Fluvastatin inhibits regulated secretion of endothelial cell von Willebrand factor in response to diverse secretagogues

Richard J. FISH, Hong YANG¹, Christelle VIGLINO, Raoul SCHORER, Sylvie DUNOYER-GEINDRE and Egbert K. O. KRUIHOF²

Service of Angiology and Haemostasis, Department of Internal Medicine, Geneva University Hospital, 24 Rue Micheli-du-Crest, CH-1205 Geneva, Switzerland

Regulated secretion of EC (endothelial cell) vWF (von Willebrand factor) is part of the haemostatic response. It occurs in response to secretagogues that raise intracellular calcium or cAMP. Statins are cholesterol-lowering drugs used for the treatment of cardiovascular disease. We studied the effect of fluvastatin on regulated secretion of vWF from HUVEC (human umbilical-vein ECs). Secretion in response to thrombin, a protease-activated receptor-1 agonist peptide, histamine, forskolin and adrenaline (epinephrine) was inhibited. This inhibition was reversed by mevalonate or geranylgeranyl pyrophosphate, and mimicked by a geranylgeranyl transferase inhibitor, demonstrating that the inhibitory mechanism includes inhibition of protein geranylgeranylation. To investigate this mechanism further, calcium handling and NO (nitric oxide) regulation were studied in fluvastatin-treated HUVEC. Intracellular calcium mobilization did not correlate with vWF secretion. Fluvastatin increased

eNOS [endothelial NOS (NO synthase)] expression, but NOS inhibitors failed to reverse the effect of fluvastatin on vWF secretion. Exogenous NO did not inhibit thrombin-induced vWF secretion. Many small GTPases are geranylgeranylated and some are activated by secretagogues. We overexpressed DN (dominant negative) Rho GTPases, RhoA, Rac1 and Cdc42 (cell division cycle 42), in HUVEC. DNCdc42 conferred inhibition of thrombin- and forskolin-induced vWF secretion. We conclude that, via inhibition of protein geranylgeranylation, fluvastatin is a broad-spectrum inhibitor of regulated vWF secretion. Geranylgeranylated small GTPases with functional roles in regulated secretion, such as Cdc42, are potential targets for the inhibitory activity of fluvastatin.

Key words: calcium, cAMP, endothelial cell, regulated secretion, statin, von Willebrand factor.

INTRODUCTION

Regulated secretion of vWF (von Willebrand factor) from EC (endothelial cell) WPBs (Weibel–Palade bodies) occurs in response to raising the intracellular calcium concentration or cAMP, and is important for haemostasis and inflammation [1]. WPBs are a storage pool for a number of proteins involved in responses to acute endothelial activation. vWF contributes to WPB structure and is packaged to enable rapid unfurling of vWF multimers upon regulated secretion [2]. vWF secretion has been measured in studies of responses to calcium-raising agonists, such as thrombin and histamine, and for agents that increase cAMP levels, such as adrenaline (epinephrine) or forskolin [3]. Vasopressin, a further cAMP-raising vWF secretagogue [4], is exploited therapeutically in patients with von Willebrand's disease [5].

HMG-CoA reductase (3-hydroxy-3-methylglutaryl-CoA reductase) inhibitors, or statins, inhibit cholesterol biosynthesis. Statins are used therapeutically to reduce low-density lipoprotein cholesterol, and they reduce myocardial infarction and death rates in patients with high cholesterol [6]. Statins also prevent re-infarction and reduce mortality in patients without elevated cholesterol [7], suggesting additional beneficial effects beyond lipid lowering. Such 'pleiotropic effects' probably reflect inhibition of isoprenoid synthesis and subsequent protein prenylation.

Isoprenoid geranylgeranyl and farnesyl moieties are attached to proteins to enable membrane association and protein–protein interactions [8]. Targets for prenylation include the small GTPase family and the γ subunits of heterotrimeric G-proteins, linking prenylation to gene regulation, cytoskeletal organization, intracellular vesicle trafficking, secretion and signal transduction.

One pleiotropic effect of statins is their ability to raise NO (nitric oxide) levels by increasing eNOS [endothelial NOS (NO synthase)] mRNA stability [9]. NO influences the nitrosylation of the NSF [NEM (*N*-ethylmaleimide)-sensitive factor], a protein involved in vesicle trafficking and membrane fusion [10]. Simvastatin inhibits thrombin-induced vWF secretion from cultured arterial ECs [11], partly in an NO-dependent manner. However, the physiological relevance of NO as a regulator of vWF secretion has been contested [12].

Several mechanistic details of calcium- and cAMP-induced vWF secretion are known. For example, calmodulin activity is required for thrombin-induced secretion [13], and protein kinase A is required for secretion in response to cAMP-inducing agonists [4]. vWF secretagogues induce cytoskeletal rearrangements in ECs [14], via activation of distinct small GTPases [15]. Increased cAMP also leads to clustering of a group of WPBs to the microtubule-organizing centre. This does not occur with calcium-mobilizing stimuli [16]. In addition, agonist-specific secretion of heterogeneous subsets of WPBs was recently demonstrated [17].

Abbreviations used: AUC, area under curves; Cdc42, cell division cycle 42; cGK, cGMP-dependent protein kinase; DN, dominant negative; EC, endothelial cell; NO, nitric oxide; NOS, NO synthase; eNOS, endothelial NOS; fura 2/AM, fura 2 acetoxymethyl ester; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GGPP, geranylgeranyl pyrophosphate; HBSS, Hanks balanced salt solution; HEK-293 cell, human embryonic kidney cell; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA reductase; HSA, human serum albumin; HUVEC, human umbilical-vein ECs; IBMX, isobutylmethylxanthine; L-NAME, *N*^G-nitro-L-arginine methyl ester; L-NMMA, *N*^G-monomethyl-L-arginine; NEM, *N*-ethylmaleimide; NSF, NEM-sensitive factor; PAR, protease-activated receptor; RT, reverse transcriptase; SNAP, S-nitroso-*N*-acetyl-DL-penicillamine; vWF, von Willebrand factor; WPB, Weibel–Palade body.

¹ Present address: St. Michael's Hospital, Toronto, ON, Canada M5B 1W8.

² To whom correspondence should be addressed (email Egbert.Kruihof@hcuge.ch).

We set out to determine whether inhibition of HMG-CoA reductase could inhibit vWF secretion in response to a broad range of secretagogues. Using the HUVEC (human umbilical-vein ECs) model, we demonstrate that fluvastatin inhibits regulated vWF secretion in response to all calcium-mobilizing and cAMP-raising secretory stimuli tested. The inhibitory mechanism acts via inhibition of protein geranylgeranylation, but does not depend on changes in intracellular calcium handling with calcium-mobilizing agents, or NO-based regulation. We also demonstrate a potential role for the GTPase Cdc42 (cell division cycle 42) in regulated vWF secretion. The broad inhibition spectrum of fluvastatin implies that statin-treated endothelium is less responsive to diverse secretory stimuli.

EXPERIMENTAL

Materials

Fine chemicals and secretagogues, including adrenaline, forskolin, histamine, IBMX (isobutylmethylxanthine), GGPP (geranylgeranyl pyrophosphate), GGTI-298, L-NAME (*N*^G-nitro-L-arginine methyl ester), L-NMMA (*N*^G-monomethyl-L-arginine) and thrombin were obtained from Sigma–Aldrich (Schnellendorf, Germany). SNAP (*S*-nitroso-*N*-acetyl-DL-penicillamine) and buffers were from Invitrogen (Paisley, Renfrewshire, Scotland, U.K.). Fluvastatin was from Calbiochem (San Diego, CA, U.S.A.). The TFLLR-NH₂ peptide was purchased from an in-house peptide synthesis facility.

Cells

HUVEC were isolated as described in [18], and cultured in EGM-2 medium (Cambrex, Walkersville, MD, U.S.A.). Umbilical cords were obtained by written consent, with approval from the local ethics committee. Cells were used at passage 1–3. HEK-293 cells (human embryonic kidney cells), for adenoviral vector amplification, were cultured in DMEM (Dulbecco's modified Eagle's medium) and 5 % (v/v) foetal calf serum (Invitrogen).

Regulated release of vWF

Confluent HUVEC in 24-well plates were incubated in EGM-2 medium with fluvastatin and other agents, as described in the Results section. Cells were washed four times with HBSS (Hanks balanced salt solution)/0.1 % HSA (human serum albumin). Secretory stimuli were added in HBSS/0.1 % HSA for 10 or 40 min and vWF was quantified by ELISA [18] in cell-conditioned buffer.

Intracellular calcium measurements

Confluent HUVEC in 96-well plates were incubated with or without fluvastatin, and then with calcium buffer (143 mM NaCl, 6 mM KCl, 1 mM MgSO₄, 0.1 % glucose, 1 mM CaCl₂, 0.125 mM Sulfinpyrazone, 0.1 % HSA and 20 mM HEPES, pH 7.4) with 2 μ M fura 2/AM (fura 2 acetoxymethyl ester) and 0.03 % Pluronic F-127 (both Invitrogen), for 40 min at 37°C. Cells were washed once and incubated for 30 min at 37°C in calcium buffer, to allow for esterase cleavage of fura 2/AM. A FlexStation instrument (Molecular Devices, Sunnyvale, CA, U.S.A.) was used for fluorescence measurements. Fluorescence was measured at 510 nm with excitation at 340 and 380 nm. After a 20 s baseline, agonists were added and fluorescence was detected for 2 min.

Quantitative RT (reverse transcriptase)–PCR

eNOS mRNA was quantified by RT–PCR using the $\Delta\Delta C_T$ (comparative change in threshold cycle) method, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the housekeeping gene, as described previously [19]. Primer sequences (5'–3') were GAPDH forward GGTGAAGGTCGGAGTCAAC and GAPDH reverse CCATGGGTGGAATCATATTG, and eNOS forward TGGTACATGAGCACTGAGATCG and eNOS reverse CCACGTTGATTTCCACTGCTG.

NO measurements

NO in cell-conditioned medium was measured indirectly by quantification of nitrites, using the Griess reagent kit and the manufacturer's instructions (Invitrogen).

Flow cytometry and detection of PAR-1 (protease-activated receptor-1)

After treatment with or without fluvastatin, HUVEC were detached in PBS/EDTA (1 mM), washed in PBS/1 % BSA (PBS/BSA) and incubated for 75 min, on ice, in PBS/BSA with 10 μ g/ml mouse anti-human PAR-1 antibodies (Zymed Laboratories, San Francisco, CA, U.S.A.), or isotype-matched antibodies (Dako, Baar, Switzerland). After two washes, cells were incubated for 45 min in PBS/BSA containing 4 μ g/ml phycoerythrin-labelled goat anti-mouse antibodies (BD Biosciences, San Jose, CA, U.S.A.). After two washes, samples were analysed using an FACStar instrument (BD Biosciences). For intracellular staining, cells were fixed in 1 % paraformaldehyde/PBS and permeabilized in 0.1 % saponin/PBS, prior to antibody staining. Data were analysed using WinMDI v2.8. Overlaid histograms were smoothed for clarity (setting 3).

Adenoviral vectors

Adenoviral vectors for expression of Myc-tagged N19RhoA, N17Rac1 and N17Cdc42 [referred to as DN (dominant negative)] were from Dr A. J. Ridley (Ludwig Institute, London, U.K.). Vectors were amplified in HEK-293 cells and titrated using the Adeno-X Rapid Titre kit (Clontech–Takara, Saint-Germain-en-Laye, France). For experiments, vectors were used at a multiplicity of infection of 1500, as described previously [15]. Myc-tagged DN small GTPases were detected by flow cytometry with anti-c-Myc antibodies (Zymed Laboratories) in permeabilized cells, as described for intracellular PAR-1 detection.

Graphics and statistics

Data representations and statistical tests were made using Prism software (GraphPad, San Diego, CA, U.S.A.). All error bars represent S.E.M.

RESULTS

Fluvastatin inhibits regulated secretion of vWF

We investigated the effects of fluvastatin on regulated vWF secretion from cultured human ECs. We incubated cells with a range of fluvastatin doses and incubation times, and measured vWF secretion in response to thrombin (9 nM or 1 unit/ml, for 40 min). A 24 h incubation with 2.5 μ M fluvastatin reproducibly gave over 50 % inhibition of thrombin-induced vWF secretion, was more effective than shorter incubation times, did not disrupt the HUVEC culture monolayer and did not change vWF antigen levels measured in cell lysates (results not shown). In addition, 2.5 μ M is close to peak fluvastatin concentrations measured in the

Figure 1 Fluvastatin inhibits regulated secretion of vWF

vWF was measured in cell-conditioned buffer after a 40 min stimulation with thrombin (A), TFLLR-NH₂ (B), histamine (C) and forskolin (+ 100 μ M IBMX) (D), using HUVEC control cells (■) and cells treated with fluvastatin (2.5 μ M, 24 h) (▼). Results are expressed as ng of vWF per well after a 40 min stimulation. Results are from three independent experiments ($n = 3$), each performed in duplicate, except control cells in (A), where $n = 2$.

plasma of healthy subjects 1 h after an 80 mg dose of fluvastatin [20].

HUVEC were stimulated with secretagogues after a 24 h incubation with fluvastatin (2.5 μ M). vWF secretion was measured after stimulation with thrombin, TFLLR-NH₂ (a selective agonist peptide for PAR-1, the predominant EC thrombin receptor) and histamine, as calcium-mobilizing ligands. A combination of forskolin and IBMX was used to raise cAMP. Forskolin activates adenylate cyclase and IBMX inhibits cAMP phosphodiesterases. Fluvastatin inhibited vWF secretion induced by all secretagogues (Figure 1). vWF secretion in response to thrombin, TFLLR-NH₂ and forskolin was inhibited at all five secretagogue concentrations. For histamine, we observed a shift in EC₅₀ values (with 95 % confidence interval) from 0.76 μ M (0.13–4.4) in control cells to 3.8 μ M (2.1–6.6) in fluvastatin-treated cells. At histamine concentrations above 10 μ M, vWF secretion by fluvastatin-treated cells was comparable with that of control cells.

Our results demonstrate inhibition of regulated vWF secretion after a 40 min secretagogue stimulation in fluvastatin-treated cells. To assess whether the inhibitory effects of fluvastatin were also measurable after a shorter stimulation, we compared vWF secretion induced by thrombin and forskolin/IBMX after 10 and 40 min in control and fluvastatin-treated cells. Fluvastatin treatment (2.5 μ M, 24 h) inhibited vWF secretion induced by both stimuli after 10 or 40 min stimulations (Supplementary Figure S1 at <http://www.BiochemJ.org/bj/405/bj4050597add.htm>). When the vWF secreted from non-stimulated cells was subtracted from the stimulated cell values, the percentage inhibition of regulated secretion by fluvastatin was approx. 65 % for both stimuli. There

was not a significant difference between the percentage inhibition after 10 or 40 min stimulations with either stimuli.

Fluvastatin inhibits regulated vWF secretion via inhibition of HMG-CoA reductase

To investigate whether HMG-CoA reductase is the target for fluvastatin in our secretion experiments, we studied the effect of adding mevalonate, a metabolite downstream of HMG-CoA reductase, with fluvastatin (Figure 2). Mevalonate (0.5 mM) reversed the inhibitory effects of fluvastatin on vWF secretion induced by thrombin, histamine and forskolin, as representative secretagogues. We also included adrenaline as a secretagogue in this analysis. Adrenaline activates adenylate cyclase via adrenergic receptors and is a weak EC secretagogue *in vitro* (Figure 2D), but has physiological relevance for acute systemic vWF release [21].

Fluvastatin inhibits regulated vWF secretion via inhibition of protein geranylgeranylation

Addition of the geranylgeranyl lipid moiety to proteins is catalysed by geranylgeranyl transferase, and requires GGPP. To investigate if fluvastatin inhibits regulated vWF secretion by inhibiting protein geranylgeranylation, we added GGPP in combination with fluvastatin and measured regulated vWF secretion. Thrombin and forskolin were used as calcium-mobilizing and cAMP-raising agents respectively. GGPP completely reversed the inhibitory effect of fluvastatin in response to both stimuli (Figure 3). To confirm that the fluvastatin-mediated inhibition of

Figure 2 Fluvastatin inhibits vWF secretion via inhibition of HMG-CoA reductase

HUVEC were treated for 24 h in medium (Ctrl), 2.5 μ M fluvastatin (F2.5 μ M), 0.5 mM mevalonate (Mev 0.5 mM) or 2.5 μ M fluvastatin with 0.5 mM mevalonate (F + Mev). Secretion of vWF was measured in non-stimulated cells (empty bars) or after a 40 min stimulation (black bars) with 9 nM (1 unit/ml) thrombin (A), 1 μ M histamine (B), 30 μ M forskolin/100 μ M IBMX (C), or 100 μ M adrenaline/100 μ M IBMX (D). A representative experiment performed in triplicate or quadruplicate is shown.

regulated vWF secretion acts via geranylgeranylation, we added a geranylgeranyl transferase inhibitor, GGTI-298, to HUVEC prior to secretagogue stimulation. GGTI-298 mimicked the effect of fluvastatin on regulated vWF secretion induced by thrombin and forskolin (Figure 3). Our results demonstrate that fluvastatin inhibits regulated secretion of vWF by inhibition of protein geranylgeranylation.

Effects of fluvastatin on intracellular calcium

We studied whether changes in calcium handling could explain, at least in part, the inhibition of vWF secretion by calcium-mobilizing secretagogues. Using fura 2 fluorescence, baseline intracellular calcium concentrations were approx. 30% higher in fluvastatin-treated cells, compared with controls (Figure 4A and [22]). We monitored intracellular calcium mobilization in response to thrombin, TFLLR-NH₂ and histamine. Calcium mobilization can be biphasic, reflecting release from intracellular stores and extracellular influx. Measurements of peak calcium and AUC (area under curves) account for these phases, which have both been correlated with regulated vWF secretion [12,23].

The EC₅₀ for peak calcium mobilization induced by thrombin and TFLLR-NH₂, both PAR-1 ligands, was marginally increased in fluvastatin-treated cells (Figures 4B and 4F). For example,

EC₅₀ values for thrombin (with 95% confident limits) were 1.3 nM (1.0–1.6) in control cells and 3.6 nM (2.6–4.9) after fluvastatin. Peak histamine responses were comparable in control and fluvastatin-treated cells. AUC dose–response curves for thrombin and TFLLR-NH₂ suggest slightly higher EC₅₀ values in fluvastatin-treated cells, compared with controls (Figures 4C and 4G). AUC histamine responses were comparable in control and fluvastatin-treated cells (Figure 4E). At the highest concentration of each secretagogue the AUC was increased in fluvastatin-treated cells. This increase was measured in the extracellular influx phase of the calcium response (Figure 4H). In the absence of extracellular calcium, this did not occur (results not shown). For all stimuli, the minor fluvastatin-mediated changes in peak and AUC calcium flux could not be correlated with fluvastatin-mediated inhibition of vWF secretion.

The increased calcium mobilization EC₅₀ for thrombin and TFLLR-NH₂, with fluvastatin, might be explained by reduced cell surface expression of PAR-1, observed in smooth-muscle cells [24]. Decreased receptor density could reduce responses to sub-saturation agonist concentrations. Using PAR-1-specific antibodies and flow cytometry, reduced cell surface PAR-1 was measured in fluvastatin-treated cells. This reduction was due to altered PAR-1 distribution, as permeabilized cells had similar total PAR-1 staining with or without fluvastatin (Supplementary Figure S2 at <http://www.BiochemJ.org/bj/405/bj4050597add.htm>).

Fluvastatin and NO

A previous report has shown that simvastatin increases NO in aortic EC and, via nitrosylation and inhibition of NSF, inhibits thrombin-induced vWF secretion [11]. We investigated whether changes in NO regulation account for the effects of fluvastatin on regulated secretion in HUVEC. By quantitative RT-PCR, we measured increased eNOS mRNA in fluvastatin-treated cells (Figure 5A). Using the Griess reaction [25] to detect nitrite in HUVEC-conditioned medium, as an indirect quantification of NO oxidation products, we were unable to measure NO. Using sodium nitrite standard curves, our nitrite detection limit was 0.8 μ M. We conclude that the nitrite concentrations attained in HUVEC-conditioned media are below this limit (results not shown).

To determine if the increase in eNOS mRNA, and by extension eNOS activity, plays a role in the inhibitory effects of fluvastatin on regulated vWF secretion, HUVEC were incubated with two NOS inhibitors, L-NAME and L-NMMA, for 30 min prior to and during 24 h with 2.5 μ M fluvastatin. Neither NOS inhibitor reversed the effects of fluvastatin on regulated secretion (Figure 5B). The inhibitors alone had no potentiating effect on thrombin-induced vWF secretion.

We tested whether exogenous NO could modify thrombin-induced vWF release. HUVEC were incubated with an NO donor, SNAP, for 4 h. A dose-dependent increase in nitrite, generated from oxidized NO, was measured. Nitrite levels reached 35 μ M with 0.5 mM SNAP (Figure 5C). Despite this increase, SNAP had no effect on thrombin-induced vWF secretion (Figure 5D). We conclude that the inhibitory effect of fluvastatin on thrombin-induced vWF secretion cannot be explained by increased NOS activity or elevated NO in HUVEC.

Rho GTPases and regulated vWF secretion

Rho GTPases function in the cytoskeletal changes that accompany stimulation of ECs with secretagogues [14,15], and inhibition of GTPases with guanosine 5'-[β -thio]diphosphate inhibits regulated vWF secretion [26]. Many Rho family GTPases are geranylgeranylated; the best characterized are RhoA, Rac1 and

Figure 3 Fluvastatin inhibits vWF secretion via inhibition of protein geranylgeranylation

HUVEC were treated for 24 h in medium [Ctrl (1), includes GGPP/fluvastatin vehicle], 2.5 μ M fluvastatin (F2.5 μ M), 10 μ M GGPP (GGPP 10 μ M), 2.5 μ M fluvastatin and 10 μ M GGPP (F2.5 μ M + GGPP 10 μ M), or 10 μ M GGTI-298 (GGTI 10 μ M). A second control was used for the GGTI vehicle. Regulated secretion of vWF was measured after 40 min. Empty bars represent vWF from non-stimulated cells and black bars represent cells treated with 9 nM thrombin (A) or 30 μ M forskolin/100 μ M IBMX (B). Results are from three independent experiments in (A) and two independent experiments in (B). In (A), unpaired Student's *t* tests were used to test significance between vWF secreted from Ctrl, F2.5 μ M, GGPP 10 μ M and F2.5 μ M + GGPP 10 μ M samples, after thrombin stimulation. *P values are given where appropriate; ns, not significant. Student's *t* tests were not used for data in (B), where *n* = 2.

Cdc42 [27]. As we identified inhibition of geranylgeranylation as a target of fluvastatin in regulated vWF secretion, we tested whether inhibition of RhoA, Rac1 or Cdc42 inhibits regulated vWF secretion. HUVEC were transduced with adenoviral vectors, which lead to expression of DN mutants of the GTPases, interfering with their endogenous activity [15]. A GFP (green fluorescent protein) vector was used as a transduction control. Transduction efficiency and expression levels were monitored by flow cytometry. Transduction rates near 100% and very similar DN GTPase expression levels were measured (Supplementary Figures S3A and S3B, at <http://www.BiochemJ.org/bj/405/bj4050597add.htm>). Regulated secretion of vWF was measured in transduced HUVEC, in response to thrombin (Figure 6A) and forskolin/IBMX (Figure 6B) as calcium- and cAMP-raising secretagogues respectively. Transduction with vectors for GFP, DNRhoA and DNRac1 had no effect on vWF secretion induced by either stimuli. However, overexpression of DNCdc42 partially, but significantly, inhibited vWF secretion in response to both secretagogues. We conclude that Cdc42 plays a role in regulated secretion induced by calcium- and cAMP-raising stimuli. However, the inhibitory effects of DNCdc42 expression are less pronounced than those of fluvastatin or GGTI-298 (compare Figure 3 with Figure 6), suggesting that fluvastatin and GGTI-298 target other proteins important for secretion.

DISCUSSION

Our principal finding is that fluvastatin inhibits regulated secretion of vWF induced by a wide range of secretagogues. vWF secretion induced by agents that provoke intracellular calcium mobilization, or factors that raise cAMP levels, is subject to this inhibition. Secretion from WPBs results in the release of vWF, a protein that is essential for platelet aggregation, and exposure of cell surface P-selectin, a key leucocyte adhesion molecule. The reduced secretion of vWF from WPBs by statin treatment

may therefore have marked effects on both coagulation and inflammation.

We found no link between calcium handling and fluvastatin-mediated inhibition of regulated secretion. Minor changes in peak responsiveness to thrombin and TFLLR-NH₂ did not correlate with regulated vWF secretion for either ligand.

A role for the NSF in regulated EC secretion has been reported [10]. NSF is sensitive to nitrosylation and inhibited by increased NO. Statins have been linked to increases in eNOS expression and activity, and subsequent elevation of NO production. In turn, this was described as the source of NO for inhibition of thrombin-induced vWF secretion from arterial ECs using simvastatin [11]. However, the same study measured clear inhibition of thrombin-induced vWF secretion at concentrations of simvastatin that did not increase NO production, measured indirectly as nitrites.

We could not associate inhibition of regulated vWF secretion to NOS activity or NO in our HUVEC model. There are several explanations for the difference between the NO-dependency of the HUVEC and arterial EC models of vWF secretion. The different cell sources and their behaviour under conditions of cell culture are potential causes of biological variation. Different statins were used, and while fluvastatin and simvastatin target HMG-CoA reductase, their different structures may incur subtle differences in activity. While NSF is targeted by nitrosylation in arterial cells, NEM does not inhibit regulated vWF secretion from HUVEC [28]. This suggests that NSF activity, inhibited by NEM or nitrosylation, would not influence vWF secretion in HUVEC. Previous studies have also failed to link exogenous NO to regulated vWF secretion in the HUVEC model [29]. NO-stimulated increases in guanylate cyclase activity, leading to the downstream effects of cGMP, via cGKs [cGMP-dependent protein kinases; also known as PKG (protein kinase G)], are also unlikely to play a role in regulated release of vWF from HUVEC, because HUVEC bear little or no cGK activity [30].

Inhibition of protein geranylgeranylation is at least part of the mechanism by which fluvastatin inhibits regulated secretion.

Figure 4 Intracellular calcium handling and mobilization after fluvastatin treatment

Intracellular calcium was monitored using fura 2-associated fluorescence. Ratiometric measurements of fluorescence (340/380) are expressed as absolute F ratio in (A), or as peak F and AUC (area under curves) after baseline correction (B–G). In (A), baseline fluorescence was analysed from ten independent experiments. Results were analysed with the Wilcoxon matched pairs test ($n = 10$). In (B–G), control HUVEC (■), or cells treated with fluvastatin (▼) (24 h, 2.5 μ M) were stimulated with thrombin (B, C), histamine (D, E) or TFLLR-NH₂ (F, G). The peak fluorescence ratio (peak F ; B, D, F) or area under fluorescence curves (AUC; C, E, G) are given. Sigmoid dose–response curves were fitted for clarity. Results are from three independent experiments, each with duplicates or triplicates. (H) An example of calcium

Figure 5 Fluvastatin and NO regulation

(A) Fluvastatin increases eNOS expression in HUVEC. eNOS mRNA was measured by quantitative RT–PCR with (F2.5 μ M) and without (Ctrl) a 24 h treatment with 2.5 μ M fluvastatin. Results are from three independent experiments, each with triplicate RNA samples. Results were normalized to control cell (Ctrl) eNOS levels (100 %) and compared using an unpaired Student's t test. In (B), HUVEC were incubated for 24 h with medium (Ctrl), fluvastatin (F2.5 μ M), L-NAME (1 mM), L-NMMA (1 mM) or a combination of fluvastatin and L-NAME (F + L-NAME) or L-NMMA (F + L-NMMA). Cells were stimulated with 9 nM thrombin for 40 min and vWF secretion was measured. Empty bars are non-stimulated cells and black bars thrombin-stimulated. Results are from three independent experiments, each with duplicates. In (C), HUVEC were incubated with medium or medium containing SNAP at 0.125, 0.25 or 0.5 mM. Total nitrites (NO₂⁻) in cell-conditioned medium, after 4 h at 37 °C, were measured using the Griess reaction. In (D), HUVEC were treated as in (C) and vWF secretion was measured after a 40 min stimulation with thrombin (9 nM; black bars) or buffer (empty bars). Results for (C, D) are from three independent experiments, each with triplicates.

Exogenous mevalonate or GGPP reverses the inhibitory effects of fluvastatin. While mevalonate is a precursor to cholesterol and hydrophobic prenyl moieties, GGPP is used for geranylgeranylation, but not cholesterol synthesis. This excludes cellular cholesterol synthesis as a target of fluvastatin in regulated secretion. This is also supported by the similar inhibition of secretion seen with fluvastatin and GGTI-298. A large number of proteins undergo geranylgeranylation as a post-translational modification [8], which makes the range of possible functional targets of fluvastatin extremely broad and explains the ‘pleiotropic’ effects on ECs that have been ascribed to statins. While it seems unreasonable to imagine that the effects of fluvastatin can be attributed to the inhibition of geranylgeranylation of a single GTPase, overexpression of DNCdc42 gave a significant decrease in thrombin- and forskolin/IBMX-induced vWF secretion. Cdc42 was previously implicated in calcium-mobilizing-agent-induced vWF secretion, with PAR receptor agonist peptides as the stimuli [31]. Our results extend this finding to include secretion induced by forskolin/IBMX, which raise cAMP. We have not made a direct link between fluvastatin and Cdc42, but our results suggest that Cdc42 could be a target of fluvastatin

mobilization curves for HUVEC stimulated with 90 μ M TFLLR-NH₂, in control cells (■) and cells treated with fluvastatin (2.5 μ M, 24 h; ▼). The second phase of the curve remains elevated longer in fluvastatin-treated cells, explaining the increased AUC values in (G). Results are the means for triplicates from one of the three experiments in (G).

Figure 6 Regulated secretion of vWF is partially inhibited by DNCdc42

In (A, B), regulated secretion of vWF, from cells transduced with adenoviral vectors, was measured after stimulation with 9 nM thrombin (A) or 30 μ M forskolin/100 μ M IBMX (B). Non-stimulated secretion is shown with empty bars and secretagogue-stimulated secretion with black bars. Each data point is from three independent experiments, each with duplicates. Results were normalized to percentage non-stimulated secretion in non-transduced cells, due to large variations in the total secreted vWF between experiments. Results for thrombin- and forskolin/IBMX-induced vWF secretion were compared for non-transduced cells and cells transduced with DNCdc42, using an unpaired Student's *t* test.

treatment in HUVEC, particularly as calcium- and cAMP-raising-agent-induced vWF secretion was inhibited by Cdc42 inhibition or fluvastatin. Several other prenylated small GTPases have been implicated in EC-regulated secretion [32–34]. Of these, RalA is activated by secretagogues and is functionally involved in vWF secretion [34,35].

Our study infers that patients treated with statins may have endothelium that is less responsive to secretagogues, potentially resulting in a less thrombotic or less inflammatory phenotype. This might explain some of the beneficial effects of statins that are not directly linked to cholesterol lowering [7]. We have shown that fluvastatin blocks vWF secretion in response to a wide variety of stimuli. To our knowledge, this is the first demonstration of broad-spectrum inhibition of regulated EC secretion with an exogenous agent. Understanding which geranylgeranylated proteins are targets for this inhibition will implicate them in the endogenous regulated secretion mechanism. In the future, this knowledge could be exploited therapeutically for reducing inflammation or coagulation propagated by the release of endothelial storage granules.

This work was supported by the Swiss National Science Foundation grant 3100A0-105844. We thank Dr Claes B. Wollheim and Dr Ulrich M. Vischer (Department of Cell Physiology and Metabolism, University Medical Centre, Geneva, Switzerland) for useful discussions.

REFERENCES

- Datta, Y. H. and Ewenstein, B. M. (2001) Regulated secretion in endothelial cells: biology and clinical implications. *Thromb. Haemostasis* **86**, 1148–1155
- Michaux, G., Abbitt, K. B., Collinson, L. M., Haberichter, S. L., Norman, K. E. and Cutler, D. F. (2006) The physiological function of von Willebrand's factor depends on its tubular storage in endothelial Weibel–Palade bodies. *Dev. Cell* **10**, 223–232

- van Mourik, J. A., Romani de Wit, T. and Voorberg, J. (2002) Biogenesis and exocytosis of Weibel–Palade bodies. *Histochem. Cell Biol.* **117**, 113–122
- Kaufmann, J. E., Oksche, A., Wollheim, C. B., Gunther, G., Rosenthal, W. and Vischer, U. M. (2000) Vasopressin-induced von Willebrand factor secretion from endothelial cells involves V2 receptors and cAMP. *J. Clin. Invest.* **106**, 107–116
- Mannucci, P. M. (2004) Treatment of von Willebrand's Disease. *N. Engl. J. Med.* **351**, 683–694
- Scandinavian Simvastatin Survival Study Group (1994) Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). *Lancet* **344**, 1383–1389
- Sacks, F. M., Pfeffer, M. A., Moye, L. A., Rouleau, J. L., Rutherford, J. D., Cole, T. G., Brown, L., Warnica, J. W., Arnold, J. M., Wun, C. C. et al. (1996) The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events Trial investigators. *N. Engl. J. Med.* **335**, 1001–1009
- Roskoski, Jr, R. (2003) Protein prenylation: a pivotal posttranslational process. *Biochem. Biophys. Res. Commun.* **303**, 1–7
- Laufs, U., La Fata, V., Plutzky, J. and Liao, J. K. (1998) Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. *Circulation* **97**, 1129–1135
- Matsushita, K., Morrell, C. N., Cambien, B., Yang, S. X., Yamakuchi, M., Bao, C., Hara, M. R., Quick, R. A., Cao, W., O'Rourke, B. et al. (2003) Nitric oxide regulates exocytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor. *Cell* **115**, 139–150
- Yamakuchi, M., Greer, J. J., Cameron, S. J., Matsushita, K., Morrell, C. N., Talbot-Fox, K., Baldwin, III, W. M., Lefer, D. J. and Lowenstein, C. J. (2005) HMG-CoA reductase inhibitors inhibit endothelial exocytosis and decrease myocardial infarct size. *Circ. Res.* **96**, 1185–1192
- Zarei, S., Frieden, M., Kaufmann, J. E. and Vischer, U. M. (2006) The regulation of endothelial vWF secretion by nitric oxide: is it physiological? *J. Thromb. Haemost.* **4**, 263–265
- Birch, K. A., Pober, J. S., Zavoico, G. B., Means, A. R. and Ewenstein, B. M. (1992) Calcium/calmodulin transduces thrombin-stimulated secretion: studies in intact and minimally permeabilized human umbilical vein endothelial cells. *J. Cell Biol.* **118**, 1501–1510
- Vischer, U. M., Barth, H. and Wollheim, C. B. (2000) Regulated von Willebrand factor secretion is associated with agonist-specific patterns of cytoskeletal remodeling in cultured endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **20**, 883–891
- Wojciak-Stothard, B., Potempa, S., Eichholtz, T. and Ridley, A. J. (2001) Rho and Rac but not Cdc42 regulate endothelial cell permeability. *J. Cell Sci.* **114**, 1343–1355
- Rondaij, M. G., Bierings, R., Kragt, A., Gijzen, K. A., Sellink, E., van Mourik, J. A., Fernandez-Borja, M. and Voorberg, J. (2006) Dynein–dynactin complex mediates protein kinase A-dependent clustering of Weibel–Palade bodies in endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **26**, 49–55
- Cleator, J. H., Zhu, W. Q., Vaughan, D. E. and Hamm, H. E. (2006) Differential regulation of endothelial exocytosis of P-selectin and von Willebrand factor by protease-activated receptors and cAMP. *Blood* **107**, 2736–2744
- Vischer, U. M., Jornot, L., Wollheim, C. B. and Theler, J. M. (1995) Reactive oxygen intermediates induce regulated secretion of von Willebrand factor from cultured human vascular endothelial cells. *Blood* **85**, 3164–3172
- Fish, R. J. and Kruihof, E. K. (2004) Short-term cytotoxic effects and long-term instability of RNAi delivered using lentiviral vectors. *BMC Mol. Biol.* **5**, 9
- Siekmeier, R., Lattke, P., Mix, C., Park, J. W. and Jaross, W. (2001) Dose dependency of fluvastatin pharmacokinetics in serum determined by reversed phase HPLC. *J. Cardiovasc. Pharmacol. Ther.* **6**, 137–145
- Rickles, F. R., Hoyer, L. W., Rick, M. E. and Ahr, D. J. (1976) The effects of epinephrine infusion in patients with von Willebrand's disease. *J. Clin. Invest.* **57**, 1618–1625
- Heinke, S., Schwarz, G., Figulla, H. R. and Heinemann, S. H. (2004) The influence of statins on the free intracellular calcium concentration in human umbilical vein endothelial cells. *BMC Cardiovasc. Disord.* **4**, 4
- Birch, K. A., Ewenstein, B. M., Golan, D. E. and Pober, J. S. (1994) Prolonged peak elevations in cytoplasmic free calcium ions, derived from intracellular stores, correlate with the extent of thrombin-stimulated exocytosis in single human umbilical vein endothelial cells. *J. Cell. Physiol.* **160**, 545–554
- Yufu, T., Hirano, K., Bi, D., Hirano, M., Nishimura, J., Iwamoto, Y. and Kanaide, H. (2005) Rac1 regulation of surface expression of protease-activated receptor-1 and responsiveness to thrombin in vascular smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* **25**, 1506–1511
- Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E. and Chaudhuri, G. (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 9265–9269
- van den Eijnden-Schrauwen, Y., Atsma, D. E., Lupu, F., de Vries, R. E., Kooistra, T. and Emeis, J. J. (1997) Involvement of calcium and G proteins in the acute release of tissue-type plasminogen activator and von Willebrand factor from cultured human endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **17**, 2177–2187

- 27 Ridley, A. J. (2006) Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. *Trends. Cell Biol.* **16**, 522–529
- 28 Fayos, B. E. and Wattenberg, B. W. (1997) Regulated exocytosis in vascular endothelial cells can be triggered by intracellular guanine nucleotides and requires a hydrophobic, thiol-sensitive component. Studies of regulated von Willebrand factor secretion from digitonin permeabilized endothelial cells. *Endothelium* **5**, 339–350
- 29 Vischer, U. M., Lang, U. and Wollheim, C. B. (1998) Autocrine regulation of endothelial exocytosis: von Willebrand factor release is induced by prostacyclin in cultured endothelial cells. *FEBS Lett.* **424**, 211–215
- 30 Draijer, R., Atsma, D. E., van der Laarse, A. and van Hinsbergh, V. W. (1995) cGMP and nitric oxide modulate thrombin-induced endothelial permeability. Regulation via different pathways in human aortic and umbilical vein endothelial cells. *Circ. Res.* **76**, 199–208
- 31 Klarenbach, S. W., Chipiuk, A., Nelson, R. C., Hollenberg, M. D. and Murray, A. G. (2003) Differential actions of PAR2 and PAR1 in stimulating human endothelial cell exocytosis and permeability: the role of Rho-GTPases. *Circ. Res.* **92**, 272–278
- 32 Knop, M., Areskjold, E., Bode, G. and Gerke, V. (2004) Rab3D and annexin A2 play a role in regulated secretion of vWF, but not tPA, from endothelial cells. *EMBO J.* **23**, 2982–2992
- 33 Hannah, M. J., Hume, A. N., Arribas, M., Williams, R., Hewlett, L. J., Seabra, M. C. and Cutler, D. F. (2003) Weibel–Palade bodies recruit Rab27 by a content-driven, maturation-dependent mechanism that is independent of cell type. *J. Cell Sci.* **116**, 3939–3948
- 34 de Leeuw, H. P., Fernandez-Borja, M., Reits, E. A., Romani de Wit, T., Wijers-Koster, P. M., Hordijk, P. L., Neefjes, J., van Mourik, J. A. and Voorberg, J. (2001) Small GTP-binding protein Ral modulates regulated exocytosis of von Willebrand factor by endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **21**, 899–904
- 35 Rondaij, M. G., Sellink, E., Gijzen, K. A., ten Klooster, J. P., Hordijk, P. L., van Mourik, J. A. and Voorberg, J. (2004) Small GTP-binding protein Ral is involved in cAMP-mediated release of von Willebrand factor from endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **24**, 1315–1320

Received 22 March 2007/27 April 2007; accepted 2 May 2007

Published as BJ Immediate Publication 2 May 2007; doi:10.1042/BJ20070404