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## HDL-associated lysosphingolipids inhibit NAD(P)H oxidase-dependent monocyte chemoattractant protein-1 production

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### Abstract

**Objectives**—High density lipoprotein (HDL) levels are inversely proportional to the risk of atherosclerosis, but mechanisms of HDL atheroprotection remain unclear. Monocyte chemoattractant 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<sub>1</sub> and S1P<sub>3</sub>, but not by JTE013, an antagonist of S1P<sub>2</sub>. In addition, HDL, S1P and SPC failed to inhibit MCP1 production and ROS generation in aortas from S1P<sub>3</sub>- 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<sub>3</sub> and SR-B1 receptors.

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## INTRODUCTION

Monocyte infiltration into the vessel wall is an initial step in the formation of atherosclerotic lesion<sup>1,2</sup>. Monocyte chemoattractant protein-1 (MCP-1) is a key regulator of monocyte recruitment to sites of vascular inflammation<sup>2-4</sup>. In addition, MCP-1 induces several pro-inflammatory changes including secretion of cytokines and expression of adhesion molecules<sup>2-4</sup>. MCP-1 was detected in atherosclerotic lesion and elevated levels of MCP-1 were encountered in acute coronary syndromes<sup>2-4</sup>. 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<sup>2-4</sup>.

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

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 inhibits MCP-1 production in vascular smooth muscle cells (VSMCs) and in isolated aortas. We further demonstrate that this effect is contingent upon inhibition of NADPH-oxidase-mediated generation of reactive oxygen species (ROS). We identify the HDL receptor SR-B1 and the lysosphingolipid receptor S1P<sub>3</sub> 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, Minnesota, USA), respectively. The S1P<sub>3</sub>-null mice on a C57Bl/J6 background was generated by J.Chun. All experiments were done with 8 to 10week-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<sub>3</sub>-null mice and wild-type littermates were kept in an organ bath containing Tyrode's solution.

### Analytical 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 340nm. Rac1 and p38<sup>MAP</sup> kinase activities were determined by commercially available solid phase pull-down assay and ELISA, respectively. p47<sup>phox</sup> 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 online (<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 (Fig.1A). The presence of HDL was associated with reduced MCP-1 release (Fig.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 Supplementary Materials, <http://atvb.ahajournals.org>). 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 that was markedly reduced in the presence of HDL (Fig.1C). To assess the contribution of smooth muscle cells to thrombin-induced MCP1 production in isolated aortas, experiments were performed after mechanical removal of endothelial layer (see Supplementary Materials, <http://atvb.ahajournals.org>). As shown in Fig.1D, the thrombin-induced MCP1 production in de-endothelialized 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<sup>9–11</sup>, 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 that was reduced in the presence of HDL (Fig.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<sub>2</sub>O<sub>2</sub>. We next monitored the effect of HDL on the thrombin-induced H<sub>2</sub>O<sub>2</sub> production using a fluorogenic substrate H<sub>2</sub>DCFDA. A constant increase in DCF fluorescence was recorded in VSMCs indicating a steady-state H<sub>2</sub>O<sub>2</sub> production. Exposure of VSMCs to thrombin enhanced H<sub>2</sub>O<sub>2</sub> production and this was suppressed in cells pre-treated with HDL (Fig.2B). Since the phosphorylation of p38<sup>MAP</sup> kinase in response to thrombin occurs as a consequence of ROS generation in VSMCs<sup>12</sup>, the effect of HDL on thrombin-induced p38<sup>MAP</sup> kinase activity was examined. Fig.2C demonstrates the increase in phosphorylated p38<sup>MAP</sup> kinase in VSMCs exposed to thrombin and its suppression by HDL.

To investigate whether HDL inhibits 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 (Fig.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<sup>13,14</sup>, 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 (Fig.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 Supplementary Materials, <http://atvb.ahajournals.org>). 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 pre-treated 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 pre-treated with allopurinol, a xanthine oxidase inhibitor (see Supplementary Materials, <http://atvb.ahajournals.org>). Inhibition of p38<sup>MAP</sup> kinase with SB202190 did not prevent HDL to reduce MCP-1 production in response to thrombin (see Supplementary Materials, <http://atvb.ahajournals.org>).

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<sup>13</sup>. We next assessed the activity of Rac1 in VSMCs exposed to thrombin in the presence or absence of HDL. As shown in Fig.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<sup>phox</sup> NAD(P)H oxidase subunit is required for enzyme activation. As shown in Fig.3C, addition of thrombin to VSMCs increased and reduced, respectively, p47<sup>phox</sup> amounts associated with membrane and cytosolic VSMCs fractions. Both effects were substantially diminished in the presence of HDL. 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<sup>14,15</sup>, on MCP-1 levels, superoxide production, p38<sup>MAP</sup> 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 Supplementary Materials, <http://atvb.ahajournals.org>). 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 (Fig.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. Fig.4B illustrates that the ability of HDL<sub>3</sub>, HDL<sub>2</sub>, 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<sub>3</sub>

In agreement with previous studies, we found that both S1P<sub>2</sub> and S1P<sub>3</sub> but not S1P<sub>1</sub> are expressed in VSMCs<sup>16</sup>. 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<sub>2</sub>, and SEW2871, an agonist of S1P<sub>1</sub>. As shown in Fig. 5A, preincubation of VSMCs and aortic explants with FTY720P inhibited MCP-1 production, while 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<sub>1</sub> and S1P<sub>3</sub>, but not with JTE013 - an antagonist of S1P<sub>2</sub> (Fig.5B). These results pointed to S1P<sub>3</sub> 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<sub>3</sub>-deficient mice. Fig.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<sub>3</sub>-deficient mice. In addition, HDL, S1P and SPC failed to inhibit MCP-1 production in aortas from S1P<sub>3</sub>-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 Supplementary Materials, <http://atvb.ahajournals.org>). 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<sub>3</sub> 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 pro-atherogenic 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 pro-inflammatory signaling in the vasculature both collecting signals from pro-atherogenic factors including oxidized LDL, angiotensin II, homocysteine, or thrombin and triggering inflammatory responses such as production of cytokines and expression of adhesion molecules<sup>13,17</sup>. 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<sub>2</sub>O<sub>2</sub>, a product of superoxide decomposition, both in VSMCs and isolated aortas. Second, HDL inhibited the thrombin-induced activation of p38<sup>MAP</sup> 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 pre-treated with HDL. In addition, pre-incubation of VSMCs with HDL prevented the activation of Rac1 and the membrane translocation of p47<sup>phox</sup> 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 highly specific inhibitor of NAD(P)H oxidase but not to allopurinol - the

inhibitor of xanthine oxidase – pre-empted 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  $\alpha$ -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 apo A-I receptor, have been proposed that facilitate transfer of  $\alpha$ -tocopherol between HDL and the cell interior<sup>18,19</sup>. Whereas the direct effect of HDL-associated  $\alpha$ -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  $\alpha$ -tocopherol on cell oxidation are evident after few hours of incubation<sup>20,21</sup>. Second the purified lysophospholipids S1P and SPC, lipid components of HDL without anti-oxidative properties, mimicked HDL capacity to reduce intracellular ROS generation. Third, HDL inhibited the thrombin-induced NADPH consumption and p47<sup>phox</sup> membrane translocation, which are both located upstream to generation of superoxide. Consistent with our findings, Robbesyn et al. shown that  $\alpha$ -tocopherol-depleted HDL was still able to inhibit oxidized LDL-induced ROS generation, whereas  $\alpha$ -tocopherol failed to exert a short-term influence on the redox status of the cell<sup>21</sup>. Basing on our observations and those of Robbesyn 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 VSMCs 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 molecules<sup>14,15,22</sup>. 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<sup>16</sup>. 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  $\mu$ mol/L. As one mg of HDL contains 287 $\pm$ 17pg S1P and 290 $\pm$ 20pg SPC<sup>15</sup>, 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<sub>2</sub> and S1P<sub>3</sub>, are expressed in VSMCs<sup>16</sup>. We previously demonstrated that NO-dependent vasodilatory effects of HDL and HDL-associated lysosphingolipids were attenuated in thoracic aortas obtained from S1P<sub>3</sub>-deficient animals, suggesting that this particular receptor serves as a functional partner for HDL<sup>15</sup>. 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<sub>3</sub> and S1P<sub>1</sub> but not S1P<sub>2</sub> receptors. In addition, HDL and S1P retained its ability to inhibit thrombin-induced VSMCs activation in the presence of JTE013 – an S1P<sub>2</sub> antagonist. Conversely, elimination

of S1P<sub>3</sub> receptor by performing experiments either in the presence of S1P<sub>3</sub> inhibitor VPC23019 or in aortas from S1P<sub>3</sub>-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<sup>23</sup> suggest that the activation of the S1P<sub>3</sub> rather than S1P<sub>2</sub> 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<sub>2</sub> and inhibition of Rac1 activity<sup>16,24</sup>. However, the inhibitory effects of S1P<sub>3</sub> and the activating effects of S1P<sub>2</sub> on Rac1 activation were also reported<sup>25,26</sup>. It is noteworthy that both receptors utilize 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<sub>3</sub> has recently been shown to reside in membrane invaginations termed caveolae, which host and are stabilized by SR-B1<sup>27,28</sup>. 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<sub>3</sub> in overlapping fractions is nevertheless consistent with the notion that these receptors co-localize 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 anti-atherogenic role of this lipoprotein. Uncontrolled ROS generation is a distinguished feature of several pro-atherogenic 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<sup>29</sup>. Conversely, dramatic decrease in atherosclerotic lesions was observed in animals deficient in NAD(P)H oxidase<sup>30</sup>. Inhibition of NAD(P)H oxidase activity by HDL-associated lysosphingolipids may constitute an important mechanism by which HDL exerts its potent anti-atherogenic effects.

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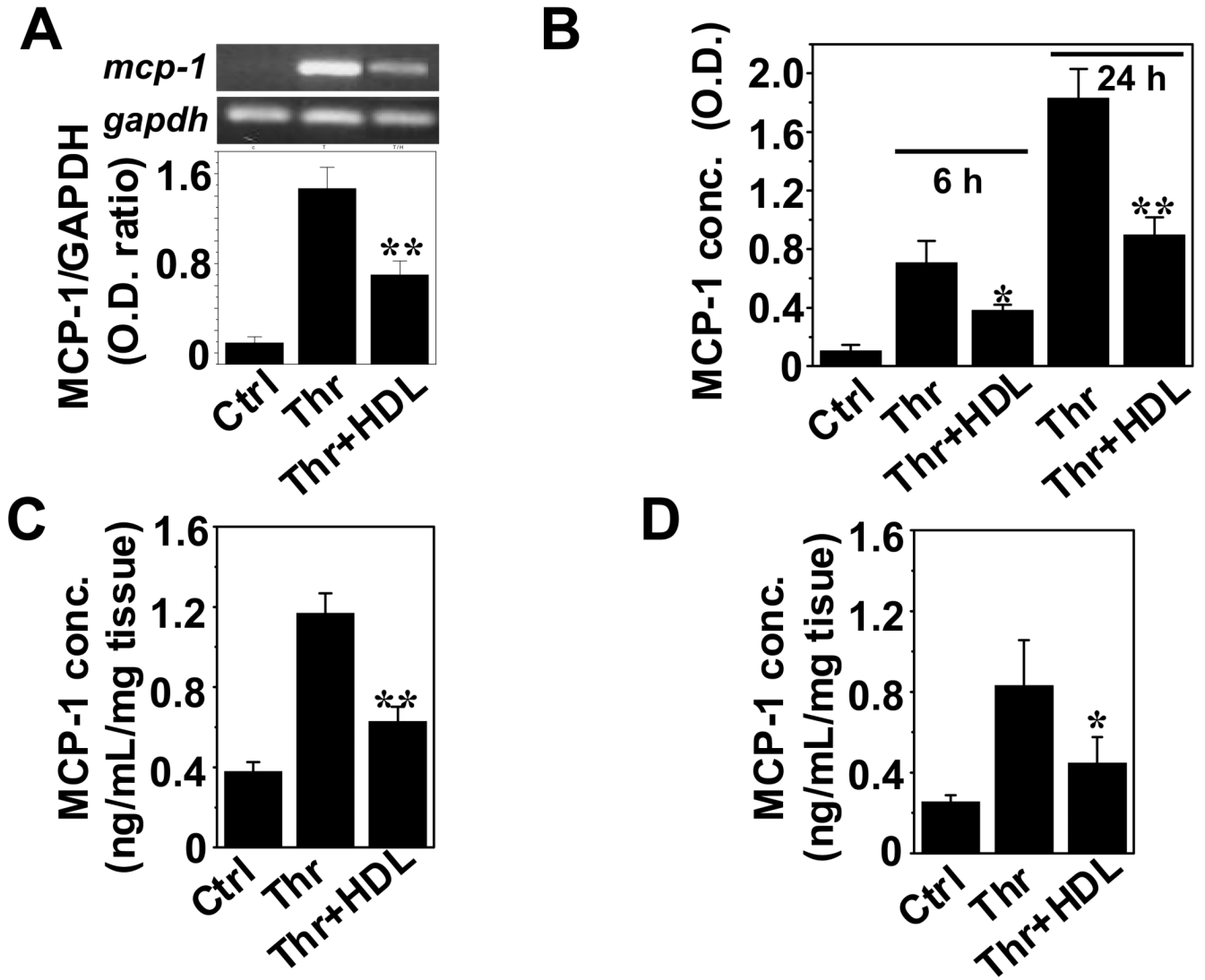
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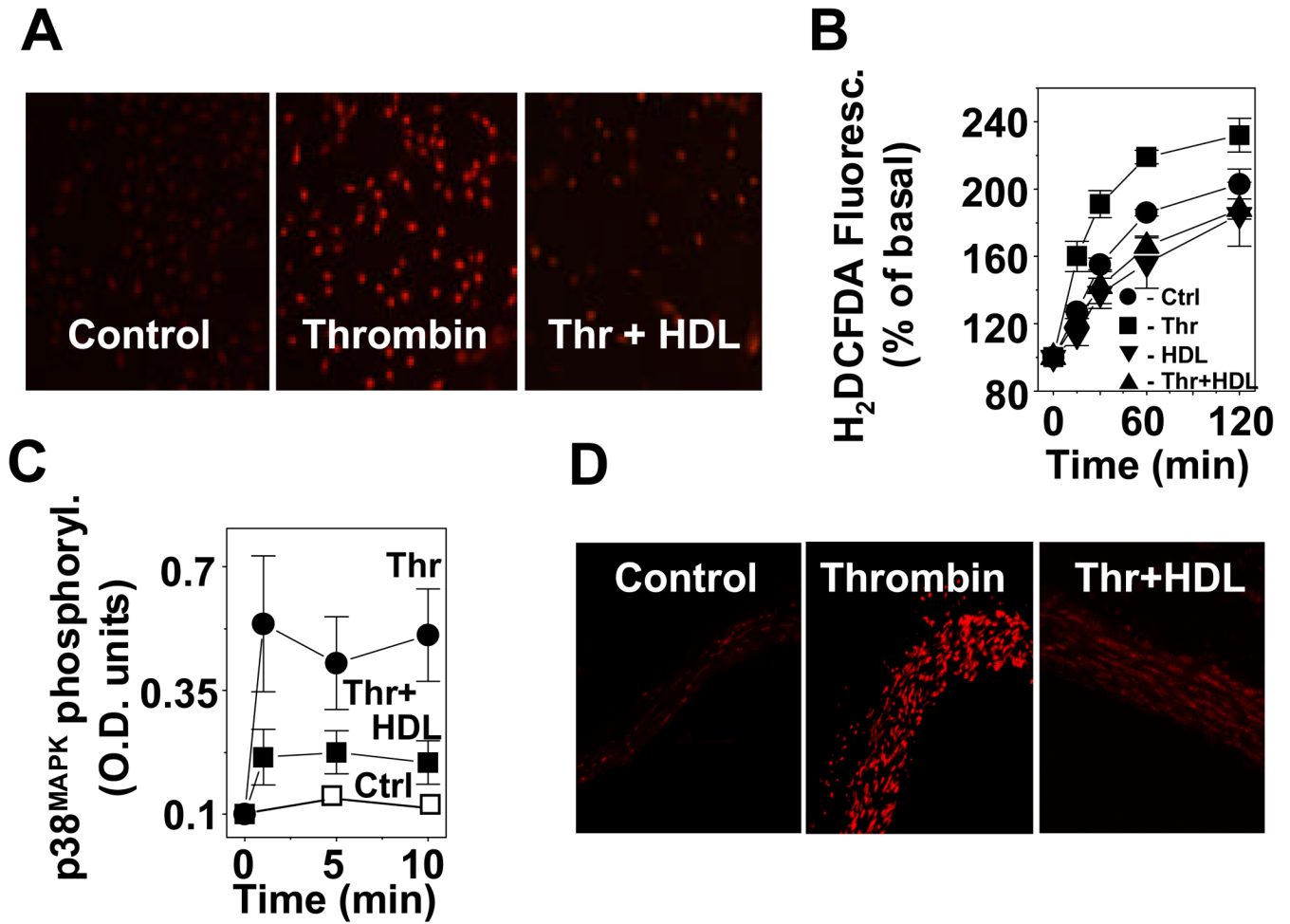
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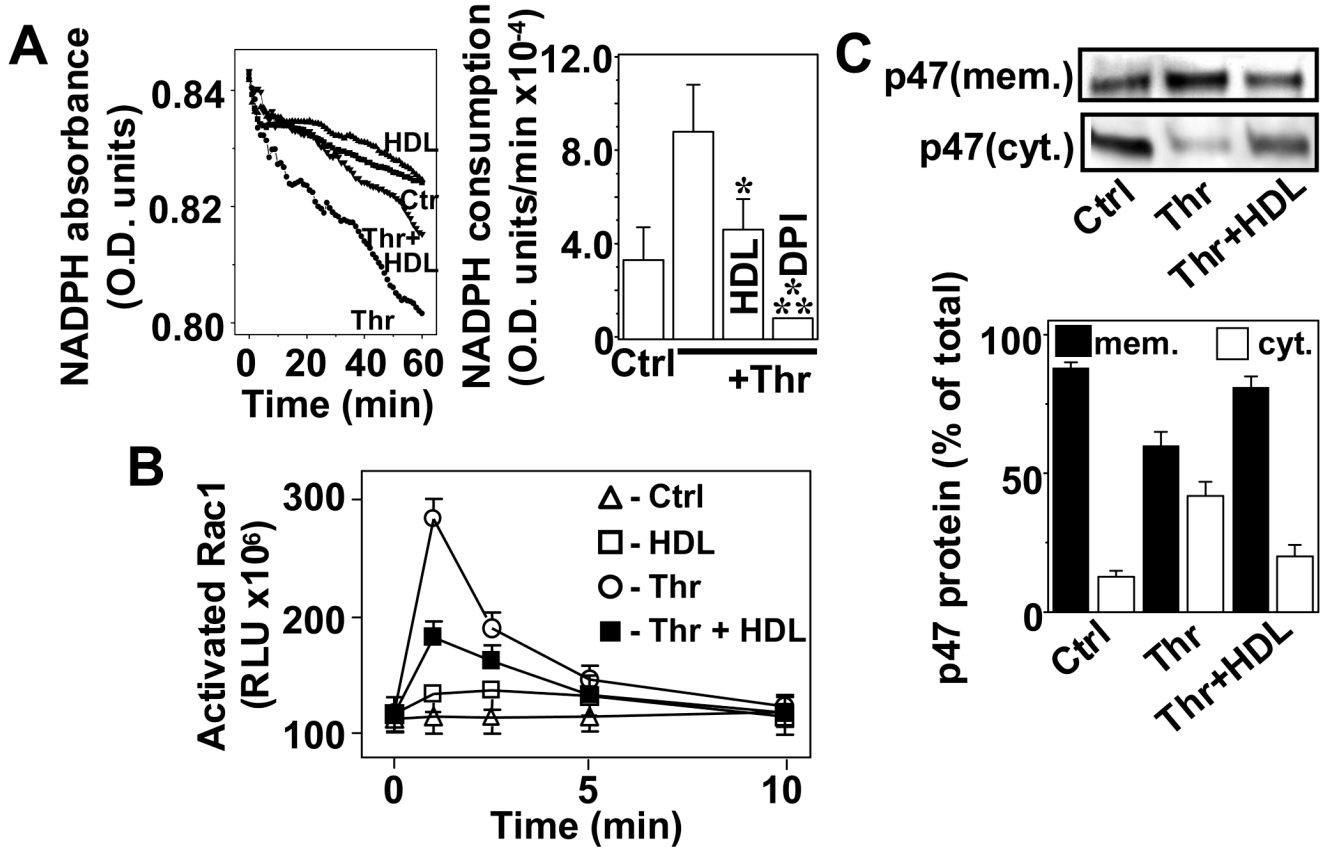


**Figure 1. Effect of HDL on the thrombin-induced MCP-1 production in VSMCs**  
VSMCs (A and B) or rat aortas (C) were exposed for 6h (B) or 24h (A–C) to thrombin (A and B–1.0U/mL; C–4.0U/mL) or thrombin/HDL (0.5g/L). **A.** mRNA levels assessed by RT-PCR. Lower panel: gel densitometry. Ratios from 3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

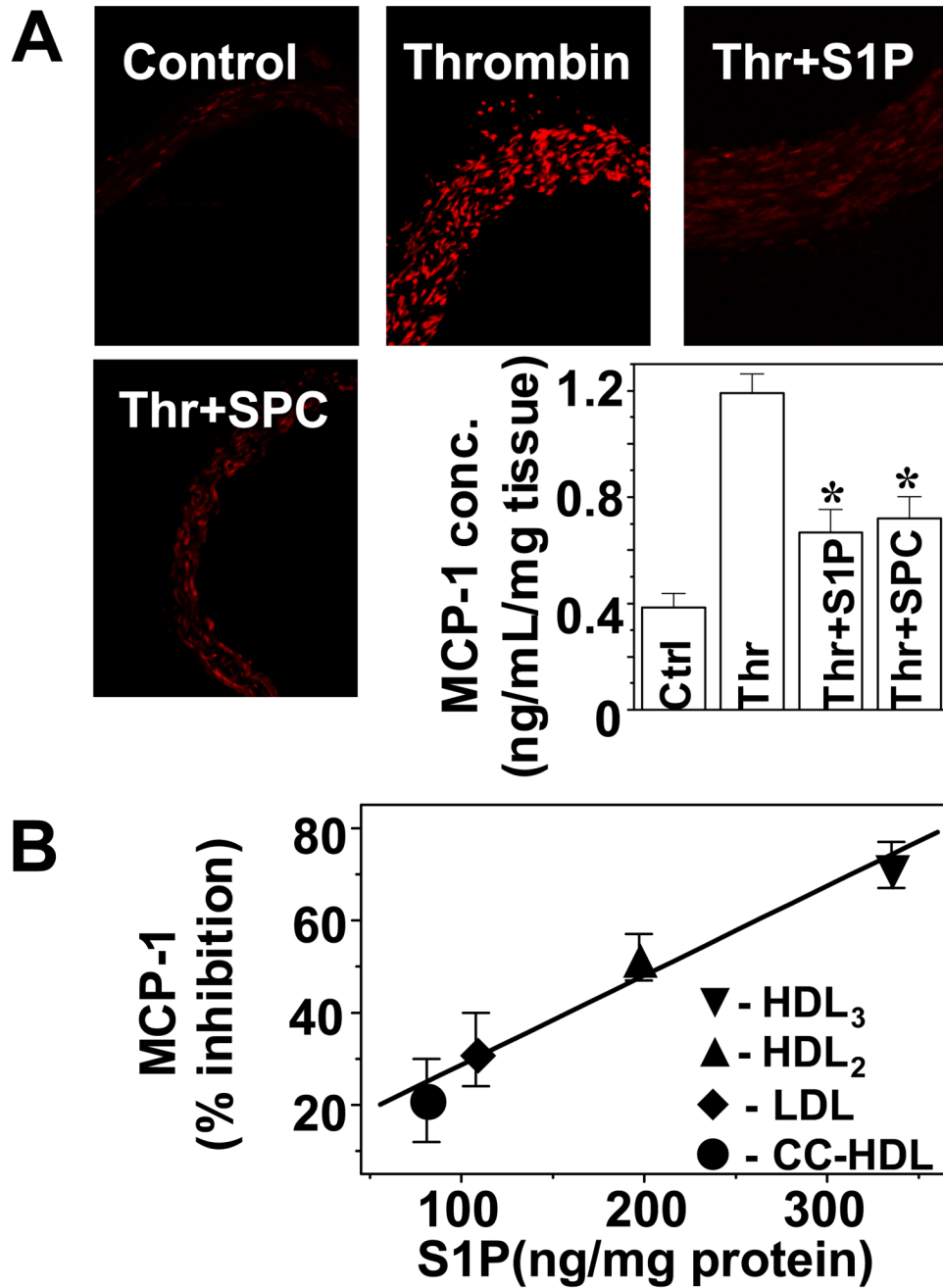


**Figure 2. Effect of HDL on the thrombin-induced ROS generation and p38<sup>MAPK</sup> kinase activation in VSMCs**

**A.** VSMCs exposed for 16h to thrombin (1.0U/mL)+HDL (0.5g/L). Superoxide detected by ethidium bromide (EtBr) fluorescence. Images ( $\times 400$ ) from 3 experiments. **B.** H<sub>2</sub>O<sub>2</sub> production in H<sub>2</sub>DCFDA-loaded VSMCs exposed to thrombin (1.0U/mL)  $\pm$  HDL (0.5g/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 (1.0U/mL)  $\pm$  HDL (0.5g/L). Phosphorylated p38<sup>MAPK</sup> assessed by ELISA. Means $\pm$ SEM from 3 to 4 experiments. **D.** Aortas were exposed for 16h to 4.0U/mL thrombin $\pm$ HDL (0.5g/L). Superoxide generation detected as above. Images from 3 experiments.

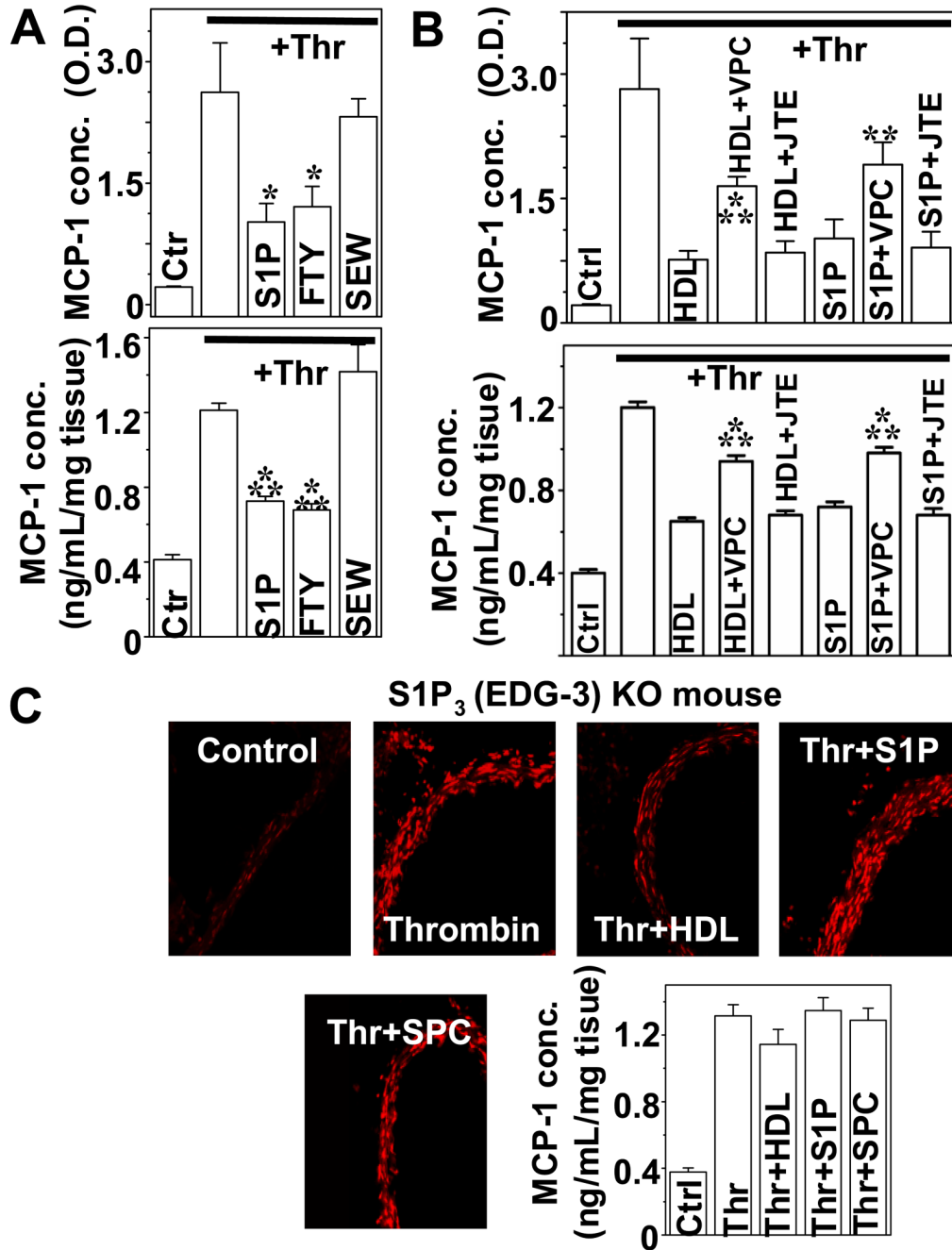


**Figure 3. Effect of HDL on the thrombin-induced NAD(P)H-oxidase activation in VSMCs**  
**A.** NADPH consumption in homogenates from VSMCs exposed for 1h to thrombin (1.0U/mL)  $\pm$  HDL (0.5g/mL). Superimposed tracings from 5 experiments. Right panel: NADPH consumption rate in VSMCs stimulated with thrombin/HDL (0.5g/L) or diphenyliodonium (DPI; 10 $\mu$ mol/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 1.0U/mL thrombin $\pm$ HDL (0.5g/mL) and assessed for (B) Rac1 activation or (C) p47<sup>phox</sup> translocation. (C). Immunoblots from 3 experiments. Lower panel: Densitometric analysis of p47<sup>phox</sup> in membrane (mem) and cytosol (cyt) fractions. Total p47<sup>phox</sup> 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 16h to 4.0U/mL thrombin  $\pm$ S1P (1.0 $\mu$ mol/L) or SPC (1.0 $\mu$ mol/L). Means $\pm$ SEM from 3 experiments. Superoxide generation detected by EtBr fluorescence. Images ( $\times$ 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—charcoal-treated HDL. Means  $\pm$ SEM from 3 experiments.



**Figure 5. Involvement of S1P<sub>3</sub> receptor in HDL- and lysophospholipids-dependent inhibition of the thrombin-induced MCP-1 production and ROS generation**

**A.** VSMCs (upper panel) or aortas (lower panel) exposed for 24h to, respectively, 1.0U/mL or 4.0U/mL thrombin±S1P (1.0μmol/L), FTY720P (1.0μmol/L) or SEW2871 (1.0μmol/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 30min with VPC2301 (20μmol/L) or JTE013 (20μmol/L) and exposed for 24h to, respectively, 1.0U/mL or 4.0U/mL thrombin±HDL (0.5g/L) or S1P (1.0μmol/L). \*\*— $p < 0.01$ , \*\*\*— $p < 0.001$  thrombin+HDL/S1P/HDL+VPC/S1P+VPC. **C.** Aortas from S1P<sub>3</sub>-deficient mice exposed for 16 h to 4.0U/mL thrombin±HDL (0.5g/L),

S1P (1.0 $\mu$ mol/L) or SPC (1.0 $\mu$ mol/L). Superoxide generation detected by EtBr fluorescence. Images ( $\times$ 400) from 3 experiments. MCP-1 levels determined by ELISA. Means $\pm$ SEM from 3 to 4 experiments.