

Endothelial-Vasoprotective Effects of High-Density Lipoprotein Are Impaired in Patients With Type 2 Diabetes Mellitus but Are Improved After Extended-Release Niacin Therapy

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Background—High-density lipoprotein (HDL)-raising therapies are currently under intense evaluation, but the effects of HDL may be highly heterogeneous. We therefore compared the endothelial effects of HDL from healthy subjects and from patients with type 2 diabetes mellitus and low HDL (meeting the criteria for metabolic syndrome), who are frequently considered for HDL-raising therapies. Moreover, in diabetic patients, we examined the impact of extended-release (ER) niacin therapy on the endothelial effects of HDL.

Methods and Results—HDL was isolated from healthy subjects (n=10) and patients with type 2 diabetes (n=33) by sequential ultracentrifugation. Effects of HDL on endothelial nitric oxide and superoxide production were characterized by electron spin resonance spectroscopy analysis. Effects of HDL on endothelium-dependent vasodilation and early endothelial progenitor cell-mediated endothelial repair were examined. Patients with diabetes were randomized to a 3-month therapy with ER niacin (1500 mg/d) or placebo, and endothelial effects of HDL were characterized. HDL from healthy subjects stimulated endothelial nitric oxide production, reduced endothelial oxidant stress, and improved endothelium-dependent vasodilation and early endothelial progenitor cell-mediated endothelial repair. In contrast, these beneficial endothelial effects of HDL were not observed in HDL from diabetic patients, which suggests markedly impaired endothelial-protective properties of HDL. ER niacin therapy improved the capacity of HDL to stimulate endothelial nitric oxide, to reduce superoxide production, and to promote endothelial progenitor cell-mediated endothelial repair. Further measurements suggested increased lipid oxidation of HDL in diabetic patients, and a reduction after ER niacin therapy.

Conclusions—HDL from patients with type 2 diabetes mellitus and metabolic syndrome has substantially impaired endothelial-protective effects compared with HDL from healthy subjects. ER niacin therapy not only increases HDL plasma levels but markedly improves endothelial-protective functions of HDL in these patients, which is potentially more important.

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Key Words: diabetes mellitus ■ endothelium ■ free radicals ■ lipids ■ nitric oxide

Reduced levels of high-density lipoprotein (HDL) are a major risk factor for coronary disease^{1,2} and are predictive of cardiovascular events in patients treated with statins who have low low-density lipoprotein (LDL) cholesterol levels.³ Numerous recent studies have suggested that HDL exerts direct endothelial-protective effects, such as stimulat-

ing endothelial cell production of nitric oxide (NO)^{4,5} and endothelium-dependent vasomotion,⁴⁻⁷ exerting antioxidant effects,⁸ and promoting endothelial progenitor cell (early EPC)-mediated endothelial repair.^{9,10} Notably, however, these studies have been performed with the use of either HDL isolated from healthy subjects or reconstituted HDL. Given

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that HDL-raising strategies are currently being intensely examined as a potential novel therapeutic approach to reduce cardiovascular events,¹¹ it is critical to further characterize the direct endothelial effects of HDL isolated from patients who are strongly considered for HDL-raising therapies.

Clinical Perspective on p 122

Inhibition of the cholesteryl ester transfer protein by torcetrapib resulted in a marked increase of HDL levels; however, this was associated with an increased risk of mortality and morbidity of unknown mechanism in the recent Investigation of Lipid Level Management to Understand its Impact in Atherosclerotic Events (ILLUMINATE) trial.¹² In addition, no beneficial effects on carotid and coronary atherosclerosis progression were observed after torcetrapib therapy.¹²⁻¹⁴ This may, at least in part, be related to inherent adverse off-target effects of torcetrapib (ie, increased blood pressure) that are not observed with anacetrapib, another potent cholesteryl ester transfer protein inhibitor.¹⁵ Conceivably, however, plasma levels of HDL may not represent a reliable surrogate end point to predict vasoprotective effects of HDL-targeted therapies.

Of note, it has been observed that the effects of HDL can be highly heterogeneous. The ability of HDL to promote cholesterol efflux from macrophages by the ATP-binding cassette transporter A-1 pathway has been suggested to be variable and to be reduced by modification of HDL by pathophysiological concentrations of myeloperoxidase.¹⁶ Moreover, HDL isolated from patients with coronary disease exhibited a proinflammatory rather than an antiinflammatory effect, which was partially attenuated in patients who were on statin therapy.¹⁷ In addition, the ability of HDL to counteract the inhibitory effect of oxidized LDL on vascular relaxation was reduced in type 2 diabetic patients.¹⁸

A careful understanding of the effects of HDL from diabetic patients with reduced HDL levels, who are frequently considered for HDL-boosting interventions, compared with HDL from healthy subjects on endothelial cell NO and superoxide production, NADPH oxidase activity, and early EPC-mediated endothelial repair is therefore required. More importantly, investigations assessing the effect of HDL-raising interventions on endothelial properties of HDL are urgently needed and may represent an attractive means to test the potential of such therapeutic approaches (ie, it is conceivable that the increase in HDL confers cardiovascular protection only if pharmacological interventions are associated with improved vasoprotective properties of HDL).

The present study therefore compared the endothelial effects of HDL isolated from patients with type 2 diabetes mellitus (meeting the criteria for metabolic syndrome) and HDL isolated from healthy subjects. In particular, the effects of HDL on endothelial cell NO and superoxide production, NADPH oxidase activity, and early EPC-mediated endothelial repair capacity were examined. Moreover, a randomized controlled clinical study was performed to assess the effect of extended-release (ER) niacin therapy on these endothelial effects of HDL in patients with type 2 diabetes mellitus. Furthermore, potential mechanisms leading to changes in the endothelial effects of HDL were examined.

Table 1. Characteristics of Healthy Subjects and Diabetic Patients

	Healthy Subjects (n=10)	Diabetic Patients (n=33)	P
Age, y	65±10	60±11	0.22
Sex, male/female	8/2	28/5	0.71
Body mass index, kg/m ²	27±3	33±4	0.0007
Waist circumference, cm	91±11	116±13	<0.0001
Mean arterial pressure, mm Hg (metabolic syndrome under antihypertensive therapy)	95±8	97±10	0.58
Hemoglobin A _{1c} , %	5.4±0.5	6.6±1.0	0.0003
Fasting glucose, mg/dL	93±18	128±34	0.009
LDL cholesterol, mg/dL (metabolic syndrome under statin therapy)	145±26	110±27	0.001
HDL cholesterol, mg/dL	53±22	36±6	0.02
Triglycerides, mg/dL	168±94	225±213	0.41
HDL composition			
Cholesterol, mg/dL	20.4±6.3	14.9±7.6	0.05
Triglycerides, mg/dL	2.1±2.9	4.3±3.1	0.07
Protein, mg/dL	65.5±22.1	57.4±12.2	0.22

Values are expressed as mean±SD or number of patients.

Methods

Patient Characteristics and Study Design

Written informed consent was obtained from all participants, and the study protocol was approved by the local ethics committee. HDL and early EPCs were isolated from peripheral blood obtained from healthy subjects and type 2 diabetic patients. Patients with type 2 diabetes mellitus and reduced HDL cholesterol levels (<40 mg/dL in men; <50 mg/dL in women) meeting the criteria for the metabolic syndrome (as defined by the American Heart Association and National Heart, Lung, and Blood Institute Scientific Statement¹⁹ and the International Diabetes Federation Metabolic Syndrome Worldwide Definition²⁰) who were on statin therapy for at least 3 weeks or healthy subjects without cardiovascular risk factors or disease and without medication were included in the study. Endothelium-dependent vasodilation was examined by high-resolution ultrasound as described below. Characteristics of the study participants are shown in Table 1.

Diabetic patients (n=33) were then randomized (1:1) to receive 3-month treatment with ER niacin (Niaspan, Merck KGaA, Darmstadt, Germany) or matching placebo. HDL was isolated after ER niacin or placebo therapy, and endothelium-dependent vasodilation was determined. ER niacin was started at 500 mg/d, and the dosage was increased every month to achieve 1000 and 1500 mg/d, respectively. Placebo was started with 1 tablet per day, and the dosage was increased every month to achieve 2 and 3 tablets per day, respectively. The dose and timing of ER niacin therapy were based on previous observations²¹ to achieve a significant increase of HDL plasma levels. One patient in the placebo group and 2 patients in the ER niacin group discontinued therapy. There was no change in the medication of diabetic patients during the treatment period. Characteristics of the patients in both treatment groups are shown in Table 2.

Isolation of HDL

HDL was isolated from diabetic patients (n=33) and healthy subjects (n=10) from fresh, fasting plasma by ultracentrifugation ($d=1.063$ to 1.21 g/mL) as described previously.^{4,22} Protein, cholesterol, and triglyceride contents of HDL were measured after isolation and are shown in Tables 1 and 2. The concentrations of HDL used in the present study were based on protein content of HDL.

Table 2. Characteristics of Diabetic Patients Before and After Placebo or ER Niacin Treatment

	Placebo (n=15)	ER Niacin (n=15)	<i>P</i> *
Age, y	62±9	58±11	
Sex, male/female	12/3	13/2	
Body mass index, kg/m ²	34±5	32±4	
Mean arterial pressure, mm Hg			0.37
Before	95±9	99±8	
After	92±11	93±9	
Hemoglobin A _{1c} , %			0.18
Before	7.1±1.0	6.3±0.9	
After	6.9±1.0	6.5±0.8	
Fasting glucose, mg/dL			0.32
Before	134±38	123±31	
After	130±28	127±31	
LDL cholesterol, mg/dL			0.96
Before	112±31	107±24	
After	101±29	101±28	
HDL cholesterol, mg/dL			0.001
Before	35±7	36±6	
After	33±5	42±5	
Triglycerides, mg/dL			0.06
Before	290±284	160±62	
After	297±232	118±61	
HDL composition			
Cholesterol, mg/dL			0.28
Before	12.2±3.5	17.5±8.9	
After	12.1±3.7	14.4±4.5	
Triglycerides, mg/dL			0.11
Before	4.5±3.2	4.0±2.6	
After	3.8±2.9	2.2±1.7	
Protein, mg/dL			0.82
Before	55.9±8.9	55.8±12.3	
After	59.2±11.8	58.7±14.1	
Medication			
Angiotensin-converting enzyme inhibitor/angiotensin receptor blocker	10/15	9/15	
Aspirin	12/15	10/15	
Diuretic	5/15	3/15	
Calcium antagonist	4/15	2/15	
β-Blocker	13/15	10/15	
Statin	15/15	15/15	
Oral antidiabetic drug	4/15	5/15	
Insulin	3/15	2/15	

Values are expressed as mean±SD or number of patients.

*The *P* values relate to the statistical analyses of changes (ie, treatment effects) of the ER niacin vs the placebo group.

Effect of HDL on Endothelial Cell NO Production

HDL was administered to cultured human aortic endothelial cells (HAECs), and the effect on endothelial NO production was examined by electron spin resonance (ESR) spectroscopy analysis with the use of the spin-trap colloid Fe(DETC)₂, as described in detail previously.^{23,24} In brief, HAECs were resuspended in 250 μL of Krebs-HEPES buffer (37°C). Then 250 μL of colloid Fe(DETC)₂ (final concentration 285

μmol/L) was added to each sample and incubated at 37°C for 60 minutes. The ESR spectra were recorded with the use of a MiniScope ESR spectrometer (Magnetech). ESR instrumental settings were as follows: center field (B₀), 3280 G; sweep, 198 G; microwave power, 4 dB; amplitude modulation, 8 G; 4096-point resolution; sweep time, 120 seconds; and number of scans, 4. Signals were quantified by measuring the total amplitude after correction of baseline and subtraction of background signals. The mean value of 3 different samples of each subject was used for statistical analysis.

HAECs were obtained from Clonetics (Clonetics Cell Systems, Germany) and cultured in endothelial cell basal medium-2 supplemented with endothelial growth medium SingleQuots, as indicated by the manufacturer.

Effect of HDL on Endothelium-Dependent, NO-Mediated Vasodilation

The effect of HDL on endothelial NO production of intact vessels (ie, the effect on endothelium-dependent, NO-mediated vasodilation) was examined by administration of increasing concentrations of HDL to aortic ring segments of C57/B16 mice (and as a control of endothelial NO synthase [eNOS]^{-/-} mice). Vasorelaxation was determined as described in detail previously.²⁵ In brief, 3-mm ring segments of thoracic aortae were mounted in an organ bath (37°C) and gradually stretched over 1 hour to a resting tension of 1.0g. Maximal vasoconstriction was elicited by depolarization with 80 mmol/L KCl, and rings were washed thereafter. After equilibration and after a submaximal precontracted tone with phenylephrine (80% of 80 mmol/L KCl-elicited contraction) was achieved, the responses of increasing concentrations of HDL were examined.

Effect of HDL on Endothelial Superoxide Production and NADPH Oxidase Activity

The effect of HDL on endothelial superoxide production was assessed in tumor necrosis factor-α-stimulated (100 U/mL, 24 hours) HAECs with the use of ESR spectroscopy and the spin-trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine, as described in detail previously.^{24,26} The effect of HDL on the activity of NADPH oxidase, a major endothelial cell oxidant enzyme system, was examined by ESR spectroscopy, as described previously.^{24,26}

Effect of HDL on Endothelial Repair Capacity of Early EPCs

Early EPCs were isolated and cultured as described in detail previously.^{24,27–29} In brief, peripheral blood mononuclear cells were isolated by density gradient centrifugation with Biocoll (Biochrome, Berlin, Germany), and 10⁷ cells were cultured on fibronectin-coated 6-well plates in endothelial cell basal medium-2 (containing 5 mmol/L glucose) supplemented with endothelial growth medium SingleQuots exactly as indicated by the manufacturer except for hydrocortisone (Clonetics, Inc). After 4-day culture, nonadherent cells were removed by washing plates with PBS. Remaining cells were trypsinized and used for *in vivo* functional analysis.

Early EPCs from diabetic subjects were exposed to placebo (PBS); HDL from healthy subjects, HDL from diabetic subjects, and *in vivo* reendothelialization capacity were assessed as described below. The effects of different HDLs were compared with the use of early EPCs from the same diabetic subject. Male NRM1^{tm/m} athymic nude mice, aged 7 to 10 weeks, were used to allow injection of human early EPCs. Animals were anesthetized with ketamine (100 mg/kg IP) and xylazine (5 mg/kg IP). Carotid artery electric injury was performed as described previously.^{24,30,31} In brief, the left common carotid artery was injured with a bipolar microregulator (ICC50, ERBE-Elektromedizin GmbH, Tübingen, Germany). An electric current of 2 W was applied for 2 seconds to each millimeter of carotid artery over a total length of exactly 4 mm with the use of a size marker parallel to the carotid artery. Early EPCs (5×10⁵ cells) were resuspended in 100 μL of prewarmed PBS (37°C) and transplanted 3 hours after carotid injury via tail vein injection with a 27-gauge needle. The same volume of PBS was injected into control mice. Three days after carotid injury, endothelial regeneration was evaluated by

staining denuded areas with 50 μL of solution containing 5% Evans blue dye via tail vein injection, as described previously.³² The reendothelialized area was calculated as the difference between the blue-stained area and the injured area by computer-assisted morphometric analysis. Of note, this model has been shown to allow accurate quantification of reendothelialization.^{24,30}

NO production of early EPCs was measured by ESR spectroscopy analysis with the use of the spin-trap colloid $\text{Fe}(\text{DETC})_2$, as described in detail previously.²⁴

Animals

The local animal research committee approved all animal protocols. C57/Bl6, eNOS^{-/-}, and NRM1^{tm/m} athymic nude mice were used as described above.

Lipid Peroxidation of HDL

HDL lipid peroxidation was measured by detecting the malondialdehyde content in freshly isolated HDL resulting from the decomposition of unstable peroxides, which was quantified colorimetrically after its controlled reaction with thiobarbituric acid with the use of a TBARS Assay Kit (Cayman Chemical).^{33–35} Furthermore, HDL lipid oxidation was assessed by measurement of the anionic electrophoretic mobility of HDL as determined by electrophoresis on agarose gels and staining with Sudan black (Beckman) as described previously.^{34,36,37} Migration distance was measured by reference to origin.^{34,36,37} It has been described previously that oxidized lipoproteins, in particular oxidized HDL, increase their negative charge and migrate faster in the agarose gel electrophoresis,^{34,38,39} which has been attributed, at least in part, to masking of positively charged lysine residues by lipid decomposition products.^{38,39} HDL lipid oxidation was determined in a randomly selected subgroup of study participants.

Myeloperoxidase-Catalyzed Oxidation of HDL

HDL oxidation by myeloperoxidase-generated nitrating and chlorinating oxidants was performed as described in detail previously.⁴⁰ In brief, HDL was resuspended in phosphate buffer (pH 7.4) containing 20 mmol/L sodium phosphate and 100 $\mu\text{mol/L}$ diethylenetriamine penta-acetic acid (DTPA) at 37°C, and, for modification of HDL by the myeloperoxidase/ H_2O_2 chloride system, the reaction mixture was supplemented with 50 nmol/L myeloperoxidase, 0.1 mol/L NaCl, and 125 $\mu\text{mol/L}$ of H_2O_2 . For modification of HDL by the myeloperoxidase/ H_2O_2 nitrite system, the reaction mixture was supplemented with 50 nmol/L myeloperoxidase, 100 $\mu\text{mol/L}$ nitrite, and 125 $\mu\text{mol/L}$ of H_2O_2 . Oxidative modification of HDL was initiated by adding myeloperoxidase and terminated after 60 minutes by adding 5 mmol/L methionine.

Myeloperoxidase Activity and Content

HDL-associated myeloperoxidase activity was measured as described in detail previously by UV spectrophotometry with the use of guaiacol as the substrate in a randomly selected subgroup of study participants.⁴¹ Briefly, isolated HDL (100 μg protein per well) was dissolved in 20 mmol/L phosphate buffer (pH 7.0) containing 0.34 mmol/L H_2O_2 and 200 $\mu\text{mol/L}$ DTPA. The reaction was initiated by addition of 14.4 mmol/L guaiacol, and the increase in absorbance at 470 nm due to generation of guaiacol oxidation product was recorded at 25°C. Myeloperoxidase activity was calculated from the millimolar absorbance coefficient of 26.6 $\text{mmol/L}^{-1} \cdot \text{cm}^{-1}$ (at 470 nm) for the diguaiacol oxidation product, and 1 unit of myeloperoxidase activity was defined as the amount that consumes 1 μmol of H_2O_2 per minute at 25°C. Myeloperoxidase activity was normalized per microgram of HDL protein.

Myeloperoxidase content of HDL (50 μg protein) was determined by Western blot analysis with the use of a specific antibody to human myeloperoxidase (Dako, Baar, Switzerland).

Binding of HDL to Endothelial Cells

Binding of HDL to HAECs was examined as described in detail previously.⁴² In brief, HDL was iodinated with Na^{125}I by the McFarlane monochloride procedure as modified for lipoproteins. Spe-

cific activities of ≈ 300 to 700 cpm/ng of protein were obtained. Interactions of ^{125}I -HDL (5 $\mu\text{g/mL}$, 50 minutes) with endothelial cells were examined, and specific binding was calculated by subtracting the values of the nonspecific binding from those of the total binding, as described previously.⁴² Because large amounts of HDL were required for the endothelial binding studies, additional diabetic patients with the inclusion criteria of the present study and age- and sex-matched healthy subjects ($n=7$ to 8) were recruited for these measurements.

Measurement of Flow-Mediated, Endothelium-Dependent Vasodilatation

Endothelium-dependent vasodilation of the radial artery and radial artery blood flow were examined as described in detail previously.^{43,44} In brief, radial artery diameters were measured with the use of high-resolution ultrasound (ASULAB). Then an 8-minute wrist arterial occlusion was performed, and change in radial artery diameter in response to reactive hyperemic blood flow was examined. All measurements were recorded, and 2 investigators unaware of the interventions subsequently analyzed vessel diameters. This method is well established in our laboratory and has an excellent reproducibility and variability.^{43,44}

Statistical Analysis

All data are expressed as mean \pm SD. For the statistical analysis, a comparison of endothelial effects of HDL from healthy subjects and diabetic patients was performed with the use of the Mann-Whitney *U* Test. A value of $P < 0.05$ (2 sided) was considered statistically significant. Furthermore, a comparison of the changes of endothelial effects of HDL in diabetic patients (with metabolic syndrome) after ER niacin versus placebo therapy was performed with the Mann-Whitney *U* test. The primary end point for this study was the effect of HDL on endothelial cell NO production after therapy, which was used to determine the study size. The relevant alternative was a change of 20% of endothelial NO production. With the assumption of a common SD of 15%, a sample size of 30 patients randomized 1:1 was needed to have a power of $>90\%$ to reject the null hypothesis in favor of the alternative hypothesis with a 0.05 type I error.

The primary statistical analysis for the comparison of the changes of endothelial effects of HDL in diabetic patients (with metabolic syndrome) after ER niacin versus placebo therapy was performed for the 30 patients who had completed the study. Three patients (1 in the placebo group and 2 patients in ER niacin group) discontinued therapy. A sensitivity analysis was performed assuming the “worst-case scenario,” as described in detail in Results.

The authors had full access to the data and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Participants

Baseline demographic and clinical characteristics of patients with type 2 diabetes mellitus and healthy subjects are shown in Table 1. Participants were included in the study between July 2006 and April 2009.

HDL From Diabetic Patients Loses Beneficial Effects on Endothelial NO Production and Endothelium-Dependent Vasodilation

HDL isolated from healthy subjects, but not HDL from type 2 diabetic patients, markedly stimulated endothelial cell NO production as detected by ESR spectroscopy analysis (Figure 1A and 1B). Moreover, HDL from healthy subjects dose-dependently stimulated endothelium-dependent, NO-mediated vasodilation of intact vessels, an effect that was substantially impaired when HDL from diabetic patients was examined (Figure 1C and 1D). HDL had no effect on

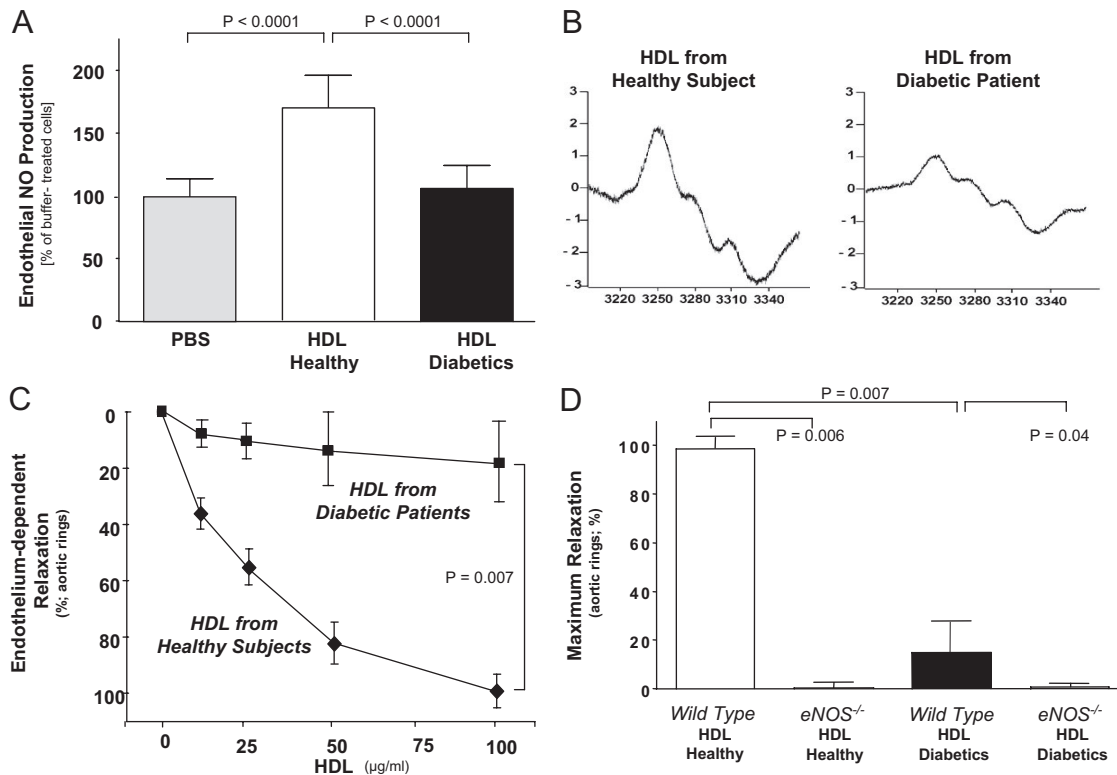


Figure 1. A, Effect of HDL (50 $\mu\text{g}/\text{mL}$, 60 minutes, 37°C) from healthy subjects ($n=10$) and diabetic patients ($n=33$) on endothelial cell NO production as determined by ESR spectroscopy analysis (representative spectra are shown in B). C, Endothelium-dependent relaxations of aortic rings of wild-type mice in response to increasing concentrations of HDL isolated from healthy subjects ($n=5$) or diabetic patients ($n=5$) are shown. The P value indicates the statistical comparison via the Mann-Whitney test between the effect of 100 μg HDL from healthy subjects and the effect of 100 μg HDL from the patient group on endothelium-dependent vasodilation. D, Maximum endothelium-dependent relaxations in wild-type and eNOS^{-/-} mice in response to HDL from healthy subjects and diabetic patients are shown ($n=5$).

endothelium-dependent vasodilation in eNOS-deficient mice, indicating that HDL-induced vasorelaxation was eNOS dependent (Figure 1C and 1D).

HDL From Healthy Subjects but Not From Diabetic Patients Inhibits Endothelial Oxidant Stress

HDL isolated from healthy subjects substantially reduced tumor necrosis factor- α -stimulated endothelial superoxide production and NADPH oxidase activity as assessed by ESR spectroscopy analysis (Figure 2A to 2C), indicating a potent antioxidant effect of HDL from healthy subjects on the endothelium. Notably, however, HDL from diabetic patients had no significant inhibitory effect on endothelial cell superoxide production or NADPH oxidase activity, indicating a loss of these antioxidant effects of HDL on the endothelium in diabetic patients (Figure 2A to 2C).

HDL From Diabetic Patients Has a Reduced Binding Capacity to Endothelial Cells Compared With HDL From Healthy Subjects

The binding capacity of HDL to endothelial cells was analyzed by labeling HDL from healthy subjects and diabetic patients with Na¹²⁵I, as described previously.⁴² Both total and specific binding of HDL from diabetic patients to endothelial cells was significantly reduced compared with HDL from healthy subjects, as shown in Figure 2D.

HDL From Healthy Subjects but Not From Diabetic Patients Stimulates Early EPC-Mediated Endothelial Repair

In vivo endothelial repair capacity of early EPCs from patients with type 2 diabetes mellitus was severely reduced compared with healthy subjects (Figure 3A and 3B), as demonstrated by transplantation of equal numbers of early EPCs from diabetic and healthy subjects into nude mice with denuded carotid arteries and measurement of the reendothelialized area by morphometric analysis 3 days after transplantation (Figure 3A and 3B).

Notably, application of HDL (50 $\mu\text{g}/\text{mL}$, 60 minutes, 37°C) from healthy subjects, but not from diabetic subjects, restored the in vivo endothelial repair capacity of early EPCs of patients with diabetes (Figure 3A and 3B), indicating a loss of the endothelial repair-promoting effect of HDL in diabetic patients. For these studies, HDL from diabetic patients and healthy subjects was added to early EPCs derived from the same diabetic patient (Figure 3A and 3B). HDL from healthy subjects also stimulated the endothelial repair capacity of early EPCs from healthy subjects (Figure 1A in the online-only Data Supplement). Recent studies have suggested that reduced NO production of early EPC is critical for their impaired endothelial repair capacity in diabetic patients.^{24,45} Notably, HDL from healthy subjects, but not HDL from diabetic patients, stimulated NO production of early EPCs (Figure 3C), suggesting that HDL may promote early EPC-mediated endothelial repair by stimulating NO pro-

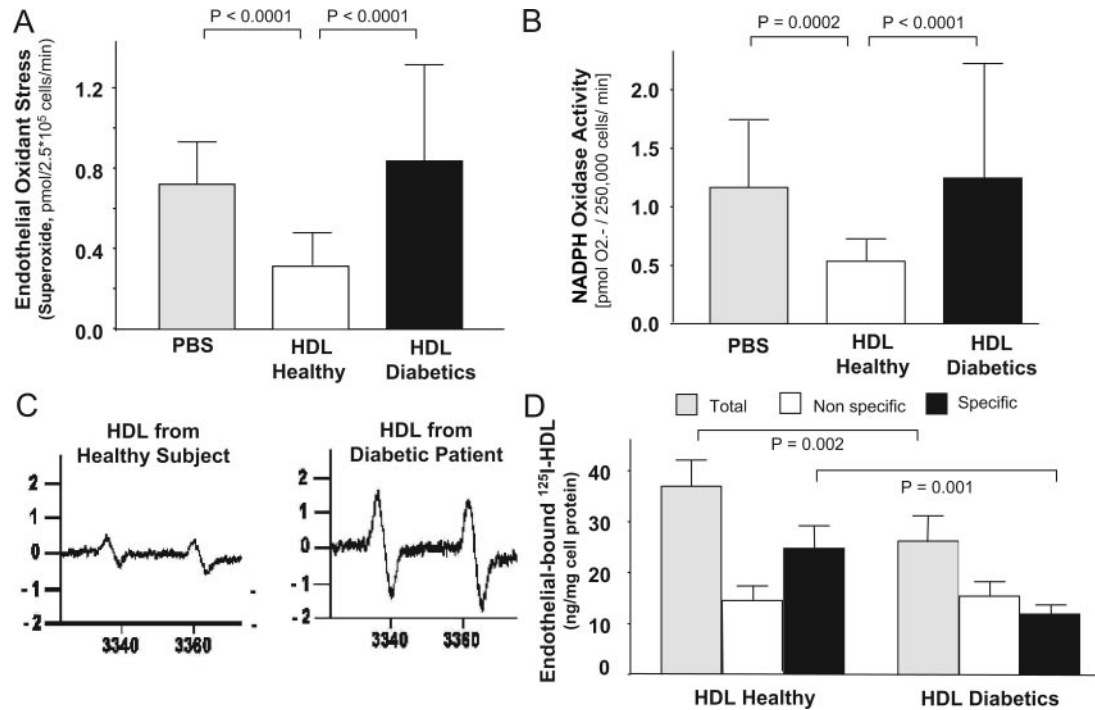


Figure 2. ESR spectroscopy analyses of the effect of HDL (50 $\mu\text{g}/\text{mL}$, 60 minutes, 37°C) isolated from healthy subjects ($n=10$) and diabetic patients ($n=33$) on endothelial oxidant stress (ie, tumor necrosis factor- α -stimulated endothelial cell superoxide production [A] and NADPH oxidase activity [B]) are shown. C, Representative ESR spectra of endothelial superoxide production in response to HDL from a healthy subject and a diabetic patient are shown. D, Total and specific endothelial binding of radioactively labeled ^{125}I -HDL from healthy subjects and diabetic patients to HAECs. Endothelial cells were incubated with ^{125}I -HDL (5 $\mu\text{g}/\text{mL}$) in the absence (total) or in the presence of an excess of unlabeled autogenic HDL (nonspecific). Specific binding of HDL was calculated by subtracting the values of nonspecific binding from the total binding ($n=7$ to 8).

duction. Moreover, inhibition of NO production in early EPCs from diabetic patients (by 1 mmol/L N^G -methyl-L-arginine methyl ester) prevented the effects of HDL from healthy subjects on early EPC-mediated endothelial repair (Figure 1B in the online-only Data Supplement), further supporting the concept that the effect of HDL on early EPC-mediated endothelial repair is, at least in part, mediated by stimulating NO production of early EPCs.

Increased Lipid Peroxidation of HDL From Diabetic Patients

Measurement of both the malondialdehyde concentration and electrophoresis mobility of HDL suggested a substantially increased lipid peroxidation of HDL isolated from diabetic patients compared with HDL from healthy subjects (Figure 4A and 4B). Myeloperoxidase-dependent oxidation of HDL has been suggested as a potential mechanism to alter the effects of HDL on cholesterol efflux from macrophages.¹⁶ Moreover, myeloperoxidase levels have been found to be closely associated with endothelial dysfunction.⁴⁶ We therefore determined the effect of myeloperoxidase-dependent oxidation of HDL on the effects of HDL on endothelial NO production.

Effect of Myeloperoxidase-Dependent Oxidation of HDL on the Capacity of HDL to Stimulate Endothelial NO Production

Exposure of HDL from healthy subjects to both myeloperoxidase-derived oxidants (chlorinating and nitrating oxidant species) rapidly impaired the capacity of HDL to stimulate endothelial NO production (Figure 4E). Moreover, HDL-associated myelo-

peroxidase activity and protein content were significantly increased in diabetic patients compared with healthy subjects (Figure 4C and 4D), compatible with the concept that myeloperoxidase-derived oxidants may contribute to the altered endothelial effects of HDL in diabetic patients.

Endothelium-Dependent Vasodilation in Healthy Subjects and Diabetic Patients

Flow-mediated, endothelium-dependent vasodilation of the radial artery was significantly impaired in diabetic patients ($n=33$) compared with healthy subjects ($n=10$; change in radial artery diameter, $5.1 \pm 1.9\%$ versus $12.0 \pm 2.3\%$; $P < 0.0001$).

ER Niacin Therapy Improves Endothelial-Protective Effects of HDL in Diabetic Patients

ER niacin therapy increased HDL levels in patients with diabetes mellitus (Table 2). Importantly, the capacity of HDL to stimulate endothelial cell NO production was increased in diabetic patients after ER niacin therapy compared with placebo treatment (Figure 5A). The primary statistical analysis was performed for the 30 patients who had completed the study. Three patients (1 in the placebo group and 2 in ER niacin group) discontinued therapy. A sensitivity analysis was performed for the primary outcome, assuming the worst-case scenario (ie, the "best" treatment effect was assumed for the missing follow-up value of the patient in the placebo group, and the "worst" treatment effect was assumed for the 2 missing follow-up values of the patients in the active treatment group).

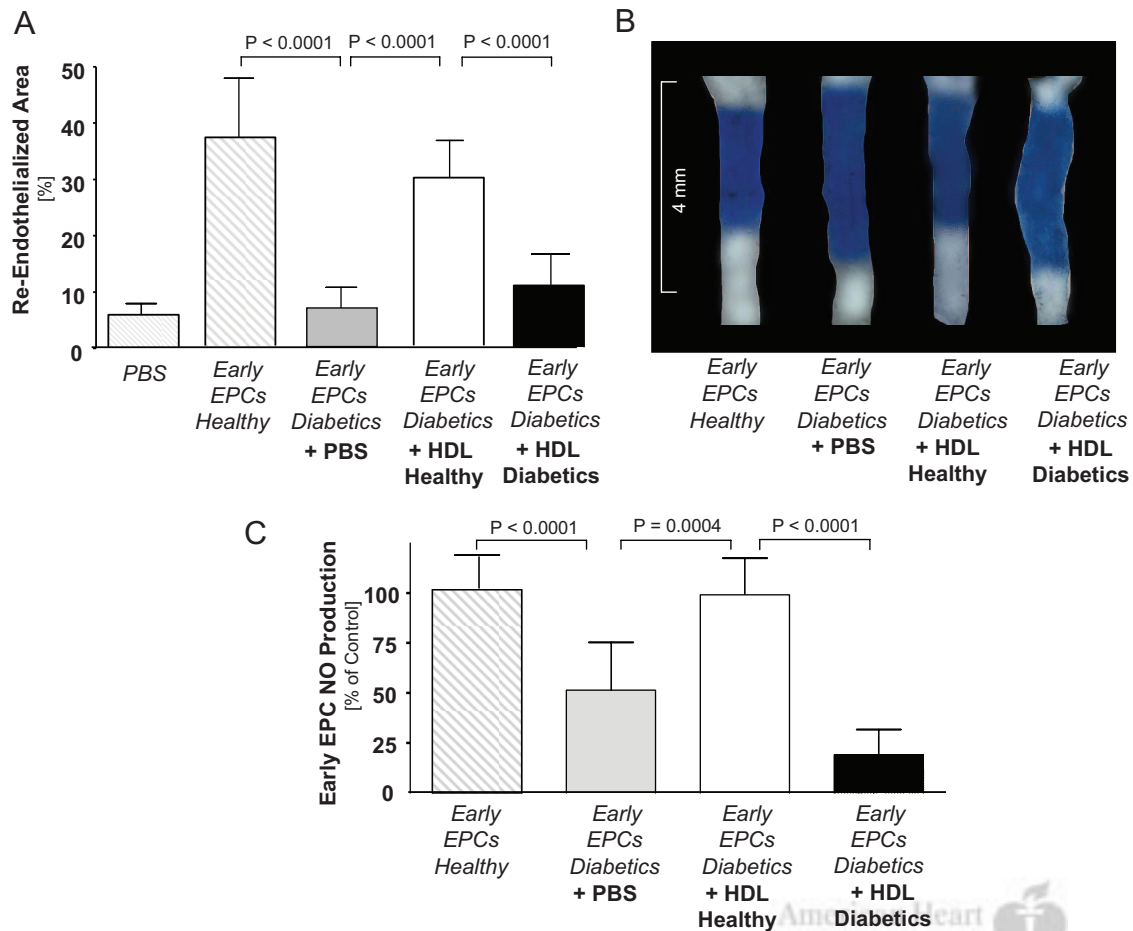


Figure 3. Effect of HDL isolated from healthy subjects and diabetic patients on endothelial repair. A, Reendothelialized area at day 3 after carotid injury in nude mice with transplantation of early EPCs from healthy subjects ($n=10$), diabetic patients ($n=33$), and diabetic patients coincubated with HDL ($50 \mu\text{g/mL}$, 60 minutes, 37°C) from healthy subjects or diabetic patients (each 5×10^5 early EPCs). B, Representative photographs of reendothelialized area. C, Effect of HDL isolated from healthy subjects and diabetic patients on NO production of early EPCs as determined by ESR spectroscopy.

This sensitivity analysis indicated that there still remained a significant effect of active treatment on HDL function (ie, HDL-stimulated endothelial NO production) ($P=0.019$).

Furthermore, HDL isolated from diabetic patients after ER niacin treatment exerted a more potent inhibitory effect on endothelial superoxide production and NADPH oxidase activity, suggesting that ER niacin therapy increased the endothelial antioxidant effects of HDL (Figure 5B to 5D). In addition, HDL isolated from ER niacin-treated patients stimulated endothelium-dependent vasodilation of intact mouse aortic rings significantly better than HDL isolated from placebo-treated diabetic patients (Figure II in the online-only Data Supplement). These findings suggest that ER niacin therapy not only increases HDL levels but exerts a beneficial effect on the endothelial-protective properties of HDL in diabetic patients, which is potentially more important.

ER Niacin Therapy Restores the Effect of HDL on In Vivo Endothelial Repair Capacity of Early EPCs in Diabetic Patients

HDL isolated from diabetic patients after a 3-month treatment with ER niacin had an increased effect on early EPC in vivo endothelial repair capacity (Figure 6A and 6B). Notably, in

diabetic patients after niacin therapy, HDL had an improved effect on early EPC NO production as determined by ESR spectroscopy (Figure 6C).

ER Niacin Therapy and Lipid Peroxidation of HDL in Diabetic Patients

The measurement of electrophoresis mobility of HDL suggested a substantial reduction of lipid peroxidation of HDL in diabetic patients after ER niacin therapy compared with placebo treatment (Figure 7A). The measurement of malondialdehyde content as an indicator of lipid oxidation revealed a lower malondialdehyde content after ER niacin therapy; however, the comparison of the changes of the malondialdehyde content between the ER niacin and placebo groups did not reach statistical significance (Figure 7B). We further determined HDL-associated activity and content of myeloperoxidase, which may promote lipid oxidation, before and after ER niacin or placebo therapy.

HDL-Bound Myeloperoxidase Activity and Content Are Reduced After ER Niacin Therapy

HDL-associated myeloperoxidase activity and content were reduced after ER niacin therapy compared with placebo therapy, compatible with the concept that ER niacin therapy

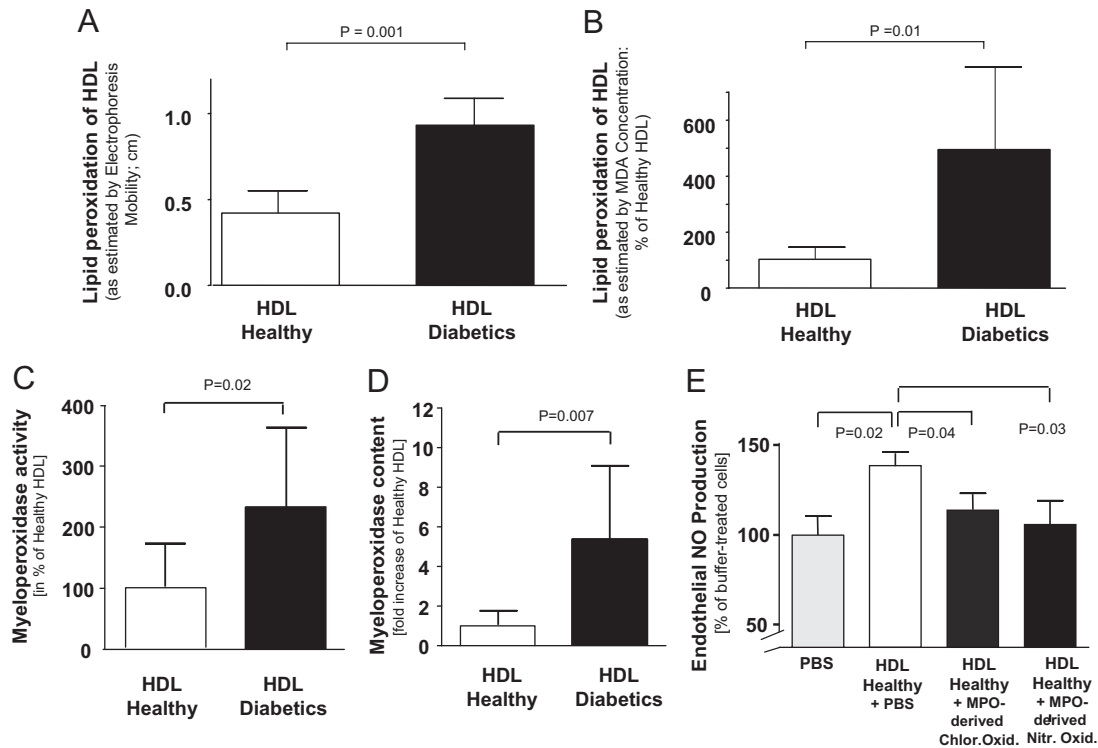


Figure 4. A and B show levels of lipid oxidation of HDL from healthy subjects or diabetic patients as determined by an electrophoresis mobility assay and malondialdehyde (MDA) measurements ($n=5$ to 10 experiments for each column). C and D show content and activity of myeloperoxidase of HDL from healthy subjects or diabetic patients ($n=5$ to 10). E shows the effect of myeloperoxidase (MPO)-derived chlorinating (Chlor. Oxid.) or nitrating (Nitr. Oxid.) oxidants on the capacity of HDL from healthy subjects to stimulate endothelial NO production ($n=4$ to 5).

may reduce the detrimental impact of myeloperoxidase-derived oxidants on HDL in diabetic patients (Figure 7C and 7D).

ER Niacin Therapy Improves Endothelium-Dependent Vasodilatation in Diabetic Patients

After 3 months of treatment with ER niacin, but not after placebo therapy, endothelium-dependent vasodilation was substantially improved in diabetic patients (Figure 8). This was not due to differences in radial artery blood flow during reactive hyperemia. Radial artery blood flow at maximal reactive hyperemia was similar in both groups before (placebo versus ER niacin group, 98 ± 11 versus 87 ± 9 mL/min; $P=NS$) and after placebo or ER niacin therapy (placebo versus ER niacin group, 95 ± 11 versus 99 ± 15 mL/min; $P=NS$).

Discussion

The present study demonstrates that HDL from patients with type 2 diabetes mellitus loses the capacity to directly stimulate endothelial NO production and to reduce endothelial oxidant stress, which is in marked contrast to the effects observed with HDL from healthy subjects. Furthermore, HDL from healthy subjects, but not from diabetic patients, promoted in vivo endothelial repair capacity of early EPCs. More importantly, ER niacin therapy not only increased HDL plasma levels in diabetic patients but also improved the endothelial-protective properties of HDL (ie, HDL isolated from diabetic patients after ER niacin therapy had an improved capacity to stimulate endothelial NO production and early EPC-mediated endothelial repair and to exert antioxi-

dant effects on endothelial cells). This was associated with an improvement of endothelium-dependent vasodilation in diabetic patients after ER niacin therapy. ER niacin therapy may therefore represent a promising strategy not only to increase HDL plasma levels but to restore direct endothelial-protective functions of HDL, which is likely more important.

Reduced plasma levels of HDL are associated with an increased risk of coronary disease and cardiovascular events, even in patients with low LDL levels on statin therapy.³ This has been attributed, at least in part, to HDL-mediated vasoprotective effects. Notably, reconstituted HDL or HDL isolated from healthy subjects has been shown to stimulate endothelial cell NO production, to improve endothelium-dependent vasodilation,⁴⁻⁷ and to exert beneficial effects on early EPC-mediated vascular repair.^{9,10} However, these effects were observed with the use of either reconstituted HDL or HDL from healthy subjects.

Notably, it has been suggested more recently that the effects of HDL can be highly heterogeneous. Modification of HDL by pathophysiological concentrations of myeloperoxidase has been observed to impair the effect of HDL on cholesterol efflux from macrophages by the ATP-binding cassette transporter A-1 pathway.¹⁶ Moreover, HDL from patients with coronary disease exerted a proinflammatory rather than an antiinflammatory effect, which was partially attenuated in patients on statin therapy.¹⁷ In addition, the capacity of HDL to counteract the oxidized LDL-induced impairment of vascular relaxation was reduced in type 2 diabetic patients.¹⁸

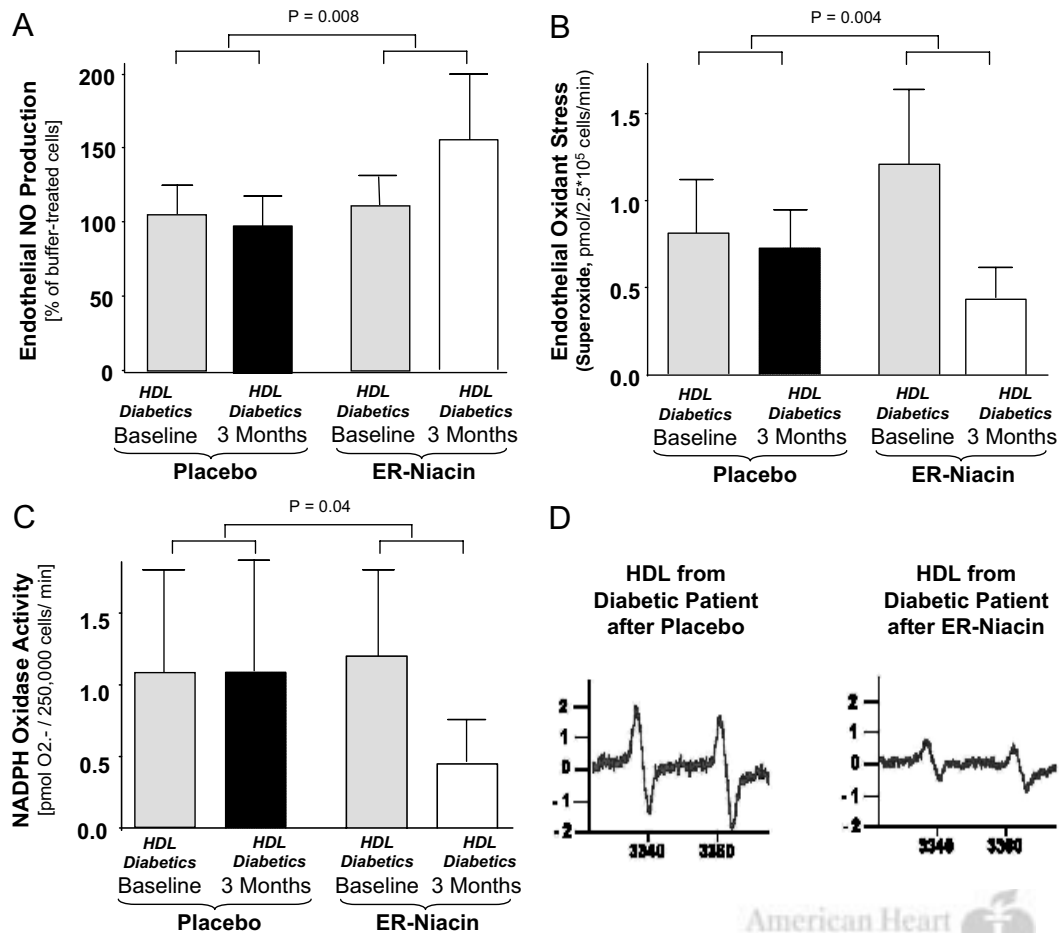


Figure 5. Effect of ER niacin therapy or placebo on endothelial-protective properties of HDL in diabetic patients. ESR spectroscopy analysis of endothelial NO bioavailability (A), superoxide production (B), and NADPH oxidase activity (C) of endothelial cells (tumor necrosis factor- α stimulated) in response to HDL (50 μ g/mL, 1 hour, 37°C) from diabetic patients before and after 3 months of ER niacin therapy (n=15) or placebo (n=15). Representative ESR spectra of superoxide production are shown (D). The P values relate to the statistical analyses of changes (ie, treatment effects) in the ER niacin vs the placebo group.

In the present study, we demonstrate that HDL from type 2 diabetic patients loses its capacity to directly stimulate endothelial NO production, in contrast to HDL isolated from healthy subjects. This is demonstrated both by a failure of HDL from diabetic patients to stimulate endothelial cell NO production and by a loss of the effect of HDL on NO-mediated vasodilation of intact arterial segments. Moreover, we and others have observed that eNOS-derived NO production plays a major role in the *in vivo* reendothelialization capacity of early EPCs,²⁴ and HDL has been suggested to promote early EPC-mediated endothelial repair.¹⁰ In the present study, we demonstrate that the beneficial effects of HDL on early EPC-mediated endothelial repair are markedly impaired in diabetic patients, likely related, at least in part, to a reduced effect of HDL from diabetic patients on NO production of early EPCs.

The present findings therefore support the concept that pharmacological HDL-raising interventions should be examined with regard to their ability to restore the vasoprotective properties of HDL. In fact, recent studies evaluating HDL-raising interventions have yielded mixed results.^{11,12}

The mechanisms underlying the loss of beneficial effects of HDL on endothelial NO production in diabetic patients are likely multifactorial and may include oxidative modification

of HDL, changes in HDL composition, and potentially altered endothelial binding of HDL. In this respect, we have observed an increased lipid peroxidation of HDL from diabetic patients in the present study. Of note, myeloperoxidase has been suggested to modify HDL and its capacity to promote cholesterol efflux from macrophages.¹⁶ Moreover, myeloperoxidase serum levels have been shown to be independently associated with endothelial dysfunction⁴⁶ and to predict risk of coronary disease.⁴¹ Notably, in the present study, we have observed that exposure of HDL from healthy subjects to both myeloperoxidase-generated nitrating and chlorinating oxidants rapidly impaired its capacity to stimulate endothelial NO production, compatible with the notion that myeloperoxidase-derived oxidants may exert effects on HDL to prevent its endothelial NO-stimulating properties. Of note, an increased binding affinity of HDL for myeloperoxidase after myeloperoxidase-mediated oxidation of HDL has been reported recently, which may lead to a vicious cycle of myeloperoxidase transport and myeloperoxidase-dependent oxidation of HDL.⁴⁷ We have therefore determined HDL-associated myeloperoxidase activity and content. Notably, both HDL-associated myeloperoxidase activity and content were increased in HDL from diabetic patients compared with healthy subjects, which is

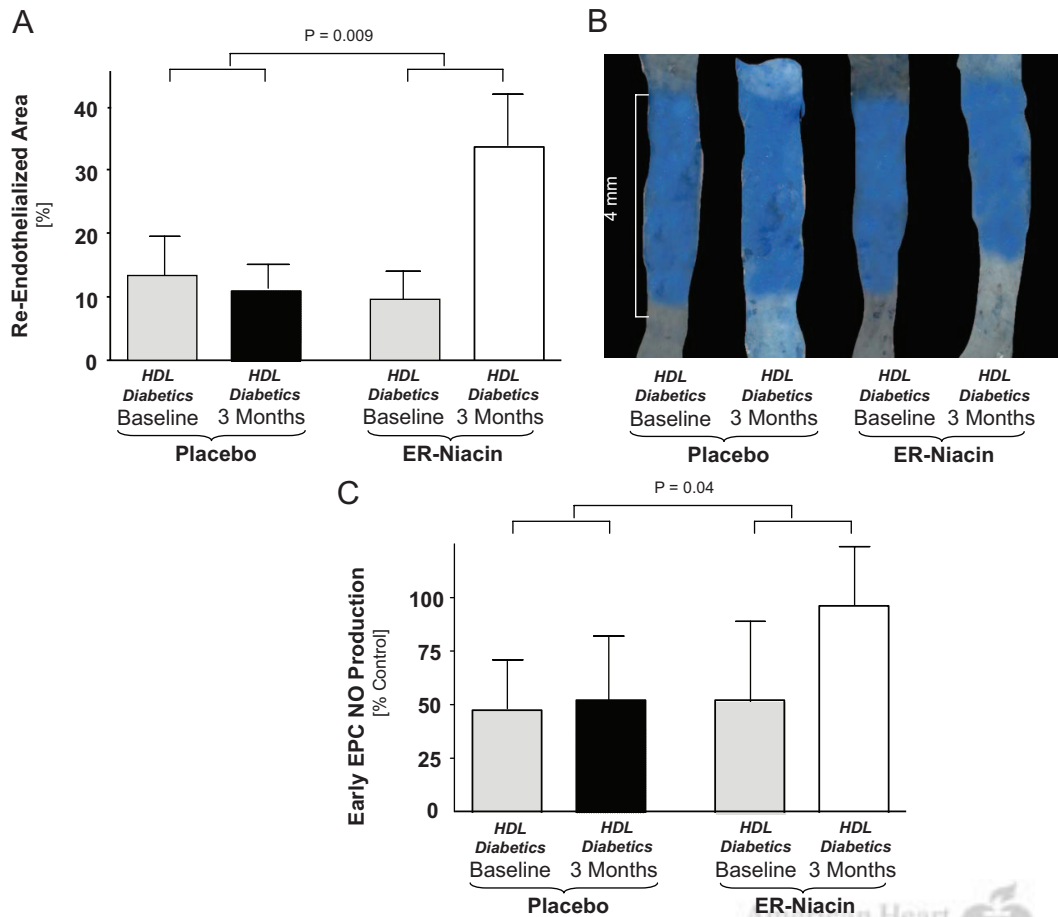


Figure 6. A, Reendothelialized area at day 3 after carotid injury in nude mice with transplantation of early EPCs from diabetic patients, coincubated with HDL (50 $\mu\text{g}/\text{mL}$, 1 hour, 37°C) from diabetic patients before and after 3 months of ER niacin therapy ($n=15$) or placebo ($n=15$). Early EPCs of the same subjects were used for analyses before and after treatment. B, Representative photographs of the effect of HDL on vascular repair. C, Effect of HDL isolated from diabetic patients before and after niacin or placebo therapy on early EPC NO production as determined by ESR spectroscopy. The *P* values relate to the statistical analyses of changes (ie, treatment effects) in the ER niacin vs the placebo group.

further compatible with a role of myeloperoxidase in altered endothelial effects of HDL from diabetic patients.

Furthermore, our endothelial binding studies suggest that HDL from diabetic patients has a reduced binding capacity toward endothelial cells, which may contribute to the altered endothelial effects of HDL from diabetic patients. However, this will likely not completely explain the lack of effect of HDL from diabetic patients on endothelial NO production because there was still detectable specific endothelial binding of HDL from diabetic patients, and a significant NO-stimulating effect of HDL from healthy subjects was observed at low HDL concentrations.

Of note, in the present study diabetic patients were on statin therapy, suggesting that this is not sufficient to restore an endothelial-protective phenotype of HDL. This is in agreement with a recent study by Ansell et al,¹⁷ suggesting that statin therapy attenuates the proinflammatory effect of HDL from patients with coronary disease, but this is not sufficient to lead to HDL with antiinflammatory properties, as is observed in healthy subjects. Therefore, the manner in which the vasoprotective properties of HDL can be further improved in patients at risk is of particular interest.

The present study demonstrates for the first time that ER niacin therapy promotes the endothelial-protective properties

of HDL in type 2 diabetic patients. HDL from diabetic patients after ER niacin therapy, but not after placebo treatment, had an improved capacity to stimulate endothelial NO production, to promote early EPC-mediated endothelial repair, and to inhibit endothelial cell oxidant stress. The mechanisms by which niacin therapy exerts these effects on the vascular properties of HDL remain to be further evaluated; however, they may include reduced lipid oxidation of HDL after ER niacin therapy. In particular, HDL-associated myeloperoxidase activity and content were reduced after ER niacin therapy, which may contribute to reduced oxidation of HDL. The mechanisms whereby niacin may exert these antioxidant effects remain to be determined. Interestingly, recent studies have suggested an upregulation of peroxisome proliferator-activated receptor- γ in monocytes/macrophages and adipocytes after niacin therapy,^{48,49} and peroxisome proliferator-activated receptor- γ agonism has been observed to inhibit hypercholesterolemia-induced leukocyte myeloperoxidase activation,⁵⁰ raising the possibility that this may represent a potential pathway contributing to the antioxidant effects of niacin therapy. Moreover, Green et al⁵¹ have suggested recently that niacin therapy may reverse changes of the

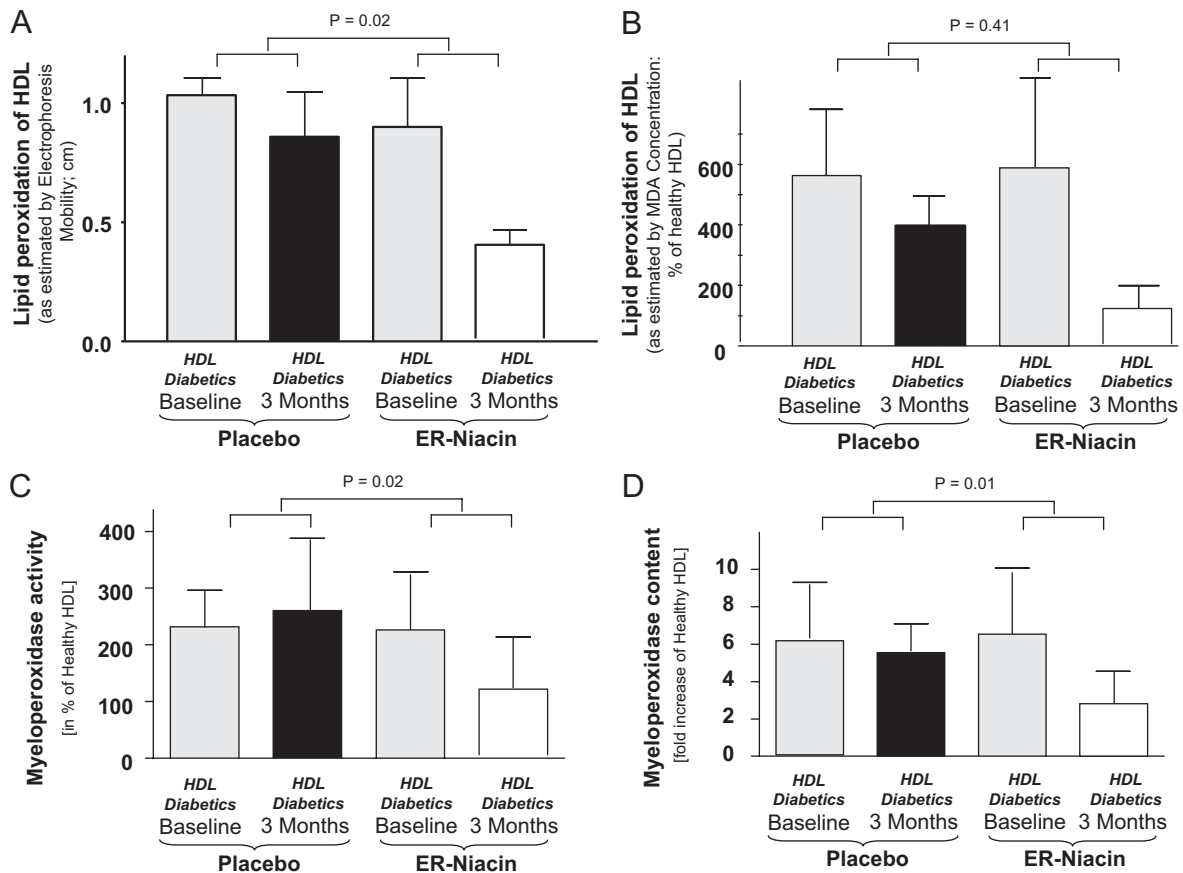


Figure 7. A and B show levels of lipid oxidation of HDL from diabetic patients before and after 3 months of ER niacin or placebo therapy (n=3 to 7 experiments for each column). MDA indicates malondialdehyde. C and D show the content and activity of myeloperoxidase of HDL from diabetic patients before and after niacin or placebo therapy (n=5). The P values relate to the statistical analyses of changes (ie, treatment effects) in the ER niacin vs the placebo group.

HDL proteome, as observed in 6 patients with coronary disease, representing another potential explanation for the different vascular effects of HDL after niacin therapy. In particular, a partial reversal of changes of the HDL-associated proteins apolipoprotein E, apolipoprotein CII, apolipoprotein J, apolipoprotein F, and phospholipid transfer protein in coronary disease has been observed after niacin therapy; however, the underlying mechanisms and functional implications remain to be determined.⁵¹

Notably, ER niacin has been shown to reduce the progression of carotid intima-media thickening over time in patients with coronary disease on statin therapy and with low HDL levels.⁵² Moreover, the combination of niacin and statin therapy has been suggested to exert beneficial clinical and coronary angiographically measurable effects in a moderate-sized study in patients with coronary disease and low HDL levels.⁵³

The present study provides a novel explanation for potential beneficial effects of ER niacin therapy on the progression of atherosclerosis (ie, the restoration of endothelial-protective properties of HDL). Currently, 2 large-scale studies examine the effect of ER niacin therapy on cardiovascular events in high-risk patients (Treatment of HDL to Reduce the Incidence of Vascular Events [HPS2-THRIVE], Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides and Impact on Global Health Outcomes [AIM-HIGH]). Whether cholesteryl ester transfer protein inhibition exerts a similar effect on vascular properties of HDL remains to be determined.

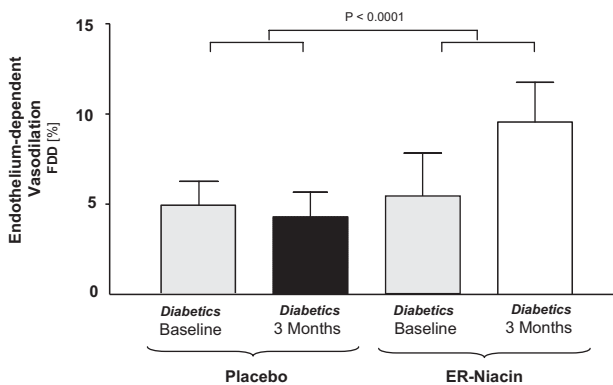


Figure 8. Change in radial artery diameter (FDD) (%) after wrist occlusion during reactive hyperemia in diabetic patients before and after 3 months of ER niacin therapy (n=15) or placebo (n=15). The P values relate to the statistical analyses of changes (ie, treatment effects) in the ER niacin vs the placebo group.

Study Limitations

Given that most study participants of the present study were male, we cannot exclude that there may be different endothelial responses of HDL from female patients compared with male patients with and without ER niacin therapy. A subgroup analysis did not reveal significant differences between the

endothelial responses of HDL from male and female patients; however, this will have to be analyzed further in future studies.

The present study has included healthy subjects and patients with type 2 diabetes mellitus with reduced HDL levels (meeting the criteria for metabolic syndrome). As shown in Table 1, the characteristics of diabetic patients and healthy subjects were significantly different in terms of body mass index, waist circumference, and LDL and HDL cholesterol levels. Therefore, the differences in study outcomes may not necessarily be related to the fact that one group had diabetes mellitus and the other did not but may also be related to the aforementioned factors (ie, to differences in adiposity and lipid levels). The rationale for examining patients with both diabetes mellitus and the components of the metabolic syndrome (ie, increased weight circumference, elevated triglycerides, low HDL) was that these patients are frequently considered for HDL-raising therapies, given their low HDL levels and substantially increased cardiovascular risk. Moreover, the patients were on medication (ie, statin therapy), which may have an impact on the endothelial effects of HDL. Indeed, a recent study has suggested that statin therapy reduces the proinflammatory effects of HDL in patients with coronary disease.¹⁷ However, the rationale for including patients on statin therapy in the present study was that HDL-targeted treatment approaches are currently explored in addition to statin therapy, which represents the first-line treatment most frequently.

Conclusions

In summary, the present study provides novel evidence suggesting that HDL from diabetic patients, in contrast to HDL from healthy subjects, has markedly impaired endothelial-protective effects. Importantly, ER niacin therapy substantially improved vasoprotective properties of HDL and endothelial function in diabetic patients on statin therapy. Because recent studies evaluating HDL-raising interventions have yielded mixed results,^{11,12} circulating HDL cholesterol levels alone likely do not represent an adequate measure of therapeutic efficacy, and indexes of HDL functionality are urgently needed for assessment of the potential of HDL-targeted therapies to exert vasoprotective effects.

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Disclosures

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CLINICAL PERSPECTIVE

High-density lipoprotein (HDL) has long been considered an antiatherosclerotic lipoprotein. This has been suggested on the basis of the inverse association of plasma HDL levels with coronary disease and the risk of cardiovascular events. Furthermore, HDL has been observed to promote reverse cholesterol transport and to exert endothelial-protective effects; however, these effects were demonstrated in HDL from healthy subjects or in reconstituted HDL. The present study suggests that, in contrast to HDL from healthy subjects, HDL from patients with diabetes mellitus and metabolic syndrome loses important endothelial-protective effects, such as the capacity to stimulate endothelial nitric oxide production or to promote endothelial progenitor cell-mediated endothelial repair. These observations are likely important for the understanding of the vascular effects of HDL-raising therapies, which may be particularly vasoprotective if the “on-treatment” HDL exerts beneficial vascular effects. Notably, extended-release niacin therapy, at present the most effective agent in clinical use for increasing HDL, improved the capacity of HDL from diabetic patients to stimulate endothelial-protective effects.

Endothelial-Vasoprotective Effects of High-Density Lipoprotein Are Impaired in Patients With Type 2 Diabetes Mellitus but Are Improved After Extended-Release Niacin Therapy

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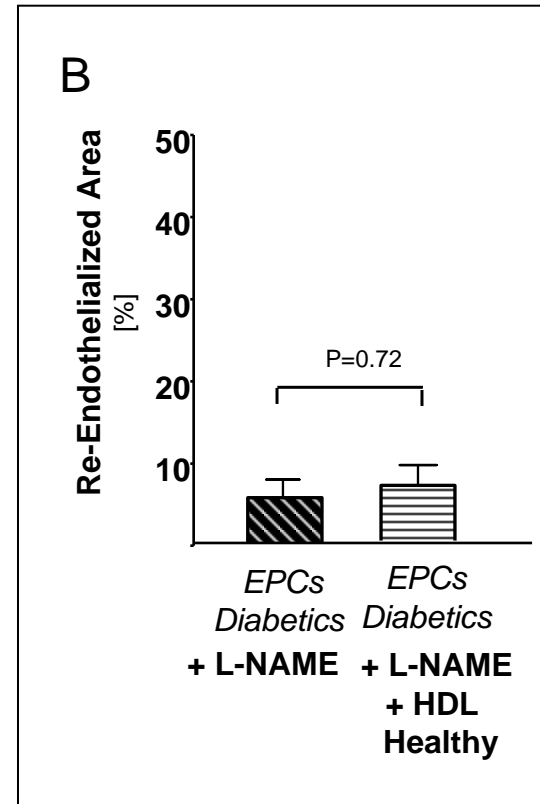
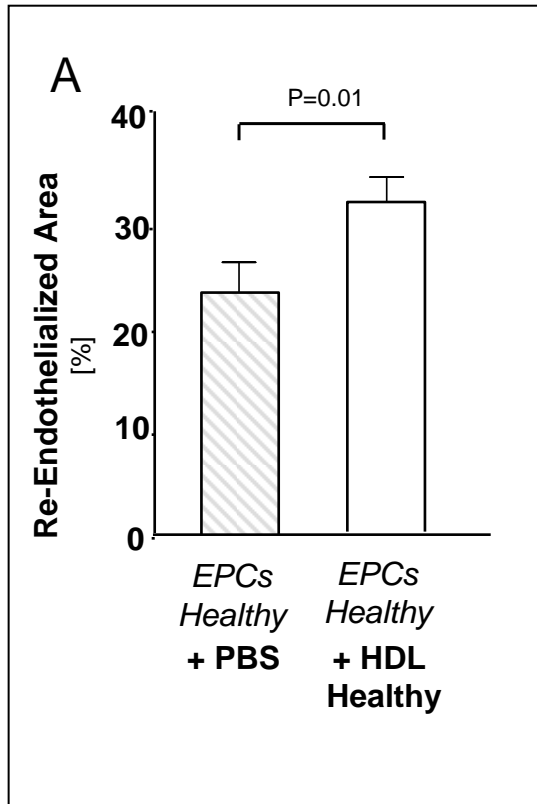
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Supplementary Figure legends

Supplementary Figure 1. A, Effect of HDL isolated from healthy subjects on endothelial repair capacity of endothelial progenitor cells from healthy subjects. Re-endothelialised area at day 3 after carotid injury in nude mice with transplantation of EPCs from healthy subjects with co-incubation with PBS or HDL (50 µg/ml, 60 min, 37°C) from healthy subjects (each 5×10^5 EPCs; n=3). **B,** Role of NO-synthase for the effect of HDL from healthy subjects on endothelial repair capacity of endothelial progenitor cells from diabetic patients. Re-endothelialised area at day 3 after carotid injury in nude mice with transplantation of EPCs from diabetic patients with co-incubation with the NO-synthase inhibitor L-NAME (1 mM, 60 min, 37°C) and coincubation of L-NAME (1 mM) and HDL (50 µg/ml, 60 min, 37°C) isolated from healthy subjects (each 5×10^5 EPCs; n=4-5).

Supplementary Figure 2. Endothelium-dependent relaxation of aortic rings of mice in response to HDL (100 µg/ml) isolated from diabetic patients after 3 months of placebo or extended-release niacin therapy (n=5).

Supplemental Figure 1



Supplemental Figure 2

