been attributed to alterations of the HDL proteome and lipidome (4,6,20) as well as HDL protein and lipid modifications (e.g., oxidation and glycation) (4,5). Recently, we have shown that CAD-HDLs have lower S1P content than healthy HDLs (12). Considering that HDL-S1P contributes to several potentially atheroprotective HDL effects (3,9,21,22) and our findings of CAD-HDL–impaired S1P-dependent signaling, we suggest that reduced S1P content may be another cause for HDL dysfunction in CAD. Indeed, low HDL-S1P in CAD-HDLs translated into reduced ERK1/2, Akt, and eNOS activation, both in $S1P_1$-overexpressing CHO cells and human endothelial cells. Additionally, the potency of HDL-mediated vasodilation in intact arteries depended on their S1P content.

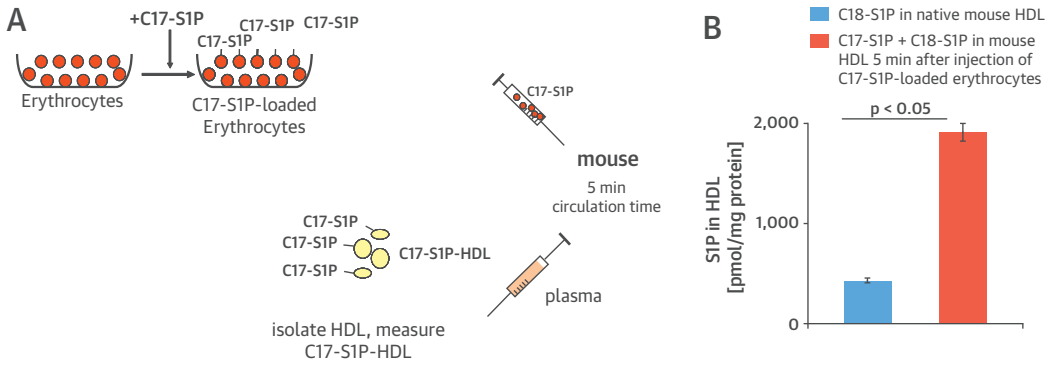
Another novel observation was that healthy HDLs and CAD-HDLs with the same S1P content were equally efficient in inducing signaling. This suggested, at least in our system, that HDL signaling was mainly dependent on their ability to carry, retain, and present S1P. Accordingly, it is important to uncover the molecular determinants of the lower S1P content in CAD-HDL. Although alterations of apo M may be the culprit (especially as its genetic deletion in mice results in low HDL-S1P), variations in plasma apo M have been excluded as a cause of differences in plasma S1P (23), and we did not find HDL–apo M content to differ between healthy and CAD-HDLs (Online Figure 2). Here, we have shown that a reduction of HDL-S1P can be caused by oxidative modifications such as those known to occur in CAD in vivo (4,5). Another cause of lower S1P content in CAD-HDLs may be the prevalence of HDL_3 over HDL_2 in CAD (16) because we observed lower S1P content and inferior S1P uptake in HDL_3 compared to HDL_2. When expressed per mol HDL instead of mg HDL protein (and considering the lower molecular weight and higher protein concentration of HDL_3 compared to HDL_2), our data align with previous observations, in which HDL_3 have been shown to carry more S1P per mol (24). Although apo M and apo AI were altered with HDL oxidation, their levels were similar in HDL_2 and HDL_3, thus excluding simple stoichiometric changes in apolipoprotein/S1P ratios as plausible causes.

While the in vivo reasons for low S1P in CAD-HDL need to be further characterized, the direct consequence for us was to test whether CAD-HDL can be loaded externally with S1P and whether this compensated for innate signaling defects. We have

FIGURE 7 Continued

(A) Time and concentration kinetics of S1P uptake by healthy HDL and CAD-HDL from erythrocytes loaded with different sphingosine concentrations (n = 3 each). All loading times and concentrations resulted in HDL-S1P values higher than those of native HDL. Values obtained with 0.1 and 1.0 μM sphingosine differed only after 45 min, whereas those with 10 μM differed from all others at all times. HDL-S1P was different between 10 and 20 min for 0 and 0.1 μM sphingosine, and between 10 min and 45 min for 0 μM sphingosine. **(B)** ERK1/2 and Akt activation by representative native HDL and low-dose S1P-loaded HDL (same individual) from Figure 7A (the HDL loading was performed with 0.1 μM sphingosine-laden erythrocytes for 10 min). **(C)** ERK1/2, Akt and eNOS phosphorylation by native and S1P-loaded CAD-HDL (direct loading) from the same individuals in HUVEC. **(D)** ERK1/2, Akt and eNOS activation by native and S1P-loaded (direct loading) healthy HDL and CAD-HDL. The **red arrowhead** compares the response to healthy and CAD-HDL before, and the **blue arrowhead** after S1P-loading. **(E)** Vasodilation of NE–precontracted rat mesenteric arteries by native and S1P-loaded CAD-HDL (direct loading) from the same individuals. S1P-loaded CAD-HDLs were used at 10-fold lower protein concentration than native CAD-HDL to show their better effectiveness. The chamber concentrations of HDL-S1P are depicted below the protein concentrations. The original tracings show the response to native CAD-HDL at 0.3 mg/ml and S1P-loaded CAD-HDL at 0.03 mg/ml (10-fold less). Abbreviations as in Figure 1.

FIGURE 8 S1P-Loading of HDL by Erythrocyte Infusion



(A) C17-S1P-loading and administration of erythrocytes and the detection of C17-S1P transfer to HDL. **(B)** S1P content of native HDL and HDL isolated 5 min after infusion of C17-S1P-erythrocytes (sum of natural C18-S1P and administered C17-S1P). Abbreviations as in Figure 1.

provided affirmative answers to both questions (**Central Illustration**). We have excluded a role for apo M in this process by showing that apo M–deficient HNF $1A^{-/-}$ HDLs containing virtually no S1P could be successfully (and to the same extent as control HDLs) loaded with S1P both in vitro and in vivo and that this, too, corrected their severely defective signaling. A recent study also showed that HDLs from regular apo $M^{-/-}$ mice took up S1P normally from erythrocytes (25).

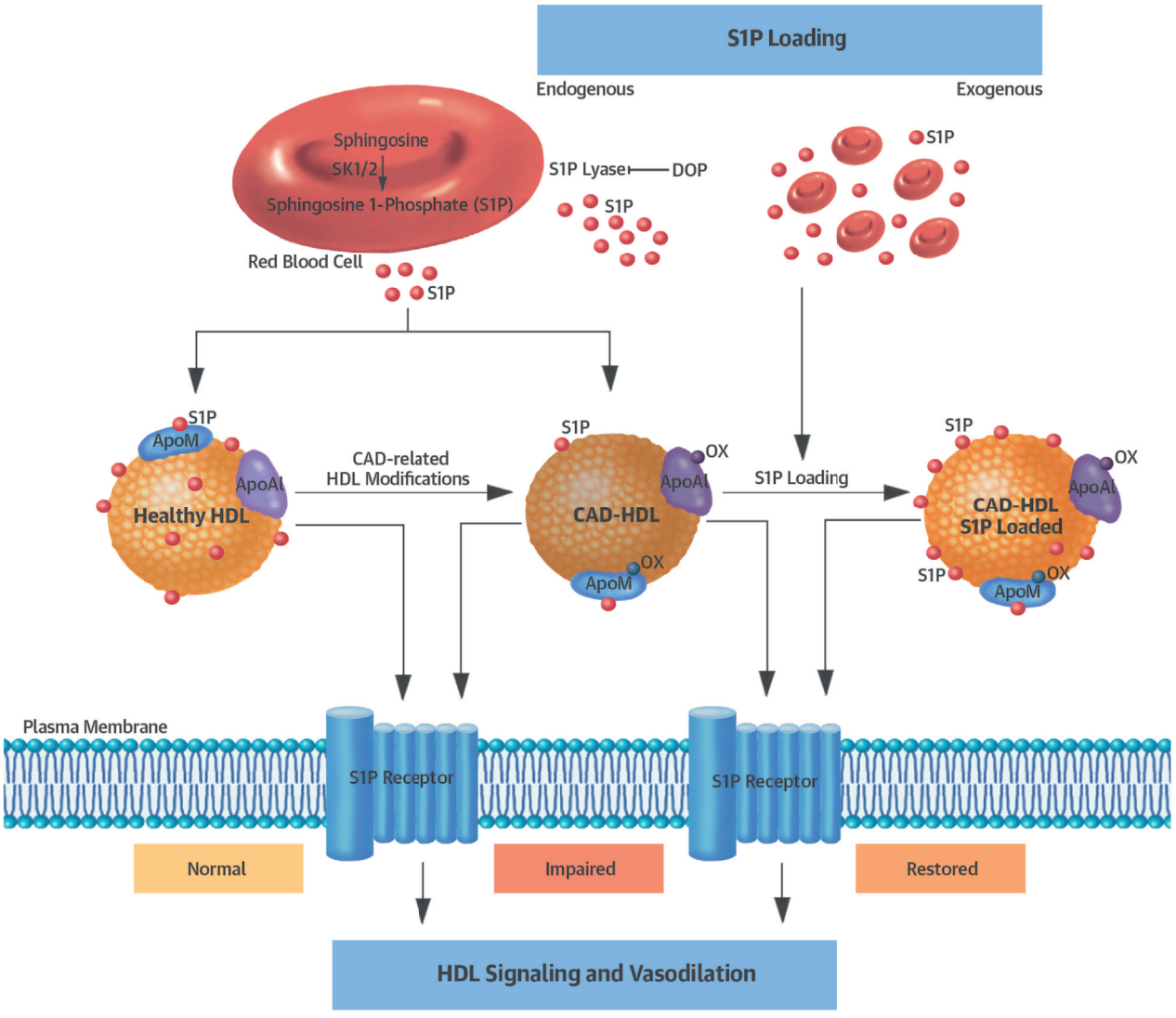
Most intriguing was our observation that increasing HDL-S1P promoted HDL-mediated vasodilation both in healthy HDLs and CAD-HDLs. This provided functional relevance to our findings particularly in respect to studies that have shown an impaired flow-mediated vasodilation (FMD) in CAD that was related solely to low HDL cholesterol (26) and studies showing FMD improvement by administration of reconstituted HDL (27). Considering the speed and effectiveness by which native HDL acquires S1P from cellular donors or plasma, it is conceivable that administering reconstituted HDL (devoid of S1P) would rapidly lead to their uptake of S1P from the environment, thereby improving the HDL-S1P–conveyed part of HDL-mediated vasodilation. The concept of S1P-loading of HDL to achieve better function is supported by recent findings that S1P supplementation improved the impaired ability of glycated HDL to induce cyclooxygenase-2 in endothelial cells (28).

We have shown that S1P-loading of HDL can be successfully achieved in vitro but also in vivo even with apo M–deficient S1P-lacking HDL after pharmacological inhibition of the S1P-degrading enzyme S1P lyase. Most importantly, in vivo loading completely corrected the intrinsic HDL-signaling defect in HNF $1A^{-/-}$ mice caused by low HDL-S1P. To apply this strategy in vivo (while circumventing the undesirable effects of lyase inhibition or direct S1P administration) (3) we injected S1P-loaded erythrocytes to preferentially increase HDL-S1P. This administration route proved to be extremely efficient as almost all of the exogenous erythrocyte-associated S1P was taken up by the HDL fraction after only 5 min. Furthermore, the 4- to 5-fold increase of HDL-S1P we observed was high enough to equalize any differences in S1P existing between healthy HDL and CAD-HDL in humans and might thus suffice to improve CAD-HDL signaling in vivo.

Interestingly, previous research in anemic patients receiving erythrocyte transfusion very similar to our erythrocyte injection showed increased levels of plasma and HDL-S1P (29), hinting at potential applicability in humans. However, despite the efficient S1P-loading of HDLs in vivo, we do not know for how long HDLs retain a therapeutically elevated S1P content. Of note, intra-individual HDL-S1P concentrations remain stable over a period of up to 18 months as we have recently reported for CAD patients (30). Whether S1P-loading will have an impact on general S1P-dependent functions of HDL in vivo, such as cardioprotection, vasodilation, and cell survival (9,21,22), will be the focus of future studies.

STUDY LIMITATIONS. CAD manifests with multiple symptoms and grades of severity prompting us to define clinical inclusion criteria (6 months without

CENTRAL ILLUSTRATION **S1P-Loading Corrects HDL Dysfunction in CAD**



Sattler, K. et al. J Am Coll Cardiol. 2015; 66(13):1470–85.

S1P is generated by phosphorylation of sphingosine by SK1/2 in red blood cells that store and release S1P for uptake by HDLs. In HDL, S1P binds to apo M (and possibly other components when in excess). CAD-related modifications of HDL such as OX diminish HDL-S1P content resulting in impaired HDL-S1P signaling through endothelial S1P receptors. CAD-HDL loading with S1P achieved by pharmacological blockade (DOP) of its degradation by the S1P lyase or by administration of S1P-loaded erythrocytes replenishes HDL-S1P content and corrects HDL dysfunction. apo = apolipoprotein; CAD = coronary artery disease; DOP = 4-deoxypyridoxine; HDL = high-density lipoprotein; OX = oxidation; S1P = sphingosine-1-phosphate; SK1/2 = sphingosine kinases 1 and 2.

the need for revascularization and clinically stable disease). This selection bias has to be corrected in the future by evaluating patients with other CAD conditions as well as by population-wide studies. CAD patients were under medication while controls received none, and the effect of drugs on HDL-S1P remains unknown. Our systems were designed for the highest possible sensitivity to S1P and thus do not account for other functional differences between healthy and CAD-HDL. The measures we have applied to increase S1P content in mice in vivo are not easily transferable in humans. Ideally,

FMD measurements should be used to correlate (and, in case of HDL-S1P–increasing therapeutic intervention, monitor) vasoreactivity and HDL-S1P content in the same individuals, both with normal and impaired FMD. At the same time, these challenges define the next endeavors in this promising research area.

CONCLUSIONS

We have shown that reduced HDL-S1P content contributes to HDL dysfunction in CAD. Our findings suggest that CAD-HDL can be efficiently loaded with S1P, leading to HDL function comparable to that of healthy HDL. This provides a potentially novel therapeutic approach to the correction of HDL dysfunction.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: HDLs' anti-atherogenic effects are mediated, in part, by the bioactive lipid S1P. Dysfunctional HDL has been implicated in the pathogenesis of atherosclerotic CAD, and reduced S1P content is a cause of HDL dysfunction. Increasing S1P restores HDL function.

TRANSLATIONAL OUTLOOK: Clinical studies are needed to investigate the efficacy and safety of increasing the S1P content of HDL in patients with CAD.

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KEY WORDS apolipoprotein M, dysfunction, lipids, lipoproteins, signaling, sphingolipids, vasodilation

APPENDIX For a supplemental Methods section, figures, and tables, please see the online version of this article.