

Circulation

JOURNAL OF THE AMERICAN HEART ASSOCIATION



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Circulation 2006, 114:1403-1409: originally published online September 18, 2006
doi: 10.1161/CIRCULATIONAHA.105.607135

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75214

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High-Density Lipoproteins and Their Constituent, Sphingosine-1-Phosphate, Directly Protect the Heart Against Ischemia/Reperfusion Injury In Vivo via the S1P₃ Lysophospholipid Receptor

Gregor Theilmeier, MD*; Christoph Schmidt, MD*; Jörg Herrmann, MD; Petra Keul, BS; Michael Schäfers, MD; Ilka Herrgott, MD; Jan Mersmann, MD; Jan Larmann, MD; Sven Hermann, MD; Jörg Stypmann, MD; Otmar Schober, MD, PhD; Reinhard Hildebrand, MD; Rainer Schulz, MD; Gerd Heusch, MD; Michael Haude, MD; Karin von Wnuck Lipinski, PhD; Christine Herzog, PhD; Martina Schmitz, PhD; Raimund Erbel, MD; Jerold Chun, MD, PhD; Bodo Levkau, MD

Background—All treatments of acute myocardial infarction are aimed at rapid revascularization of the occluded vessel; however, no clinical strategies are currently available to protect the heart from ischemia/reperfusion injury after restitution of blood flow. We hypothesized that some of the cholesterol transport-independent biological properties of high-density lipoprotein (HDL) implied in atheroprotection may also be beneficial in settings of acute myocardial reperfusion injury.

Methods and Results—In an in vivo mouse model of myocardial ischemia/reperfusion, we observed that HDL and its sphingolipid component, sphingosine-1-phosphate (S1P), dramatically attenuated infarction size by ≈20% and 40%, respectively. The underlying mechanism was an inhibition of inflammatory neutrophil recruitment and cardiomyocyte apoptosis in the infarcted area. In vitro, HDL and S1P potently suppressed leukocyte adhesion to activated endothelium under flow and protected rat neonatal cardiomyocytes against apoptosis. In vivo, HDL- and S1P-mediated cardioprotection was dependent on nitric oxide (NO) and the S1P₃ lysophospholipid receptor, because it was abolished by pharmacological NO synthase inhibition and was completely absent in S1P₃-deficient mice.

Conclusions—Our data demonstrate that HDL and its constituent, S1P, acutely protect the heart against ischemia/reperfusion injury in vivo via an S1P₃-mediated and NO-dependent pathway. A rapid therapeutic elevation of S1P-containing HDL plasma levels may be beneficial in patients at high risk of acute myocardial ischemia. (*Circulation*. 2006;114:1403-1409.)

Key Words: lipoproteins ■ inflammation ■ apoptosis ■ endothelium ■ sphingolipids ■ microcirculation ■ reperfusion

The main therapeutic goals in patients with acute myocardial infarction are to minimize myocardial damage, improve cardiac repair, and reduce myocardial remodeling. State-of-the-art therapy is rapid reperfusion of the infarcted myocardium through revascularization of the occluded vessel. However, the benefit of reperfusion is compromised by the endothelial injury and inflammation that follow reinstitution of blood flow, leading to additional myocardial damage, a process termed “ischemia/reperfusion injury.” Despite all efforts to prevent the sequelae of reperfusion injury in

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patients,¹ there are currently no clinical strategies available to effectively protect cardiac tissue from the inflammatory damage inherent to reperfusion.²

High-density lipoproteins (HDLs) are the most powerful independent negative predictor of cardiovascular events evident in all large prospective epidemiological studies.^{3,4} Clinical trials designed to elevate HDL levels have shown that with each increment of 1 mg/dL in HDL cholesterol (HDL-

Received December 9, 2005; revision received July 25, 2006; accepted July 27, 2006.

From the Institute for Anatomy (G.T., I.H., J.M., J.L., R.H., C.H., M. Schmitz), Department of Anesthesiology and Intensive Care (G.T., C.S., I.H., J.M., J.L., C.H.), Department of Nuclear Medicine (M. Schäfers, S.H., O.S.), and Department of Cardiology and Angiology (J.S.), IZKF Münster, University Hospital, Münster, Germany; Department of Internal Medicine (J.H.), Mayo Clinic, Rochester, Minn; Institute of Pathophysiology, Center of Internal Medicine (P.K., R.S., G.H., K.v.W.L., B.L.) and Department of Cardiology (M.H., R.E.), West German Heart Center, University Hospital, Essen, Germany; and Department of Molecular Biology (J.C.), Helen L. Dorris Child and Adolescent Neuropsychiatric Disorder Institute, The Scripps Research Institute, La Jolla, Calif.

*The first 2 authors contributed equally to this work.

Correspondence to Bodo Levkau, MD, Institute of Pathophysiology, Center of Internal Medicine, University Hospital Essen, Hufelandstrasse 55, 45122 Essen, Germany (e-mail levkau@uni-essen.de), or Gregor Theilmeier, MD, Institute for Anatomy, Department of Anesthesiology and Intensive Care, University Hospital Münster, Vesaliusweg 2-4, 48149 Münster, Germany (e-mail theilmeier@anit.uni-muenster.de).

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Circulation is available at <http://www.circulationaha.org>

DOI: 10.1161/CIRCULATIONAHA.105.607135

C), the risk for complications of coronary heart disease decreased by 2% to 3%.⁵ The long-term beneficial effect of HDL has been largely attributed to their key role in reverse cholesterol transport that leads to lipid unloading of the plaque.⁶ However, short-term HDL elevation has also been shown to be beneficial: In patients with acute coronary syndromes, each 1-mg/dL increment of HDL-C during the course of a 16-week treatment with atorvastatin resulted in a 1.4% risk reduction for recurrent adverse events.⁷ Because of such short-term beneficial effects, the potent antioxidative, antiinflammatory, and vasodilatory properties of HDL have been implicated in the stabilization of vulnerable coronary lesions.⁸ We hypothesized that in addition to atheroprotection, such properties may be also beneficial for the coronary microcirculation in settings of myocardial reperfusion injury. Direct effects of HDL on postischemic myocardium *in vivo* have not yet been reported.

The constituents of the HDL particle that mediate its diverse biological effects are still under investigation.⁸ Recently, we and others have identified several sphingolipids, such as sphingosine-1-phosphate (S1P), as constituents of human HDL and have found them responsible for part of the nitric oxide (NO)-mediated vasodilatory effect of HDL.^{9–11} Acute administration of reconstituted HDL has been shown to normalize the endothelial dysfunction of hypercholesterolemic patients and individuals heterozygous for ABC1 in an NO-dependent manner.^{12,13} Because NO is also a crucial player in myocardial ischemia/reperfusion injury,¹⁴ we hypothesized that HDL in general and its S1P content in particular may have a beneficial effect on myocardial damage after ischemia/reperfusion.

Methods

Materials

HDL ($d=1.125$ to 1.210 g/mL) and low-density lipoprotein (LDL; $d=1.019$ to 1.069 g/mL) was isolated from human plasma as described previously¹⁰ and administered intravenously to mice bred in the central animal facility of the University Hospital Münster in 100 μ L of 0.9% saline per 10 g of body weight. S1P (Sigma, Taufkirchen, Germany) from methanol stocks was air-dried and dissolved in phosphate-buffered saline/1% bovine serum albumin and administered intravenously in 100 μ L/10 g. *N*^G-nitro-L-arginine methyl ester (L-NAME) administration (1.5 μ g/mL drinking water) was initiated 21 days before ischemia and continued until the end of the reperfusion. Mortality due to the ischemia/reperfusion procedure was not different among treatments.

Myocardial Ischemia/Reperfusion in Mice

HDL and S1P treatments were assessed in an outbred Swiss strain of mice. All studies were repeated in the C57Bl6 background of the S1P₃^{-/-} mice to ensure strain independence of the effects. A total of 74 Swiss, 23 C57Bl6/N, and 19 S1P₃^{-/-} mice grouped in age- and sex-matched clusters of the different treatment modalities were used for infarct-size measurements. For histology, 17 additional Swiss mice were used. Transient myocardial ischemia (30 minutes) followed by 24-hour reperfusion was inflicted with the approval of the Institutional Review Board. Briefly, in barbiturate-anesthetized mice, thoracotomy and ligation of the left anterior descending coronary artery at the level of the left atrium was performed with silk 7-0 sutures tied transiently over PE10 tubing for 30 minutes. The chest was closed, and the animals were weaned from the ventilator. For leukocyte depletion, 2 mg of hydroxycarbamide per gram was administered intraperitoneally 2 days before ligation, with another 1 mg/g given on the following day (Bristol-Myers Squibb, Brussels, Belgium). For infarct-size measurements, animals were reanesthetized and perfused with 0.9% saline through the abdominal aorta.

The coronary ligation was retied, and 2% Coomassie Blue was injected to delineate the area at risk. The heart was sectioned into 5 equal slices and immersed in 2,3,5-triphenyltetrazolium chloride (Sigma, Taufkirchen, Germany) at 37°C for 10 minutes. Left ventricular area, area at risk (AAR), and area of infarction were determined morphometrically as previously published with NIH Image software.¹⁵ In untreated mice, left ventricular cross-sectional area was 13.9 ± 0.7 mm². Ligation resulted in an ischemic area of 7.6 ± 0.5 mm² ($n=7$), which constituted the AAR, and the infarcted area was 3.4 ± 0.4 mm² ($n=7$). Treatment with S1P reduced infarct size to 2.9 ± 0.4 mm² ($n=8$). Neither left ventricular area nor AAR was statistically different between treatment groups. Immunohistochemistry for polymorphonuclear leukocytes was performed with a monoclonal antibody (MCA771G, Serotec, Oxford, England) and terminal dUTP nick end-labeling assays with the ApoptTag kit (Chemicon, Temecula, Calif). The number of stained cells was determined semiautomatically on 3 sections per heart with morphometry software (AnalySIS, Münster, Germany).

Flow-Chamber Studies and Cardiomyocyte Apoptosis

Endothelial adhesiveness for mouse peritoneal macrophages was determined with a parallel-plate flow-chamber model as described previously.^{15,16} Thioglycolate-elicited peritoneal macrophages were labeled with Cell Tracker Green (Molecular Probes, Leiden, Netherlands) and perfused at 100 s⁻¹ across tumor necrosis factor (TNF)- α -activated immortalized murine endothelial cells (fEnd.5). The number of rolling cells was determined from 3-minute video streams captured on a confocal microscope (UltraView, Perkin Elmer, Jügesheim, Germany). Firm adhesion was quantified on pictures taken from 15 high-power fields after 3 minutes of cell perfusion followed by 3 minutes of buffer wash. Apoptosis of rat neonatal cardiomyocytes was induced by growth factor and glucose deprivation for 4 hours and evaluated by Western blotting for active caspase-3 (Cell Signaling, Frankfurt, Germany) and cleavage of poly-adenosine diphosphate (ADP)-ribose polymerase (Becton Dickinson, Heidelberg, Germany).

Statistical Analysis

Data are presented as mean \pm SEM. Because of the likely non-Gaussian distribution of the data, a nonparametric Kruskal-Wallis test was performed, followed, if $P<0.05$, by a Mann-Whitney *U* test to identify significant differences between groups at $P<0.05$ (InStat, GraphPad Inc, San Diego, Calif).

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

Results

Administration of HDL and S1P Reduces Infarct Size After Myocardial Ischemia/Reperfusion in Mice

To test whether an acute rise in HDL levels may have a beneficial effect on acute myocardial infarction, we intravenously injected HDL in a mouse model of myocardial reperfusion injury (30-minute ischemia/24-hour reperfusion). Intravenous administration of human HDL 100 μ g/g body weight 30 minutes before transient coronary artery ligation reduced infarct size by $20\pm 3.2\%$ compared with vehicle-treated controls, whereas HDL 10 μ g/g or LDL 100 μ g/g had no effect (Figure 1A and 1B). Because sphingolipids contained in HDL account for several of their biological effects,¹⁰ we tested whether S1P had an impact on reperfusion injury. Compared with vehicle control, S1P 19 and 38 ng/g body weight (which corresponds to a final calculated plasma concentration of 1.1 and 2.2 μ mol/L, respectively) dose-dependently reduced infarct size by $32\pm 6.5\%$ and $40\pm 2.5\%$, respectively, whereas 3.8 ng/g was ineffective (Figure 1C).

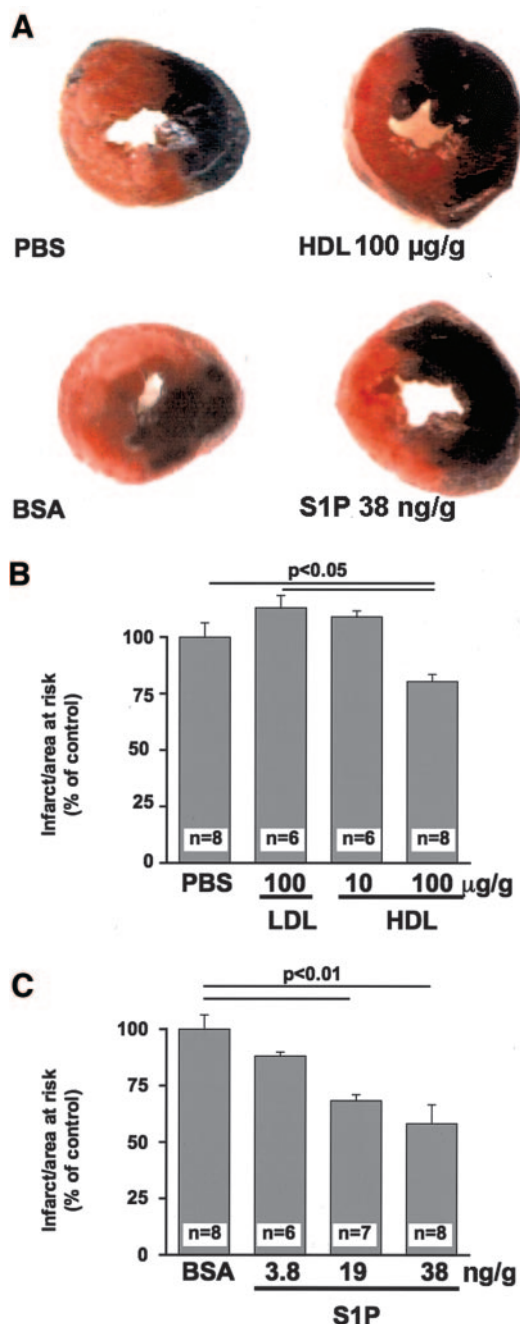


Figure 1. HDL and S1P protect against myocardial ischemia/reperfusion injury in vivo. Human HDL (10 and 100 $\mu\text{g/g}$ body weight) or S1P (3.8, 19, and 38 ng/g body weight) and their respective vehicle controls, phosphate-buffered saline and 1% bovine serum albumin or LDL (100 $\mu\text{g/g}$), were injected intravenously 30 minutes before myocardial ischemia. A, Representative sections from hearts treated with HDL, S1P, or vehicle before ischemia. B and C, Dose dependence of HDL and S1P effects and absence of LDL effects on infarct size/AAR (calculated as percent of vehicle-treated controls).

HDL and S1P Reduce Leukocyte Recruitment in the Infarcted Area and Leukocyte Adhesion to Activated Endothelium Under Flow

HDLs are known to possess potent antiinflammatory properties.^{8,17} A large part of the myocardial damage we have observed in our reperfusion injury model is due to

reperfusion-associated inflammation caused by the recruitment of polymorphonuclear leukocytes (PMNs). Accordingly, induction of panleukopenia by hydroxyurea abolished the typical increase of infarct size between 3 and 24 hours of reperfusion (Figure 2A). To test whether HDL and S1P may protect by inhibiting inflammation, we assessed their impact on PMN recruitment into the infarcted area 24 hours after ischemia/reperfusion. Morphometric quantification revealed that HDL 100 $\mu\text{g/g}$ body weight decreased PMN recruitment to $\approx 75\%$ of vehicle-treated controls, and S1P 38 ng/g body weight decreased PMN recruitment to $< 50\%$ of controls (Figure 2B). To test whether HDL and S1P have an effect on leukocyte adhesion under flow in vitro, we used a parallel-plate flow-chamber model in which murine macrophages were perfused over a confluent monolayer of TNF- α -activated murine endothelial cells. Stimulation with TNF- α (100 ng/mL) increased macrophage firm adhesion by $480 \pm 60\%$ compared with unstimulated endothelial cells ($3423 \pm 408/\text{mm}^2$ versus $713 \pm 103/\text{mm}^2$, $P < 0.001$). Cotreatment of endothelial cells with TNF- α and HDL 100 $\mu\text{g/mL}$ or S1P 1 $\mu\text{mol/L}$ for 4 hours reduced macrophage adhesion by 31% and 51%, respectively, compared with TNF- α alone (Figure 2C). Macrophage rolling was not affected, and pretreatment of macrophages with S1P had no effect on adhesion (data not shown).

HDL and S1P Protect Cardiomyocytes Against Apoptosis In Vitro and In Vivo

The damage inflicted by leukocytes during myocardial reperfusion is mediated by the oxidative burst and release of cytotoxic mediators, which leads to both necrosis and apoptosis in cardiomyocytes. Because S1P receptors are present and functional in cardiomyocytes,¹⁸ and both HDL and S1P are potent antiapoptotic signaling mediators in a number of experimental systems,^{19,20} we tested whether they may protect cardiomyocytes against apoptosis. In vivo, HDL-treated mice and S1P-treated mice had $\approx 35\%$ and $\approx 45\%$ less apoptotic cell death, respectively, in the myocardial infarction area than vehicle-treated controls, as measured by terminal dUTP nick end-labeling staining (Figure 3A). In vitro, HDL 1 mg/mL and S1P 10 $\mu\text{mol/L}$ potentially protected rat neonatal cardiomyocytes from apoptosis induced by glucose and growth factor withdrawal, as seen by the inhibition of caspase-3 processing and poly-ADP-ribose polymerase cleavage (Figure 3B). In contrast, the major protein component of HDL, apolipoprotein A1, had no effect (Figure 3B).

HDL- and S1P-Mediated Protection From Reperfusion Injury Depends on NO

NO plays an important role in the protection of the myocardium against ischemia/reperfusion injury. Because both HDL and S1P have been shown to generate NO in endothelial cells, we tested whether the beneficial effects of HDL and S1P on reperfusion injury were due to their ability to generate NO. Treatment of endothelial cells with 10 $\mu\text{mol/L}$ L-NAME completely abolished the antiadhesive effect of HDL and S1P on leukocytes under flow in vitro (Figure 4A). In vivo, L-NAME administration in mice for 21 days before myocardial ischemia/reperfusion completely eliminated the protec-

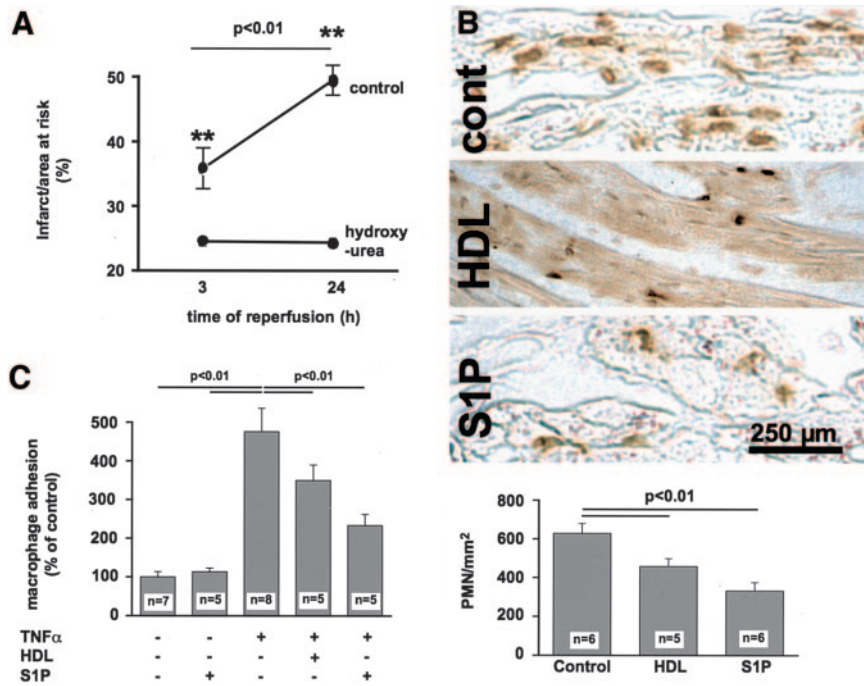


Figure 2. HDL and S1P inhibit PMN recruitment in the infarction area in vivo and macrophage adhesion to activated endothelium under flow in vitro. A, Panleukopenia (through hydroxyurea treatment) abolishes the increase of infarct size/AAR after reperfusion (** $P < 0.01$ vs hydroxyurea). B, Representative immunohistochemistry and morphometric quantification of PMNs in the infarction area of vehicle-treated, HDL-treated, and S1P-treated mice, respectively, 24 hours after ischemia/reperfusion. cont. indicates control. C, Macrophage adhesion to TNF- α -activated endothelial cells in vitro in the presence and absence of 100 μ g/mL HDL or 1 μ mol/L S1P as quantified in a parallel-plate flow-chamber system.

tion afforded by HDL and S1P, which resulted in infarcts as large as those in untreated mice (Figure 4B).

Protection From Reperfusion Injury by HDL and S1P Is Mediated by the S1P₃ Lysophospholipid Receptor

We have previously reported on the requirement of the S1P₃ receptor for NO-dependent vasodilation by HDL and S1P.¹⁰ Therefore, we tested its role in mediating the protective effect of HDL and S1P on infarct size using S1P₃-deficient mice. In S1P₃^{-/-} mice, neither HDL nor S1P conferred any protection against reperfusion injury compared with the respective wild-type controls (Figure 5).

Discussion

Increasing evidence suggests that HDLs may be atheroprotective through several inherent properties that are separable

from and independent of their role in reverse cholesterol transport.^{8,21} The present study has identified another unique biological property of HDL: the ability to directly protect ischemic myocardium from reperfusion injury in vivo. A recent study has shown improved functional postischemic recovery of isolated rat hearts by HDL and has attributed it to scavenging of myocardially released TNF- α .²¹ In contrast, we have observed that the protective effect of HDL depended on the S1P₃ receptor and was mimicked by exogenous S1P, which suggests that the S1P content of HDL was responsible for cardioprotection. In previous studies, we have shown that this content is sufficient for engaging the S1P₃ receptor (287 ± 17 pmol S1P/mg HDL).¹⁰

In the present study, blockade of NO generation abolished the beneficial effect of HDL and S1P on reperfusion injury.

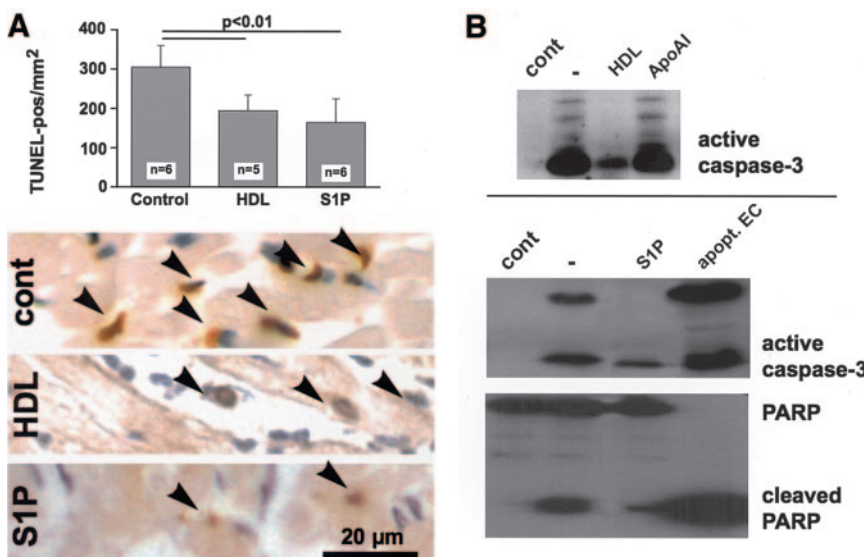


Figure 3. HDL and S1P inhibit apoptosis of cardiomyocytes in vitro and in the infarction area in vivo. A, Representative terminal dUTP nick end-labeling (TUNEL) staining from the infarction area of control, HDL-treated, and S1P-treated mice 24 hours after ischemia/reperfusion; morphometric quantification is presented above. B, Western blots for active caspase-3 and poly-ADP-ribose polymerase (PARP) cleavage in rat neonatal cardiomyocytes deprived of glucose and growth factors for 4 hours in the presence or absence of 1 mg/mL HDL or 1 mg/mL apolipoprotein A1 (ApoA1; upper panel) and 10 μ mol/L S1P (lower panel). TUNEL-pos indicates positive TUNEL staining; cont, cardiomyocytes under regular growth conditions; -, cardiomyocytes deprived of glucose and growth factors; and apopt. EC, apoptotic endothelial cells as positive control.

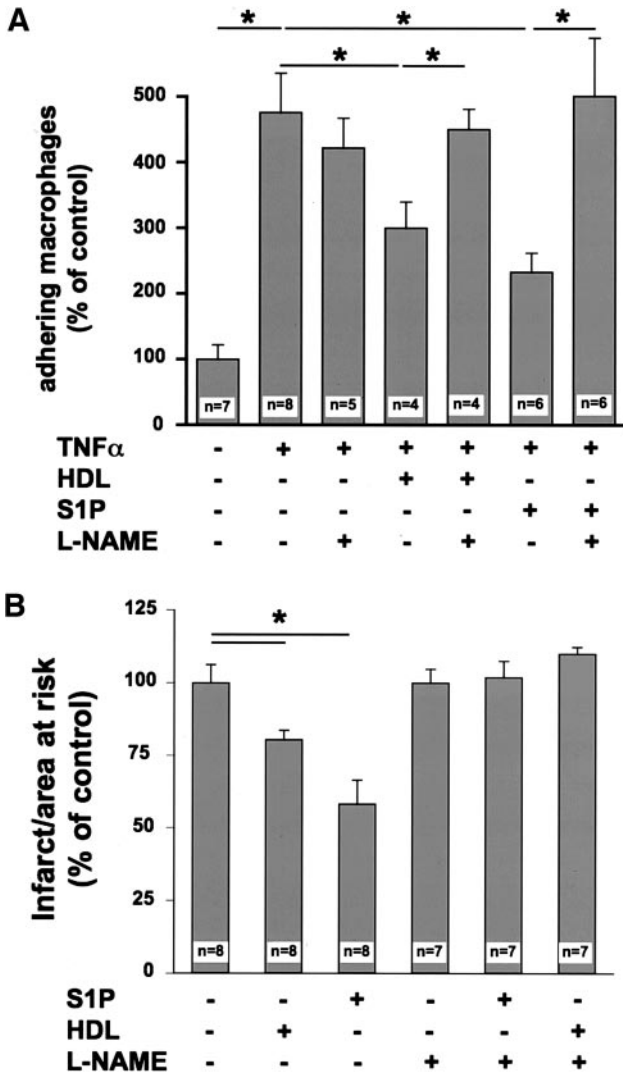


Figure 4. Inhibition of adhesion and reperfusion injury by HDL and S1P depend on NO in vitro and in vivo. A, Macrophage adhesion to TNF- α -activated endothelial cells incubated with or without 100 μ g/mL HDL and 1 μ mol/L S1P, respectively, in the presence and absence of 10 μ mol/L L-NAME was quantified in a parallel-plate flow-chamber system. B, Effect of HDL and S1P on infarct size/AAR after oral treatment with L-NAME or vehicle (* P <0.05).

NO exerts a plethora of beneficial biological effects in the myocardial microcirculation, and its decreased bioavailability has been implied in all aspects of microcirculatory reperfusion injury: (1) decreased endothelium-dependent vasodilator capacity, (2) no/low-reflow, and (3) increased microvascular permeability.^{1,14,22} Conversely, NO donors or interventions aimed at increasing NO bioavailability have been shown to attenuate myocardial reperfusion injury in mice, rabbits, and humans by a mechanism related to pharmacological preconditioning.^{1,2,23,24} Release of NO and S1P receptor signaling are closely linked: Several groups, including ours, have shown that engagement of S1P receptors including S1P₃ by HDL and S1P leads to NO generation and vasodilation.^{10,25-27} In addition, S1P regulates the endothelial cell barrier by potently inhibiting transcellular and microvascular perme-

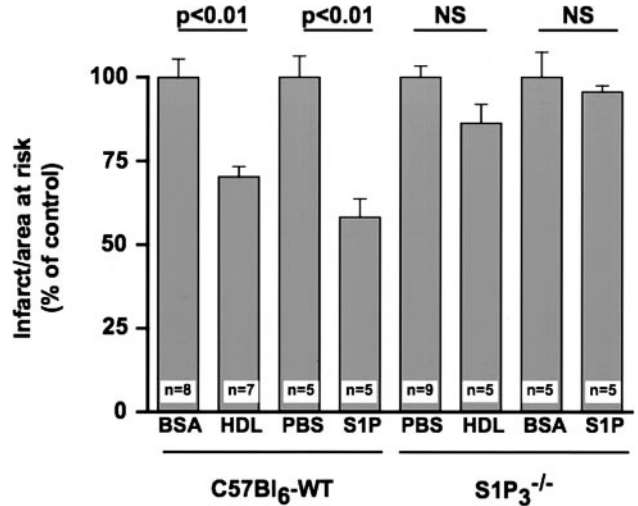


Figure 5. The S1P₃ lysophospholipid receptor is required for cardioprotection by HDL and S1P. Infarct size/AAR was determined in S1P₃-deficient mice and their matching wild-type controls (C57Bl₆) after treatment with 100 μ g/g HDL and 38 ng/g S1P, respectively (* P <0.05). NS indicates not significant.

ability and leakage,^{28,29} effects that are also ascribed to NO.^{19,20,30} In fact, NO may be mediating these S1P effects, as suggested by a recent study in which S1P inhibited TNF- α -mediated monocyte adhesion to aortic endothelium in mice.¹⁸ We provide mechanistic evidence that NO is indeed causally involved in mediating the antiadhesive effect of S1P in vitro and in reducing inflammatory cell infiltration during reperfusion injury in vivo. Thus, the dramatic decrease of reperfusion injury caused by HDL and S1P may be due to both attenuation of endothelial dysfunction and inhibition of leukocyte extravasation through NO generation. In addition, HDL and S1P may have direct beneficial effects on the myocardium itself, on the basis of the following: (1) both agents protected cardiomyocytes against apoptosis in vitro and in vivo in the present study; (2) sphingosine kinase-1, the key enzyme in S1P synthesis, has been shown to mediate ischemic preconditioning in Langendorff hearts and its genetic deficiency sensitized the myocardium to ischemia/reperfusion injury^{31,32}; and (3) several S1P receptors are present and functional in cardiomyocytes,³³ which suggests the possibility of direct receptor-mediated antiapoptotic effects. Thus, S1P may protect the heart by acting on both the endothelial cells and cardiomyocytes, and S1P₃ may be involved in both processes.

Indisputably, HDL is the most potent endogenous anti-atherogenic factor inversely correlated to long-term cardiovascular risk. However, by identifying HDL as a direct guardian of the myocardium against reperfusion injury, we put forward the hypothesis that a patient's current HDL level may influence the extent of myocardial damage he or she would suffer during acute ischemia. Indeed, short-term pharmacological elevation of HDL has been shown to reduce the high risk for recurrent adverse events in patients with acute coronary syndromes.⁷ By broadening the scope of beneficial HDL effects beyond "mere" atheroprotection, the present study suggests that the direct cardioprotective effect of HDLs

may independently contribute to their inverse correlation with cardiovascular risk.⁷

The implications of our observations may open a new field of clinical research to address important questions on the timing, extent, and means for elevating HDL in a way that best exploits its direct cardioprotective effect. Yet unknown are the patient collectives that would most benefit from HDL-raising interventions: Is it “only” the high-risk patient with an acute coronary syndrome,⁷ certain patients in preoperative settings (cardiac or noncardiac), or maybe even anyone scheduled for a routine percutaneous coronary intervention? In any case, rapid-HDL-elevation strategies or pharmacological HDL surrogates would be required. Niacins, fibrates, and statins are known to increase HDL-C levels, but other drugs that preferentially affect HDL, such as the cholesteryl ester transfer protein inhibitors, are under investigation.^{6,34} Exogenous HDL mimetics, such as reconstituted HDL particles, recombinant apolipoprotein A1, or apolipoprotein A1 Milano, have the advantage of rapid bioavailability and immediate effects¹³ and have been shown to reduce ischemia/reperfusion injury in rabbits (ETC-216).³⁵ In this respect and on the basis of the present study, S1P analogues may also be considered as functional HDL mimetics, especially because structural S1P homologs such as FTY720, currently in phase III clinical trials for immunosuppression in kidney transplant patients,³⁶ potentially generate NO via the same mechanism as S1P contained in the HDL particle.³⁷ Furthermore, the S1P content of HDL may not only be a target for intervention but also constitute a novel predictor of cardiovascular risk, because 54% of the total plasma S1P is contained in HDL.^{38,39}

Clearly, strategies designed to rapidly elevate HDL levels in general and their S1P content in particular may improve the prognosis of the myocardium at risk for ischemia and reperfusion. The concept of exploiting the direct beneficial effects of HDL for immediate cardioprotection may become very attractive for both patients and physicians, because it potentially applies to any clinical setting with imminent myocardial ischemia, from interventional cardiology and cardiac surgery to perioperative care for the cardiovascular high-risk patient.

Acknowledgments

We gratefully acknowledge the technical assistance of M. Lox, S. Brodner, G. Gaede, M. Greiwe, S. Lütke Enking, D. Bürger, K. Abouhamed, S. Mersmann, and V. Brinkmann.

Sources of Funding

This study was supported in part by the Deutsche Forschungsgemeinschaft (Th667/3-1, LE 940/3-1, and SFB656, projects A1, C3, and Z2, Germany), Federal Ministry of Education and Research (Fö.01KS9604/0), Interdisciplinary Center for Clinical Research Münster (project C21, The1/68/04, and ZPG 4a), Innovative Medizinische Forschung (Th110319 to Dr Theilmeier), the National Institute of Mental Health, the National Institute of Neurological Disorders and Stroke, and the National Institute of Drug Abuse (MH51699, MH07123, NS048478, and DA019674 to Dr Chun), and the H.-H. Deichmann Foundation for Atherosclerosis Research (to Dr Levkau).

Disclosures

None.

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CLINICAL PERSPECTIVE

Myocardial infarction is the leading cause of heart failure and cardiac death in Western industrialized countries. Reperfusion therapy greatly improves the outcome of myocardial infarction, but its benefit is still limited by the lack of measures to protect the myocardium from reperfusion injury after reinstatement of blood flow. High-density lipoproteins (HDLs) owe their reputation as the best negative predictor of cardiovascular events to their role in reverse cholesterol transport and their potent antioxidative, antiinflammatory, and vasodilatory properties. We have shown that acute administration of HDL and its constituent, sphingosine-1-phosphate (S1P), dose-dependently reduced reperfusion injury in a mouse model of transient myocardial ischemia, which resulted in a major reduction of infarction size. This was due to an inhibition of both leukocyte recruitment and cardiomyocyte apoptosis in the infarction area. Through genetic and pharmacological studies, we have identified the S1P-receptor S1P₃ and its nitric oxide-generating capacity as crucial for cardioprotection by HDL and S1P. Thus, in patients at risk of myocardial ischemia, the individual levels of HDL and HDL-associated S1P may influence the extent of myocardial damage after acute ischemia. In addition, therapeutic interventions to rapidly elevate HDL levels and their S1P content and/or administration of pharmacological HDL and S1P mimetics may represent novel and effective treatment strategies against reperfusion injury. Such strategies potentially may be very attractive to clinical medicine, because they apply to any setting with imminent myocardial ischemia, from daily interventional cardiology and cardiac surgery to perioperative care for the cardiovascular high-risk patient.