Shear Stress Regulates Endothelial Microparticle Release

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Rationale: Endothelial activation and apoptosis release membrane-shed microparticles (EMP) that emerge as important biological effectors.

Objective: Because laminar shear stress (SS) is a major physiological regulator of endothelial survival, we tested the hypothesis that SS regulates EMP release.

Methods and Results: EMP levels were quantified by flow cytometry in medium of endothelial cells subjected to low or high SS (2 and 20 dyne/cm²). EMP levels augmented with time in low SS conditions compared with high SS conditions. This effect was sensitive to extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and Rho kinases inhibitors but unaffected by caspase inhibitors. Low SS-stimulated EMP release was associated with increased endothelial Rho kinases and ERK1/2 activities and cytoskeletal reorganization. Overexpression of constitutively active RhoA stimulated EMP release under high SS. We also examined the effect of nitric oxide (NO) in mediating SS effects. L-NG-nitroarginine methyl ester (L-NAME), but not D-NG-nitroarginine methyl ester, increased high SS-induced EMP levels by 3-fold, whereas the NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) decreased it. L-NAME and SNAP did not affect Rho kinases and ERK1/2 activities. Then, we investigated NO effect on membrane remodeling because microparticle release is abolished in ABCA1-deficient cells. ABCA1 expression, which was greater under low SS than under high SS, was augmented by L-NAME under high SS and decreased by SNAP under low SS conditions.

Conclusions: Altogether, these results demonstrate that sustained atheroprotective low SS stimulates EMP release through activation of Rho kinases and ERK1/2 pathways, whereas atheroprotective high SS limits EMP release in a NO-dependent regulation of ABCA1 expression and of cytoskeletal reorganization. These findings, therefore, identify endothelial SS as a physiological regulator of microparticle release. (Circ Res. 2013;112:1323-1333.)

Key Words: ABCA1 ■ endothelial cells ■ microparticles ■ nitric oxide ■ rho kinase ■ shear stress

Hemodynamic forces are the major determinant of atherosclerotic plaque localization. Shear stress (SS), a mechanical force generated by blood flow on the vascular endothelial cells, plays a fundamental role in regulating the phenotype of the endothelium. Plaques form preferentially at areas where blood flow is low and oscillatory, such as arterial bifurcations or curvatures, whereas blood vessels exposed to high laminar SS are less prone to develop atherosclerotic plaques.1 The atheroprotective effect of SS on endothelial cells is explained by the local release of nitric oxide (NO), an important mediator, which reduces endothelial permeability, leukocyte adhesion, and increases endothelial survival.2-4

Microparticles (MPs) are submicron membrane vesicles (0.1–1 µm) released on cell activation or apoptosis. They are characterized by the exposure of negatively charged phospholipids, such as phosphatidylserine, on their outer membrane leaflet after loss of membrane asymmetry.5 Phosphatidylserine externalization contributes to MPs procoagulant potential. MPs found in human plasma originate from different cell types, mainly platelets, leukocytes, and red blood cells, but also from endothelial cells.6 Endothelial MPs (EMPs) have been identified as a marker of endothelial dysfunction and as a marker of systemic vascular remodeling.7 So far, the pathophysiological mechanisms regulating EMP formation in vivo remain unknown. In vitro, endothelial apoptosis and activation by proinflammatory mediators increase EMP basal release by activating either caspase-, Rho kinase-, or mitogen-activated protein (MAP) kinase–dependent pathways.7 Because low SS (LSS) is an

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Importantly, this study highlights that different SS levels might regulate in vivo endothelial apoptosis. The aim of this study was, therefore, to evaluate the effects of SS on EMP release.

We first investigated whether or not EMP plasma levels associate with arterial shear rate, the major determinant of SS, in healthy subjects presenting at least 1 cardiovascular risk factor. We previously observed in patients with end-stage renal failure that EMP plasma levels are inversely correlated with local arterial SS. This relationship was independent of age and blood pressure, but this observation was made in a group of highly selected patients. We also examined the effects of different SS profiles on EMP release in vitro conditions and investigated the signaling pathways involved.

**Methods**

See the Online Data Supplement for expanded version.

**Clinical Study**

The study approved by the local ethical committee included 74 asymptomatic subjects without cardiovascular disease,10,11 and their cardiovascular risk factors were assessed according to current guidelines.12,13 The risk of coronary heart disease was estimated by entering age, male sex, systolic pressure, total and high-density lipoprotein–cholesterol, presence or absence of smoking in the Framingham model equations,13 and defined the 10-year coronary risk as low (<10%), intermediate (10%-20%), or high (20%). All participants gave written informed consent.

Subjects were examined after 12 hours fasting in the supine position. The brachial artery was visualized longitudinally by high-resolution ultrasound. Resting wall shear rate was calculated as 4x the ratio between the pulsed Doppler-measured mean blood velocity and the diastolic diameter.

Platelet-free plasma was obtained from citrated blood after centrifugation at 1500g (15 minutes) followed by 11 000g (2 minutes) within 1 hour after blood withdrawal, as described earlier.14

**Plasmid Electroporation**

DNA vector corresponding to pSG5-RhoA-V14G was used to transiently express the constitutively active V14-RhoA mutant.15

**Microparticle Generation and Isolation**

Unidirectional laminar SS was applied to confluent HUVECs using a parallel plate chamber system.16 Local SS was calculated using Poiseuille law and averaged 0, 2, or 20 dynecm², corresponding to static, LSS, and high SS (HSS) conditions, respectively. All inhibitors were added to the cells 1 hour before tumor necrosis factor α (TNF-α) or SS exposure and were kept during the indicated experimental time. The perfusion medium was collected, centrifuged to eliminate any cell debris (600g; 15 minutes; 4°C), and the resulting supernatant was then ultracentrifuged (20500g; 90 minutes; 4°C) to pellet MP, which were resuspended in 200 μL of 0.1-μm filtered medium and stored at −80°C.

**Microscopy**

HUVECs were observed by contrast phase microscopy to confirm correct response to SS using Zeiss Primo Vers (magnitude: 10x5). Transmission electron microscopy was performed at the IFR83-Jussieu-PARIS core facility.

In situ cell death detection kit from ROCHE (Neuilly-sur-Seine, France) was used to stain apoptotic cells and quantify the number of green fluorescent nuclei on DAPI-blue–stained cell nuclei. Actin localization at the plasma membrane was assessed by containing fluorescent phallolidin (Invitrogen, Carlsbad, CA) and anti-CD31 antibody (BD Pharmingen, Franklin Lakes, NJ). Actin localization at the plasma membrane was quantified by a score system for each image (0: no colocalization to 3: full colocalization) by 3 different blinded operators.

**Microparticle Characterization**

MPs were labeled with FITC–annexin-V in calcium-dependent manner, VE-cadherin antibody (CD144-PE, Immunotech; Marseille, France), or isotypic control. MPs were defined as elements with a size <1 μm and >0.5 μm that were positively labeled with either FITC–annexin V or CD144-PE antibody by flow cytometry.14 Unless otherwise indicated, samples were analyzed using EPICS XL (Beckman Coulter; Brea, CA); some samples were measured again using a highly sensitive flow cytometer (Galios, Beckman Coulter) to assess small (<0.5 μm) and large (>0.5 μm) EMP populations.17 Levels of apoptotic bodies were estimated as annexin V + propidium iodide + vesicles in the 1- to 4-μm range according to Hristov et al.15 Annexin V + propidium iodide + apoptotic bodies averaged 6±1, 7±1, and 6±1 events/μL in LSS, LSS, and static conditions, respectively (n=5), and were therefore negligible in each experimental condition. In addition, the presence of exosomes was estimated using the specific marker TSG101.19 TSG101 levels were hardly detectable in endothelial MP preparations, when compared with a positive control (Online Figure I).

**RNA Extraction and Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction**

RNA was extracted using TRIzol (Invitrogen). RT-PCR was performed with 0.1 μg of RNA (Qiagen); quantitative polymerase chain reaction was performed using SYBRgreen (Sigma). Ubiquitin C was used as housekeeping gene as previously described20 to normalize the expression of ABCA1. Normalization was done using the 2-delta CT method.

**Western Blotting**

HUVECs were scraped off in radio immunoprecipitation assay or saline buffer, and MP pellets were resuspended in radio immunoprecipitation assay buffer.

**Statistical Analysis**

**Clinical Study**

Clinical continuous parameters are expressed as mean±SDs with ranges and qualitative parameters as number of subjects (%). Quantitative
variables with non-normal distribution were log-transformed before correlations analysis. Normal distribution was assessed by means of the Shapiro–Wilk test. The relationship between MP levels and brachial artery baseline shear rate was analyzed by univariate and multivariable linear regression by adjusting for each of the risk factors (one by one), with MP as the dependent variable, and with shear rate and each risk factors as independent variables.

**In Vitro Study**

Data obtained from at least 5 independent experiments are expressed as means±SEM. Statistics were performed using PRINS software. Significant differences between >2 conditions were determined by 1-way ANOVA followed by post hoc analysis using Bonferroni test. Comparisons between different SS conditions or between control and treatment conditions were performed using a Wilcoxon signed-rank test. For ABCA1 mRNA expression only, controls were not paired with treatment, so a Mann–Whitney U test was used. Significance was accepted for a value of P<0.05.

**Results**

**Atheroprone Low Laminar SS Augments Endothelial MP Shedding**

Circulating EMPs were quantified as CD144-positive events in platelet-free plasma obtained from 74 asymptomatic subjects. As shown in Online Table I, 93% of the patients were at 10-year low to intermediate coronary risk. CD144+MPs and brachial artery baseline shear rate did not differ between men and women. In univariate analysis, CD144+MP plasma levels were inversely related to brachial artery baseline shear rate (r=−0.25; P=0.009), which is directly proportional to SS, assuming that blood viscosity does not significantly vary in healthy subjects (Figure 1A). This inverse relationship remained significant in multivariable analyses after adjusting for the following: (1) either age, male sex, hypertension, hypercholesterolemia, low high-density lipoprotein, smoking, or diabetes mellitus; or (2) the integration of multiple risk factors as expressed by the number of cardiovascular risk factors or by the 10-year Framingham risk score (P<0.05). However, no significant correlation was observed between brachial artery baseline shear rate and CD11a+ (r=0.041; P=0.084) leukocyte MP levels, which have been associated with subclinical atherosclerosis. In view of this observation in healthy subjects, in vitro experiments on cultured endothelial cells were designed to evaluate the effect of different levels of SS on the subsequent EMP release.

Low-passage endothelial cells were maintained for up to 24 hours under sustained atheroprotective high laminar SS (20 dyne/cm²), atheroprone low laminar SS (2 dyne/cm²), or static conditions. EMP levels were quantified in cultured medium up to 24-hour exposure to the different SS levels. After 12 hours, both LSS and static conditions significantly augmented EMP release when compared with controls.

Figure 1. Relationship between endothelial microparticle (EMP) and shear stress levels. A, Correlation between baseline brachial artery shear rate and CD144+ EMP level in patients (n=74; r=−0.25; P=0.009). B, Timecourse of annexin V + EMP release in vitro (static: black diamond; low shear stress (LSS): gray square; high SS (HSS): white circle; 2–6 hours: n=4; 12 hours: n=5; 24 hours: n=17). C, Comparison of annexin V + EMP level and CD144+ EMP level under different SS profiles in vitro (annexin V: black circle, n=17; CD144+: white square, n=6). EMPs were measured by flow cytometry (size of 0.1–1.0 μm that stained positively for annexin V or CD144). Annexin V labeling: ***P<0.001; **P<0.01 (compared with HSS); CD144 labeling: $$$P<0.01; $P<0.05 (compared with HSS). D, Membrane blebbing of human umbilical vein endothelial cells under different shear stress profiles (from left to right: HSS; LSS; static condition [static]) was observed by transmission electron microscopy (×9600; representative experiment out of 5). Black arrows indicate presence of membrane blebs in LSS and static conditions.
atheroprotective HSS profile, reaching a 2.5- and 3-fold increase at 24 hours, respectively ($P<0.001$; Figure 1B). This finding was confirmed when labeling EMPs with an anti-VE-cadherin (CD144) antibody (Figure 1C). Electron microscopy investigations demonstrated that endothelial membrane blebs were formed under static conditions, and even more under LSS, whereas plasma membrane of cells exposed to HSS seemed more regular (Figure 1D). As expected, cells were aligned under HSS but not under LSS or static conditions (Online Figure IIA). Furthermore, apoptosis evidenced by terminal deoxynucleotidyl transferase dUTP nick end labeling staining was more pronounced under LSS or static conditions than under HSS (Online Figure IIB).

Then, we tested the hypothesis that EMP release results from increased endothelial apoptosis in LSS and static conditions. The pan-caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-FMK) abolished endothelial apoptosis in both conditions ($P=0.049$; Online Figure IIB) and significantly decreased the release of EMP under static conditions ($P=0.0078$; Figure 2A). However, Z-VAD-FMK had no effect under LSS condition ($P=0.8125$; Figure 2A). These results demonstrate that endothelial caspase activity does not contribute to LSS-associated EMP release.

**Pathways Implicated in EMP Release Under LSS**

Next, we examined the potential contribution of Rho kinases (ROCKs), MAP-kinases, and nuclear factor κB pathways, as previous studies identified the role of these pathways in EMP release under proinflammatory conditions. All of the 5 inhibitors tested affected basal EMP release in the absence of TNF-α (static conditions; Online Table II). In agreement with previous reports, nuclear factor κB and p38 MAP-kinase pathway inhibitors abolished TNF-α-induced EMP release, whereas ROCK, extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), and Jun-kinase inhibitors had no effect on EMP release under LSS ($P<0.05$ both), whereas inhibitors of p38 MAP-kinase, Jun-kinase, and nuclear factor κB were without effect (Figure 2B). Concomitant inhibition of ROCK and caspases using Y27632 and Z-VAD-FMK showed no additional effect when compared with experiments in the presence of ROCK inhibitor alone (41±17% versus 42±3% inhibition, respectively; n=4). Exposure to LSS for 12 hours significantly increased phospho-myosin light chain (MLC) (a ROCK substrate) and phospho-ERK1/2, when compared with HSS (Figure 3A), showing that a sustained exposure to LSS increased endothelial ROCK and ERK1/2 activities, whereas no difference was observed between cells exposed to HSS and those maintained under static conditions ($P=0.90$ and 0.55, respectively). The ROCK inhibitor Y27632 significantly decreased phospho-MLC ($P=0.045$) and phospho-ERK1/2 ($P=0.034$) levels in endothelial cells exposed to LSS, whereas the ERK1/2 inhibitor PD98059 reduced only the level of phospho-ERK1/2 ($P=0.01$) without affecting that of phospho-MLC ($P=0.43$; Figure 3B). Finally, the ROCK inhibitor Y27632 did not affect EMP release on HSS (24 hours; control: 123±33; Y27632: 112±43 annexin V + MPs/μL; n=4; $P=0.95$). Conversely, increasing ROCK activity by endothelial cell transfection with the constitutively active RhoA-V14 mutant increased EMP release under HSS, when compared with mock-transfected cells (Figure 3C).

Actin depolymerization at the plasma membrane is a key factor in circulating MPs formation. Exposure of endothelial cells to cytochalasin D under static condition
reduced intracellular actin stress fiber formation, augmented actin localization at the plasma membrane, and decreased EMP release (P<0.035; Figure 4A). Fewer amounts of F-actin were localized at the plasma membrane under LSS, when compared with HSS conditions (P<0.032; Figure 4B). To demonstrate that activation of the ROCK and ERK1/2 pathways was associated with F-actin delocalization from the plasma membrane, we evaluated the level of F-actin at the plasma membrane after inhibition of ROCK and ERK1/2. Both inhibitors restored F-actin localization at the plasma membrane in endothelial cells chronically exposed to LSS (ROCK inhibition: P<0.043; ERK1/2 inhibition: P=0.049), whereas they had no effect under HSS condition (Figure 4B). As expected, transfection with the constitutively active RhoA-V14 mutant increases stress fiber in endothelial cytoplasm both under HSS and LSS conditions (Figure 4C). When we examined the plasma membrane, RhoA overexpression clearly decreased the level of polymerized actin, confirming that LSS-induced EMP release is mediated by membrane weakening (Figure 4C).

Because μ-calpain activation mediates platelet MP shedding, we also examined its potential contribution to increased EMP release under LSS. Cleavage of α-fodrin (150 kDa fragment versus 250 kDa) and expression of total μ-calpain (D4) and cleaved μ-calpain (D1) were monitored on different SS profiles as an index of endothelial calpain activity.25 The expression of latent μ-calpain, its cleavage, and the cleavage of endothelial α-fodrin were not different among HSS, LSS, and static exposure (Online Figure IIIA and IIIB), demonstrating that μ-calpain activity is not associated with the increased in EMP release under LSS.

Effect of NO on Endothelial MP Release on Different SS Profiles

Because NO is an important mediator in the response of endothelial cells to different SS profiles, we tested the hypothesis that endogenous NO might affect the release of EMPs under these conditions. L-NG-nitroarginine methyl ester (L-NAME) significantly increased EMP release under atheroprotective HSS, whereas D-NG-nitroarginine methyl ester has no effect (HSS: 367±37; HSS+D-NG-nitroarginine methyl ester: 270±22; N=6; P=0.0625). Furthermore, L-NAME did not modify EMP release in atheroprone LSS conditions (Figure 5A). Similarly, exposure to the NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) during sustained exposure of endothelial cells to LSS significantly decreased the release of EMPs (Figure 5B). These results, therefore, show that SS-induced NO production prevents EMP formation. To evaluate a possible effect of L-NAME on caspase activation,
endothelial cells were treated with both L-NAME and Z-VAD-FMK under HSS; no significant effect was observed on EMP levels (HSS+L-NAME: 378±44; HSS+L-NAME+Z-VAD-FMK: 288±67 annexin V + EMPs/μL; P=0.56; n=6), therefore ruling out the possible contribution of caspase activation to EMP release after NO synthase (NOS) inhibition.

Then, we tested the possible repressive effect of NO on the ROCK-ERK1/2 pathway. In HSS endothelial cells, L-NAME did not significantly affect the levels of phospho-MLC and phospho-ERK1/2 (Online Figure IV A and IVB). Similarly, in LSS conditions, exposure to SNAP did not modify the levels of phospho-MLC and phospho-ERK1/2 (Online Figure IVC and IVD). However, cotreatment with SNAP and the ERK1/2 inhibitor further reduced EMP release from 268±99 to 188±89 annexin V + EMPs/μL when compared with ERK1/2 inhibition alone (P=0.031; n=6), but had no significant effect when compared with SNAP alone (222±98 versus 188±89 annexin V + EMPs/μL, respectively; P=0.18, n=6). These observations suggest that NO and ERK1/2 have common effectors regarding endothelial MP release. To demonstrate that NO prevents F-actin delocalization, we evaluated the level of F-actin at the plasma membrane after NOS inhibition. L-NAME treatment decreased F-actin localization at the plasma membrane in endothelial cells chronically exposed to HSS (P=0.031), whereas this inhibitor had no effect under LSS condition (Figure 5C).

Then, we focused on plasma membrane remodeling and phosphatidylserine externalization as the other key factor in MP release and investigated the contribution of scramblase-1 and ABCA1 flippase to NO effects. Scramblase-1 expression was increased under LSS condition as compared with HSS (P=0.047) but was not affected by L-NAME (Online Figure V). Sustained exposure to LSS also significantly increased endothelial ABCA1 expression, when compared with HSS (P=0.047; Figure 6). Exposure to L-NAME augmented ABCA1 protein expression under HSS, to reach a value not different from that of cells exposed to LSS (P=0.87; Figure 6A). Quantitative polymerase chain reaction analysis of ABCA1 mRNA expression demonstrated that LSS increased ABCA1 mRNA levels compared with HSS, and NOS inhibition increased ABCA1 mRNA levels under both LSS and HSS conditions (Figure 6B). SNAP addition reduced mRNA expression under LSS (n=5; P=0.031; data not shown). When endothelial cells were exposed to the liver X receptor agonist TO901317 (10−7 mol/L) to pharmacologically reproduce the effect of LSS, ABCA1 mRNA expression increased by 18-fold (n=3, data not shown) and EMP release augmented by 1.4-fold under HSS condition (Figure 7A). We also silenced ABCA1 under the different shear profiles to further confirm the contribution of ABCA1 to EMP release. Transfection efficacy averaged 55%, and ABCA1 expression levels in LSS conditions were no longer different from those observed under HSS (P=0.81; Figure 7B). Under these experimental conditions, ABCA1 silencing significantly reduced EMP release by 40% under LSS (P=0.015; Figure 7C). Finally, expression of ABCA1 and scramblase-1 were not affected by ROCK inhibition under LSS (P=0.43 and P=0.68, respectively; Online Figure VI).

**Microparticle Characteristics**

In a subset of experiments, we quantified EMPs using the next generation flow cytometer (Galios, Beckman Coulter) to quantify MPs <0.5 μm as shown by Robert et al.17 LSS

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**Figure 4. Actin localization and endothelial microparticle (EMP) release.** EMPs were measured by flow cytometry using annexin V staining, and actin localization at the plasma membrane was measured by a score system (0: no colocalization to 3: total colocalization; F-actin: green, CD31: red). **A**, Effect of cytochalasin D treatment under static condition (24 hours; 2.5×10−7 mol/L; n=5); experiments with cytochalasin D could not be performed under shear stress as cells detached from their matrix. **B**, Effect of ERK1/2 and Rho kinases inhibitor on actin localization under high-shear stress (HSS) or low-SS (LSS; 24 hours; n=5). **C**, Effect of RhoA overexpression on actin localization under high- or low-SS. *P<0.05 (compared with static condition); **P<0.01 (compared with HSS condition); $P<0.05 (compared with LSS).
increased the release of both small and large EMPs, when compared with HSS conditions (n=13; P=0.033 and P=0.013, respectively; Online Table III). Under LSS, ROCK inhibition decreased EMP release for the large and the total population, significantly (n=7; P=0.047 and P=0.016), but not for the small population (P=0.36). Under HSS, L-NAME increased EMP release for the large and the total population (n=8; P=0.023 and P=0.023), but this effect was not significant for the small population (P=0.054; Online Table II). These results demonstrate that the molecular mechanism of SS we identify in the present study contributes mostly to the regulation of large EMPs (size >0.5 μm).

Discussion

The present results demonstrate for the first time that atheroprone SS conditions stimulate the formation and release of EMPs and identify hemodynamic forces as an important determinant of their plasma levels in healthy subjects.

Endothelial cells in culture shed membrane MPs in response to apoptotic stimuli (such as serum deprivation or exposure to camptothecin)26 or after activation by TNF-α, thrombin, plasminogen activator inhibitor-1, angiotensin II, reactive oxygen species, uremic toxins, or oxidized low-density lipoproteins.7,21,27–30 Because SS is a major determinant of endothelial survival,11 we tested the hypothesis that LSS augments the EMP release in vitro. We quantified EMPs in endothelial cell supernatant using either calcium-dependent annexin V-labeling or CD144 antibody (versus isotype). Sustained exposure to atheroprone LSS conditions increased both endothelial apoptosis and the release of MPs in the medium, when compared with physiological HSS conditions. EMP release was already detectable at 12 hours and further increased after a 24-hour exposure to LSS. Interestingly, a pan-caspase inhibitor decreased endothelial apoptosis but did not modify EMP release, demonstrating that caspase activation was not involved in LSS-induced MP formation.

Our in vitro findings on the effect of SS on EMP release are corroborated by the negative association we observed specifically between plasma EMP levels and arterial shear forces in apparently healthy subjects, independently of traditional risk factors. We previously made a similar observation in patients with end-stage renal diseases,9 who are known for having endothelial dysfunction. Interestingly, circulating EMP levels are also elevated in healthy subjects with enforced physical inactivity, where a decrease in blood flow and arterial SS has been reported.32 Furthermore, while the present article was under revision, Jenkins et al33 reported that acute reduction in blood flow increased circulating
levels of EMPs in healthy subjects. Taken all together, these in vivo results reinforce the pathophysiological relevance of our in vitro data.

Previous studies have reported activation of different signaling pathways during EMP release, depending on the agonist triggering membrane vesiculation. EMP formation requires activation of the RhoA-ROCK pathway during thrombin and angiotensin II stimulation,21,29 whereas TNF-α–dependent EMP release is controlled by the p38-MAP kinase pathway. 22 We observed in the present study that ROCK and ERK1/2 inhibitors decreased LSS-induced EMP release, whereas inhibitors of p38-MAP kinase, nuclear factor κB, and Jun-kinase pathways were without effect. The contribution of the ROCK pathway was supported by the observation that the ROCK activity was stimulated after a 12-hour exposure to LSS, as evidenced by increases in MLC phosphorylation. Interestingly, the modest EMP release observed on sustained HSS conditions was insensitive to inhibition of Rho-kinase activity. Furthermore, increased ROCK activity after constitutively active RhoA overexpression in endothelial cells exposed to HSS stimulated EMP release by ≈2-fold, to reach levels comparable with those observed on LSS condition. Taken altogether, these findings identify the ROCK pathway as a major determinant of EMP release on sustained exposure to atheroprone LSS (Figure 8). Sustained LSS activation of the ROCK pathway increased ERK1/2 activity because the preferential ROCK inhibitor Y27632 impaired ERK1/2 phosphorylation, leading to cytoskeletal reorganization and subsequent EMP release. Interestingly, Latham et al34 reported that Rho kinase–dependent formation of β-actin fibers in the perinuclear region facilitates agonist-induced endothelial membrane protrusions and, then, EMP release.

Our findings also demonstrate that endogenous NO release from endothelial cells chronically exposed to atheroprotective HSS prevented the release of EMPs. The NOS inhibitor L-NAME increased EMP release under HSS, whereas the NO donor decreased EMP release under LSS. Part of the HSS effect might result from an increased presence of polymerized actin at the plasma membrane, which results from the augmented NO release under these HSS conditions. Downregulation of ABCA1 expression by endogenously released NO could also contribute to limit the release of EMPs in HUVECs exposed to HSS (Figure 8). Deletion of the ABCA1 locus in mice is an experimental model for the human Tangier dyslipidemia, a naturally occurring loss of function mutation in man characterized by low or undetectable plasma levels of high-density lipoprotein and increased deposition of cholesteryl esters in tissues.35,36 The control of cellular lipid efflux by ABCA1 is a consequence of the intrinsic flippase activity of the ABCA1 transporter that modulates the distribution of phosphatidylinerse at the plasma membrane.5,35,37 Indeed, cells from ABCA1-deficient mice expose lower amounts of phosphatidylinerse and release fewer MPs.35,38,39 Our finding of increased EMP release after endothelial cell stimulation with a potent liver X receptor activator, which increases ABCA1 expression,40 reinforces the role of ABCA1 flippase in regulating EMP release in a NO-dependent manner. Although the present results identify for the first time a NO-dependent regulation of ABCA1-mediated release of EMPs, they do not exclude the possibility that other regulators of membrane remodeling and phosphatidylinerse exposure might be affected by endothelial NO. However, the present data rule out a NO-dependent regulation of scramblase-1 expression in endothelial cells; like ABCA1, scramblase-1 expression was decreased under HSS, but unlike ABCA1, its expression was not restored by L-NAME.

Taken together, the present findings demonstrate that physiological atheroprotective HSS hampers EMP release by limiting phosphatidylinerse exposure, resulting from endogenous NO release and subsequent downregulation of ABCA1 expression (Figure 8). However, under pathological conditions (ie, sustained atheroprone LSS),
endothelial ROCK activity is augmented and the cytoskeleton is reorganized. Concurrently, endothelial NOS expression is decreased,41 resulting in the loss of the NO-mediated repression of ABCA1, leading to membrane remodeling and phosphatidylserine exposure. Both increased Rho-kinase activity and ABCA1 expression concur to increase EMP release under LSS pathological conditions. HSS has been shown previously to rapidly, but transiently, activate endothelial ERK signaling,42 but this does not result in a large release of EMPs. Activation of ERK pathway by HSS might not be sustained enough to promote the subsequent release of EMPs. In addition, the concomitant increased NO release in HSS conditions will limit cytoskeletal reorganization and ABCA1-dependent loss of membrane asymmetry, 2 necessary steps in MP formation. The specific NO targets regulating these events remain so far unidentified. NO might affect signal transduction in many ways, through either changes in the redox balance or posttranslational modifications of proteins, such as S-nitrosylation. Interestingly, SS has been shown to increase S-nitrosoylation of multiple proteins in endothelial cells,43 particularly, caspase-3.2 This mechanism likely contributes to the NO-mediated protection of endothelial cells from programmed cell death. However, this effect is unlikely to decrease the release of EMPs, for EMP levels are not affected by a pan-caspase inhibitor in both low and high shear conditions in the present study. Further studies are required to identify NO molecular targets involved in MP generation.

In conclusion, this study identifies SS as a major determinant of EMP formation and release, both in vitro and in vivo.

Figure 7. ABCA1 expression in human umbilical vein endothelial cells modulates endothelial microparticle (EMP) release. EMP release was quantified by flow cytometry using annexin V staining (A–C). ABCA1 protein (B) levels were assessed by Western blot. A, Effect of T090131 on EMP release under different shear stress profiles (24 hours; n=5; 10−7 mol/L). B, Efficient ABCA1 silencing under low shear stress (LSS; 24 hours; n=7; 5×10−8 mol/L), expressed compared with high SS (HSS; 1 AU). GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase. C, Effect of ABCA1 silencing on EMP release under different shear stress profiles (24 hours; n=7; 5×10−8 mol/L). *P<0.05 (compared with HSS); $P<0.05 (compared with LSS).

Figure 8. Signaling pathways regulating shear stress–induced endothelial microparticle (EMP) release. Under physiological conditions (ie, sustained exposure to high laminar shear stress; left) endothelial cells release nitric oxide (NO), which clamps down endothelial ABCA1 expression, limiting membrane remodeling and phosphatidylserine exposure. In addition, NO limits cytoskeletal reorganization by stabilizing actin at the plasma membrane. Both mechanisms concur to limit EMP release. On the contrary, under pathological conditions (ie, sustained exposure to low laminar shear stress; right), increased endothelial Rho kinases and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) activities associated with cytoskeleton reorganization augment EMP release. This effect is reinforced by the loss of NO-induced downregulation of ABCA1 expression.

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References


What Is Known?

• The mechanical shear forces generated by blood flow play a fundamental role in regulating vascular endothelial survival and phenotype.
• Endothelial activation and apoptosis release microparticles that emerge as important biological effectors.

What New Information Does This Article Contribute?

• Exposure to atheroprotective laminar high shear stress hampers endothelial microparticles release, when compared with low shear stress conditions.
• Atheroprone low shear augments endothelial microparticles release through Rho kinases-dependent cytoskeletal reorganization, independently of caspase activation.
• Nitric oxide hampers endothelial microparticle formation in high shear stress conditions by limiting ABCA1 expression and membrane remodeling.

Novelty and Significance

Endothelial activation causes the release of microparticles that emerge as important biological effectors. Their formation requires cellular membrane remodeling and caspase-, Rho kinases-, or mitogen-activated protein kinase–dependent cytoskeletal reorganization. Shear stress, a mechanical force generated by blood flow on vascular endothelial cells, plays a fundamental role in regulating endothelial survival and phenotype. We tested the hypothesis that shear stress regulates endothelial microparticle formation. Both augmented RHOK-dependent cytoskeletal reorganization and membrane remodeling increased microparticle release in atheroprone low shear conditions. In atheroprotective conditions, nitric oxide impaired cytoskeletal reorganization and ABCA1-dependent membrane remodeling. This study, therefore, identifies shear stress as a major determinant of endothelial microparticle release.

References

Shear Stress Regulates Endothelial Microparticle Release
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SUPPLEMENTAL MATERIAL

Materials and Methods

Clinical study
Eighty-one asymptomatic subjects without known cardiovascular disease from the MiPRA-Met cohort underwent flow-mediated vasodilatation (FMD) measurement to assess endothelial function as part of their cardiovascular risk stratification.

Risk factors assessment.
Resting brachial blood pressure was the mean of three sphygmomanometric measurements, measured with an arm size-adapted cuff by a trained physician. Hypertension was defined as blood pressure at or above 140 or 90mmHg or presence of current antihypertensive treatment. Fasting blood lipids and glucose were measured by enzymatic methods [after precipitation of low-density lipoprotein (LDL) and very-low-density lipoprotein for high-density lipoprotein (HDL) measurement]. Hypercholesterolemia was defined as total cholesterol at or above 5.2mmol/l or presence of current lipid-lowering drug treatment. Low HDL was defined as HDL cholesterol below 1.0mmol/l (women) or 0.9mmol/l (men). Diabetes was defined as fasting blood glucose at or above 7mmol/l or current anti-diabetic treatment. Current smoking was defined as daily consumption of at least one cigarette for at least 3 months. We estimated the risk of coronary heart disease by entering age, male sex, systolic pressure, total and HDL-cholesterol, presence or absence of smoking into the Framingham model equations and defined the 10-year coronary risk as low (<10%), intermediate (10-20%) or high (>20%). The study was approved by the local ethical committee and all participants gave written informed consent.

Brachial artery shear rate assessment.
Subjects were examined in the morning, after 12 h fasting, in a quiet and temperature controlled room, and in the supine position for at least 5 min with the right arm in extension inside a cushion designed to avoid lateral movements. The brachial artery was visualized longitudinally by high-resolution ultrasound (ATL 5000) with a 7–12 MHz transducer probe positioned above the elbow and fixed in a robotic arm. Prior to the FMD manoeuvre previously described in detail, the brachial artery resting wall shear rate was calculated as four times the ratio between the pulsed Doppler-measured mean blood velocity and the diastolic diameter. A study of repeatability in 20 subjects previously showed that two manoeuvres of shear rate measurement performed 10 min apart were highly correlated (r=0.82, p<0.001) with a coefficient of variation of 12%.

Cell culture
Human Umbilical Vein Endothelial cells (HUVEC) were bought from Promocell (Heidelberg, Germany) and grown to confluence on 0.2% gelatin-coated culture slides (Menzel Glazer; Braunschweig, Germany) at 37°C in a humidified 5% CO₂ incubator. Cells (passage 2 to 4) were cultured in endothelium cell basal medium containing growth factors (human epidermal growth factor, human basic fibroblast growth factor, human endothelial cell growth supplement and 1% foetal calf serum; Promocell), supplemented with the antibiotics streptomycin (100u/ml), and penicillin (100u/ml) and 10µg/L of antifungal (Amphotericin B, Promocell).

SiRNA transfection
SiRNA targeting ABCA1 mRNA was used to transiently suppress ABCA1 protein expression and non-targeting siRNA was used as control (both from Dharmacon, ON-target plus smart pool siRNA). siRNA was transfected to endothelial cells by lipofection using hyperfect. Briefly, siRNA and hyperfect were resuspended separately in medium without antibiotics (OptiMEM, Gibco), when mixed together in order to obtain liposomes containing siRNA and added onto the cells. After 6 hours, medium was change for endothelium cell basal medium and shear stress experiments were performed 18 hours later.
**Plasmid electroporation**

DNA vector corresponding to pSG5-RhoA-V14G has been used to transiently expressed the constitutively active V14-RhoA mutant. Cells were used at 50 to 60% confluence for cDNA transfection by use of Nucleofector (Lonza/Amaxa) according to the manufacturer's instructions. Briefly, 5×10^6 cells were electroporated with 2 μg of plasmid, using the U-01 program, and then replated in endothelial cell growth medium containing 1% fetal calf serum (FCS) for 6h, then medium was changed and cells reached confluence within 42 h. Transfection efficiency was assessed by RhoA and Phospho-MYPT1 (Rho-kinase target substrate) western blot.

**Microparticle generation and isolation**

A unidirectional controlled level of laminar shear stress (SS) was applied to confluent HUVECs using a parallel plate chamber system, which was constituted of a Plexiglas block with an entrance and an exit port for the medium fitted on the top cells and clamped tightly as described earlier. The space between the block and the cells was set by a 250 μm–thick sealing rubber gasket. Each perfusion system was connected to a closed perfusion circuit consisting of a 3-port reservoir and the medium was perfused at different rates (2 to 20 ml/min) using a peristaltic pump (Gilson). Shear stress was calculated using Poiseuille’s law for parallel-plate geometry: \( \tau = \frac{6\mu Q}{W h^2} \), where \( \mu \) is the viscosity of the medium (0.01 poise), \( Q \) is the flow rate, \( W \) is the width of the channel (25 mm) and \( h \) is the separation between the parallel plates (0.25 mm).

The circuit was placed in a 5% CO\(_2\) incubator set at 37°C. HUVECs were exposed for different times (24, 8, 6 or 2 h) to 0, 2 or 20 dynes/cm\(^2\) in the perfusion system. Cells were treated with either the nitric oxide (NO) synthase inhibitor N-nitro-L-arginine-methyl-ester (L-NAME, 10\(^{-5}\) mol/L), or its D-enantiomer (D-NAME) (from Interchim, Montluçon, F), the NO donor S-nitroso-N-acetylenicilamine (SNAP, 10\(^{-5}\) mol/L), the ERK1/2 inhibitor PD98059 (10\(^{-5}\) mol/L), the Jun-kinase inhibitor SP600125 (10\(^{-6}\) mol/L), the p38 pathways inhibitor SB203580 (10\(^{-5}\) mol/L), the Rho-associated protein kinase (ROCK) inhibitor Y27632 (10\(^{-6}\) mol/L), the NFκB inhibitor pyrrolidine dithiocarbamate (PDTC, 10\(^{-5}\) mol/L), cytochalasin D (cytoD, 2.5x10\(^{-4}\) mol/L), the LXR agonist TO901317 (10\(^{-5}\) mol/L) (all from Sigma Aldrich; St Louis, MO), or the general caspase inhibitor Z-VAD-FMK (2.10\(^{-5}\) mol/L) (R&D systems; Minneapolis, MN). Non-treated cells were used as controls. Stock solutions of PD98059, SB203580, SP600125, TO901317 and Z-VAD-FMK were obtained in pure DMSO and then further diluted in medium. Pilot experiments (n=8) demonstrated that DMSO (0.1%, corresponding to the concentration present in medium + inhibitor solution) did not affect basal release of EMPs (p=0.9453, Wilcoxon test). In some experiments, EMP release was stimulated by TNFα exposure (10\(^{-5}\) g/L, Calbiochem; Darmstadt, Germany). All inhibitors were added to the perfusion medium, which was then filtered (0.1 μm) and added to the cells 1h prior to TNFα treatment or shear exposition and left during the SS during the indicated experimental times. The perfusion medium was collected following the TNFα and shear stress experiments. A 15 minute (min) centrifugation at 600 g at 4°C was carried out to eliminate any cell debris. The supernatant was then ultra-centrifugated for 90 min at 20500 g at 4°C to pellet the MP fraction, which was resuspended in 200 μL of medium and stored at -80°C before analysis.

**Microscopy**

**Immunofluorescent microscopy**

HUVECs were washed twice with PBS. They were then fixed in 4% paraformaldehyde for 10 minutes and washed again three times in PBS. In order to permeabilize the cells, 0.1% Triton solution was added for 10 min. In situ cell death detection kit from ROCHE (Neuilly-sur-seine, Fr) was used to stain apoptotic cells. Actin localization at the plasma membrane was assessed by co-staining for polymerized actin, using fluorescent phalloidin (Invitrogen, Carlsbad, CA) and CD31 (BD Pharmingen, Franklin Lakes, NJ). Fluorescent labeling was analyzed using a Zeiss fluorescent microscope. Apoptosis was quantified by reporting the number of green fluorescent nuclei on DAPI-blue stained cell nuclei expressed in percentage of apoptotic cells. Actin localization at the plasma membrane was quantified by a score system for each image (0: no colocalization to 3: full colocalization) by three different blinded operators.

**Transmission electron microscopy (TEM)**
Transmission electron microscopy observations were performed at the IFR83-Jussieu-PARIS core facility. Briefly, cells were fixed with Karnovsky’s fixative adapted for vascular profusion (2% Paraformaldehyde, 2.5% Glutaraldehyde and 0.1mol/L sodium phosphate Buffer, Electron Microscopy Sciences, Hatfield, PA). After dehydration in ethanol, cells were embedded in Epon. Grids were analyzed with a transmission electron microscope (EM 912 OMEGA, ZEISS) equipped with a LaB6 filament at 80kV and images were captured with digital camera (SS-CCD, Proscan 1kx1k), magnification: 1936x12, with item software.

**Contrast phase microscopy**
HUVECs were observed by contrast phase microscopy after each SS experiment to confirm correct response to SS (high SS: cells were aligned in the direction of medium circulation, low SS and static condition: no alignment). Observations were performed using Zeiss Primo Vers, magnitude: 10x5.

**Flow cytometry analysis**
Twenty microliters of MP resuspended pellet were incubated with 100 µL of annexin-V coupled to fluorescein isothiocyanate (FITC) (ROCHE) in AnnV binding buffer (10mmol/L HEPES, pH 7.4, 140mM NaCl, 2.5 mMol/L CaCl$_2$) for 30 min at room temperature in the dark. As calcium is required for AnnV binding, control experiments were performed as described above, but in calcium-free buffer (10mmol/L Hepes, pH 7.4, 140mMol/L NaCl, 3mmol/L EDTA). In some experiments, MPs were labeled using VE-cadherin antibody (CD144-PE, Immunotech; Marseille, Fr) and control experiments were performed in the presence of isotypic control (PE-IG2A) as previously described$^9$. All buffers were filtered through a 0.1 µm filter. Stained samples were well mixed and analyzed by flow cytometry (EPICS XL, Beckman Coulter; Brea, CA). Flow-count fluorospheres (Beckman Coulter) were used to establish MP concentration in the sample. Data were analyzed using Flow-Jo software. Events less than 1µm diameter were identified in forward scatter and side scatter intensity dot representation, in comparison with fluorescent microbeads (0.5, 0.9 and 3 µm in diameter; Megamix Biocytex; Marseille, Fr). MPs were defined as elements with a size less than 1 µm and greater than 0.1 µm that were positively labeled by AnnV in a calcium-dependent manner or by CD144.

**RNA Extraction and Real-Time quantitative Reverse-Transcription Polymerase Chain Reaction**
RNA was extracted and purified from HUVECs exposed to different flow patterns with TRizol (Invitrogen). Briefly, cells were lysed in TRizol, and then chloroform was added in order to separate RNA in the aqueous phase. Isopropanol was added to precipitate RNA. The RNA was next washed and redissolved in purified RNase-free water. After measurement of concentration and purity, RNA was kept at -80°C before use. RT-PCR was performed with 0.1µg of RNA using Qiagen products and protocols; qPCR was performed using SYBRgreen products and protocol (Sigma). Primer sequences were as follows: ABCA1 sense, GAGACTAACCAGGCAATCCG, ABCA1 antisense, GCTTGTTCAGGTTGACACACT; Ubiquitin C sense, ACATTGGTCCTGCGCTTGA, Ubiquitin C antisense, TTTTGCGAATGCAACAACTTT (obtained from Eurogentec), used at final concentration of 3.3x10$^{-7}$mol/L. Ubiquitin C was used as housekeeping gene as previously described by Vandesompele et al$^{10}$ to normalize the expression of ABCA1. Normalization was done using the 2deltaCT method.

**Western blotting**
HUVECs were washed with cold PBS and scraped off in RIPA buffer (150mmol/L NaCl, 50mmol/L TrisHCl pH7.4, 2 mmol/L EDTA, 0.5% sodium deoxycholate, 0.2% sodium deoxychyl sulfate, 2mmol/L activated orthovanadate, complete protease inhibitor cocktail tablet and complete phosphatase inhibitor cocktail tablet (Roche, Fr)) or NETF buffer (100mmol/L NaCl, 50mM TrisHCl pH7.4, 2 mmol/L EDTA, 50mmol/L NaF, 1% NP-40, complete protease inhibitor cocktail tablet and complete phosphatase inhibitor cocktail tablet). Lysates were sonicated (15 seconds, 40 watts, Vibra Cell, Bioblock) and protein content was quantified using the Lowry protein assay (Bio-Rad; Hercules, CA). Lysates were mixed with the reducing sample buffer for electrophoresis and subsequent transfer onto nitrocellulose membranes (Bio-Rad). Equal loading was verified using Ponceau red solution. Membranes were incubated in milk TBS-T with anti-α-fodrin (Enzo life sciences, Plymouth Meeting,
PA, 1/1000), anti-µ-calpain (recognizing D1 (latent form) and D4 domain (latent + aminoprocessed forms), Abcam, Cambridge, UK, 1/1000), anti-phospho-ERK1/2, anti-Phospho-MYPT1 (Santa Cruz, CA; 1/1000), anti-phospho-MLC, anti-ERK1/2, anti-MLC (Cell Signaling Technology; Beverly, MA; 1/1000), anti-scramblase-1 (Acris, San Diego, CA, 1/100), anti-ABCA1 (Millipore, 1/1000) antibodies or in BSA TBS with anti-Phospho-MYPT1 antibody (Santa Cruz, CA; 1/500). After secondary antibody incubation (Amersham, GE Healthcare, UK 1/3000), immunodetection was performed using an enhanced chemiluminescence kit (Immun-Star Western C kit, Bio-Rad) and bands were revealed using the Las-4000 imaging system and Image Gauge software (Fujifilm, Tokyo, Japan). After initial immunodetection, membranes were stripped of antibodies and re-probed with anti-GAPDH antibody (Chemicon, Millipore; Billerica, MA, 1/8000). Values reported from Western blots were obtained by band density analysis using Image Gauge software (Fujifilm) and expressed as the ratio protein of interest/GAPDH expression.

**Statistical analysis**

**Clinical study:** Clinical continuous parameters are expressed as means ± standard deviations with ranges and qualitative parameters as number of subjects (%). Quantitative variables with non normal distribution (CD144+ microparticles) were log-transformed to achieve normal distribution before correlations analysis. Normal distribution was assessed by means of the Shapiro-Wilk test. The relationship between CD144+ MP and brachial artery baseline shear rate was analysed by linear regression, both in univariate analysis and in multivariable analysis by adjusting for each of the risk factors (one by one), with CD144+ MP as the dependent variable, and with shear rate and each risk factors as the independent variables.

**In vitro study:** Data obtained from at least 5 independent experiments are expressed as mean±SEM. Statistics were performed using Prims software. Significant differences between the 3 SS conditions (static, low and high SS) were determined by one-way ANOVA followed by post hoc analysis using Bonferroni’s test. Comparisons between control and treatment conditions were performed using a Wilcoxon signed-rank test (paired and non-parametric T-test). For ABCA1 mRNA expression only, controls were not paired with treatment, so a Mann-Whitney U-test was used. Significance was accepted for a value of p<0.05.
Online Figure I: Characterization of the endothelial microparticle pellet. Western blot for the exosomal marker TSG101 in EMP pellet. Results are normalized using ponceau red and expressed compared to exosomes fraction (100%).
Online Figure II: Shear stress effect on endothelial cell alignment and apoptosis.

A: cell morphology by contrast phase microscopy (x50); B: Representative experiment of Tunel assay to investigate endothelial apoptosis: (blue for the nucleus of alive cells, bright light blue corresponding to apoptotic nuclei (x100)). ZVAD-FMK(2.10^{-5} mol/L) reduces apoptosis in HUVECs under static or low SS profiles. C: Bar graph averaging SS effect on endothelial apoptosis (24h, N=5), evidenced by Tunel assay in the presence of absence of ZVAD-FMK pan-caspase inhibitor. Data represent means±SEM. *: p<0.05 (compared to HSS), $: p<0.05 (compared to untreated condition).
Online Figure III: \( \mu \)-calpain activity under different SS profiles.

A: WB analysis of \( \alpha \)-fodrin-120kD relative expression compared to total \( \alpha \)-fodrin-250kD (N=8) under different SS profiles. B: WB analysis of \( \mu \)-calpain latent form (lat \( \mu \)-calpain : D1 fragment, relative expression normalized to total GAPDH, N=4) and activated \( \mu \)-calpain (D4, relative expression compared to lat \( \mu \)-calpain, N=4) under different SS profiles. Data represent means±SEM. Blots originated from a same membrane.
Online Figure IV: Absence of effect of NO on ROCK and ERK1/2 activation in endothelial cells exposed to HSS.

Western Blot analysis of P-ERK1/2 or P-MLC (relative expression compared to GAPDH) at time 12 hours. **A**: effect of L-NAME under high SS condition on P-ERK1/2 (N=5; p=0.875). **B**: effect of L-NAME under high SS condition on P-MLC (N=5; p=0.313). **C**: effect of SNAP under low SS condition on P-ERK1/2 (N=5; p=0.99). **D**: effect of SNAP under low SS condition on P-MLC (N=5; p=0.563). Bars represent means±SEM. Blots originated from a same membrane.
Online Figure V: Scramblase-1 expression under different SS profiles.
Western Blot analysis of scramblase-1 (relative expression normalized to GAPDH) under different SS profile treated or not with L-NAME (24h, N=7). Data represent means±SEM. *p<0.05 (compared to untreated HSS). Blots originated from a same membrane but were re-arranged to correspond to the current graphic presentation.
Online Figure VI: Effect of ROCK inhibition on Scramblase-1 and ABCA1 expression under low SS.

Western Blot analysis of scramblase-1 and ABCA1 (relative expression compared to GAPDH) under low SS profile in endothelial cells treated or not with Y27632 (24h, N=5). Data represent means±SEM. Blots originated from the same membrane.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>50 ± 11 (22-77)</td>
</tr>
<tr>
<td>Male gender, n (%)</td>
<td>57 (69)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>37 (45)</td>
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<tr>
<td>Hypercholesterolemia, n (%)</td>
<td>32 (39)</td>
</tr>
<tr>
<td>Low HDL, n (%)</td>
<td>24 (29)</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>7 (8)</td>
</tr>
<tr>
<td>Current smoking, n (%)</td>
<td>18 (22)</td>
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<tr>
<td>10-year Framingham risk, n (%)</td>
<td></td>
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<tr>
<td>&lt;10% at 10 years</td>
<td>51 (63)</td>
</tr>
<tr>
<td>10-20% at 10 years</td>
<td>24 (30)</td>
</tr>
<tr>
<td>&gt;20% at 10 years</td>
<td>6 (7)</td>
</tr>
<tr>
<td>Brachial artery shear rate, s⁻¹</td>
<td>58.2 ± 35.0 (3.7-172.6)</td>
</tr>
<tr>
<td>CD144+ microparticles, events/µL</td>
<td>450 ± 409 (0-2127)</td>
</tr>
</tbody>
</table>

**Online Table I:** Clinical characteristics. Risk of coronary heart disease was estimated by entering age, male sex, systolic pressure, total and HDL-cholesterol, presence or absence of smoking into the Framingham model equations and defined the 10-year coronary risk as low (<10%), intermediate (10-20%) or high (>20%). Data are mean ± s.d. or number of subjects, (n) with percentages.
<table>
<thead>
<tr>
<th></th>
<th>Static</th>
<th>Static + TNFα</th>
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<tr>
<td>Untreated</td>
<td>100±0</td>
<td>171.0±9.6</td>
</tr>
<tr>
<td>PDTC</td>
<td>97.1±4.9</td>
<td><strong>117.8±7.4</strong></td>
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<tr>
<td>PD98059</td>
<td>117.2±6.7</td>
<td>176.5±20.8</td>
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<tr>
<td>SB203580</td>
<td>86.9±8.3</td>
<td><strong>88.9±10.6</strong></td>
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<tr>
<td>SP600125</td>
<td>98.2±6.2</td>
<td>164.9±24.9</td>
</tr>
<tr>
<td>Y27632</td>
<td>97.4±8.2</td>
<td>181.5±25.1</td>
</tr>
</tbody>
</table>

**Online Table II: Effect of different inhibitors on EMP release under TNFα treatment (static condition).**

EMPs were measured by flow cytometry using AnnexinV staining (24h, N=7, TNFα: 10nmol/L; NFκB inhibitor: PDTC, 10^{-5}mol/L; ERK1/2 inhibitor: PD98059, 10^{-5}mol/L; Jun-kinases inhibitor: SP600125, 10^{-6}mol/L; p38 MAP-kinase inhibitor: SB203580, 10^{-5}mol/L; ROCK inhibitor: Y27632, 10^{-6}mol/L). Data are expressed as means±SEM. **p<0.01 (compared to TNFα treatment).**
**Online Table III: EMP characteristics**

EMPs were measured by flow cytometry (Gallios) using AnnexinV staining (24h, shear stress N=13, L-NAME: 10⁻⁵mol/L, N=8; Y27632, 10⁻⁶mol/L, N=7). EMP size was analyzed according to that of calibration beads (above or below 0.5μm in diameter). Data are expressed as means±SEM. *p<0.05 (compared to High SS), $p<0.05 (compared to Low SS)

<table>
<thead>
<tr>
<th>Condition</th>
<th>total EMPs</th>
<th>&gt;0.5μm EMPs</th>
<th>&lt;0.5μm EMPs</th>
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<tbody>
<tr>
<td>High SS</td>
<td>4414±792</td>
<td>3781±677</td>
<td>633±117</td>
</tr>
<tr>
<td>Low SS</td>
<td>7750±1602 *</td>
<td>6437±1335 *</td>
<td>1312±287 *</td>
</tr>
<tr>
<td>High SS + L-NAME</td>
<td>4392±1076</td>
<td>3674±877</td>
<td>717±203</td>
</tr>
<tr>
<td>Low SS</td>
<td>7418±2133</td>
<td>6175±1821</td>
<td>1243±365</td>
</tr>
<tr>
<td>Low SS + Y27032</td>
<td>6283±2235 $</td>
<td>5419±194 $</td>
<td>863±308</td>
</tr>
</tbody>
</table>
References:

4. Third report of the national cholesterol education program (ncep) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel iii) final report. *Circulation.* 2002;106:3143-3421