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Shear Stress Regulates Endothelial Microparticle Release

Anne-Clémence Vion, Bhama Ramkhelawon, Xavier Loyer, Gilles Chironi, Cecile Devue, Gervaise Loirand, Alain Tedgui, Stéphanie Lehoux,* Chantal M. Boulanger*

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/cm^2). 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 atheroprone 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

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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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Nonstandard Abbreviations and Acronyms	
EMP	endothelial microparticles
HSS	high SS
HUVEC	human umbilical vein endothelial cells
MAP	mitogen-activated protein
MP	microparticles
NOS	NO synthase
siRNA	small interfering RNA
SS	shear stress
TNF	tumor necrosis factor

important determinant of endothelial apoptosis in vivo, we hypothesized that different SS levels might regulate in vivo endothelial vesiculation.[8] 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.[9] 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 4× 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 1500*g* (15 minutes) followed by 11 000*g* (2 minutes) within 1 hour after blood withdrawal, as described earlier.[14]

Cell Culture

Confluent human umbilical vein endothelial cells (HUVEC; passage 2–4; Promocell, Heidelberg, Germany) were cultured on 0.2% gelatin-coated slides (Menzel Glazer; Braunschweig, Germany) in endothelium cell basal medium containing growth factors, 1% fetal calf serum (Promocell), streptomycin (100 U/mL), penicillin (100 U/mL), and amphotericin B (10 μg/L).

Small Interfering RNA Transfection

Small interfering RNA (siRNA) targeting ABCA1 mRNA was used to transiently suppress ABCA1 protein expression, and nontargeting siRNA was used as control (both from Dharmacon, ON-target plus smart pool siRNA).

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 dyne/cm^2, 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 (600*g*; 15 minutes; 4°C), and the resulting supernatant was then ultracentrifugated (20 500*g*; 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: 10×5). 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 costaining using fluorescent phalloidin (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.[18] Annexin V + propidium iodide + apoptotic bodies averaged 6±1, 7±1, and 6±1 events/μL in HSS, 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 described[20] 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

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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 mean±SEM. Statistics were performed using Prims 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.[10] 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/cm^2), atheroprone low laminar SS (2 dyne/cm^2), 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

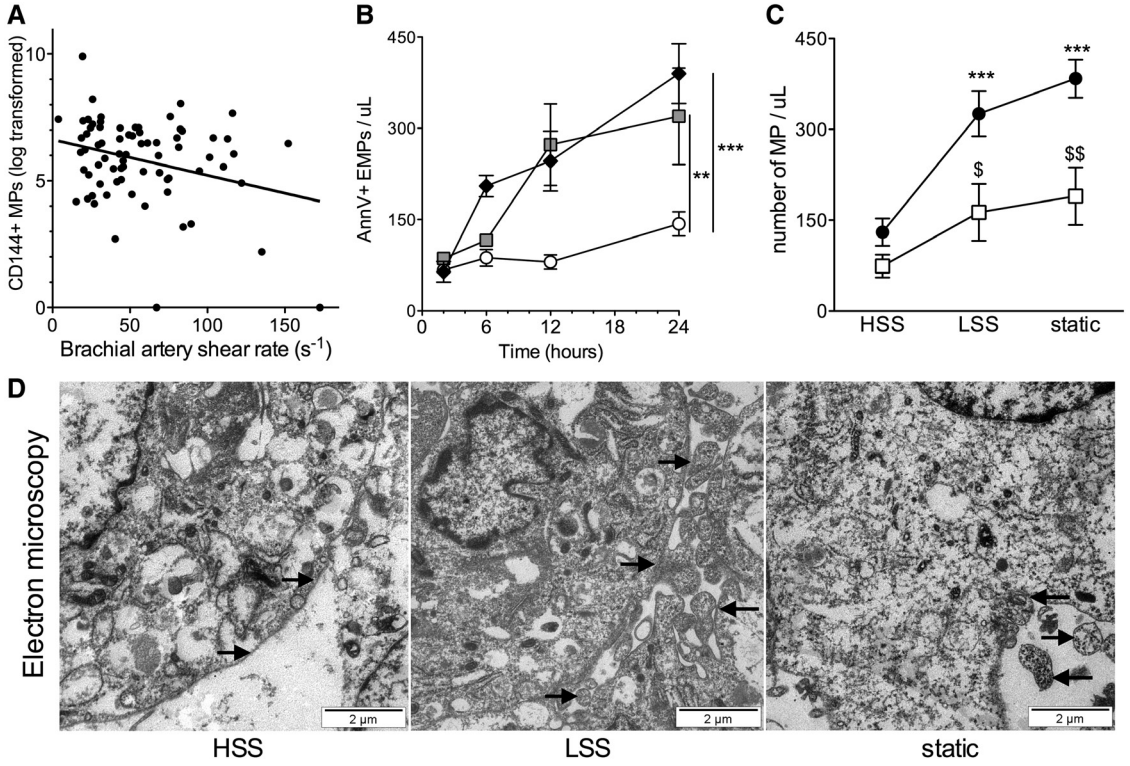


Figure 1. Relationship between endothelial microparticle (EMP) and shear stress levels. **A**, Correlation between baseline brachial artery shear rate and CD144+ EMP level in patients (n=74; $r=-0.25$; $P=0.009$). **B**, Timecourse of annexin V + EMP release in vitro (static: black diamond; low shear 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.

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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).

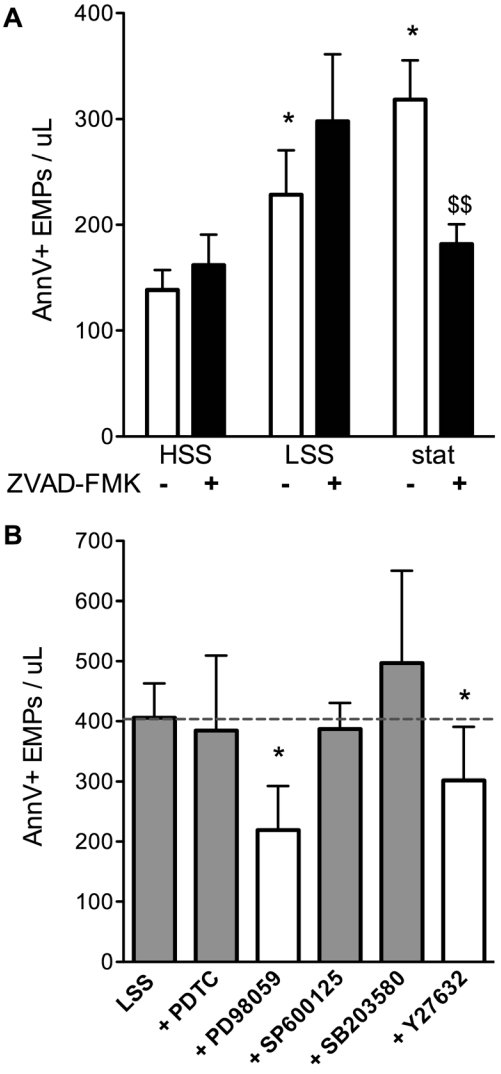


Figure 2. Signaling pathways associated with endothelial microparticle (EMP) release. **A**, EMP level under different shear stress profile treated or not with Z-VAD-FMK (pan-caspase inhibitor; 24 hours; n=8; 2×10^{-5} mol/L). $$P$<0.05 (compared with high shear stress [HSS]), $$$P$<0.01 (compared with untreated condition). **B**, Effect of different inhibitors on EMP release under low SS (LSS; 24 hours; n=6; nuclear factor κB inhibitor: pyrrolidine dithiocarbamate (PDTC), 10^{-5} mol/L; ERK1/2 inhibitor: PD98059, 10^{-5} mol/L; Jun-kinase inhibitor: SP600125, 10^{-6} mol/L; p38 mitogen-activated protein [MAP]-kinase inhibitor: SB203580, 10^{-5} mol/L; Rho kinases inhibitor: Y27632, 10^{-6}mol/L).*P<0.05 (compared with untreated condition). ERK1/2 indicates extracellular signal-regulated protein kinases 1 and 2; and Z-VAD-FMK, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone.

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.[21,22] None 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[14,22] (Online Table II). However, unlike what was observed for TNF-α–induced stimulation, inhibitors of ROCK (Y27632) and ERK1/2 (PD98059) significantly decreased the release of EMP on exposure to 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.[23,24] Exposure of endothelial cells to cytochalasin D under static condition

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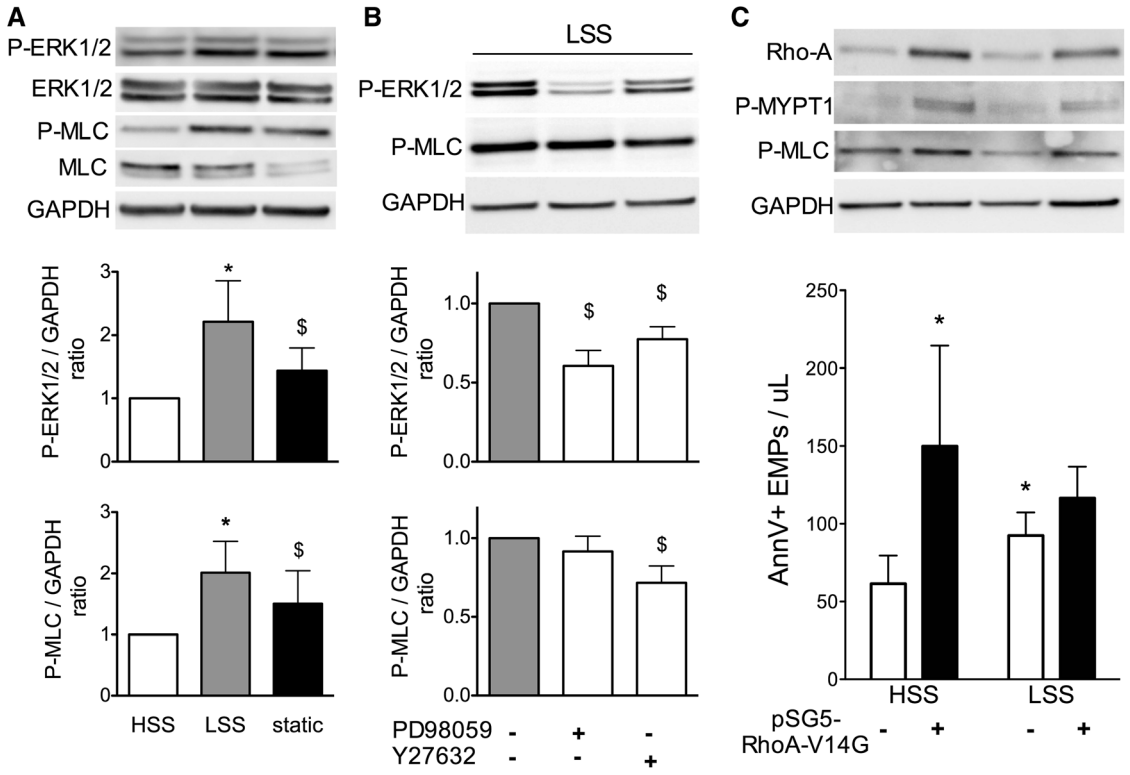


Figure 3. Mechanisms involved in low shear stress (LSS)-induced endothelial microparticle (EMP) release. **A**, ERK1/2 and Rho kinases (ROCK) phosphorylation (12 hours; n=6), total ERK1/2 and MLC expression under different SS profiles (n=4; representative blot) normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and expressed compared with high SS (HSS; 1 AU). **B**, ERK1/2 and ROCK phosphorylation under low SS treated or not with ERK1/2 or ROCK inhibitors (12 hours; n=6). **C**, Bar graph showing the effect of constitutively active RhoA overexpression on EMPs level (n=5) and representative blots of RhoA, phosphorylated myosin phosphatase targeting protein (P-MYPT-1), and GAPDH expression in mocked- and pSG5-RhoA-V14G-transfected endothelial cells showing the efficacy of plasmid expression in human umbilical vein endothelial cells under low and high SS. *P<0.05 (compared with HSS); \$$P$<0.05 (compared with LSS).

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,

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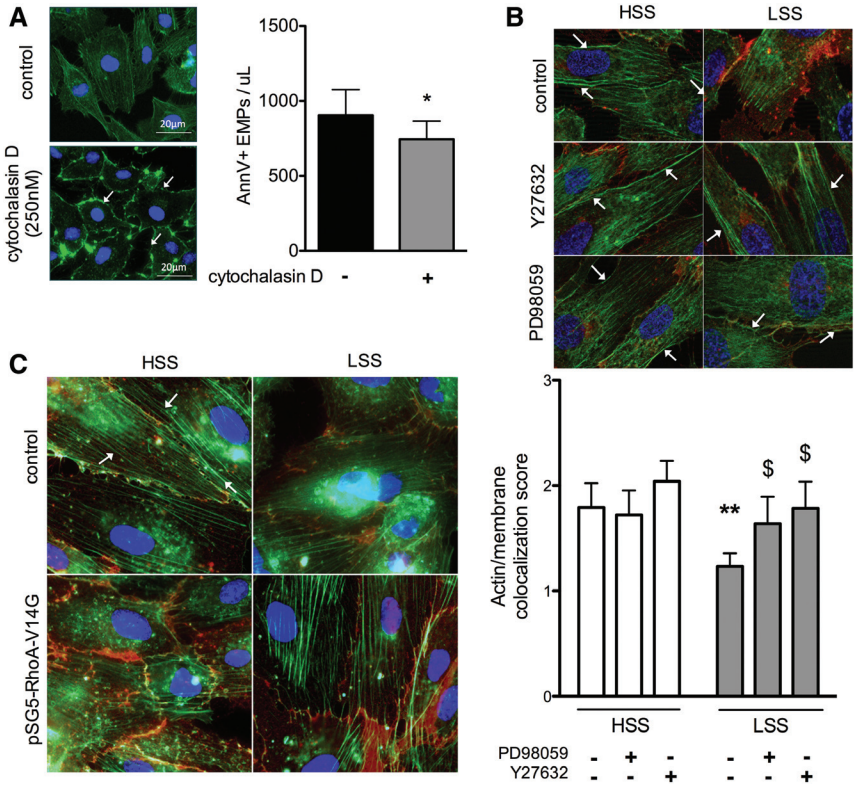


Figure 4. Actin localization and endothelial microparticle (EMP) release. EMPs were measured by flow cytometry using annexin V staining, and actin localization at the plasma membrane was measured by a score system (0: no colocalization to 3: total colocalization; F-actin: green, CD31: red). **A**, Effect 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).

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 IVA 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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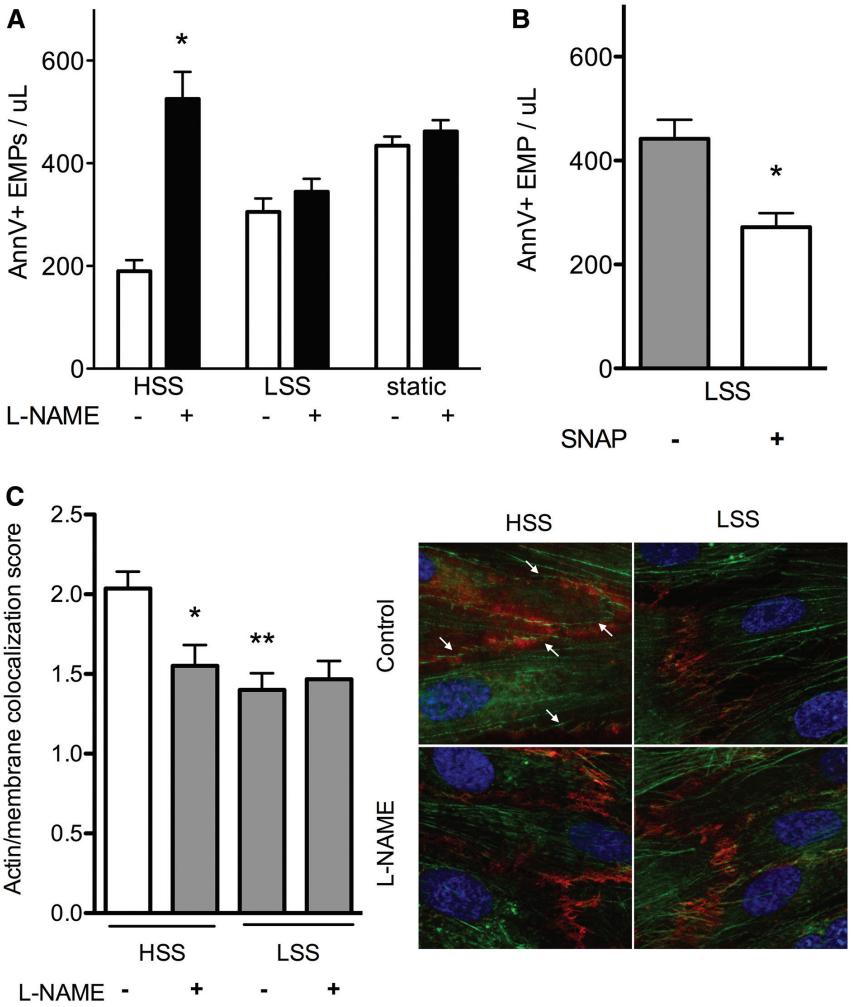


Figure 5. NO contribution to endothelial microparticle (EMP) release by increasing polymerized actin at the plasma membrane. Actin localization at the plasma membrane was measured by a score system (0: no colocalization to 3: total colocalization; F-actin: green, CD31: red). **A**, Effect of L-NG-nitroarginine methyl ester (L-NAME) treatment on EMP release (24 hours; n=7; 10^{-5} mol/L). **B**, Effect of S-nitroso-N-acetyl-D,L-penicillamine (SNAP) treatment on EMP release (24 hours; n=6; 10^{-5} mol/L). **C**, Effect of L-NAME on actin localization (24 hours; n=5; 10^{-5} mol/L). **P*<0.05; ***P*<0.01 (compared with untreated high shear stress [HSS] condition). LSS indicates low SS.

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.[7,21,22,27–30] Because SS is a major determinant of endothelial survival,[31] 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 al[33] reported that acute reduction in blood flow increased circulating

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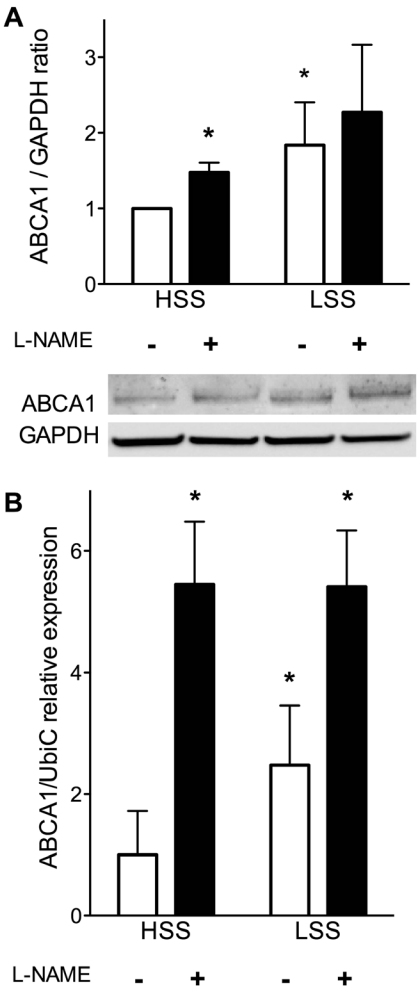


Figure 6. ABCA1 expression in human umbilical vein endothelial cells is dependent on shear stress level and NO production. ABCA1 protein (**A**) and mRNA (**B**) levels were assessed by Western blot and quantitative Polymerase Chain Reaction (qPCR) analysis, respectively. **A**, Effect of SS and L-NAME treatments on ABCA1 protein expression (24 hours; n=6), **B**, Effect of SS and L-NAME treatments on ABCA1 mRNA expression (24 hours; n=7); **P*<0.05 (compared with high SS [HSS]). GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase; L-NAME, L-NG-nitroarginine methyl ester; LSS, low SS; qPCR, quantitative polymerase chain reaction.

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 al[34] 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 phosphatidylserine at the plasma membrane.[5,35,37] Indeed, cells from ABCA1-deficient mice expose lower amounts of phosphatidylserine 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 phosphatidylserine 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 phosphatidylserine exposure, resulting from endogenous NO release and subsequent downregulation of ABCA1 expression (Figure 8). However, under pathological conditions (ie, sustained atheroprone LSS),

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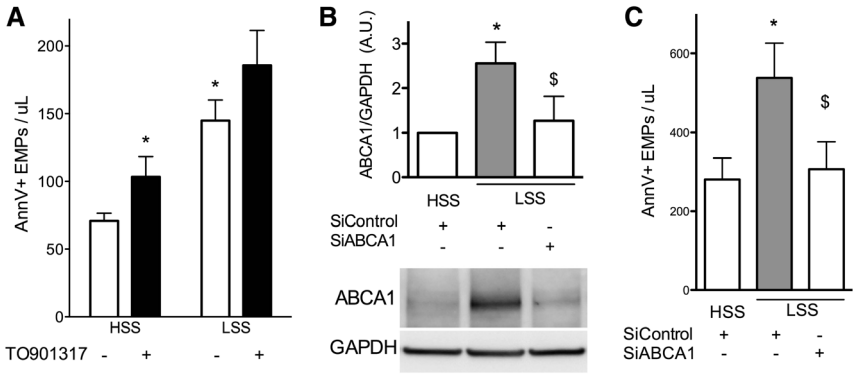


Figure 7. ABCA1 expression in human umbilical vein endothelial cells modulates endothelial microparticle (EMP) release. EMP release was quantified by flow cytometry using annexin V staining (**A**–**C**), ABCA1 protein (**B**) levels were assessed by Western blot. **A**, Effect of TO90131 on EMP release 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).

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-nitrosylation 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.

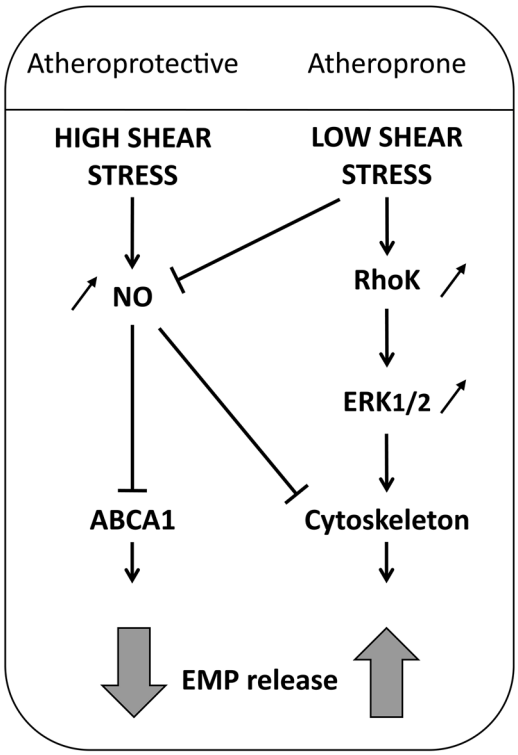


Figure 8. Signaling pathways regulating shear stress–induced endothelial microparticle (EMP) release. Under physiological conditions (ie, sustained exposure to high laminar shear stress; **left**) endothelial cells release nitric oxide (NO), which clamps down endothelial ABCA1 expression, limiting membrane 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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Disclosures

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Novelty and Significance

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.

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.

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