HMG-CoA Reductase Inhibitors Inhibit Endothelial Exocytosis and Decrease Myocardial Infarct Size

Munekazu Yamakuchi, James J.M. Greer, Scott J. Cameron, Kenji Matsushita, Craig N. Morrell, Karen Talbot-Fox, William M. Baldwin III, David J. Lefer, Charles J. Lowenstein

Abstract—Three-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors protect the vasculature from inflammation and atherosclerosis by cholesterol dependent and cholesterol independent mechanisms. We hypothesized that HMG-CoA reductase inhibitors decrease exocytosis of Weibel-Palade bodies, endothelial cell granules whose contents promote thrombosis and vascular inflammation. We pretreated human aortic endothelial cells with simvastatin for 24 hours, then stimulated the cells with thrombin, and measured the amount of von Willebrand factor (vWF) released into the media. We then measured the effect of simvastatin on myocardial infarction in mice. Simvastatin decreased thrombin-stimulated Weibel-Palade body exocytosis by 89%. Simvastatin inhibited exocytosis in part by increasing synthesis of nitric oxide (NO), which S-nitrosylated N-ethylmaleimide sensitive factor (NSF), a critical regulator of exocytosis. Simvastatin treatment attenuated myocardial infarct size by 58% in wild-type but not eNOS knockout mice. Furthermore, simvastatin decreased endothelial exocytosis and neutrophil infiltration into ischemic-reperfused myocardium, which was mediated in part by P-selectin contained in Weibel-Palade bodies. However, simvastatin did not affect exocytosis and inflammation in myocardial infarcts of eNOS knockout mice. Inhibition of endothelial exocytosis is a novel mechanism by which HMG-CoA reductase inhibitors may reduce vascular inflammation, inhibit thrombosis, and protect the ischemic myocardium. These findings may explain part of the pleiotropic effects of statin therapy for patients with cardiovascular disease. (Circ Res. 2005;96:1185-1192.)

Key Words: endothelium • nitric oxide • myocardial infarction • hypercholesterolemia

Therapy with 3-hydroxymethylglutaryl coenzyme A reductase inhibitors (statins) decreases myocardial infarctions and death in patients with elevated levels of cholesterol.1–3 However, statins also improve clinical outcomes in patients with average levels of cholesterol.4–9 Part of the beneficial effect of statins is because of their ability to decrease cholesterol levels, inhibiting low density lipoprotein (LDL) cholesterol synthesis, and increasing LDL particle clearance.10,11 In addition, statins appear to have cholesterol independent effects on the cardiovascular system: statins improve endothelial function, stabilize fibrous plaques, and decrease vascular inflammation.12–23 Statins reduce plasma levels of C-reactive protein (CRP), a biomarker of the acute phase inflammatory response that correlates with cardiovascular disease risk.24,25 Statins diminish the expression of major histocompatibility (MHC) class II on T-cells, block leukocyte function antigen-1 (LFA-1) mediated adhesion and costimulation of lymphocytes, and regulate T-cell differentiation.26–29 Statins inhibit interactions between leukocytes and endothelial cells and also decrease the number of inflammatory cells within atherosclerotic plaques.30,31 Statins decrease myocardial infarct size in part by inhibition of leukocyte trafficking into postischemic myocardium.32–34 However, the molecular basis for the antiinflammatory properties of statins is not completely understood.

Statins may inhibit vascular inflammation by interfering with leukocyte trafficking. The initial stages of vascular inflammation are characterized by leukocyte rolling along endothelial cells.35–37 Leukocyte rolling is mediated by selectins and their glycoprotein ligands expressed on the surface of leukocytes and endothelial cells. P-selectin is normally contained within endothelial cells inside granules called Weibel-Palade bodies, but inflammatory agonists trigger endothelial exocytosis of these granules, translocating P-selectin to the external surface of endothelial cells, where it can trigger leukocyte rolling.38–42 Exocytosis thus plays a critical role in the initial stages of vascular inflammation.

We hypothesized that statins inhibit vascular inflammation by regulating endothelial cell exocytosis. Exocytosis is a process mediated by N-ethylmaleimide sensitive factor (NSF) and soluble NSF attachment receptor proteins (SNAREs).43–45 We previously showed that nitric oxide (NO) inhibits NSF.46,47 Others have shown that statins increase eNOS expression and activity in
an Akt dependent manner.\textsuperscript{12,48,49} We now show that statins decrease endothelial exocytosis by increasing NO synthesis which inhibits NSF. These results may explain in part how statins protect blood vessels from inflammation.

**Materials and Methods**

**Materials**

Simvastatin was the kind gift of Merck, Inc. (Rahway, NJ). Simvastatin was activated before use by dissolving in ethanol (50 mg/mL), followed by the addition of 0.813 mL of 1 mol/L NaOH and correction to pH 7.2 by the addition of 1 mol/L HCl. N-nitro-l-arginine methylester (L-NAME), geranylgeranyl pyrophosphate (GGPP), farnesyl pyrophosphate (PPP), L-mevalonate, and thrombin were purchased from Sigma Chemicals (St. Louis, Mo). Mouse monoclonal antibodies to NSF, eNOS, and phospho-eNOS (S1177) were from BD Biosciences (Bedford, Mass). Goat polyclonal antibody to GAPDH (V-18) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif). Rabbit antibody to nitrosocysteine was from Calbiochem (San Diego, Calif).

**Cell Culture**

Human aortic endothelial cells (HAEC) and EGM-2 media were obtained from Clonetics (Walkersville, Md). HAEC were grown to confluence in EGM-2 media supplemented with growth factors and 2% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO\textsubscript{2}.

**Measurement of vWF Release**

HAEC were seeded into a 96-well plate and incubated for 24 hours. Cells were pretreated as described and then stimulated with thrombin for 1 hour. The amount of vWF released into the media was measured by an ELISA (American Diagnostica).

**Western Blotting**

Cells were seeded into a 6-well plate and incubated with simvastatin. Proteins were extracted in lysis buffer (10 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L sodium orthovanadate, and proteinase inhibitor cocktail). After centrifugation at 16 000 x g for 10 minutes to remove insoluble material, protein concentrations in the supernatant were determined using a Bio-Rad DC protein assay kit. Cell lysates were boiled for 5 minutes. Samples containing 20 μg of total protein were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, blocked with TBS with 0.1% Tween 20 (TBST) and 5% nonfat milk, and then immunoblotted with antibody. The proteins were detected by ECL Plus chemiluminescence kit (Amersham Biosciences).

**Determination of S-Nitrosylation of NSF**

Measurement of nitrosylated NSF in cultured cells was performed by immunoprecipitation with antibody to nitrosocysteine, followed by immunoblotting with antibody to NSF.

**NSF-SNARE Pull-down Assay**

The disassembly activity of NSF was measured by a coprecipitation assay as described previously.\textsuperscript{46} Recombinant RGS-(His)\textsubscript{6}-NSF (0.1 μg/μL) was pretreated with buffer or 1.0 mmol/L DEA or DEA- NONOate for 10 minutes at 22°C. Recombinant RGS-(His)\textsubscript{6}-α-SNAP (0.1 μg/μL), and SNAP polypeptides (0.1 μg/μL each of VAMP-3, SNAP-23, and GST-Syntaxin-4) were added, followed by either 5 mmol/L ATP/10 mmol/L MgCl\textsubscript{2} or 5 mmol/L ATP/s/10 mmol/L MgCl\textsubscript{2}. This mixture of NSF and SNAP polypeptides was then incubated in binding buffer (4 mmol/L HEPES pH 7.4, 0.1 mmol/L NaCl, 1 mmol/L EDTA, 3.5 mmol/L CaCl\textsubscript{2}, 3.5mmol/L MgCl\textsubscript{2}, and 0.5% Nonidet P-40) and glutathione-agarose beads for 1 hour at 4°C with rotation. The beads were washed with binding buffer 4 times, mixed with SDS-PAGE sample buffer, boiled for 3 minutes, and analyzed by immunoblotting.

**Measurement of Nitric Oxide Metabolites (Griess Reaction)**

Nitrite was measured by the Griess reaction adapted for a 96-well plate reader. Briefly, 100 μL of sample media was incubated with 100 μL of Griess reagent (2.7% sulfanilamide, 4% ortho-phosphoric acid and 0.1% N-(l-naphthyl)ethylenediamine) for 5 minutes in 96-well plates. Absorbance at 550 nm was measured using a plate reader. Nitrite concentrations were calculated by comparison with the absorbance of standard solutions of sodium nitrite.

**Animals**

All of the mice used in the present studies were C57BL/6J at 8 to 10 weeks of age obtained from the Jackson Laboratories (Bar Harbor, Me). In additional experiments, we used mice completely deficient in endothelial nitric oxide synthase (eNOS). These eNOS\textsuperscript{−/−} mice were originally donated from Dr. Paul Huang (Massachusetts General Hospital; Boston, Mass) and generated in our breeding colony at LSU-Health Sciences Center (Shreveport, La). eNOS\textsuperscript{−/−} mice were used at 8 to 10 weeks of age.

**Intravital Video Microscopy and Leukocyte Adhesion In Vivo**

Mice were anesthetized with ketamine/xylazine, and 0.05% rhodamine 6G (excitation: 528 nm; emission: 551 nm, Molecular Probes) was injected retro-orbitally and allowed to circulate for 15 minutes to stain leukocytes in vivo. The animals were then prepared for intravital microscopy with an externalized mesentery. One mesenteric venule (~100 to 200 μm in diameter) per animal was filmed for 15 minutes after a topical superfusion of 20 μL histamine 1 mmol/L to induce Weibel-Palade-body exocytosis.\textsuperscript{46} Leukocyte adherence was expressed as the number of adhering fluorescent cells per square millimeter of venular surface, normalized to the diameter and length of segment viewed.

**Myocardial Ischemia-Reperfusion (I/R) Protocol**

Mice were treated with PBS or with simvastatin, which was dissolved in PBS and administered via intraperitoneal injection at a final dose of 0.25 mg/kg 18 hours before the onset of myocardial ischemia. Surgical ligation of the left main coronary artery (LCA) was performed similar to methods described previously. Briefly, mice were anesthetized with intraperitoneal injections of ketamine (50 mg/kg) and pentobarbital sodium (50 mg/kg). The animals were then attached to a surgical board with their ventral side up. The mice were orally intubated with PE-90 polyethylene tubing connected to PE-240 tubing and then connected to a Model 683 rodent ventilator (Harvard Apparatus). The tidal volume was set at 2.2 milliliters and the respiratory rate was set at 122 breaths per minute. The mice were supplemented with 100% oxygen via the ventilator side port. A median sternotomy was performed using an electric cautery and the proximal left main coronary artery was visualized and completely ligated with 7 to 0 silk suture mounted on a tapered needle (BV-1, Ethicon). Coronary artery occlusion was maintained for 30 minutes followed by removal of suture, closure of the chest, and reperfusion of the ischemic myocardium for 24 hours.

**Myocardial Infarct Size Determination**

At 24 hours of reperfusion, the mice were anesthetized as described previously, intubated, and connected to a rodent ventilator.\textsuperscript{50} A catheter (PE-10 tubing) was placed in the common carotid artery to allow for Evans Blue dye injection. A median sternotomy was performed and the left main coronary artery was re-ligated in the same location as before. Evans Blue dye (1.2 mL of a 2.0% solution, Sigma) was injected into the carotid artery catheter into the heart to delineate the ischemic zone from the nonischemic zone. The heart was rapidly excised and serially sectioned along the long axis in five, 1 mm thick sections that were then incubated in 1.0% 2,3,5-triphenyltetrazolium chloride (Sigma) for 5 minutes at 37°C to demarcate the viable and nonviable myocardium within the risk zone. Each of the 5, 1 mm thick myocardial slices were weighed and the areas of...
infarction, risk, and nonischemic left ventricle were assessed by a blinded observer using computer-assisted planimetry (NIH Image 1.57). All of the procedures for the left ventricular area-at-risk and infarct size determination have been previously described.50

Immunohistochemistry of Murine Myocardial Infarction
Following the induction of myocardial ischemia and reperfusion, the heart specimens were fixed in acid methanol (10% glacial acetic acid; 60% methanol, and 30% water) and embedded in paraffin. Sections were reacted with polyclonal rabbit antibodies to human myeloperoxidase (Biomed, Foster City, Calif). The primary rabbit antibodies were localized with a biotin conjugated donkey antibody to rabbit IgG Biotin (Jackson ImmunoResearch Laboratories, West Grove, Pa) followed by Vectastain ABC Elite enhancement method (Vector Labs, Inc., Burlingame, Calif), and the staining was developed using diaminobenzidine with nickel. Hematoxylin (Richard Allen Scientific, Kalamazoo, Mich) was used as a counterstain. The area infiltrated and concentration of cells staining myeloperoxidase were scored in a masked fashion on a scale from 0 to 3, in which 0 represented minimal (0% to 10%), 1 represented minor (10% to 25%), 2 represented moderate (25% to 50%), and 3 represented major (>50%) involvement of the left ventricle free wall in neutrophil infiltration. The area of vWF expression was scored in a similar fashion.

Statistical Analysis
Results were expressed as mean±standard deviation (S.D.). Comparison between groups was performed by Student’s paired 2-tailed t test. Two-way ANOVA was used to describe differences between groups, with post hoc analysis performed by the methods of Student-Newman-Keuls. A value of P<0.05 was considered significant.

Results
Simvastatin Inhibits Endothelial Exocytosis of Weibel-Palade Bodies
To determine the effect of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) on endothelial cell exocytosis, we pretreated HAEC with activated simvastatin for 24 hours, then stimulated the cells with thrombin for 60 minutes, and measured the amount of vWF released into the media. Thrombin activated endothelial cell release of vWF (Figure 1A). However, simvastatin decreased vWF release in a dose-dependent manner, with an IC50 between 10 to 100 nmol/L (Figure 1A). The inhibitory effect of simvastatin began within 14 hours of treatment (Figure 1B).

NO Mediates Statin Inhibition of Endothelial Cell Exocytosis
We postulated that simvastatin decreases endothelial cell exocytosis by increasing endothelial NO production. To measure the effect of statins on eNOS expression, HAEC were pretreated with increasing amounts of activated simvastatin for 24 hours, and cell lysates were immunoblotted for eNOS. Simvastatin increased steady-state levels of eNOS in a dose-dependent manner (Figure 2A). Lysates were also blotted for eNOS phosphorylated on Ser1177. Simvastatin also increased steady-state levels of phosphorylated eNOS (Figure 2A). We then used the Griess assay to measure the amount of NO produced by endothelial cells. Simvastatin increased the amount of synthesized NO in a dose-responsive manner (Figure 2B).

Statins Increase Nitrosylation of NSF
We next determined whether or not NO mediates statin inhibition of endothelial cell exocytosis. We pretreated HAEC with activated simvastatin and L-NAME for 24 hours, and then stimulated the cells with thrombin and measured vWF release. Thrombin increased vWF release in control cells as expected (Figure 2C). Statin decreased thrombin stimulated release of vWF also as expected (Figure 2C). However, L-NAME blocked much of the inhibitory effects of statins on exocytosis (Figure 2C).

Statins Increase Nitrosylation of NSF
NO inhibits exocytosis by S-nitrosylation of NSF, which inhibits the ability of NSF to disassemble SNARE molecules and to drive fusion of the Weibel-Palade body membrane with the plasma membrane.46 Accordingly, we next explored the effect of statins on NSF nitrosylation. HAEC were treated with increasing concentrations of simvastatin, and cell lysates were immunoprecipitated with antibody to nitrosocysteine and immunoblotted with antibody to NSF. Simvastatin increased the nitrosylation of NSF in a dose-dependent manner (Figure 3A). Simvastatin increased NSF nitrosylation by activating endothelial NOS, since L-NAME inhibited simvastatin induced nitrosylation (Figure 3B). Nitrosylation of NSF blocked the ability of NSF to disassemble SNARE complexes in vitro (Figure 3C).

Statins Inhibit Exocytosis by Inhibition of HMG-CoA Reductase
To confirm that the effects of statins on exocytosis are mediated by inhibition of HMG-CoA reductase, we measured thrombin induced exocytosis in HAEC treated with simva-
Statins and L-mevalonate. Simvastatin blocked exocytosis, and mevalonate reverses this inhibition (Figure 4). However, mevalonate metabolites formed downstream of HMG-CoA reductase restored exocytosis (Figure 4).

**Statins Inhibit Leukocyte Rolling In Vivo**

We next explored the effect of statins on vascular inflammation in mice. Mice were pretreated with activated simvastatin 0.25 mg/kg or saline, and after 24 hours the mice were anesthetized and injected with rhodamine 6G to label leukocytes. The mesentery was externalized, superfused with 20 μL histamine 1 mmol/L, and rolling leukocytes were imaged with a fluorescent digital camera. Before histamine treatment, simvastatin decreased adherent leukocytes (Figure 5A and 5B). Histamine treatment increased leukocyte rolling (Figure 5B). However, simvastatin blunted histamine-induced leukocyte rolling (Figure 5B).

Figure 2. NOS and NO mediated effects of simvastatin on Weibel-Palade body exocytosis. A, HMG CoA-reductase inhibitor increased eNOS steady-state protein levels. HAEC were treated with activated simvastatin for 24 hours, and cell lysates were immunoblotted with (top) antibody to eNOS or (middle) antibody to eNOS phosphorylated on S1177 (eNOS-P). (This experiment was repeated twice with similar results.) B, HMG CoA-reductase inhibitor increased NO₂⁻ production. HAEC were treated with activated simvastatin for 24 hours, and media was analyzed for nitrite with the Griess reaction (n=3±SD. *P<0.01 vs 0 μmol/L). C, L-NAME decreased simvastatin inhibition of Weibel-Palade body exocytosis. HAEC were pretreated with 0.1 mmol/L L-NAME or 1.0 μmol/L simvastatin as above, stimulated with media or thrombin, and the amount of vWF released into the media was measured by ELISA. (n=3±SD. *P<0.05)

Figure 3. Simvastatin increased S-nitrosylation of NSF. A, Simvastatin increased S-nitrosylation of NSF. HAEC were treated with activated simvastatin for 24 hours, and cell lysates were immunoprecipitated with antibody to nitrosocysteine, followed by immunoblotting with antibody to NSF (upper). To confirm that simvastatin did not change NSF expression, total cell lysates were also immunoblotted with antibody to NSF (lower). This experiment was repeated twice with similar results. B, Statins increased NSF nitrosylation by activating NOS. HAEC were treated with 1 μmol/L activated simvastatin for 24 hours with 0, 0.1, or 1.0 mmol/L L-NAME; and cell lysates were immunoprecipitated with antibody to nitrosocysteine, followed by immunoblotting with antibody to NSF (top). To confirm that simvastatin did not change NSF expression, total cell lysates were also immunoblotted with antibody to NSF (lower). This experiment was repeated twice with similar results. C, NO inhibited NSF disassembly activity. Recombinant NSF was pre-treated or not with DEA-NONOate, and then mixed with α-SNAP, GST-syntaxin-4, VAMP-3, and SNAP-23. ATP or ATP-γ-S was added. The mixture was precipitated with glutathione-sepharose beads, and precipitants were immunoblotted with antibody to NSF (top) or syntaxin-4 (bottom).

Figure 4. Simvastatin inhibited Weibel-Palade body exocytosis by inhibition of HMG-CoA reductase. HAEC were treated with activated simvastatin for 24 hours, and mevalonate (MVA) or geranylgeranyl-pyrophosphate (GGPP) or farnesylpyrophosphate (FPP) were added to some cells. HAEC were then stimulated with media or thrombin, and the amount of vWF released into the media was measured by ELISA. (n=3±SD. *P<0.05 vs thrombin+MVA, GGPP, or FFP.)
We then confirmed that simvastatin affects NSF nitrosylation in vivo. Mice were treated or not with simvastatin as above, spleen lysates were immunoprecipitated with antibody to nitrosocysteine, and precipitants were immunoblotted with antibody to NSF. Simvastatin increased S-nitrosylation of NSF (Figure 5C, left). To confirm that eNOS mediated the effects of simvastatin by producing NO which led to S-nitrosylation of NSF, we added vehicle or simvastatin to eNOS knockout mice, and then repeated the assay for nitrosylated NSF. Mice lacking eNOS did not nitrosylate NSF at rest, and simvastatin did not increase levels of nitrosylated NSF (Figure 5C, right).

**Statins Inhibit Myocardial Infarction Size**

We next explored the effect of statins on myocardial infarction in mice. Mice were pretreated with activated simvastatin 0.25 mg/kg or saline, and after 18 hours the left main coronary artery was surgically ligated for 30 minutes, at which time reperfusion was initiated by release of the occlusion. After 24 hours the mice were euthanized and their hearts were analyzed for infarct size and neutrophil infiltration.

Simvastatin decreased the size of the myocardial infarction (Figure 6A). Simvastatin decreased infarct size measured as infarct size (INF) relative to the entire left ventricle (LV) or INF relative to the area at risk (AAR). Simvastatin did not affect the area at risk (AAR/LV), only the infarct size. To investigate the potential role of NO in the protective effects of simvastatin, we subjected eNOS−/− mice to the myocardial ischemia-reperfusion protocol as above. Simvastatin did not affect myocardial infarct size in eNOS−/− mice (Figure 6B).

**Statins Inhibit Exocytosis and Neutrophil Infiltration In Murine Myocardial Infarction**

We next explored the mechanism by which statins decrease myocardial infarction in mice. Because neutrophils mediate early reperfusion injury, we reasoned that statins would
decrease neutrophil infiltration. We therefore stained sections from treated and nontreated mice with antibodies to the neutrophil myeloperoxidase (MPO), and counterstained the sections with hematoxylin. A pathologist graded the neutrophil infiltration in a masked fashion (Methods). Simvastatin decreased neutrophil infiltration into infarcted myocardium (Figure 7A and 7B). However, when we repeated these experiments using eNOS knockout mice, simvastatin had no effect on neutrophil infiltration (Figure 7C).

We hypothesized that statins decrease endothelial exocytosis and the release of vWF in vivo. To test this hypothesis, we stained murine hearts with myocardial infarction with antibody to vWF. Simvastatin decreased vWF staining in murine hearts after myocardial infarction (Figure 7D and 7E). However, simvastatin had no effect on vWF staining in the hearts of eNOS knockout mice after myocardial infarction (Figure 7F).

Discussion

The major finding of this study is simvastatin inhibited endothelial exocytosis by increasing synthesis of NO which S-nitrosylated NSF. Simvastatin decreased neutrophil infiltration into myocardial infarct and decreased the extent of myocardial infarcts in mice. However, simvastatin failed to decrease inflammation in myocardial infarcts of eNOS knockout mice. These data imply that statins decreased vascular inflammation through a NO dependent mechanism.

Statin Inhibition of Neutrophil Infiltration

Our data suggest a novel mechanism by which statins regulate vascular inflammation. Simvastatin increased NO production (Figure 2) which covalently modifies NSF (Figure 3), a key regulator of endothelial exocytosis. Nitrosylation of NSF in turn blocked externalization of P-selectin to the endothelial surface, which activates leukocyte rolling, the first step in leukocyte inflammation. Our data show that simvastatin blocked neutrophil rolling along murine venules (Figure 6). Finally, simvastatin decreased neutrophil infiltration into infarcted myocardium; in contrast, lack of eNOS abrogated the protective effects of simvastatin (Figure 7A through 7C). Taken together, these findings demonstrate that statins decrease vascular inflammation and myocardial necrosis through a cholesterol-independent effect.

Antiinflammatory Effects of Statins

Our work complements the studies of others showing that statins can inhibit leukocyte trafficking and inflammation. We observed that statins inhibited P-selectin externalization, which mediates leukocyte rolling, the first step in leukocyte trafficking. Statins can bind to LFA-1, inducing an allosteric effect that blocks the interaction of LFA-1 with intercellular adhesion molecule-1 (ICAM-1). The interaction between LFA-1 and ICAM-1 mediates leukocyte adherence, the second step in leukocyte trafficking. Thus statins can selectively target individual steps in leukocyte trafficking, thereby inhibiting vascular inflammation.

NO and eNOS Mediate the Antiinflammatory Effects of Simvastatin

Our pharmacological and genetic data suggest that the anti-inflammatory effects of simvastatin are mediated by NO
produced by eNOS. The NOS inhibitor L-NAME blocked simvastatin inhibition of endothelial exocytosis (Figure 2C). L-NAME also decreased simvastatin-induced S-nitrosylation of NSF (Figure 3B). Mice lacking eNOS failed to show S-nitrosylation of NSF, and simvastatin failed to increase S-nitrosylated NSF (Figure 5C). In contrast to its effects in wild-type mice, simvastatin did not decrease exocytosis (Figure 7F), neutrophil infiltration (Figure 7C), and infarct size (Figure 6B) in eNOS knockout mice. These data illustrate 2 points: NO derived from eNOS S-nitrosylates NSF in vivo; and the effect of simvastatin is mediated by an eNOS dependent pathway.

**Beneficial Effects of Statins in Clinical Trials**

Recent clinical trials show that statins markedly decrease ischemia, recurrent myocardial infarctions, and mortality following an acute coronary event. The benefit of statins in some clinical trials is seen remarkably early, suggesting that a cholesterol independent effect of statins may contribute to the vascular protective effects of statins. Part of the beneficial effects of statins is because of the reduction in cholesterol levels. However, our data suggest that statins may reduce ischemia and infarction by blocking endothelial release of mediators that promote vascular inflammation, which in turn may lead to plaque instability and myocardial infarction. These findings suggest that endothelial exocytosis is a potential therapeutic target. Novel drugs that inhibit endothelial exocytosis may be useful in the treatment of cardiovascular diseases in which inflammation and thrombosis play a prominent role.52,53

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In an article published by Yamakuchi et al (Circ Res, 2005:96:1185–1192.), Figure 5 contained an error in Panel B. The corrected figure is below:

**Figure 5.** Simvastatin inhibited leukocyte rolling in vivo. A, Simvastatin decreased leukocyte adhesion to murine venules. Mice were treated with vehicle (PBS) or simvastatin (1 mg/kg) IP, and after 24 hours the mice were anesthetized and injected with rhodamine 6G to label leukocytes. The mesentery was exteriorized, superfused with 20 μL histamine 1 mmol/L, and rolling leukocytes were imaged with a fluorescent digital camera. B, Quantification of simvastatin inhibition of leukocyte adhesion in mice (n=3 to 5±SEM. *P<0.04 vs vehicle). C, Simvastatin increased S-nitrosylation of NSF in vivo. Wild-type or eNOS knockout mice were treated with simvastatin for 24 hours, and spleen lysates were immunoprecipitated with antibody to nitrosocysteine. Precipitants were fractionated and immunoblotted with antibody to NSF (upper) to detect nitrosylated NSF (NSF-NO). To confirm that simvastatin did not change total NSF expression, total cell lysates were also immunoblotted with antibody to NSF (lower).