HDL-Associated Lysosphingolipids Inhibit NAD(P)H Oxidase-Dependent Monocyte Chemoattractant Protein-1 Production

Markus Tölle, Alicja Pawlak, Miriam Schuchardt, Akira Kawamura, Uwe J. Tietge, Stefan Lorkowski, Petra Keul, Gerd Assmann, Jerold Chun, Bodo Levkau, Markus van der Giet and Jerzy-Roch Nofer

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/28/8/1542

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2008/06/09/ATVBAHA.107.161042.DC1.html

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at
http://atvb.ahajournals.org//subscriptions/

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, a division of Wolters Kluwer Health, 351 West Camden Street, Baltimore, MD 21202-2436. Phone: 410-528-4050. Fax: 410-528-8550. E-mail:
journalpermissions@lww.com

Reprints: Information about reprints can be found online at
http://www.lww.com/reprints

HDL-Associated Lysosphingolipids Inhibit NAD(P)H Oxidase–Dependent Monocyte Chemoattractant Protein-1 Production

Markus Tölle, Alicja Pawlak, Miriam Schuchardt, Akira Kawamura, Uwe J. Tietge, Stefan Lorkowski, Petra Keul, Gerd Assmann, Jerold Chun, Bodo Levkau, Markus van der Giet, Jerzy-Roch Nofer

Objectives—High-density lipoprotein (HDL) levels are inversely proportional to the risk of atherosclerosis, but mechanisms of HDL atheroprotection remain unclear. Monocyte chemoatractant protein-1 (MCP-1) constitutes an early component of inflammatory response in atherosclerosis. Here we investigated the influence of HDL on MCP-1 production in vascular smooth muscle cells (VSMCs) and rat aortic explants.

Methods and Results—HDL inhibited the thrombin-induced production of MCP-1 in a concentration-dependent manner. The HDL-dependent inhibition of MCP-1 production was accompanied by the suppression of reactive oxygen species (ROS), which regulate the MCP-1 production in VSMCs. HDL inhibited NAD(P)H oxidase, the preponderant source of ROS in the vasculature, and prevented the activation of Rac1, which precedes NAD(P)H-oxidase activation. The HDL capacity to inhibit MCP-1 production, ROS generation, and NAD(P)H-oxidase activation was emulated by sphingosine 1-phosphate (S1P) and sphingosylphosphorylcholine (SPC), two lysosphingolipids present in HDL, but not by apolipoprotein A-I. HDL-, S1P-, and SPC-induced inhibition of MCP-1 production was attenuated in VSMCs pretreated with VPC23019, an antagonist of lysosphingolipid receptors $S1P_1$ and $S1P_3$, but not by JTE013, an antagonist of $S1P_2$. In addition, HDL, S1P, and SPC failed to inhibit MCP1 production and ROS generation in aortas from $S1P_3$- and SR-B1–deficient mice.

Conclusion—HDL-associated lysosphingolipids inhibit NAD(P)H oxidase-dependent ROS generation and MCP-1 production in a process that requires coordinate signaling through $S1P_3$ and SR-B1 receptors. (*Arterioscler Thromb Vasc Biol.* 2008;28:1542-1548)

Key Words: HDL ■ sphingosine-1-phosphate ■ MCP-1 ■ ROS ■ NADPH-oxidase

Monocyte infiltration into the vessel wall is an initial step in the formation of atherosclerotic lesion.[1,2] Monocyte chemoattractant protein-1 (MCP-1) is a key regulator of monocyte recruitment to sites of vascular inflammation.[2–4] In addition, MCP-1 induces several proinflammatory changes including secretion of cytokines and expression of adhesion molecules.[2–4] MCP-1 was detected in atherosclerotic lesion, and elevated levels of MCP-1 were encountered in acute coronary syndromes.[2–4] Animals genetically modified to lack MCP-1 or its receptor, CCR2, displayed reduced atherosclerotic lesions, whereas overexpression of MCP-1 in macrophages led to increased susceptibility to atherosclerosis.[2–4]

Numerous studies documented an inverse relationship between high-density lipoprotein (HDL) levels and the progression of atherosclerosis and suggested that antiatherogenic effects of HDL are related to inflammation and its sequel.[5] For instance, HDL inhibits expression of adhesion molecules and reduces leukocyte homing to arterial endothelium.[5,6] Suppression of cytokine and chemokine production by HDL was observed after infusion of reconstituted HDL in animal models of inflammation.[7] The inverse relationship between HDL and acute phase proteins was repeatedly reported.[8]

Despite the central role played by MCP-1 in vascular inflammation, little information is available concerning the effect of HDL on MCP-1 production. In this study, we show that HDL-associated lysosphingolipids inhibit MCP-1 production in vascular smooth muscle cells (VSMCs) and in isolated aortas. We further demonstrate that this effect is contingent on inhibition of NADPH-oxidase-mediated generation of reactive oxygen species (ROS). We identify the HDL

receptor SR-B1 and the lysosphingolipid receptor S1P$_3$ as integral components of HDL-mediated inhibition of MCP-1 production.

Methods

Animals

C57BL/J6 and heterozygous SR-B1 mice on C57Bl/J6 background were obtained from Charles River Laboratories (Sulzfeld, Germany) and Jackson Laboratories (Bar Harbor, Me), respectively. The S1P$_3$-null mice on a C57Bl/J6 background was generated by J. Chun (Department of Molecular Biology, Scripps Research Institute, La Jolla, California, USA). All experiments were done with 8- to 10-week-old male homozygous animals and wild-type littermates.

Cells and Aortic Explants

VSMCs derived from rat thoracic aortas from 6-month-old male normotensive Wistar–Kyoto were maintained in DMEM containing 10% FCS and antibiotics. Aortic explants obtained from SR-B1–null mice, S1P$_3$-null mice, and wild-type littermates were kept in an organ bath containing Tyrode solution.

Analytic Procedures

MCP-1 RNA and protein levels were determined by RT-PCR and ELISA, respectively. Superoxide and hydrogen oxide production were assessed using fluorescence microscopy or spectroscopy with hydroethidin or 2′,7′-dichlorofluorescein, respectively. NADPH consumption was followed by light spectrometry at 340 nm. Rac1 and p38^{MAP}kinase activities were determined by commercially available solid phase pull-down assay and ELISA, respectively. p47^{phox} translocation was assessed by Western blot after fractionation of cytosolic and membrane proteins by sequential protein extraction.

Statistical Analysis

Data are presented as means±SEM. Comparisons between the groups were performed with Mann–Whitney U test, unless indicated otherwise.

Detailed Methods can be found in the supplemental materials (available online at http://atvb.ahajournals.org).

Results

HDL Inhibits Thrombin-Induced MCP-1 Production

To investigate whether HDL directly influences the agonist-induced MCP-1 gene expression, VSMCs were stimulated with thrombin in the absence or presence of the lipoprotein. Addition of thrombin led to an accumulation of MCP-1 mRNA in VSMCs in the absence but not in the presence of HDL (Figure 1A). The presence of HDL was associated with reduced MCP-1 release (Figure 1B). The inhibitory effect of HDL on the thrombin-induced MCP-1 production was concentration-dependent. The inhibitory effects of HDL on thrombin-induced MCP-1 production were observed also in endothelial (HMEC-1) and macrophage (RAW264.7) cell lines (see supplemental materials). To test the effect of HDL on MCP-1 production in a setting more akin to the situation in vivo, experiments with isolated mouse aortas were performed. There was an increase in MCP-1 levels in supernatants from aortic segments from C57Bl/J6 mice exposed to thrombin, which was markedly reduced in the presence of HDL (Figure 1C). To assess the contribution of smooth muscle cells to thrombin-induced MCP-1 production in isolated aortas, experiments were performed after mechanical removal of endothelial layer (see supplemental materials). As
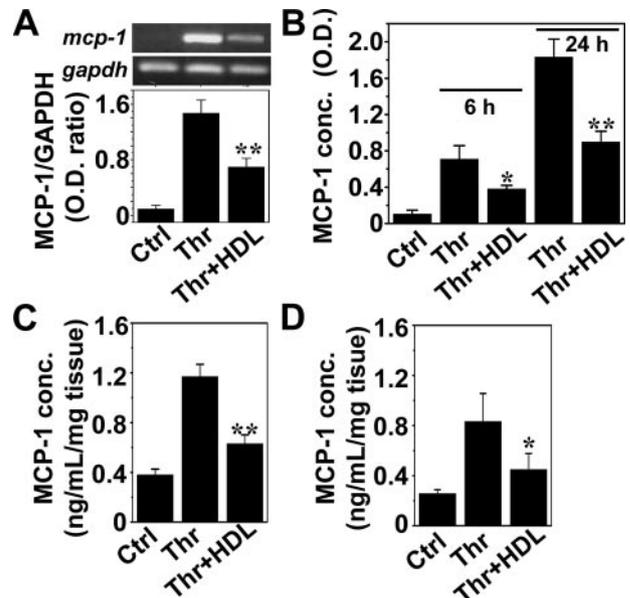

Figure 1. Effect of HDL on the thrombin-induced MCP-1 production in VSMCs. VSMCs (A and B) or rat aortas (C) were exposed for 6 hours (B) or 24 hours (A-C) to thrombin (A and B, 1.0 U/mL; C, 4.0 U/mL) or thrombin/HDL (0.5 g/L). A, mRNA levels assessed by RT-PCR. Lower panel, gel densitometry. Ratios from 3 experiments. B and C, MCP-1 determined by ELISA. Means±SEM from 3 to 6 experiments. D, MCP-1 in media from thrombin-stimulated aortas±HDL (0.5 g/L). Means±SEM from 3 experiments. For all panels: *$P<0.05$, **$P<0.01$ thrombin vs thrombin/HDL.

shown in Figure 1D, the thrombin-induced MCP1 production in de-endotelialized aortas was significantly inhibited in the presence of HDL.

HDL Inhibits Thrombin-Induced ROS Generation in VSMCs

As the agonist-induced MCP-1 expression in VSMCs is controlled by the intracellular redox status,[9–11] we next examined whether HDL affects thrombin-induced ROS generation. VSMCs were loaded with hydroethidine (HE), which is converted to ethidium bromide in the presence of superoxide, and exposed to thrombin. This resulted in a substantial increase in ethidium fluorescence, which was reduced in the presence of HDL (Figure 2A). The inhibitory effects of HDL on superoxide generation were concentration-dependent with a maximum at 0.5g/L HDL.

Superoxide generated in cells is converted to H$_2$O$_2$. We next monitored the effect of HDL on the thrombin-induced H$_2$O$_2$ production using a fluorogenic substrate H$_2$DCFDA. A constant increase in DCF fluorescence was recorded in VSMCs indicating a steady-state H$_2$O$_2$ production. Exposure of VSMCs to thrombin enhanced H$_2$O$_2$ production, and this was suppressed in cells pretreated with HDL (Figure 2B). Because the phosphorylation of p38^{MAP}kinase in response to thrombin occurs as a consequence of ROS generation in VSMCs,[12] the effect of HDL on thrombin-induced p38^{MAP}kinase activity was examined. Figure 2C demonstrates the increase in phosphorylated p38^{MAP}kinase in VSMCs exposed to thrombin and its suppression by HDL.
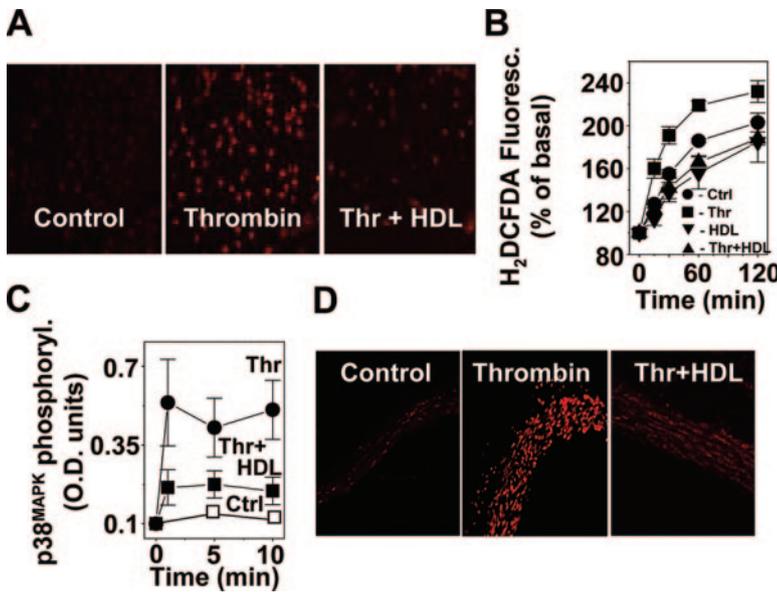
Figure 2. Effect of HDL on the thrombin-induced ROS generation and p38^{MAP}kinase activation in VSMCs. A, VSMCs exposed for 16 hours to thrombin (1.0 U/mL) $\pm$HDL (0.5 g/L). Superoxide detected by ethidium bromide (EtBr) fluorescence. Images ($\times$400) from 3 experiments. B, H$_2$O$_2$ production in H$_2$DCFDA-loaded VSMCs exposed to thrombin (1.0 U/mL) $\pm$HDL (0.5 g/L). Data are percent fluorescence increase relative to the intensity of unstimulated cells. Means$\pm$SEM from 6 to 8 experiments. C, VSMCs exposed to thrombin (1.0 U/mL) $\pm$HDL (0.5 g/L). Phosphorylated p38^{MAP} assessed by ELISA. Means$\pm$SEM from 3 to 4 experiments. D, Aortas were exposed for 16 hours to 4.0U/mL thrombin$\pm$HDL (0.5 g/L). Superoxide generation detected as above. Images from 3 experiments.

To investigate whether HDL inhhibits ROS generation in isolated mouse aortas, aortic segments were incubated with HE, exposed to thrombin, and examined by confocal microscopy. Exposure to thrombin resulted in a substantial increase in ethidium fluorescence indicating enhanced superoxide production (Figure 2D), which was reduced in the presence of HDL.

HDL Inhibits Thrombin-Induced NAD(P)H-Oxidase Activation in VSMCs

As the agonist-inducible NAD(P)H-oxidase is a predominant source of ROS in the vasculature,[13,14] we next investigated whether the suppressing effect of HDL on the intracellular ROS production is mediated via inhibition of NAD(P)H-oxidase. We measured the NADPH consumption rate in VSMCs, which occurs contemporaneously with ROS generation. Thrombin increased the NADPH consumption as compared to untreated cells, and this effect was reduced by HDL and blocked by diphenyliodonium (DPI), an inhibitor of NAD(P)H-oxidase (Figure 3A). To gain further evidence pointing to NAD(P)H oxidase as a target of HDL, we made use of gp91ds—a cell-permeable peptide specifically inhibiting NAD(P)H oxidase (see supplemental materials). gp91ds but not gp91scr, its inactive analogue, abolished thrombin-induced NADPH consumption and significantly reduced superoxide generation and MCP-1 production both in VSMCs and isolated aortas. HDL failed to further reduce thrombin-induced NADPH consumption, superoxide generation, and MCP-1 production in VSMCs and aortas pretreated with gp91ds, but retained its inhibitory activity in the presence of gp91scr. These results indicate that intact NAD(P)H oxidase is necessary and sufficient for HDL to exert its inhibitory effects. By contrast, xanthine oxidase does not serve as molecular target of HDL, as these lipoproteins blocked thrombin-induced superoxide generation and MCP-1 production in VSMCs pretreated with allopurinol, a xanthine oxidase inhibitor (see supplemental materials). Inhibition of p38^{MAP}kinase with SB202190 did not prevent HDL from reducing MCP-1 production in response to thrombin (see supplemental materials).

The induction of NAD(P)H-oxidase requires activation and translocation of GTPase Rac1 to cell membrane, where the assembly of NAD(P)H-oxidase is accomplished.[13] We next assessed the activity of Rac1 in VSMCs exposed to thrombin in the presence or absence of HDL. As shown in Figure 3B, exposure of cells to thrombin led to a marked increase in active Rac1, and this effect was alleviated by HDL. Similarly to Rac1, translocation of p47^{phox} NAD(P)H oxidase subunit is required for enzyme activation. As shown in Figure 3C, addition of thrombin to VSMCs increased and reduced, respectively, p47^{phox} amounts associated with membrane and cytosolic VSMCs fractions. Both effects were substantially diminished in the presence of HDL.
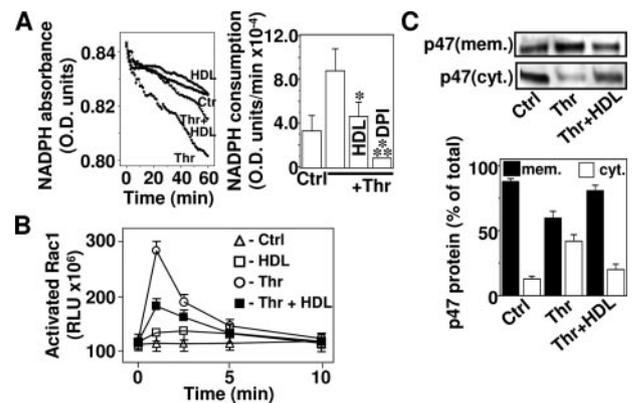
Figure 3. Effect of HDL on the thrombin-induced NAD(P)H-oxidase activation in VSMCs. A, NADPH consumption in homogenates from VSMCs exposed for 1 hour to thrombin (1.0 U/mL) $\pm$HDL (0.5 g/mL). Superimposed tracings from 5 experiments. Right panel, NADPH consumption rate in VSMCs stimulated with thrombin/HDL (0.5 g/L) or diphenyliodonium (DPI; 10 μmol/L). Means$\pm$SEM from 3 to 6 experiments. *$P<0.05$, ***$P<0.001$ thrombin vs thrombin+HDL/DPI. B and C, VSMCs exposed to 1.0 U/mL thrombin$\pm$HDL (0. 5g/mL) and assessed for (B) Rac1 activation or (C) p47^{phox} translocation. C, Immunoblots from 3 experiments. Lower panel, Densitometric analysis of p47^{phox} in membrane (mem) and cytosol (cyt) fractions. Total p47^{phox} set as 100%.

Figure 4. Effect of HDL-associated lysosphingolipids on the thrombin-induced MCP-1 production, ROS generation, and NAD(P)H oxidase activation in VSMCs and mouse aortic segments. A, MCP-1 determined in media from aortic segments exposed for 16 hours to 4.0 U/mL thrombin±S1P (1.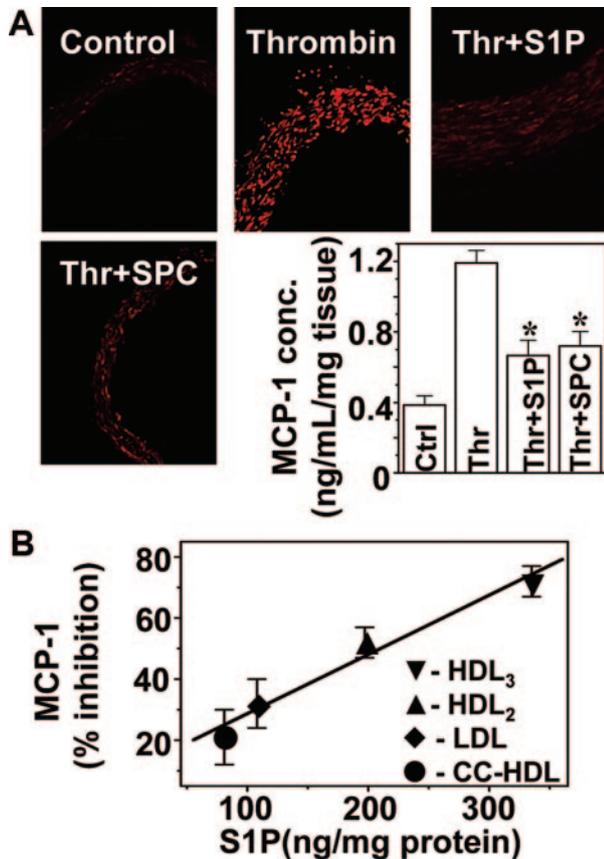0 μmol/L) or SPC (1.0 μmol/L). Means±SEM from 3 experiments. Superoxide generation detected by EtBr fluorescence. Images (×400) from 3 experiments. *$P<0.05$ thrombin vs thrombin+S1P/SPC. B, Effect of lipoprotein fractions with variable S1P amounts on thrombin-induced MCP1 production in VSMCs. CC-HDL indicates charcoal-treated HDL. Means±SEM from 3 experiments.

HDL-Associated Lysophospholipids S1P and SPC Inhibit MCP-1 and ROS Production

To determine HDL entities responsible for the inhibition of MCP-1 production and ROS generation we tested the effects of apo A-I, the constitutive protein of HDL, as well as S1P and SPC, lysosphingolipids previously identified in HDL,[14,15] on MCP-1 levels, superoxide production, p38[MAP]kinase phosphorylation, NAD(P)H consumption, and Rac1 activation in VSMCs. All tested responses to thrombin were inhibited in the presence of S1P or SPC but not apo A-I (see supplemental materials). We also tested the effects of S1P and SPC on the thrombin-induced MCP-1 production and superoxide generation in isolated aortas. Preincubation of HE-loaded aortic explants with lysosphingolipids inhibited the thrombin-induced ROS generation (Figure 4A). In addition, the pretreatment with S1P or SPC reduced MCP-1 production in explants stimulated with thrombin. To further assess the propensity of lipoprotein-associated lysosphingolipids to suppress VSMCs activation, we examined the effect of lipoprotein fractions containing various amounts of S1P on thrombin-induced MCP-1 production. Figure 4B illustrates that the
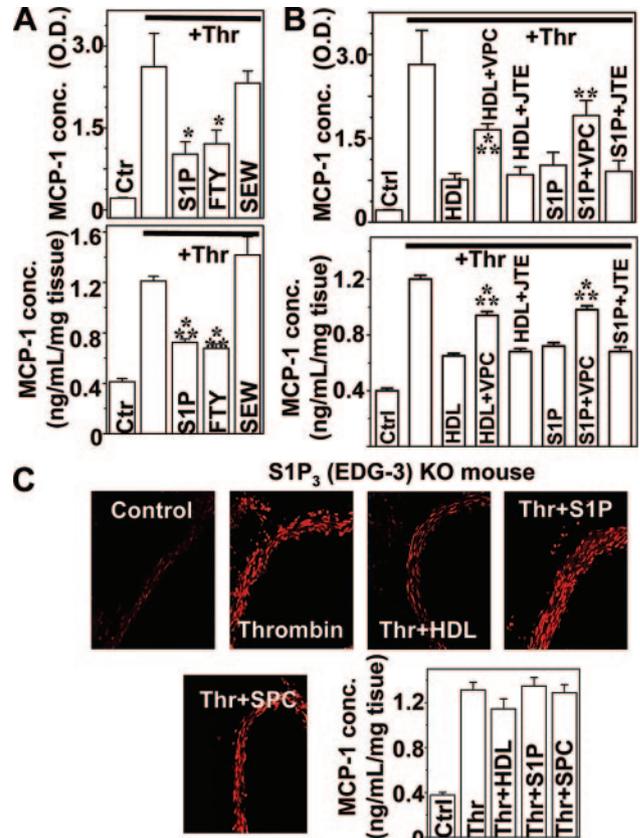
Figure 5. Involvement of S1P$_3$ receptor in HDL- and lysophospholipid-dependent inhibition of the thrombin-induced MCP-1 production and ROS generation. A, VSMCs (upper panel) or aortas (lower panel) exposed for 24 hours to, respectively, 1.0 U/mL or 4.0 U/mL thrombin±S1P (1.0 μmol/L), FTY720P (1.0 μmol/L), or SEW2871 (1.0 μmol/L). MCP-1 in media determined by ELISA. Means±SEM from 3 to 5 experiments. *$P<0.05$, ***$P<0.001$ thrombin vs thrombin+S1P/ FTY720P. B, MCP1 in media from VSMCs (upper panel) or aortas (lower panel) preincubated for 30 minutes with VPC2301 (20 μmol/L) or JTE013 (20 μmol/L) and exposed for 24 hours to, respectively, 1.0 U/mL or 4.0 U/mL thrombin±HDL (0.5 g/L) or S1P (1.0 μmol/L). **$P<0.01$, ***$P<0.001$ thrombin+HDL/S1P/ HDL+VPC/S1P+VPC C, Aortas from S1P$_3$-deficient mice exposed for 16 hours to 4.0 U/mL thrombin±HDL (0.5 g/L), S1P (1.0 μmol/L), or SPC (1.0 μmol/L). Superoxide generation detected by EtBr fluorescence. Images (×400) from 3 experiments. MCP-1 levels determined by ELISA. Means±SEM from 3 to 4 experiments.

ability of HDL$_3$, HDL$_2$, and LDL to inhibit MCP-1 production increased proportionally to their S1P content. Conversely, the inhibitory effects of HDL were diminished after reduction of their S1P content by charcoal treatment.

The Inhibitory Effects of HDL on MCP-1 Production and ROS Generation Are Mediated by S1P$_3$

In agreement with previous studies, we found that both S1P$_2$ and S1P$_3$ but not S1P$_1$ are expressed in VSMCs.[16] To examine which S1P receptor mediates inhibitory effects of HDL and lysosphingolipids on MCP-1 production, we used FTY720P, an agonist of all S1P receptors except S1P$_2$, and SEW2871, an agonist of S1P$_1$. As shown in Figure 5A, preincubation of VSMCs and aortic explants with FTY720P inhibited MCP-1

production, whereas SEW2871 had no effect. We also found that the inhibitory effects of HDL and S1P on MCP-1 production were partially reversed in VSMCs and aortas preincubated with VPC23019—an antagonist of $S1P_1$ and $S1P_3$, but not with JTE013—an antagonist of $S1P_2$ (Figure 5B). These results pointed to $S1P_3$ as a mediator of inhibitory effects of HDL and HDL-associated lysosphingolipids. To address this issue more specifically, we examined the influence of HDL, S1P, and SPC on the thrombin-induced ROS generation and MCP-1 production in aortic explants obtained from $S1P_3$-deficient mice. Figure 5C demonstrates that the capacity of HDL to inhibit ROS generation was reduced and that of S1P and SPC abolished in aortic rings from $S1P_3$-deficient mice. In addition, HDL, S1P, and SPC failed to inhibit MCP-1 production in aortas from $S1P_3$-deficient mice.

HDL and S1P Fail to Inhibit Thrombin-Induced MCP-1 Production and ROS Generation in Aortas From SR-B1–Deficient Mice

As scavenger receptor type B1 (SR-B1) is critically involved in several physiological effects of HDL, we next examined its involvement in the HDL-mediated downregulation of MCP-1 production (see supplemental materials). Aortic explants from SR-B1–deficient mice responded to thrombin stimulation with MCP-1 production and superoxide generation that was affected neither by HDL nor by S1P. SR-B1 deficiency did not affect $S1P_3$ expression and vice versa. In addition, fractionation of VSMCs plasma membrane revealed that both receptors were recovered from overlapping fractions characterized by low lipid content and distinct from caveolae.

Discussion

Activated smooth muscle cell is an abundant source of proatherogenic cytokine and chemokines including MCP-1. The present study provides evidence that MCP-1 expression is directly inhibited by HDL in VSMCs. In addition, the inhibitory effects of HDL were seen in endothelial cells and macrophages as well as in isolated whole aortas. Dose-response studies demonstrated the significant reduction of MCP-1 production by HDL concentrations close to physiological. Cumulatively, these results suggest that HDL reduces the chemotactic stimulus attracting leukocytes into the arterial wall. Along this way HDL may locally limit the inflammation and thereby inhibit development of atherosclerosis.

The enhanced MCP-1 production is an integral part of a larger response to various pathological situations. Concerted productions of inflammatory mediators such as interleukins, chemokines and adhesive proteins represent other components of this response negatively regulated by HDL. It is now established that NAD(P)H oxidase is located at the cross-road of proinflammatory signaling in the vasculature both collecting signals from proatherogenic factors including oxidized LDL, angiotensin II, homocysteine, or thrombin and triggering inflammatory responses such as production of cytokines and expression of adhesion molecules.[13,17] The present study for the first time documents that NAD(P)H oxidase–dependent ROS generation is negatively regulated by HDL. The evidence underlying the inhibitory effect of HDL proceeded along several pathways of investigations. First, HDL reduced

the thrombin-induced generation of superoxide, a common progenitor of ROS, and the formation of H_2O_2, a product of superoxide decomposition, both in VSMCs and isolated aortas. Second, HDL inhibited the thrombin-induced activation of $p38^{MAP}$ kinase, which occurs as a consequence of NAD(P)H oxidase activation and is partially required for MCP-1 induction. Third, the increase in NAD(P)H consumption caused by thrombin was diminished in VSMCs pretreated with HDL. In addition, preincubation of VSMCs with HDL prevented the activation of Rac1 and the membrane translocation of $p47^{phox}$ NAD(P)H oxidase subunit, which are both required for the assembly of NAD(P)H oxidase complex. Fourth, exposure of VSMCs to p91ds—a higly specific inhibitor of NAD(P)H oxidase— but not to allopurinol—the inhibitor of xanthine oxidase—preempted the inhibitory effects of HDL. Collectively, these data demonstrate that HDL suppresses the agonist-induced ROS production at cellular level. As modulation of the redox status constitutes an integral element of signal transduction processes, inhibition of ROS generation by HDL represents a novel mechanism by which this lipoprotein affects intracellular signaling pathways.

A question arises, by which mechanism HDL influences intracellular ROS generation. As HDL is known to carry α-tocopherol, the supplementation of cells with this compound could account for inhibitory effects exerted by these lipoproteins on intracellular ROS generation. Mechanisms involving both phospholipid transfer protein (PLTP), an enzyme associated with HDL, and ATP-binding cassette protein A1 (ABCA1), an apoA-I receptor, have been proposed that facilitate transfer of α-tocopherol between HDL and the cell interior.[18,19] Whereas the direct effect of HDL-associated α-tocopherol on the cellular redox status cannot be entirely dismissed, this study supports the contention that the inhibitory effect of HDL on ROS generation is independent from supplying cells with antioxidants. First, the inhibitory effect of HDL on ROS generation was seen within minutes after treatment. By contrast, the inhibitory effects of α-tocopherol on cell oxidation are evident after few hours of incubation.[20,21] Second the purified lysophospholipids S1P and SPC, lipid components of HDL without antioxidative properties, mimicked HDL capacity to reduce intracellular ROS generation. Third, HDL inhibited the thrombin-induced NADPH consumption and $p47^{phox}$ membrane translocation, which are both located upstream to generation of superoxide. Consistent with our findings, Robbesyn et al shown that α-tocopherol–depleted HDL was still able to inhibit oxidized LDL–induced ROS generation, whereas α-tocopherol failed to exert a short-term influence on the redox status of the cell.[21] Basing on our observations and those of Robesyn et al we postulate that HDL inhibits ROS generation by directly influencing the activation of NADPH oxidase via inhibition of Rac1 activation.

The present study provides support to the contention that the substantial portion of inhibitory effects exerted by HDL on VSMC activation can be attributed to S1P and SPC. Both compounds were previously shown to account for several pleiotropic effects of HDL including inhibition of endothelial apoptosis, activation of endothelial nitric oxide synthase (eNOS), and inhibition of the expression of adhesion mole-

cules.[14,15,22] Current findings extend these observations by showing that HDL lysosphingolipids inhibited NADPH-oxidase activation, ROS generation, and MCP-1 production in VSMCs, whereas apo A-I, a major protein constituent of HDL, had no effect. In addition, the ability of HDL subfractions to inhibit MCP-1 production was related to their S1P content and reduced after S1P depletion. To our knowledge, this is the first report documenting the negative influence of lysosphingolipids on NAD(P)H-oxidase activation. However, the inhibition of the Rac1 activation, which is required for NADPH oxidase assembly, has been reported in VSMCs exposed to S1P at concentrations above 100 nmol/L.[16] In the present study, the inhibitory effects of lysosphingolipids on MCP1 production and ROS generation were seen in concentrations between 0.1 and 1.0 μmol/L. As 1 mg of HDL contains 287 ± 17pg S1P and 290 ± 20pg SPC,[15] these lipoproteins are likely to deliver sufficient amounts of lysosphingolipids to inhibit NAD(P)H-oxidase in vivo.

S1P and SPC mediate various physiological processes by binding to G protein–coupled receptors, two of which, $S1P_2$ and $S1P_3$, are expressed in VSMCs.[16] We previously demonstrated that NO-dependent vasodilatory effects of HDL and HDL-associated lysosphingolipids were attenuated in thoracic aortas obtained from $S1P_3$-deficient animals, suggesting that this particular receptor serves as a functional partner for HDL.[15] In the present study we show that inhibitory effects of HDL, S1P, and SPC on MCP-1 production in VSMCs and isolated aortas were emulated by FTY720P, a synthetic agonist of $S1P_3$ and $S1P_1$ but not $S1P_2$ receptors. In addition, HDL and S1P retained its ability to inhibit thrombin-induced VSMC activation in the presence of JTE013—an $S1P_2$ antagonist. Conversely, elimination of $S1P_3$ receptor by performing experiments either in the presence of $S1P_3$ inhibitor VPC23019 or in aortas from $S1P_3$-deficient animals led to reversal of inhibitory effects of HDL and lysosphingolipids. These observations together with previous findings showing reduced MCP-1 levels in apoE-deficient mice treated with FTY720[23] suggest that the activation of the $S1P_3$ rather than $S1P_2$ receptor by HDL led to the inhibition of NAD(P)H-oxidase and MCP-1 production in VSMCs. Previous studies suggested a link between activation of $S1P_2$ and inhibition of Rac1 activity.[16,24] However, the inhibitory effects of $S1P_3$ and the activating effects of $S1P_2$ on Rac1 activation were also reported.[25,26] It is noteworthy that both receptors use similar intracellular signaling machinery and turn on either activating or inhibitory signals depending on cellular context.

The HDL-dependent inhibition of ROS generation and MCP-1 production was not observed in aortas from SR-B1–deficient mice suggesting that binding to this receptor is also required for inhibitory effects of HDL. Interestingly, SR-B1 deficiency equally effectively abolished inhibitory effects exerted by S1P, though the latter compound is not regarded SR-B1 ligand. It is worth notice that $S1P_3$ has recently been shown to reside in membrane invaginations termed caveolae, which host and are stabilized by SR-B1.[27,28] Whereas in the present study we failed to detect SR-B1 in cell membrane fractions expected to contain structural components of caveolae, the demonstration of the presence of both SR-B1 and $S1P_3$ in overlapping fractions is nevertheless consistent with the notion that these receptors colocalize in certain plasmalemmal compartment(s). It would, therefore, be tempting to speculate that the primary role of SR-B1 in HDL signaling is to secure plasma membrane microenvironment optimal for effective signal transduction over S1P receptors.

The direct inhibitory effect of HDL-associated lysosphingolipids on the agonist-induced NAD(P)H oxidase activation for the first time demonstrated in this study is important for better understanding of the antiatherogenic role of this lipoprotein. Uncontrolled ROS generation is a distinguished feature of several proatherogenic processes including lipid oxidation, NO degradation, and altering cell functions such as adhesion, motility, proliferation, and apoptosis. Increased ROS production was demonstrated in atherosclerotically changed arteries and is a marker of unstable plaques.[29] Conversely, dramatic decrease in atherosclerotic lesions was observed in animals deficient in NAD(P)H oxidase.[30] Inhibition of NAD(P)H oxidase activity by HDL-associated lysosphingolipids may constitute an important mechanism by which HDL exerts its potent antiatherogenic effects.

Disclosures

None.

References

1. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med*. 2005;352:1685–1695.
2. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med*. 2006;354:610–621.
3. Bursill CA, Channon KM, Greaves DR. The role of chemokines in atherosclerosis: recent evidence from experimental models and population genetics. *Curr Opin Lipidol*. 2004;15:145–149.
4. Boisvert WA. Modulation of atherogenesis by chemokines. *Trends Cardiovasc Med*. 2004;14:161–165.
5. Barter PJ, Nicholls S, Rye KA, Anantharamaiah GM, Navab M, Fogelman AM. Antiinflammatory properties of HDL. *Circ Res*. 2004;95:764–772.
6. Theilmeier G, De Geest B, Van Veldhoven PP, Stengel D, Michiels C, Lox M, Landeloos M, Chapman MJ, Ninio E, Collen D, Himpens B, Holvoet P. HDL-associated PAF-AH reduces endothelial adhesiveness in apoE$^{-/-}$ mice. *FASEB J*. 2000;14:2032–2039.
7. Cockerill GW, Huehns TY, Weerasinghe A, Stocker C, Lerch PG, Miller NE, Haskard DO. Elevation of plasma high-density lipoprotein concentration reduces interleukin-1-induced expression of E-selectin in an in vivo model of acute inflammation. *Circulation*. 2001;103:108–112.
8. Ridker PM, Glynn RJ, Hennekens CH. C-reactive protein adds to the predictive value of total and HDL cholesterol in determining risk of first myocardial infarction. *Circulation*. 1998;97:2007–2011.
9. de Keulaner GW, Ushio-Fukai M, Yin Q, Chung AB, Lyons PR, Ishizaka N, Rengarajan K, Taylor WR, Alexander RW, Griendling KK. Convergence of redox-sensitive and mitogen-activated protein kinase pathways in tumor necrosis factor-alpha-mediated monocyte chemoattractant protein-1 induction in vascular smooth muscle cells. *Arterioscler Thrombosis Vasc Biol*. 2000;20:385–396.
10. Brandes RP, Viedt C, Nguyen K, Beer S, Kreuzer J, Busse R, Gorlach A. Thrombin-induced MCP-1 expression involves activation of the p22phox-containing NADPH oxidase in human vascular smooth muscle cells. *Thromb Haemost*. 2001;85:1104–1110.
11. Chen XL, Zhang Q, Zhao R, Medford RM. Superoxide, H_2O_2, and iron are required for TNF-alpha-induced MCP-1 gene expression in endothe-

lial cells: role of Rac1 and NADPH oxidase. *Am J Physiol Heart Circ Physiol*. 2004;286:H1001–1007.

12. Herkert O, Diebold I, Brandes RP, Hess J, Busse R, Gorlach A. NADPH oxidase mediates tissue factor-dependent surface procoagulant activity by thrombin in human vascular smooth muscle cells. *Circulation*. 2002;105:2030–2036.

13. Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res*. 2000;86:494–501.

14. Nofer J-R, Levkau B, Wolinska I, Junker R, Fobker M, von Eckardstein A, Seedorf U, Assmann G. Suppression of endothelial cell apoptosis by high density lipoproteins (HDL) and HDL-associated lysosphingolipids. *J Biol Chem*. 2001;276:34480–34485.

15. Nofer J-R, van der Giet M, Tolle M, Wolinska I, von Wnuck Lipinski K, Baba HA, Tietge UJ, Godecke A, Ishii I, Kleuser B, Schafers M, Fobker M, Zidek W, Assmann G, Chun J, Levkau B. HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3. *J Clin Invest*. 2004;113:569–581.

16. Ryu Y, Takuwa N, Sugimoto N, Sakurada S, Usui S, Okamoto H, Matsui O, Takuwa Y. Sphingosine-1-phosphate, a platelet-derived lysophospholipid mediator, negatively regulates cellular Rac activity and cell migration in vascular smooth muscle cells. *Circ Res*. 2002;90:325–332.

17. Griendling KK, FitzGerald GA. Oxidative stress and cardiovascular injury: Part I: basic mechanisms and in vivo monitoring of ROS. *Circulation*. 2003;108:1912–1916.

18. Oram JF, Vaughan AM, Stocker R. ATP-binding cassette transporter A1 mediates cellular secretion of alpha-tocopherol. *J Biol Chem*. 2001;276:39898–39902.

19. Desrumaux C, Deckert V, Athias A, Masson D, Lizard G, Palleau V, Gambert P, Lagrost L. Plasma phospholipid transfer protein prevents vascular endothelium dysfunction by delivering alpha-tocopherol to endothelial cells. *FASEB J*. 1999;13:883–892.

20. Negre-Salvayre A, Alomar Y, Troly M, Salvayre R. Ultraviolet-treated lipoproteins as a model system for the study of the biological effects of lipid peroxides on cultured cells. III. The protective effect of antioxidants (probucol, catechin, vitamin E) against the cytotoxicity of oxidized LDL occurs in two different ways. *Biochim Biophys Acta*. 1991;1096:291–300.

21. Robbesyn F, Garcia V, Auge N, Vieira O, Frisach MF, Salvayre R, Negre-Salvayre A. HDL counterbalance the proinflammatory effect of oxidized LDL by inhibiting intracellular reactive oxygen species rise, proteasome activation, and subsequent NF-kappaB activation in smooth muscle cells. *FASEB J*. 2003;17:743–745.

22. Nofer J-R, Geigenmuller S, Gopfert C, Assmann G, Buddecke E, Schmidt A. High density lipoprotein-associated lysosphingolipids reduce E-selectin expression in human endothelial cells. *Biochem Biophys Res Commun*. 2003;310:98–103.

23. Keul P, Tölle M, Lucke S, von Wnuck Lipinski K, Heusch G, Schuchardt M, van der Giet M, Levkau B. The sphingosine-1-phosphate analogue FTY720 reduces atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol*. 2007;27:607–613.

24. Okamoto H, Takuwa N, Yokomizo T, Sugimoto N, Sakurada S, Shigematsu H, Takuwa Y. Inhibitory regulation of Rac activation, membrane ruffling, and cell migration by the G protein-coupled sphingosine-1-phosphate receptor EDG5 but not EDG1 or EDG3. *Mol Cell Biol*. 2000;20:9247–9261.

25. Sugimoto N, Takuwa N, Okamoto H, Sakurada S, Takuwa Y. Inhibitory and stimulatory regulation of Rac and cell motility by the G12/13-Rho and Gi pathways integrated downstream of a single G protein-coupled sphingosine-1-phosphate receptor isoform. *Mol Cell Biol*. 2003;23:1534–1545.

26. Lepley D, Paik JH, Hla T, Ferrer F. The G protein-coupled receptor S1P$_2$ regulates Rho/Rho kinase pathway to inhibit tumor cell migration. *Cancer Res*. 2005;65:3788–3795.

27. Singleton PA, Dudek SM, Ma SF, Garcia JG. Transactivation of sphingosine 1-phosphate receptors is essential for vascular barrier regulation. Novel role for hyaluronan and CD44 receptor family. *J Biol Chem*. 2006;281:34381–34393.

28. Frank PG, Marcel YL, Connelly MA, Lublin DM, Franklin V, Williams DL, Lisanti MP. Stabilization of caveolin-1 by cellular cholesterol and scavenger receptor class B type I. *Biochemistry*. 2002;41:11931–11940.

29. Azumi H, Inoue N, Ohashi Y, Terashima M, Mori T, Fujita H, Awano K, Kobayashi K, Maeda K, Hata K, SHinke T, Kobayashi S, Hirata K, Kawashima S, Itabe H, Hayahsi Y, Imajoh-Ohmi S, Itoh H, Yokoyama M. Superoxide generation in directional coronary atherectomy specimens of patients with angina pectoris: important role of NAD(P)H oxidase. *Arterioscler Thromb Vasc Biol*. 2002;22:1838–1844.

30. Barry-Lane PA, Patterson C, van der Merwe M, Hu Z, Holland SM, Yeh ET, Runge MS. p47phox is required for atherosclerotic lesion progression in ApoE($^{-/-}$) mice. *J Clin Invest*. 2001;108:1513–1522.

SUPPLEMENTERY MATERIALS

METHODS

Materials - Hydroethidine (HE) and 2',7'-dichlorofluorescin diacetate (H_2DCFDA) were purchased from Molecular Probes (Eugene, Oregon, USA). Polyclonal antibodies against $S1P_1$, $S1P_2$ and $S1P_3$ receptors were from Orbigen (San Diego, California, USA), and anti-Rac1 antibody was obtained from Upstate (Chicago, Illinois, USA). 5-(4-Phenyl-5-trifluoromethyltiophen-2-yl)-3-(3-trifluoromethylphenyl)-1,2,4-oxadiazole (SEW2871), a selective agonist of S1P1 receptor[1], and and (R)-phosphoric acid mono-[2-amino-2-(3-octyl-phenylcarbamoyl)-ethyl] ester (VPC23019), a competitive antagonist of $S1P_1$ and $S1P_3$ receptors[2] were obtained from Cayman Chemical (Ann Arbor, Michigan, USA) and Avanti Polar Lipids (Alabaster, Alabama, USA), respectively. Diphenyliodonium (DPI) was purchased from Merck Biosciences (Schwabach, Germany). Phosphorylated isoform of the Novartis compound 2-amino-2-[2-(4-octylphenyl)ethyl]1,3-propanediol hydrochloride (FTY720), a synthetic agonist of all S1P receptors except $S1P_2$[3]. All other chemicals were from Sigma (Taufkirchen, Germany) and were of highest purity available.

Animals - C57BL/J6 mice were obtained from Charles River Laboratories (Sulzfeld, Germany). Heterozygous SR-B1 mice on a C57Bl/J6 background were originally described by Rigotti and coworkers[4] and obtained from Jackson Laboratories (Bar Harbor, Minnesota, USA). The offspring of the mice was analyzed for the presence of targeted or wild-type SR-B1 alleles by PCR. The generation of $S1P_3$ -null mice on a C57Bl/J6 background was described elsewhere[5]. Animals were held in an air-conditioned room kept on a 12h dark/light cycle and fed standard rodent diet *ad libitum*. All experiments were performed with 8 to 10 week-old male homozygous animals and wild-type littermates as controls. All animal protocols used in this study

conformed to national law and were approved by the Ethics Committee for Animal Experiments of the Landesamt für Gesundheit und Technische Sicherheit Berlin (LAGETSI)

Cell culture and isolation of aortic segments – Most of cell culture experiments were carried out using vascular smooth muscle cells (VSMCs) derived from rat thoracic aortas from 6-month-old male normotensive Wistar-Kyoto rats as described previously[6]. These cells have been previously applied for studying various processes pertinent to atherosclerosis and are generally considered as a reliable single cell model. Cells were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (Roche, Mannheim, Germany), 100 U/ml penicillin G, 100 μg/ml streptomycin and 2 mmol/l L-glutamine. Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed initially after 24 h and then every 2–3 days. When cells had formed a confluent monolayer after about 8–10 days, they were harvested by addition of 0.05% trypsin, and the culture was continued up to eight passages. Cells exhibited typicall hill-and-valley morphology and strong propensity to proliferation. In addition, expression of smooth muscle-specific α-actin has been confirmed immunocytochemically using FITC-labeled monoclonal antibodies ASM-1 (Progen, Heidelberg, EU). Taken together, these observations suggest that cells used in this study were of medial origin[7]. Cells were made quiescent by incubation in serum-free medium containing 0.1% fetal calf serum, 100 U/ml penicillin and 10 μg/ml streptomycin for 24 h. Human endothelial cells from dermal microvessels (HMEC-1) is the first immortalized human microvascular endothelial cell line that retains the morphologic, phenotypic, and functional characteristics of normal human microvascular endothelial cells [8]. Confluent cultures of HMEC-1 showed typical cobblestone appearance and the characteristical expression of von Willebrand factor and absence of smooth muscle

α-actin staining. HMEC-1 were maintained in MCDB131 medium supplemented with 100 U/ml penicillin/streptomycin, 1% (vol/vol) L-glutamine and 7.5% (vol/vol) fetal bovine serum. Mouse macrophage cell line RAW264.7 was obtained from the German Resource Centre for Biological Materials (DSMZ), Braunschweig, Germany and maintained in RPMI1640 medium supplemented with 10% (vol/vol) fetal bovine serum and antibiotics. Aortic explants were obtained from SR-B1-null mice, S1P$_3$-null mice and wild-type littermates as described previously[8]. Aortic explants were kept in an organ bath containing Tyrode's solution (pH 7.4, 10 mL) at 37°C continuously gassed with 95% O$_2$/CO$_2$.

Fractionation of plasma membranes - VSMCs were harvested after reaching 90% confluence in a disruption buffer containing NaCl (0.3 mol/L), EDTA (5 mmol/L), Tris-HCl (50 mmol/L, pH 7.4), Complete® protease inhibitor and pepstatin A (0.7 µg/mL), and subsequently homogenized using a Emulsflex-C5 at 4°C. Homogenates were treated with 1% Triton X-100 for 30 minutes, diluted with an equal volume of sucrose solution (2.4 mol/L) and centrifuged at 50000 rpm for 18 h at 4°C in a SW-40 rotor (Beckman Instruments, Krefeld, Germany) over a sucrose step gradient containing following layers: 1.2 mol/L, 1.0 mol/L, 0.8 mol/L, 0.6 mol/L, 0.2 mol/L mol/L. Fractions of 1.0 mL from top to bottom of the gradient were subjected to precipitation with trichloroactetate. Pellets were washed and resuspended in electrophoresis (Laemmli) buffer by shaking overnight at 4°C.

HDL and lysophospholipids - HDL$_3$ (d = 1.125 - 1.210 g/mL), HDL$_2$ (d = 1.075 - 1.125 g/mL), and LDL (d = 1.020 - 1.063 g/mL) were prepared from fresh human plasma pooled from at least three random and anonymous donors by sequential ultracentrifugation as previously described[10]. HDL was stored at 4 °C for no longer than 14 days and was not frozen and thawed before use. HDL$_3$ was used in all but one experiment in this study and is referred to as HDL throughout the manuscript.

HDL was deprived of S1P by overnight incubation with charcoal as described[11]. Sphingosine 1-phosphate (S1P) and sphingosylphosphorylcholine (SPC) were purchased from Sigma (Taufkirchen, Germany). The purity of S1P and SPC was checked by HPLC.

Determination of MCP-1 mRNA level - Following incubation with agonists, VSMCs were washed twice with ice-cold PBS and total RNA was isolated using RNeasy Mini Kits (Qiagen, Hilden, Germany). First-strand cDNA was synthesized from 1 µg or total RNA with the use of iScript cDNA Synthesis Kit (Bio-Rad, München, Germany). The resulting cDNA was subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) with gene-specific primers for rat MCP-1 gene and mouse/rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) obtained from R&D Systems (Minneapolis, Minneapolis, USA). 2 µg of cDNA were amplified with 2.5 U of HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany) in a 50 µL reaction mixture containing 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dTNP mix, and each of the primer pairs (7,5 µmol/L). The amplification protocol comprised 25 cycles of denaturation at 95°C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were visualized by agarose gel electrophoresis and quantified using the AlphaEase FC software system (Alpha Innotech, San Leandro, California, USA). MCP-1 mRNA was standardized against the level of the respective GAPDH control.

Determination of MCP-1 protein levels - VSMCs plated in 24-well plates were incubated in serum-free DMEM for indicated times with agonists, and the media were collected after 8h or 24h. 5 mm aortic segments of wild-type mice, SR-B1-null mice or $S1P_3$-null mice were incubated in an organ bath in the presence of agonists for 16h. MCP-1 levels in incubation solutions were determined using a commercially

available ELISA kit according to the manufacturer instructions (R&D Systems, Minneapolis, MN, USA) .

Determination of superoxide production - The oxidative fluorescent dye hydroethidine (HE) was used to evaluate *in situ* production of superoxide. HE is freely permeable to cells and in the presence of superoxide is oxidized to ethidium bromide (EtBr), where it is trapped by intercalating with DNA[12.] EtBr shows an excitation at 488 nm and has an emission spectrum of 610 nm. VSMCs grown on cover slides or aortic segments were incubated for 30 min in serum-free DMEM or 16 hours in an organ bath, respectively, in the presence of absence of agonists. After incubation, HE (1.0 µmol/L) was applied to each cover slide and the slide was cover-slipped. Unfixed frozen ring slices were cut into 20-µm-thick sections and placed on a glass slide. HE (2.0 µmol/L) was applied to each tissue section and cover-slipped. Slides were incubated in a light-protected humidified chamber at 37°C for 30 minutes. Images were obtained with a Zeiss LSM510 laser scanning confocal microscope equipped with a krypton/argon laser. Tissues were processed and imaged in parallel. Laser settings were identical for acquisition of images. Fluorescence was detected with a 585-nm long-pass filter.

Determination of hydrogen peroxide production - Intracellular hydrogen peroxide levels were determined as described previously[13]. Briefly, VSMCs in suspension (1 x 10^6 cells/mL) were incubated with 5 µmol/L H_2DCFDA for 30 min and then washed and resuspended in phosphate-buffered saline (PBS). H_2DCFDA is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed and oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF). The level of DCF fluorescence reflecting the H_2O_2 concentration was monitored using spectral fluorimeter (F-2000 Hitachi, Tokyo, Japan) at 534 nm after excitation at 488 nm. Following stimulation with an agonist, the percent fluorescence increase was determined every 15 min.

5

HDL and lysophospholipids neither showed autofluorescence nor quenched the DCF fluorescence.

Measurement of NAD(P)H oxidase activity - Measurement of NAD(P)H oxidase activity was performed as described with minor modifications[14]. Briefly, following to the stimulation with agonists, VSMCs were washed and scrapped at 0°C into Hanks's buffered salt solution (HBSS) containing Complete® protease inhibitor cocktail. Cells were disrupted by three freeze-thawing cycles and cell homogenates were prepared using Dounce homogenizer. 0.05 µg of homogenate were added to 0.5 mL HBSS containing 5 mmol/L NADPH. Diphenyliodonium (DPI), an unspecific inhibitor of flavine oxidases, was added immediately before assaying. The decrease of the absorbance of NADPH at 340 nm was monitored for 60 min onwards. The NADPH activity was calculated as a first-order derivative for the time range, in which NADPH consumption was linear. The activity of the NAD(P)H oxidase under control conditions was set to 100%. Neither HDL nor lysophospholipids alone exhibited absorbance at 340 nm.

Determination of p38^MAPK phosphorylation - VSMCs were incubated for indicated times with agonists and the whole cell lysates were collected after indicated time periods. The amounts of the phosphorylated isoform of p38^MAPK in the cell lysates were determined using a commercially available ELISA kit according to the manufacturer instructions (Biosource, Solingen, Germany)

SDS-PAGE and Western Blotting - SDS-PAGE and Western blotting were performed exactly as described previously[10].

Pull-down solid-phase Assay for Rac1 Activation – starved VSMCs were stimulated with agonists for different time points. Protein lysates were collected and activated (GTP-bound) Rac1 was analyzed with a G-LISA luminesence assay kit (Cytoskeleton, Denver, CO) according to the manufacturer´s instructions.

Assay for p47^phox^ translocation – VSMCs were starved in serum-free medium for 48 h. The cells were stimulated with thrombin (4.0 U/mL) in the presence or absence of HDL (0.5 g/L). Sequential protein extraction was done using the Qproteome Cell Compartment Kit (Qiagen) according to the manufacturer´s instructions. The fraction 1 with cytosolic proteins and the second fraction containing membrane proteins were used for western blot analysis. Samples (50 µg protein/lane for membrane fraction and cytoplasmic fraction) were electrophoresed on 12% gel (Pierce) and transferred to a polyvinylidene difluoride membrane (Bio-Rad) by semidry electroblotting. The membrane was incubated with a specific polyclonal rabbit anti-p47phox antibody (1:1000, Upstate) overnight at 4°C and after washing with HRP-conjugated anti-rabbit IgG antibody (1:5000) for 1 h at room temperature. The bands were detected using Super Signal Dura West Substrate (Pierce) and the Chemi-Smart (Vilber Lourmat) imaging system. The density of the corresponding bands was measured quantitatively using Bio1D Image software (Vilber Lourmat).

Aortic relaxation - The effect of acetylcholine on arterial relaxation were evaluated in 2-mm rings of thoracic aortae from 3-month-old male C57BL/J6 mice. The wall tension of the vasculature was measured in mouse aortas with small vessel myograph using established methodology[10]. The endothelium was mechanically removed by gently rubbing the internal layer with a thin wire. Following equilibration and submaximal precontraction with phenylephrine (PE) (1.0 µmol/l), relaxation to 1 µmol/l acetylcholine was tested to confirm the loss of integrity of the endothelium. The maintenance of functional smooth muscle cell integrity after manipulation was confirmed by evaluation of the endothelium-independent relaxation to sodium nitroprusside (1.0 µmol/l).

Determination of endotoxin concentration in apoA-I – Endotoxine content in apoA-I was determined using limulus amebocyte lysate (LAL) endpoint assay (Cambrex Inc.,

East Rutherford, NJ) according to manufacturer's instruction. ApoA-I used in the present study contained <20 pg endotoxin/μg of apoprotein as determined by the LAL method.

Statistical analysis - Data are presented as means ± SEM. form three separate experiments or as results representative for at least three repetitions, unless indicated otherwise. Where error bars do not appear on figures error was within the symbol size. Comparisons between the groups were performed with two-tailed Mann-Whitney U-test. *p* values less than 0.05 were considered significant.

RESULTS

HDL inhibits thrombin-induced MCP-1 production in endothelial cells and mavcrophages - To investigate whether HDL influences the thrombin-induced MCP-1 production in cells other than smooth muscle cells, HMEC-1 endothelial cells and RAW264.7 macrophages were stimulated with thrombin in the absence or presence of the lipoprotein. As shown in Fig. I, addition of thrombin led to a marked accumulation of MCP-1 in cell media, while HDL significantly reduced thrombin-induced MCP-1 increases.

Endothelium removal

Fig.II (demonstrates that in contrast to intact vessels pre-contracted de-endotelialized aortas failed to dilate in response to acetylcholine (ACH), but showed normal relaxation to sodium nitroprusside (SNP), suggesting the maintenance of functional smooth muscle cell integrity after removal of endothelium.

Involvement of NADPH oxidase, xanthine oxidase and p38^{MAP} kinase in the inhibitory effects of HDL on thrombin-induced ROS generation and MCP-1 production in VSMCs - To gain further evidence pointing to NAD(P)H oxidase as a molecular target of HDL, we made use of gp91ds – a cell–permeable peptide specifically inhibiting

NAD(P)H oxidase activity. As shown in Fig. III, application of gp91ds but not gp91scr, its inactive analogue, to VSMCs or isolated rat aortas completely abolished thrombin-induced NADPH consumption and significantly reduced superoxide generation (determined as ethidium fluorescence) and MCP-1 production both in VSMCs and isolated aortas. HDL failed to further reduce thrombin-induced NADPH consumption, superoxide generation and MCP-1 production in VSMCs and aortas pre-treated with gp91ds, but fully retained its inhibitory activity in the presence of gp91scr. These results indicate that intact NAD(P)H oxidase activity is necessary and sufficient for HDL to exert its inhibitory effects. As xanthine oxidase is another source of ROS in the vasculature[15], we next investigated, whether the suppressing effect of HDL on the intracellular ROS generation and MCP-1 production are related to inhibition of this enzyme. To this aim, we made use of allopurinol – a cell–permeable xanthine oxidase inhibitor. As shown in Fig. IV, application of allpourinol to VSMCs failed to affect thrombin-induced ROS generation and MCP-1 production. Moreover, HDL fully retained its ability to inhibit ROS generation and MCP-1 production in VSMCs pre-treated with allopurinol. These results indicate that intact xanthine oxidase activity is not required for HDL to exert their inhibitory effects.

Similarly to xanthine oxidase, the involvement of p38^{MAP} kinase in HDL-induced inhibition of MCP-1 production in VSMCs was studied using SB202190, a specific p38^{MAP} kinase blocker. As shown in Fig. V, thrombin-induced MCP-1 production was partially reduced in SB202190 pre-treated cells, and this effect was further increased in the presence of HDL. Hence, inhibition of p38^{MAP} kinase does not prevent HDL to reduce MCP-1 production in response to thrombin and, consequently, this enzyme does not seem to constitute primary target of HDL inhibitory effects in VSMCs.

HDL-associated lysophospholipids S1P and SPC inhibit MCP-1 production and ROS generation in VSMCs - To determine HDL entities responsible for the inhibition of

MCP-1 production and ROS generation we tested the effects of apo A-I, the constitutive protein of HDL, as well as S1P and SPC, lysosphingolipids previously identified in HDL[17,18], on MCP-1 levels as well as on superoxide production, p38[MAP] kinase phosphorylation, NAD(P)H consumption, and Rac1 activation in VSMCs. As shown in Fig. VIA, all tested responses to thrombin were inhibited in the presence of S1P or SPC but not apo A-I. Fig. VIB shows that the inhibitory effects of both S1P and SPC on MCP-1 production and superoxide generation occurred within physiological concentration ranges.

HDL and S1P fail to inhibit thrombin-induced MCP-1 production and ROS generation in aortas from SR-B1-deficient mice - As scavenger receptor type B1 (SR-B1) is critically involved in several physiological effects exerted by HDL, we next examined its involvement in mediating the HDL inhibitory effects on MCP-1 production. As shown in Fig. VIIA, aortic explants obtained from SR-B1-deficient mice responded to thrombin stimulation with MCP-1 production that was affected neither by HDL nor by S1P. Moreover, both HDL and S1P failed to inhibit thrombin-induced increase in superoxide generation in explants from SR-BI-deficient mice (Fig. VIIB). Fig. VIIC demonstrates that SR-B1-deficiency did not affect $S1P_3$ expression and vice versa. In addition, fractionation of VSMCs plasma membrane on sucrose step gradient revealed that both receptors were to large extent recovered from overlapping fractions characterized by low lipid content and distinct from caveolae (Fig. VIID).

REFERENCES TO SUPPLEMENTARY MATERIALS

1. Davis MD, Clemens JJ, Macdonald TL, Lynch KR Sphingosine 1-phosphate analogs as receptor antagonists. _J Biol Chem_ 2005:280:9833-9841.

2. Sanna MG, Liao J, Jo E, Alfonso C, Ahn MY, Peterson MS, Webb B, Lefebvre S, Chun J, Gray N, Rosen H.. Sphingosine 1-phosphate (S1P) receptor subtypes S1P1 and S1P3, respectively, regulate lymphocyte recirculation and heart rate. *J Biol Chem* 2004;279:13839-13848.

3. Brinkmann V, Davis MD, Heise CE, Albert R, Cottens S, Hof R, Bruns C, Prieschl E, Baumruker T, Hiestand P, Foster CA, Zollinger M, Lynch KR. The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *J Biol Chem* 2002;277:21453-21457.

4. Rigotti A, Trigatti BL, Penman M, Rayburn H, Herz J, Krieger M. A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. *Proc Natl Acad Sci U S A* 1997;94:12610-12615.

5. Ishii I, Friedman B, Ye X, Kawamura S, McGiffert C, Contos JJ, Kingsbury MA, Zhang G, Brown JH, Chun J. Selective loss of sphingosine 1-phosphate signaling with no obvious phenotypic abnormality in mice lacking its G protein-coupled receptor, LP(B3)/EDG-3. *J Biol Chem* 2001;276:33697-33704.

6. Tepel M, Jankowski J, Schluter H, Bachmann J, van der Giet M, Ruess C, Terliesner J, Zidek W. Diadenosine polyphosphates' action on calcium and vessel contraction. *Am J Hypertens* 1997;10:1404-1410.

7. Villaschi S, Nicosia RF, Smith MR. Isolation of a morphologically and functionally distinct smooth muscle cell type from the intimal aspect of the normal rat aorta. Evidence for smooth muscle cell heterogeneity. *In Vitro Cell Dev Biol Anim.* 1994;30A:589-95

8. Ades EW, Candal FJ, Swerlick RA, George VG, Summers S, Bosse DC, Lawley TJ. HMEC-1: establishment of an immortalized human microvascular endothelial cell line. *J Invest Dermatol* 1992;99:683-690

11

9. Tolle M, Levkau B, Keul P, Brinkmann V, Giebing G, Schonfelder G, Schafers M, von Wnuck Lipinski K, Jankowski J, Jankowski V, Zidek W, van der Giet M. Immunomodulator FTY720 Induces eNOS-dependent arterial vasodilatation via the lysophospholipid receptor S1P3. *Circ Res* 2005;96:913-920.

10. Nofer J-R, Levkau B, Wolinska I, Junker R, Fobker M, von Eckardstein A, Seedorf U, Assmann G. Suppression of endothelial cell apoptosis by high density lipoproteins (HDL) and HDL-associated lysosphingolipids. *J. Biol. Chem.* 2001;276:34480-5

11. Schaphorst KL, Chiang E, Jacobs KN, Zaiman A, Natarajan V, Wigley F, Garcia JG. Role of sphingosine-1 phosphate in the enhancement of endothelial barrier integrity by platelet-released products. *Am J Physiol Lung Cell Mol Physiol.* 2003;285:L258-67

12. Rothe G, Valet G. Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescin. *J Leukoc Biol* 1990;47:440-448.

13. Tepel M, Echelmeyer M, Orie NN, Zidek W. Increased intracellular reactive oxygen species in patients with end-stage renal failure: effect of hemodialysis. *Kidney Int* 2000;58:867-872.

14. van der Giet M, Erinola M, Zidek W, Tepel M. Captopril and quinapril reduce reactive oxygen species. *Eur J Clin Invest* 2002;32:732-737.

15. Matesanz N, Lafuente N, Azcutia V, Martin D, Cuadrado A, Nevado J, Rodriguez-Manas L, Sanchez-Ferrer CF, Peiro C. Xanthine oxidase-derived extracellular superoxide anions stimulate activator protein 1 activity and hypertrophy in human vascular smooth muscle via c-Jun N-terminal kinase and p38 mitogen-activated protein kinases. *J Hypertens.* 2007;25:609-18
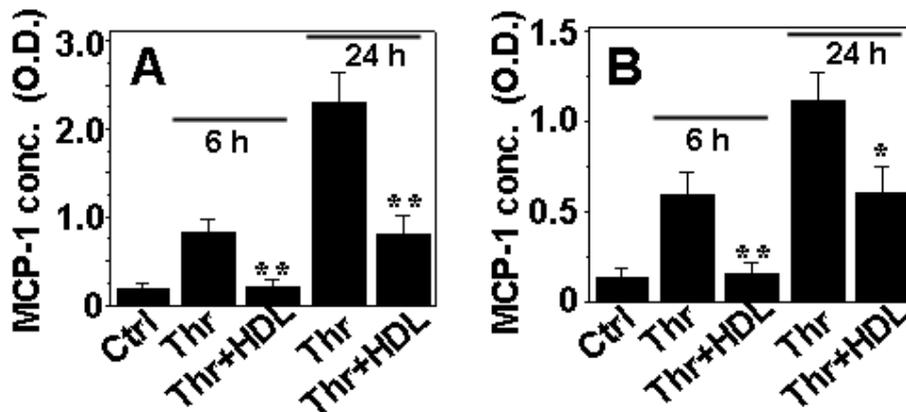
FIGURES

Figure I. *Effect of HDL on the thrombin-induced MCP-1 production in endothelial cells and macrophages* - Endothelial cells (HMEC-1; A) and macrophages (RAW264.7; B) were exposed for 6 h or 24h to thrombin (1.0 U/mL) in the presence or absence of HDL (0.5 g/L). Media were collected and MCP-1 protein levels were determined by ELISA. Data represent mean ± SEM from 4 experiments.
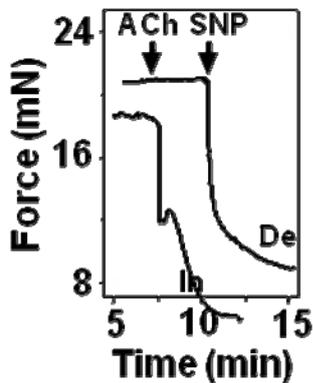

Figure II. Effect of acetylcholine in deendothelialized aortae

De-endothelialized (de) or intact (in) aortas were precontracted with phenylephrine and endothelium- or VSMCs-dependent relaxation was induced by 1.0µmol/L acetylcholine (Ach) or 1.0µmol/L sodium nitroprusside (SNP), respectively.
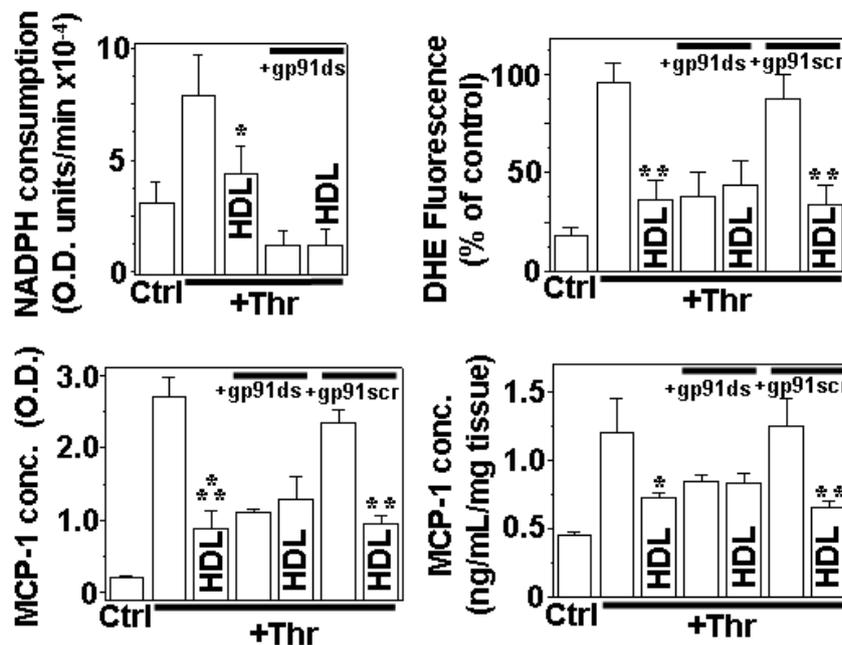
Figure III. *Effect of gp91ds on the HDL-induced inhibition of ROS generation and MCP-1 production in VSMCs* - Thrombin-induced NADPH-consumption (upper left panel), superoxide production (upper right panel), and MCP-1 production in VSMCs (lower leftt panel) and isolated aortas (lower right panel) were determined in the presence of HDL (0.5 g/L) and/or gp91ds (10.0 µg/mL) or its inactive analogue gp91scr (10.0 µg/mL). Shown are data from 3 to 5 experiments. * - p<0.05, ** - p<0.01, *** - p<0.001 treatment without HDL vs. treatment with HDL.
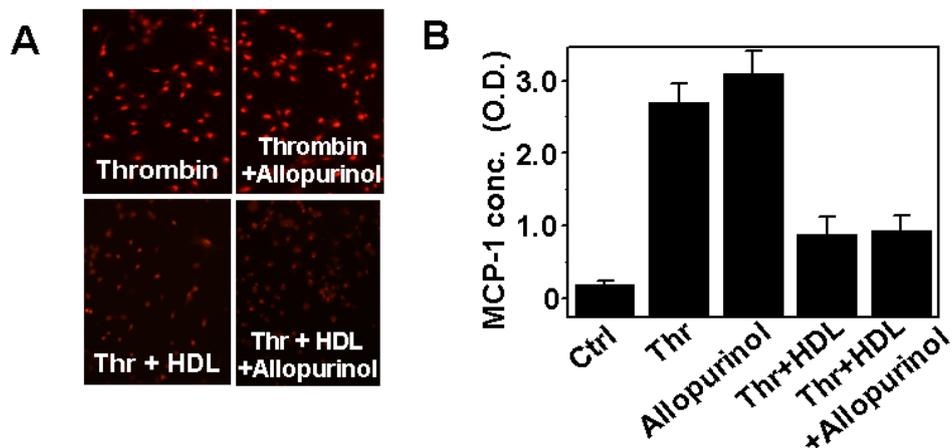
Figure IV. *Effect of allopurinol on the HDL-induced inhibition of ROS generation and MCP-1 production in VSMCs* - VSMCs were exposed for 16 h or 24 h to thrombin (1.0 U/mL) in the absence or presence of allopurinol (5.0 µmol/L), HDL (0.5 g/L), or both. **A**. Superoxide generation was detected by EtBr fluorescence using laser scanning confocal microscopy. **B**. Conditioned media were collected and MCP-1 protein levels were determined by ELISA. Data represent mean $\pm$ SEM from 3 experiments.
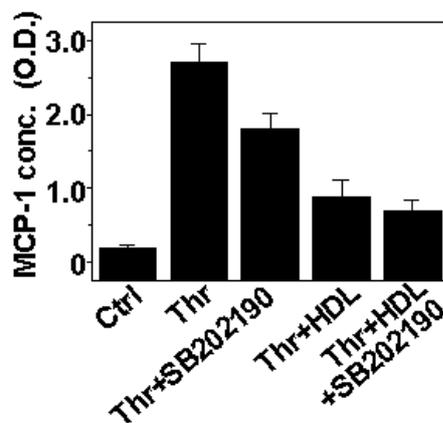

Figure V. *Effect of SB202190 on the HDL-induced inhibition of MCP-1 production in VSMCs* - VSMCs were exposed for 24 h to thrombin (1.0 U/mL) in the absence or presence of SB202190 (20 µmol/L), HDL (0.5 g/L), or both. Conditioned media were collected and MCP-1 protein levels were determined by ELISA. Data represent mean $\pm$ SEM from 3 experiments.
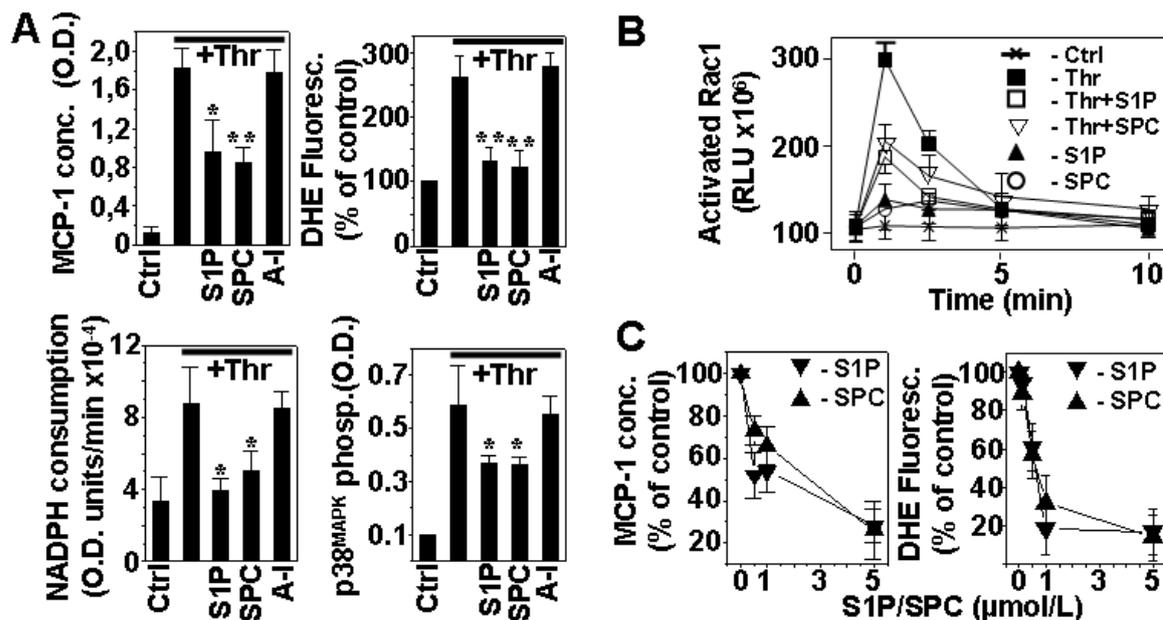
Figure VI. *Effect of HDL-associated lysosphingolipids on the thrombin-induced MCP-1 production, ROS generation, and NAD(P)H oxidase activation in VSMCs and mouse aortic segments* – **A and B**. VSMCs were exposed to 1.0 U/mL thrombin in the presence or absence of S1P (1.0 µmol/L), SPC (1.0 µmol/L) or apoA-I (50 µg/L) and **A**: MCP-1 production, superoxide generation, p38^MAPK phosphorylation, NAD(P)H consumption rate and **B**: Rac1 activation were determined as described above. Data represent ± SD from at least 6 experiments. * - p<0.05, ** - p<0.01, thrombin vs. thrombin + S1P or SPC **C**. Dose-dependent inhibitory effect of S1P and SPC on the thrombin-induced MCP-1 production (left panel) and superoxide generation (right panel). Values represent mean ± SEM from 3 experiments.
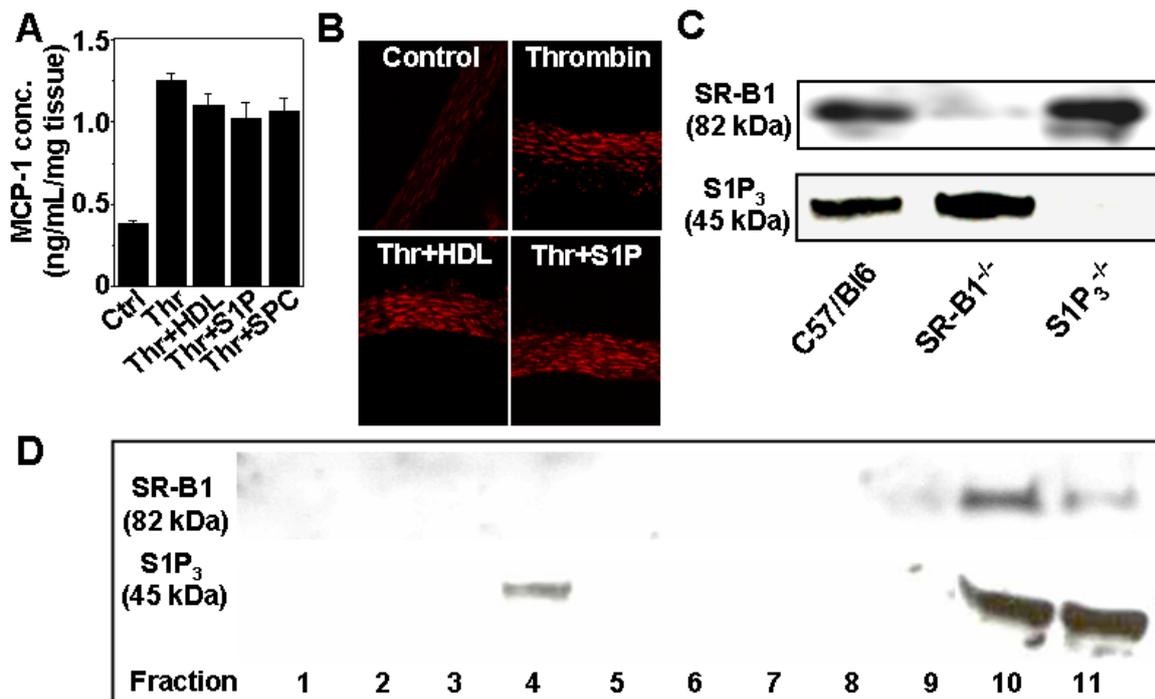
Figure VII. *Effect of HDL on the thrombin-induced MCP-1 production and ROS* *generation in SR-B1-deficient mice* – Aortic segments from wild type and SR-B1-deficient mice were exposed for 16 h to 4.0 U/mL thrombin in the absence or presence of HDL (0.5 g/L), S1P (1.0 µmol/L) and SPC (1.0 µmol/L). **A.** MCP-1 levels in media were determined by ELISA. Data represent mean ± SEM from 3 to 4 experiments. **B.** Superoxide generation was detected by EtBr fluorescence. Shown are representative images (magnification, x 400) from three experiments. **C**. Aortic homogenates from SR-B1^{-/-} and S1P$_3$[-/-] mice were probed with antibodies for expression of SR-B1 and S1P$_3$. **D**. VSMCs homogenates were fractionized over sucrose gradient and SR-B1 and S1P$_3$ presence in each fraction was investigated by Western blot. Shown are representative results of one experiment out of two.