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Statin decreases endothelial microparticle release from human coronary artery endothelial cells: implication for the Rho-kinase pathway

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Abstract

Objective. Elevated plasma levels of endothelial microparticles (EMPs) are associated with the presence of clinical atherosclerosis. Considering the anti-inflammatory properties of HMG-CoA reductase inhibitors on the endothelium, we studied the effect of fluvastatin on the release of EMPs in cultured human coronary artery endothelial cells (HCAEC). *Methods and results.* EMPs were generated in TNF-α-activated HCAECs. The absolute number of EMPs was enumerated using a novel two-color flow cytometric immunostaining technique with TruCount beads as an internal reference. EMPs are defined as EC membrane vesicles (1–2 μm in size) with a characteristic immunophenotype. The addition of fluvastatin to TNF-α-activated HCAECs significantly suppressed EMP release. Fluvastatin suppressed TNF-α-induced Rho activation.The Rho-kinase inhibitor, Y-27632, reproduced the effect of statin. *Conclusion.* EMP release from TNF-α-activated HCAECs is suppressed by fluvastatin. In addition, the Rho/Rho-kinase may play an important role in modulating EMP release.

Keywords: Atherosclerosis; Endothelial microparticle; HMG-CoA reductase inhibitor; Rho GTPase

Atherosclerosis is a condition characterized by elevated inflammatory cytokines and endothelial cell (EC) dysfunction [1]. Recently, it has been shown that upon activation, ECs release microparticles (EMPs) and elevated serum levels of EMPs are demonstrable in patients with thrombotic disorders including coronary artery disease (CAD) [2]. In addition to indicating EC injury, EMPs may play a direct role in thrombogenesis by virtue of tissue factor, platelet factor-3 activity, and a distinctive antigenic profile of cellular adhesion molecules (CAM) that may preferentially bind to and activate leukocytes [3]. In vitro, ECs release EMPs when activated by inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) [4]. Thus, EMP release reflects EC activation, and analysis of EMPs can serve as a marker of EC activation.

HMG-CoA reductase inhibitor (statin) therapy targeted at lowering of atherogenic low-density lipoprotein can significantly reduce cardiovascular morbidity and mortality [5]. Furthermore, studies with statins have demonstrated that this survival benefit precedes any significant reduction in serum cholesterol levels suggesting an additional effect on the ECs, independent of cholesterol reduction. Among the non-lipid lowering actions of statins are plaque stabilization, reduced inflammation, reversal of endothelial dysfunction, and decreased thrombogenicity [6].

In ECs, the Rho family of small GTPases are key regulators of both cell adhesion and the cytoskeleton [7].

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Interestingly, the anti-inflammatory properties of statins may be mediated by inhibition of Rho protein isoprenylation, thus preventing membrane attachment of Rho proteins and the subsequent activation of downstream effectors such as Rho-kinase (ROK) [8].

Improved endothelial function is one of the earliest clinical markers of successful atherogenic risk factor modification and statins have been shown to positively modulate endothelial function [9]. We therefore examined the in vitro effect of fluvastatin on EMP release and RhoA activation in human TNF-α-activated human coronary artery endothelial cells (HCAECs).

Methods

Materials. Culture products were from Cambrex Bio Science (Walkersville, MD). TNF-α was purchased from Sigma (St. Louis, MO). Rho-kinase inhibitor (Y-27632) and fluvastatin were purchased from Calbiochem. Fluorescent TruCount bead lyophilized pellets were from Becton–Dickinson Biosciences (San Jose, CA). Calibration beads were obtained from Molecular Probes (Eugene OR). Monoclonal anti-human CD62E-fluorescein isothiocyanate (FITC), CD105-FITC, CD51-FITC, CD106-phycoerythrin (PE), CD31-PE, and CD54-PE antibodies were obtained from Ancell (Bayport, MN). Rhotekin Rho binding domain agarose, GTPγS, and GDP were obtained from Upstate Biotechnology (Lake Placid, NY). RhoA antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). SDS–PAGE gel (8–16%) was obtained from Cambrex (Rockland, ME). HRP-conjugated goat anti-rabbit IgG was obtained from Cell Signaling (Beverly, MA).

Cell culture and EMP preparation. HCAECs were purchased from Cambrex Bio Science (Walkersville, MD) and cultured according to the supplier's specifications. All experiments were performed on confluent cells. ECs were treated with 10 ng/mL TNF-α alone or with the addition of fluvastatin (0.1 μM/mL) or ROK inhibitor, Y-27632 (10 μM/L). Controls were untreated. After 24 h culture supernatants from flasks containing HCAEC were collected and centrifuged for 30 min at 17,570*g*, (4 °C). Pellets were resuspended in 1 mL PBS and analyzed on the same day.

Flow cytometric analysis. For single platform method, 3 μL each of FITC-conjugated and PE-conjugated monoclonal antibodies against the above-mentioned CAMs plus 50 μL of re-suspended pellet was added to tubes (two antibodies/tube) preloaded with fluorescent TruCount bead lyophilized pellets. Tubes were incubated for 20 min at room temperature. Analysis of EMPs was performed using a FACScan flow cytometer (Becton–Dickinson Biosciences, San Jose, CA). The EMPs were excited with 488 nm light from a 15 mW argon laser. Logarithmic green and red fluorescences of FITC and PE were measured through 530/30 and 585/42 nm bandpass filters, respectively. Data from 2000 events were acquired and analyzed with the use of CELLQuest software (version 3.3, Becton–Dickinson). The absolute number of EMPs was calculated from the appropriate dot plot values entered into the following formula:

$$\text{Absolute number of EMPs} \times 10^6/\text{mL} = \frac{\#\text{ of events in EMP region(R2)}}{\#\text{ of beads collected (R1)}} \times \frac{\text{total }\#\text{ of beads per tube}}{\text{test volume(50 μL)}}.$$

The total number of beads per tube is supplied by the manufacturer and varies among lot numbers.

RhoA activity. RhoA activity was determined by a pull-down assay as previously described [10]. Cell lysates were incubated with Rhotekin Rho binding domain agarose for 45 min. The agarose beads were collected and electrophoresed on 8–16% SDS–PAGE gel (Cambrex, Rockland, ME). GDP and GTP-labeling of cell lysates served as negative and positive controls, respectively. Western blotting was performed with RhoA antibody at a dilution of 1:200. The blot densities were analyzed with Mocha Image Analysis (version 1.2.10).

Statistical analysis. Statistical analysis was performed using SPSS 10.0.7 statistical software (SPSS, Chicago, IL). Values are stated as means ± SEM. One-way ANOVA was used to compare means from three or more groups. In cases where the data were not normally distributed, the Mann–Whitney rank sum test was used. A value of $p < 0.05$ was considered as significant for all tests.

Results

Quantitation of EMP

EMPs are sub-microscopic membranous particles (size range about 1.5 μm), which are shed from TNF-α-activated ECs [11]. We defined EMPs as elements ranging in size between 1 and 2 μm as assessed by their logarithmic amplification of the forward scatter (FSC) and side scatter (SCC) signals. Bivariant FSC/SCC efficiently distinguished calibration beads of 1 and 2 μm in R2 from TruCount beads that were scattered in R1 (Figs. 1A and B) and thus EMPs were defined as particles gated in R2. To evaluate the number of EMPs released, two-color flow cytometric immunostaining of prepared samples from HCAEC culture was performed. The number of TruCount beads in addition to EMP count was enumerated from the R1 and R2 regions, respectively (Fig. 1C). The purity as well as the proportion of EMP in this gate was identified further using FITC/PE fluorochrome-conjugated monoclonal antibodies to the above-mentioned CAMs. A representative dot plot of double-stained EMPs (Fig. 1D) demonstrates logarithmic green and red fluorescences of FITC (FL1) and PE (FL2), respectively.

Release of CAM-specific EMP in TNF-α-activated HCAEC

TNF-α is a pivotal inflammatory cytokine in patients with hypercholesterolemia [12]. In addition, TNF-α generates the release of EMPs from EC in vitro [4]. Data presented in Table 1 show that ECs release species of EMP exhibiting quantitative differences in antigenic phenotypes in response to activation. As assessed by flow cytometry, EMPs specific for CD31, CD105, CD51, CD54, CD62E, and CD106 were detectable from cultured HCAEC. Treatment of HCAEC with TNF-α (10 ng/mL) for 24 h significantly increased the total number (EMP × 10^6/mL) of CD105, CD31, and CD51 specific EMPs (CD105: 43 ± 4.7 to 124 ± 34, CD31: 145 ± 36 to 233 ± 77, and CD51: 29.8 to 76.4 ± 46, $p < 0.05$). CD62 and CD54 positive EMP increased by 15% and 57%, respectively, however, this was not significant. Levels of CD106 specific EMPs remained unchanged after treatment with TNF-α.

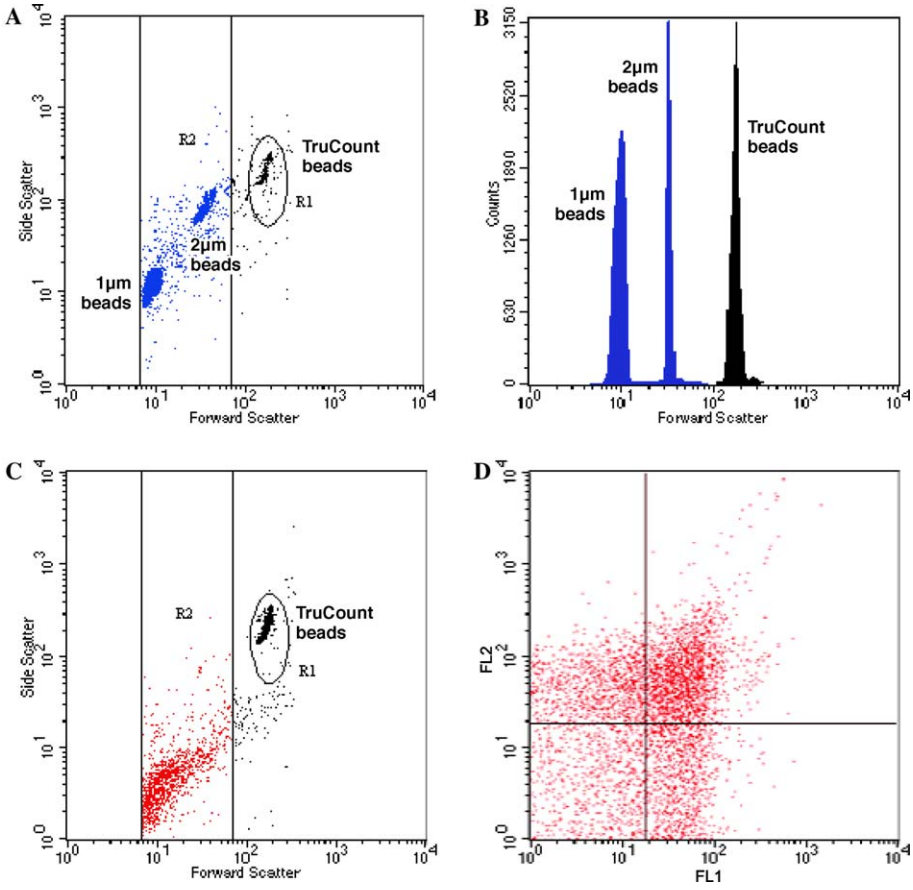

Fig. 1. Representative flow cytometric density plots demonstrating the gating protocol used to identify EMP and TruCount bead populations. Labels indicate the gate used to display the data. TruCount Tube Beads are in black. Calibration beads (1 and 2 μm) are in blue. EMPs are in red. (A,B) TruCount beads can be counted and differentiated by size from TruCount bead population (R1), 1 and 2 μm beads = R2 (gate for EMP). (C) representative dot blot demonstrating gated EMPs (R2) and TruCount beads (R1). (D) Representative scatter blot demonstrating double-staining of EMP population with both a FITC- and PE-conjugated monoclonal antibody. (Logarithmic fluorescences of FITC [FL1] and PE [FL2], respectively.)

Table 1
TNF-α-activated HCAECs release species of EMP exhibiting quantitative differences in antigenic phenotypes

	Control	TNF-α	TNF-α/Flu	TNF-α/Y-27632
CD31 (PECAM-1)	145 ± 36	233 ± 77*	231 ± 80	255 ± 104
CD105 (endoglin)	43 ± 5	124 ± 34*	45 ± 8**	56 ± 13***
CD51 (vitronectin)	29 ± 4	76 ± 46*	43 ± 7**	44 ± 10***
CD 54 (ICAM-1)	82 ± 15	129 ± 44	125 ± 35	159 ± 24
CD62E (ELAM)	86 ± 2	99 ± 3	80 ± 7	65 ± 13
CD106 (VCAM-1)	128 ± 21	109 ± 41	167 ± 45	228 ± 80

$CD105^+$ and $CD51^+$ EMP are reduced by fluvastatin or the Rho inhibitor Y-27632. The data are of six individual experiments and are represented as means ± SEM of absolute count of EMP ($\times 10^6$/mL). Results are shown after 24 h of no treatment or treatment with TNF-α or TNF-α plus fluvastatin 0.1 μM/L or TNF-α plus Y-27632.

* $p < 0.05$ between TNF-α treatment and control.

** $p < 0.05$ between TNF-α and TNF-α plus fluvastatin treatment.

*** $p < 0.05$ between TNF-α and TNF-α plus Y-27632.

Role of Rho/ROK

Inactivation of Rho GTPases by statins is known to regulate the expression of several endothelial genes whose products play key roles in thrombosis and vascular biology [13]. TNF-α (10 ng/mL) induced RhoA activation in HCAEC at 24 h. Fluvastatin (0.1 μM/L) significantly prevented TNF-α-induced RhoA activation (Fig. 2).

Statin and ROK inhibitor suppresses TNF-α-induced EMP release

Statins have been shown to have anti-thrombotic properties [14]. Table 1 shows that fluvastatin (0.1 μM/L) significantly suppresses the release of $CD105^+$ and $CD51^+$ EMP (124 ± 34 to 45 ± 7.6 and 76.4 ± 46 to 43 ± 7.3, $p < 0.05$). Treatment with Y-27632 (10 μM/L)

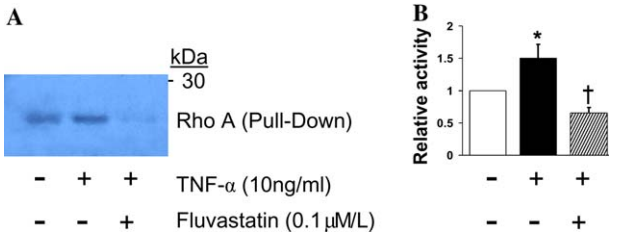

Fig. 2. Effect of fluvastatin on TNF-α-induced RhoA activation. Confluent HCAECs in culture were treated with TNF-α (10 ng/mL) alone or with fluvastatin (0.1 μM/L). (A) Representative immunoblot of active RhoA. (B) Graph represents densitometric analyses of active RhoA immunoblots; $n = 3$ (*$p < 0.05$ vs control, †$p < 0.05$ vs TNF-α).

reproduced the effect of fluvastatin demonstrated by a significant decrease in $CD105^+$ and $CD31^+$ EMP (124 ± 32 to 56 ± 13, and 76 ± 26 to 44 ± 10, $p < 0.05$). $CD31^+$ and $CD54^+$ EMP remained elevated after treatment with fluvastatin and Y-27632. $CD62^+$ EMP was suppressed by 19% but this was not statistically significant. The total number of EMPs specific for CD106 increased after treatment with fluvastatin and Y-27632, however this was not statistically significant. Preliminary studies did not demonstrate a synergistic effect between fluvastatin and Y-27632.

Discussion

Recent evidence highlights the importance of inflammation in the development of atherosclerosis and its clinical manifestations [15]. Although the specific relationship between plasma levels of EMPs and atherosclerosis is unclear, our study strengthens the link between EC activation and EMP production. In our study, we found a significant TNF-α-dependent elevation of $CD31^+$, $CD51^+$, and $CD105^+$ EMP from HCAEC in vitro. These findings paralleled those of a previous study [4]. A clinical correlate is the finding of elevated $CD31^+$ and $CD51^+$ EMP in patients with CAD [2].

Statin-treatment reduces pro-inflammatory CAM synthesis by endothelial cells after TNF-α stimulation [16]. In this study, we found a significant suppression of $CD51^+$ and $CD105^+$ EMP after treatment with statin. Fluvastatin was chosen for this study because of its known anti-inflammatory properties and ability to inhibit adhesion molecule expression on ECs [17–19]. The effect of fluvastatin was at 0.1 μM/L, a level that is close to peak plasma concentration in humans after administration of relatively high doses of fluvastatin, suggesting that the effects of fluvastatin observed in this study are clinically relevant. Although it is known that EC activation results in an increased expression of CD62 (E-selectin), we found no significant increase in $CD62^+$ EMP at 24 h. This is explainable considering that E-selectin is synthesized de novo in response to TNF-α, with maximum cell surface expression at only 4–6 h [20]. The relationship between CAM protein synthesis and the antigenic phenotype of EMPs may not be predicted easily, since the latter is also influenced by the rate of surface shedding.

Although recent studies have identified a role for Rho/ROK activation in experimental models of vascular inflammation and the early development of atherosclerosis, nothing is known about the function of Rho in EMP release [21,22]. In this study, we have explored the role of Rho/ROK in the regulation of EMP release from activated HCAEC in vitro. Involvement of Rho/ROK in EMP release is supported by the down-regulatory effect of both statin and Y-27632 on EMP release. Although these data do prove the contribution of Rho proteins to the regulation of EMP release, they do not rule out the participation of other prenylated proteins. These findings suggest that inactivation of Rho/ROK pathway by statins may impair actin cytoskeleton organization, which further leads to the suppression of EMP release.

Currently, cardiovascular risk assessment is based on established risk factors, including gender, age, smoking, diabetes, and serum concentrations of cholesterol; however, at present, there are only a few markers for the detection of endothelial activation ex vivo. The circulating levels of plasma EMPs may have a direct pathophysiological role in the development of CAD and thus quantitation of EMP levels may serve as an early diagnostic screening tool for CAD in addition to measuring atherosclerotic burden in the management of patients with known CAD.

A difficult problem in EMP studies has been comparing results between laboratories. Since EMPs exist as heterogeneous species in proportions varying with the nature of the EC injury, it is unlikely that any single marker will efficiently label total EMPs. Thus, the need for precise and reproducible quantification is necessary if EMPs are to be a reliable marker for atherosclerosis. We developed a new, two-color flow cytometric technique to quantitate the absolute number of EMPs in vitro with TruCount beads as an internal standard. This technique was reproducible and required a small amount of sample thus lending itself to the testing of clinical specimens.

In summary, fluvastatin prevents EMP release from TNF-α-activated HCAEC in vitro. In addition, our data strongly suggest a link between statins, the Rho/ROK pathway, and the regulation of EMP release. The direct interference of statins with the release of EMPs, factors related to the development of atherosclerosis, may thus be a novel mechanism underlying the beneficial effects of statins in CAD. These findings provide new insights into the cholesterol-independent effects of statin on the EC. However, similar to their effects on EMP release, the links connecting the regulation of their expression to

Rho GTPases remain unknown. Clearly, further studies are required to unravel the molecular mechanisms underlying the action of the Rho/ROK pathway on the release of EMPs.

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