High-density lipoprotein determines adult mouse cardiomyocyte fate after hypoxia-reoxygenation through lipoprotein-associated sphingosine 1-phosphate

Rong Tao,1,4 Holly E. Hoover,1 Norman Honbo,1 Mikaila Kalinowski,1 Conrad C. Alano,3 Joel S. Karliner,1,5 and Robert Raffai2

1Cardiology Section, 2Surgical Service, and 3Neurology Service, Veterans Affairs Medical Center and Department of Medicine, University of California, San Francisco, California; 4Department of Cardiology, Ruijin Hospital, Jiao Tong University, Shanghai, China; and 5Cardiovascular Research Institute, University of California, San Francisco, California

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Tao R, Hoover HE, Honbo N, Kalinowski M, Alano CC, Karliner JS, Raffai R. High-density lipoprotein determines adult mouse cardiomyocyte fate after hypoxia-reoxygenation through lipoprotein-associated sphingosine 1-phosphate. Am J Physiol Heart Circ Physiol 298: H1022–H1028, 2010. First published January 8, 2010; doi:10.1152/ajpheart.00902.2009.—The lipid mediator sphingosine 1-phosphate (S1P) is bound to HDL (54%) and is also released by cells during ischemia (27, 28). HDL-associated S1P also mediates endothelial cell survival, inhibition of adhesion molecule expression, and cell migration (22, 30, 34, 37). HDL also affects functions of other cell types, such as smooth muscle cells and neural cells (22, 30, 34, 37). Kimura et al. (16, 17) recently reported that HDL-induced endothelial cell migration and survival in response to the absence of serum and growth factors are mediated by the S1P1 receptor subtype, which couples to ERK (40). It also has been demonstrated that S1P3 receptor mediates HDL protection of the heart against ischemia-reperfusion injury in vivo (40).

However, it has not been fully established that S1P associated with HDL directly mediates cardiomyocyte survival produced by this lipoprotein in the absence of effects on the vasculature. In the present study, we tested the hypothesis that HDL induces cardiomyocyte survival and signal activation in vitro. HDL was treated or not with autologous mouse HDL, which significantly increased myocyte viability as measured by trypan blue exclusion. This survival effect was abrogated by the S1P1 and SIP3 receptor antagonist VPC 23019. The selective S1P1 antagonist CAY10444, the G, antagonist pertussis toxin (PTX) inhibited the pro-survival effect of HDL. We observed that HDL activated both Akt (protein kinase B) and the MEK1/2-ERK1/2 pathway and also stimulated phosphorylation of glycogen synthase kinase-3β. ERK1/2 activation was through an S1P1 subtype receptor-G protein-dependent pathway, whereas the activation of Akt was inhibited by CAY10444, indicating mediation by S1P3 subtype receptors. We conclude that HDL, via its cargo of S1P, can directly protect cardiomyocytes against simulated oxidative injury in the absence of vascular effects and that pro-survival signal activation is dependent on both S1P1 and S1P3 subtype receptors.

Materials and Methods

Materials. S1P, PD-98059, wortmannin, and pertussis toxin (PTX) were obtained from Biomol International (Plymouth Meeting, PA). VPC 23019 was obtained from Avanti Polar Lipids (Alabaster, AL). CAY10444 was acquired from Cayman Chemical (Ann Arbor, MI). Antibodies directed against phosphorylated and total endogenous Akt (serine 473) and GSK-3β (serine 21/9) were purchased from Cell Signaling Technology (Danvers, MA).

Animals. Male C57Bl/6 mice (20–25 g) were purchased from Charles River Laboratories (Hollister, CA). All studies were approved by the Institutional Animal Care and Use Committee of the San Francisco Veterans Affairs Medical Center. This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Isolation of HDL from plasma. C57Bl/6 mice were anesthetized with pentobarbital sodium and bled by retro-orbital puncture. HDLs (density 1.063–1.21 g/ml) were isolated from fresh plasma by preparative ultracentrifugation, as described previously (10), and dialyzed against phosphate-buffered saline. HDLs were stored on ice and used for experiments within 2 wk.

Address for reprint requests and other correspondence: J. S. Karliner, Cardiology Section (111C5), 4150 Clement St., San Francisco, CA 94121 (e-mail: joel.karliner@med.va.gov).

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Adult mouse cardiomyocyte isolation and culture. Adult mouse cardiomyocytes were isolated and cultured using a modification of the collagenase dissociation method described by Zhou et al. (45) as previously described in our laboratory (38, 44). Mice were treated with heparin (50 units) and anesthetized by intraperitoneal injection with pentobarbital sodium (200 mg/kg). The heart was quickly excised, and the aorta was cannulated for retrograde perfusion in a Langendorff apparatus at a constant flow rate of 3 ml/min at 37°C. The heart was perfused for 9–10 min with isolation buffer [120 mM NaCl, 5.4 mM KCl, 1.2 mM MgSO4, 1.2 mM NaH2PO4, 5.6 mM glucose, 5 mM NaHCO3, 10 mM HEPES, 50 μM CaCl2, 10 mM 2, 3-butanedione monoxime (BDM) and 5 mM taurine], followed by digestion for 9 min with collagenase II (1.5 mg/ml; Worthington, Lakewood, NJ) in isolation buffer. After digestion, the soft and flaccid heart was removed, and myocytes were suspended in isolation buffer. A series of four centrifugations (40 g, 1 min) and resuspensions were used for stepwise Ca2+ reintroduction from 50 μM to 1.0 mM, which was the final medium Ca2+ concentration.

Isolated cardiomyocytes were plated for 2 h on 35- and 60-mm tissue culture dishes coated with 10 μg/ml laminin. The cells were suspended in minimum essential medium (MEM) with Hanks’ buffered salt solution (HBSS), 10 μg/ml penicillin, 1.5 μM vitamin B12, and 10 mM BDM. After this period of attachment, the medium was changed to MEM-HBSS containing 10 μg/ml penicillin, 1.5 μM vitamin B12, and 1 mM BDM and was incubated overnight at 37°C in a humidified atmosphere of 1% CO2 and air. The culture protocol yielded an average of 80% rod-shaped myocytes at a plating density of 50 cells/mm² that were viable at pH 7.2 for 48 h. Experiments were performed the day following isolation and culture.

Hypoxia–reoxygenation protocol. On the day after isolation and culture, cardiomyocytes were rinsed and subsequently incubated in a Bactron I anaerobic chamber containing a humidified atmosphere of 1% CO2 and 99% N2 for 3 h. This procedure produced a Po2 of <4 Torr. Experimental medium was changed to serum-free, glucose-free MEM with HBSS that did not contain BDM. This medium was preequilibrated for 16 h in an anaerobic chamber containing 1% CO2 and 99% N2. Normoxic experimental medium also was preequilibrated for 16 h in a water-jacketed incubator in a humidified atmosphere of 1% CO2 and air.

Measurement of cell survival. Cardiomyocyte survival was measured by staining cells in tissue culture dishes with trypan blue solution (Sigma Chemical, St. Louis, MO) diluted to a final concentration of 0.04% (wt/vol). Myocytes were visualized using bright-field microscopy at ×100 magnification. The number of viable (unstained) and nonviable (blue stained) cardiac myocytes in 10 random microscopic fields was recorded, and at least 300 cells were counted in each dish. Percent survival was defined as the number of unstained myocytes counted in each corresponding normoxic control dish. This calculation accounted for the detachment and loss of nonviable cells during the experimental hypoxia protocols.

Western blot analysis. On the day after isolation and culture, cardiomyocytes were treated with pharmacological agonists and antagonists under normoxic conditions for the indicated times. For whole cell extraction, cells were lysed in buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, 0.1% protease inhibitor mixture (Roche Applied Science, Indianapolis, IN), and phosphatase inhibitor cocktails (Sigma). Protein concentration was determined using the Bradford method. Equal amounts of protein were resuspended in 4X Laemmli sample buffer, boiled for 5 min, and subjected to sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis. After transfer to a polyvinylidene fluoride membrane, the extract was blocked in 5% nonfat milk in Tris-buffered saline (TBS) plus 0.1% Tween 20 for 1 h. The membranes were probed overnight with primary antibodies, washed three times with TBS-Tween 20 for 5 min, and probed with secondary antibodies for 1 h. The membranes were rinsed three times, and the signal was detected using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s instructions.

Statistical analysis. Data are means ± SE. Mean values were compared using one-way analysis of variance and post hoc Student-Newman-Keuls testing. P < 0.05 was considered statistically significant.

RESULTS

HDL protects adult mouse cardiomyocytes against hypoxia-reoxygenation injury. In initial studies we subjected isolated adult mouse cardiomyocytes to 3 h of hypoxia and 16 h of reoxygenation. As shown in Fig. 1, myocyte viability as determined by trypan blue exclusion was markedly diminished. Both SIP and HDL increased viability significantly and had equivalent effects. This increase was substantially but not completely inhibited by the SIP1 and SIP3 receptor antagonist VPC23019, indicating that it is the SIP associated with HDL that enhances myocyte survival. Substantial but not complete inhibition also was achieved by the selective SIP3 receptor antagonist CAY10444, the G9 antagonist PTX, the MEK kinase inhibitor PD-98059, and the phosphoinositide-3 kinase (PI3-kinase) inhibitor wortmannin. None of the inhibitors by themselves affected viability during hypoxia-reoxygenation (n = 3–4; data not shown). Interestingly, LDL by itself did not have a significant effect on cell viability (Fig. 1).

HDL activates the MEK1/2-ERK1/2 pathway. At 100 μg/ml, HDL elicited transient phosphorylation of MEK1/2, starting at 1 min and reaching a maximum at 5 min, with no change in the level of nonphosphorylated MEK1/2 (Fig. 2A). HDL at 100 μg/ml also stimulated time-dependent ERK1/2 phosphorylation, starting at 1 min and reaching a maximum between 5 and
HDL activates Akt and inactivates GSK-3β. As indicated in Fig. 3A, HDL at 100 μg/ml stimulated time-dependent Akt phosphorylation, starting at 10 min and reaching a maximum around 30 min, with no change in the level of nonphosphorylated Akt. Juhaszova et al. (14) determined that the beneficial effects of Akt activation on myocyte viability during hypoxia-reoxygenation were caused in large part by phosphorylation and inactivation of GSK-3β. In the present experiment, HDL-induced GSK-3β phosphorylation was time dependent, starting at 10 min, reaching a maximum at 30 min, and maintaining this level for at least 90 min (Fig. 3B).

HDL activates ERK1/2 through a S1P1 receptor-Gi protein-dependent pathway in adult cardiomyocytes. As shown in Fig. 4A, HDL-mediated ERK activation was markedly inhibited by PD-98059, an ERK kinase inhibitor. The ERK response to HDL was completely inhibited by PTX (Fig. 4B), suggesting an involvement of toxin-sensitive G₁ proteins. We previously reported that adult cardiomyocytes express mRNA for the S1P₁, S1P₂, and S1P₃ receptors, which couple to both G₁ and G₃ family G proteins (44). To test which S1P receptor is involved in ERK activation, we used VPC 23019, a selective S1P₁ and S1P₃ receptor antagonist. This antagonist has dissociation constants of $1.38 \times 10^{-8}$ and $1.17 \times 10^{-6}$ M for S1P₁ and S1P₃ receptors, respectively; thus, at 1 μM, it is expected to block >98% of S1P₁ receptors and approximately one-half of S1P₃ receptors (7). As shown in Fig. 4C, VPC 23019 inhibited HDL-stimulated ERK1/2 activation. In contrast, as depicted in Fig. 4D, HDL-stimulated ERK1/2 activation was insensitive to CAY10444, an S1P₃-selective antagonist (19). These observations support the conclusion that HDL stimulated ERK activation through a S1P₁ receptor-Gi protein-dependent pathway.

HDL activates Akt through a S1P₃ receptor-dependent pathway in adult cardiomyocytes. As shown in Fig. 5A, HDL-mediated Akt activation was markedly inhibited by wortmannin, a PI3-kinase inhibitor. Similarly, Akt activation in PTX-treated cardiomyocytes was markedly reduced compared with that in untreated cells (Fig. 5B). The subtype of S1P receptor responsible for the S1P-induced Akt activation was examined using S1P receptor subtype-selective antagonists. As shown in Fig. 5, C and D, activation of Akt was completely antagonized by the S1P₃-selective antagonist CAY10444. In contrast, VPC 23019, which completely inhibits S1P₁ receptors but only partially blocks S1P₃ receptors at a concentration of 1 μM (7), did not significantly inhibit Akt activation. These results suggest that S1P₁ receptor is the predominant receptor that mediates HDL-induced Akt activation.

**DISCUSSION**

The major findings of the present study are that HDL applied directly to isolated adult mouse cardiomyocytes enhances cell survival. HDL applied to isolated cardiomyocytes enhanced Akt phosphorylation, with no change in the level of nonphosphorylated Akt, and inactivated GSK-3β. Additionally, HDL increased ERK phosphorylation in a time-dependent manner. These observations support the conclusion that HDL stimulates Akt activation through a S1P₁ receptor-Gi protein-dependent pathway and ERK activation through a S1P₃ receptor-dependent pathway.

**Fig. 2.** HDL induces a time-dependent increase in the phosphorylation of MEK1/2 and ERK1/2 in adult mouse cardiomyocytes. Myocytes were exposed or not to 100 μg/ml HDL for different times (1–120 min; A and B). Western blot analyses of phosphorylated (p-)MEK 1/2, total (t-)MEK1/2, p-ERK1/2, and t-ERK1/2 representative of three independent experiments are shown. The band intensities of phosphoproteins were normalized against total MEK or ERK and expressed as fold increase over the zero time point. *P < 0.05 compared with all values without an asterisk.

**Fig. 3.** HDL induces a time-dependent increase in the phosphorylation of Akt (protein kinase B) and glycogen synthase kinase-3β (GSK-3β) in adult mouse cardiomyocytes. Myocytes were exposed or not to 100 μg/ml HDL for different times (1–120 min; A and B). Western blot analyses of p-Akt, t-Akt, p-GSK-3β, and t-GSK-3β representative of three independent experiments are shown. The band intensities of phosphoproteins were normalized against total Akt or GSK-3β and expressed as fold increase over the zero time point. *P < 0.05 compared with all values without an asterisk.
survival during hypoxia-reoxygenation. We also have shown that HDL acting via G\textsubscript{i} stimulates the prosurvival signals ERK1/2 and Akt. These prosurvival signals are mediated by S1P\textsubscript{1} and S1P\textsubscript{3} receptors located on the myocytes and are markedly attenuated by inhibitors of these receptors. We also observed that HDL causes phosphorylation and hence inactivation of GSK-3β, a molecule that acts as a key regulator of proapoptotic pathways (14). Although prior studies have im-

![Diagram](image-url)

**Fig. 4.** HDL-stimulated ERK activation is PTX sensitive, requires MEK phosphorylation, and is mediated by S1P receptors. Quiescent cardiomyocytes were treated or not for 1 h with the MEK inhibitor PD (10 \( \mu \)M; A) or for 16 h with the \( G_{\text{G}s} \) inhibitor PTX (100 ng/ml; B) before incubation with 100 \( \mu \)g/ml HDL for 10 min. The phosphorylation of ERK1/2 induced by HDL was significantly reduced by PTX or PD. Quiescent cardiomyocytes were also pretreated for 1 h with the S1P\textsubscript{1} and S1P\textsubscript{3} receptor antagonist VPC (1 \( \mu \)M; C) or the selective S1P\textsubscript{3} receptor antagonist CAY (10 \( \mu \)M; D) before stimulation with 100 \( \mu \)g/ml HDL for 10 min. Equal gel loading was assessed using an antibody against total ERK1/2. Specific bands corresponding to phosphorylated forms of ERK1/2 were quantified by densitometry and expressed as a fold change relative to the corresponding vehicle control. Data are means ± SE of 3–5 independent experiments. *\( P < 0.05 \), HDL vs. all other values.

![Diagram](image-url)

**Fig. 5.** HDL-mediated Akt activation is CAY-sensitive and is inhibited by wortmannin. Quiescent cardiomyocytes were treated or not for 1 h with the Akt inhibitor Wort (200 nM; A), for 16 h with the \( G_{\text{G}s} \) inhibitor PTX (100 ng/ml; B), for 1 h with the S1P\textsubscript{1} and S1P\textsubscript{3} receptor antagonist VPC (1 \( \mu \)M; C), or for 1 h with the S1P\textsubscript{3} receptor antagonist CAY (10 \( \mu \)M; D) before incubation with 100 \( \mu \)g/ml HDL for 30 min. Equal gel loading was assessed using an antibody against total Akt. Specific bands corresponding to phosphorylated forms of Akt were quantified by densitometry and expressed as a fold change relative to the corresponding vehicle control. Data are means ± SE of 3–5 independent experiments. *\( P < 0.05 \), HDL vs. all other values.
activated Akt via the S1P3 receptor. Thus our data provided agreement with a recent report by Means et al. (24), who reported that deletion of neither the S1P2 nor the S1P3 receptor alone affected infarct size or Akt activation. However, in double knockout mice, infarct size following ischemia-reperfusion was increased by 50% and Akt activation was markedly attenuated (23). It also has been shown that HDL impairs recruitment of polymorphonuclear cells to the infarcted area and reduces macrophage adhesion to activated endothelium in vitro (40).

Our prior in vitro work using an agonist antibody selective for the S1P1 receptor or the S1P1 receptor-specific agonist SEW2871 identified the importance of S1P1 receptor-mediated transduction of prosurvival signals in preserving myocardial viability during prolonged oxidative stress (39, 44). Using SEW2871 in desensitization studies, we also reported that ERK was activated by S1P1 receptor agonism (39). In isolated rat hearts, Tsukada et al. (42) also noted that SEW2871 protected against ischemia-reperfusion injury. In addition, the present study employing HDL implicates the S1P3 receptor in the activation of prosurvival signaling. Our findings are in agreement with a recent report by Means et al. (24), who demonstrated in adult mouse ventricular myocytes that S1P activated Akt via the S1P3 receptor. Thus our data provide evidence that both S1P1 and S1P3 receptors are required for cardioprotection by the HDL cargo of HDL and that both are necessary but each is not completely sufficient when they are separated.

Previous studies using HDL employed human preparations (16, 20, 27, 40), whereas we used autologous mouse HDL, which may exhibit different S1P-binding properties compared with the human lipoprotein. In the only prior study of cardiomyocytes, Theilmeyer et al. (40) reported that 1 mg/mg of human HDL [a concentration 10-fold higher than we used] protected neonatal rat cardiomyocytes from apoptosis induced by glucose and growth factor withdrawal as shown by inhibition of caspase-3 processing and poly(ADP-ribose) polymerase cleavage.

Other reports also have suggested that HDL might have direct effects on the myocardium. In an early study, Mochizuki et al. (25) observed that HDL decreased the incidence of ventricular arrhythmias associated with ischemia-reperfusion injury by a mechanism involving PGI2. Using reconstituted HDLs containing apolipoprotein A-I and phosphatidylcholine, Calabresi et al. (5) reported improved hemodynamic recovery and reduced creatine kinase release in isolated rat hearts. The same group (3) also noted that HDL inhibited the activation of matrix metalloproteinase-2, which was associated with reduced myocardial injury. Theilmeyer et al. (40) demonstrated that HDL and S1P equally inhibited apoptosis in the infarct area 24 h after ischemia-reperfusion injury. The mechanism by which HDL might gain access to cardiomyocytes has not been elucidated. However, it is well recognized that during reperfusion injury, vascular permeability increases markedly and large molecules such as albumin (mol wt ≈69,000, equivalent to the mol wt of small HDL species) can leak into the perivascular space and come into direct contact with cardiomyocytes (8, 33, 35, 41).

There also is evidence that synthetic HDLs, which initially are devoid of S1P, are cardioprotective in acute rodent ischemia-reperfusion models (5, 6, 9, 31). Possible mechanisms include a dose-dependent reduction of ischemia-induced tumor necrosis factor-α expression and content and enhanced prostaglandin release (5, 31) and reduced adhesion molecule (VCAM-1) expression (9). None of these studies measured prosurvival signals, and in none were there any experiments in which S1P action or its downstream consequences subjected to inhibition. Although these synthetic HDLs are prepared in the absence of S1P, the possibility has not been excluded that synthetic HDL in the perfusate of these experiments could scavenge S1P that is exported from cardiomyocytes (38) and redeem it to these cells to mediate signaling through S1P receptors on the cell surface (9).

In summary, we have provided evidence that native murine HDL induces cytoprotection against hypoxia-reoxygenation injury through the G protein-coupled sphingolipid receptors S1P1 and S1P3. As shown in Fig. 6, results of the experiments using specific inhibitors suggest that HDL-induced cell survival in cardiomyocytes involves two major pathways: MEK1/2-ERK1/2 and PI3-kinase-Akt. The S1P1 receptor transduces signals via the former pathway, and the S1P3 receptor utilizes the latter pathway.

Fig. 6. Proposed mechanisms by which HDL activates prosurvival signals in cardiomyocytes. See text for details.
HDL PROTECTS CARDIOMYOCYTES VIA S1P

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Disclosures

None of the authors declares a conflict of interest.

References


