Appl Biochem Biotechnol (2009) 157:545–553
DOI 10.1007/s12010-008-8263-7

HMG-CoA Reductase Inhibitor Regulates Endothelial Progenitor Function Through the Phosphatidylinositol 3′-Kinase/AKT Signal Transduction Pathway

Xiaohong Li · Biao Xu

Received: 24 March 2008 / Accepted: 24 April 2008 /
Published online: 18 June 2008

Abstract HMG-CoA reductase inhibitor (statins) are known to have pleiotropic effects. We examined the effect and mechanism of simvastatin on peripheral endothelial progenitor cells (EPCs). Rats were divided into simvastatin group and the control group after cardiac infarction operation. Simvastatin treatment significantly increased the number of peripheral blood CD34+ CD133+ cells, and serum concentration of vascular endothelial growth factor (VEGF) and AKT was markedly increased in vivo. In cultured EPC, simvastatin increased the concentrations of VEGF, AKT and eNOS. Western blots analysis showed that simvastatin increased the phosphorylation of eNOS and FKHRL1, which can be blocked by the PI3K/AKT pathway blocker LY294002 . Our study demonstrated that simvastatin increases the mobilization of EPCs after cardiac infarction. In in vitro study, simvastatin increases the phosphorylation of eNOS and of FKHRL1 through the PI3K/AKT signaling pathway.

Keywords Simvastatin · Endothelial progenitor cells · VEGF · AKT · NOS

Introduction

Numerous studies have demonstrated that circulating endothelial progenitor cells (EPCs) derived from bone marrow play roles in atherosclerosis, angiogenesis, and the response to vascular injury. Therefore, the stimulation of mobilization and/or differentiation of EPCs may provide a useful novel therapeutic strategy to improve postnatal angiogenesis.

X. Li · B. Xu
Department of Cardiology, Clinical Drum Tower Hospital, Nanjing Medical University,
Nanjing 210008, China

X. Li · B. Xu (✉)
Department of Cardiology, Nanjing Drum Tower Hospital, Nanjing University Medical School,
Nanjing 210008, China
e-mail: biaoxu2000@126.com

However, the mechanisms, which regulate EPCs mobilization and differentiation, are not fully elucidated.

HMG-CoA reductase inhibitors (statins) are widely prescribed to lower cholesterol in hyperlipidemic patients. Many clinical studies have confirmed that statins can decrease cardiovascular events and reduce morbidity and mortality in patients with coronary diseases [1–5]. Besides lipid lowering, statins are capable of reducing vascular inflammation, decreasing platelet aggregation and thrombus deposition, and increasing endothelium-derived nitric oxide production [6–9]. In recent years, statins have been reported to promote EPCs proliferation, migration and cell survival in vitro, and promote the restoration of epithelial cells and the genesis of new vessels [10, 11]. The mechanisms underlying the pleiotropic effects of statins are not completely understood. To gain insights into the mechanism, we investigated the effects of statins on circulating EPCs and elucidated the underlying signal transduction pathways.

Materials and Methods

Animals and Experimental Protocol

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Institutional Review Board for Animal Studies of Nanjing Drum Tower Hospital.

Healthy male Wistar rats weighing 200–250 g were provided by Shanghai Experimental Animal Center. Coronary artery ligation was performed as described previously [12–14]. In brief, rats were anesthetized by ketamine (0.3 mg/kg), intubated, and ventilated by a volume-constant rodent ventilator (KN-55 Natsume Seisakusho, Tokyo, Japan). The chest was opened from the fourth intercostal space on the left, and the left coronary artery was ligated using a 5.0 suture between the pulmonary artery outflow tract and left atrium. The heart was then returned to its normal position and the thorax was closed. After the cardiac infarction model was established, the rats were divided into two groups (n=10/group): treated group was administered 40 mg/kg simvastatin once daily; control group were gavaged with normal saline. The blood samples were taken in heparinized tubes from the tail vein before and 1, 3, 5, 7, 11, 15, and 20 days after the operation. Flow cytometric analysis was performed with 100 μl of whole blood to quantify the content of circulating EPCs, and the rest of the blood was centrifuged (1,000 rpm, 10 min) to separate the serum. It was then preserved in a −80 °C freezer for later detection of vascular endothelial growth factor (VEGF) and AKT by enzyme-linked immunosorbent assay (ELISA) according to the manual.

Evaluation of Circulating EPC Kinetics After Cardiac Infarction

EPCs were defined by expression of the two stem-cell-related surface antigens, CD34 and CD133. A volume of 100 μl peripheral blood was incubated for 15 min with 2 ml of 1X ammonium chloride lysing solution (Becton Dickinson, Heidelberg, Germany). After centrifugation at 500×g for 5 min, the cells were resuspended in 100 μl buffer and incubated for 30 min in the dark with fluorescein-isothiocyanate-labeled anti-rat CD34 monoclonal antibody (eBioscience) and monoclonal antibodies against rat-CD133 (eBioscience). Isotype-identical antibodies served as controls. After incubation, cells were

washed with 2 ml washing solution, centrifuged at 500×*g* for 5 min, and resuspended in 500 μl washing solution. Cells were analyzed by immunofluorescence flow cytometry (FACSCalibur, Becton Dickinson) and Cell Quest Software counting 100,000 events per sample. We obtained the number of EPCs per 100,000 lymphocytes (EPC/lymphocytes) and, after adjustment for the number of leukocytes in peripheral blood and the fraction of lymphocytes/leukocytes, the number of EPCs per 100 μl blood (EPC/blood).

Plasma Levels of VEGF and AKT

Plasma level of VEGF and AKT was measured by ELISA using a commercially available kit (rat Quantikine ELISA kit, R&D Systems) according to the manufacturer's instructions.

EPCs Culture Assay

EPCs were collected from the femurs of 2-week-old male Wistar rats as described [15]. The mononuclear cell fraction was obtained by density gradient centrifugation with Biocoll separating solution (density 1.077, Biochrom AG, Berlin, Germany) after centrifugation at 2,000 rpm for 20 min. The mononuclear cell fraction was carded, washed, and centrifuged at 800 rpm for 10 min. The cell pellet was inoculated into a culture bottle coated with 10 g/l gelatin at a concentration of 5×10^8 cells/l then suspended in endothelial basal medium (EBM-2®; Clonetics, Cambrex), and supplemented with 5% fetal bovine serum, 50 ng/ml VEGF (R&D Systems), 50 ng/ml human fibroblast growth factor 2 (FGF-2, R&D Systems), human epidermal growth factor, insulin-like growth factor 1 (IGF-1), and ascorbic acid. The cells were cultured at 37 °C and 5% CO_2 under saturation humidity. After 4 days in culture, non-adherent cells were removed by washing with phosphate-buffered saline (PBS), new media was applied, and the culture was maintained through day 7. Attached cells were collected and randomized into three groups: (1) the control group, with medium containing 5% calf serum; (2) simvastatin groups (activated simvastatin was added to make a series of final concentration: 0.001, 0.01, 0.1, and 1 μmol/l for the respective time points); (3) simvastatin + LY94002 groups [LY94002 (10 μmol/l)was added to the medium and incubated for 1 h, then simvastatin at a series of concentration (0.001, 0.01, 0.1, and 1 μmol/l) was added]. The cells were collected after 24 h.

Western Blot Analysis

The cells were washed and suspended in lysis buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1.7 ng/ml aprotinin, 50 μg/ml leupeptin, 2 mM sodium orthovanadate, and 20 mM sodium fluoride. After 20 min of incubation on ice, insoluble materials were removed by centrifugation at 15,000×*g* for 20 min. The protein concentrations were determined using the Bradford method. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was used to separate the proteins, which were then transferred to a nitrocellulose filter at 30 V, 4 °C. The blots were blocked with 5% skim milk in Tris-buffered saline for 1 h at room temperature and then incubated with the appropriate amount of primary antibody (anti-rat FKHRL antibody, diluted by blocking solution to 1:500, or anti-rat NOS antibody, diluted with blocking solution to 1:200) overnight at 4 °C or for 1–2 h at room temperature. The membrane was rinsed four times for 10 min each with PBST. The membrane was then incubated, with shaking, with HRP-conjugated secondary antibody (HRP-goat anti-rabbit,

diluted with blocking buffer to 1:10,000) for 1 h and then thoroughly washed with PBST. Developer was then added (0.1 ml/cm^2) to the nitrocellulose filter and incubated at room temperature for 1 min. The membrane was quickly exposed to X-ray film in a darkroom, and the film was developed in a developing machine. The exposure time was adjusted to optimize band visibility.

Statistical Method

All results are expressed as mean±SD. Comparisons between two groups were performed by unpaired Student's *t* test, and comparisons of three or more groups were performed by analysis of variance with Dunnett's posttest. A value of $P<0.05$ (two-tailed) was taken to denote statistical significance. All analyses were performed using SPSS 13.0 software (SPSS Inc.).

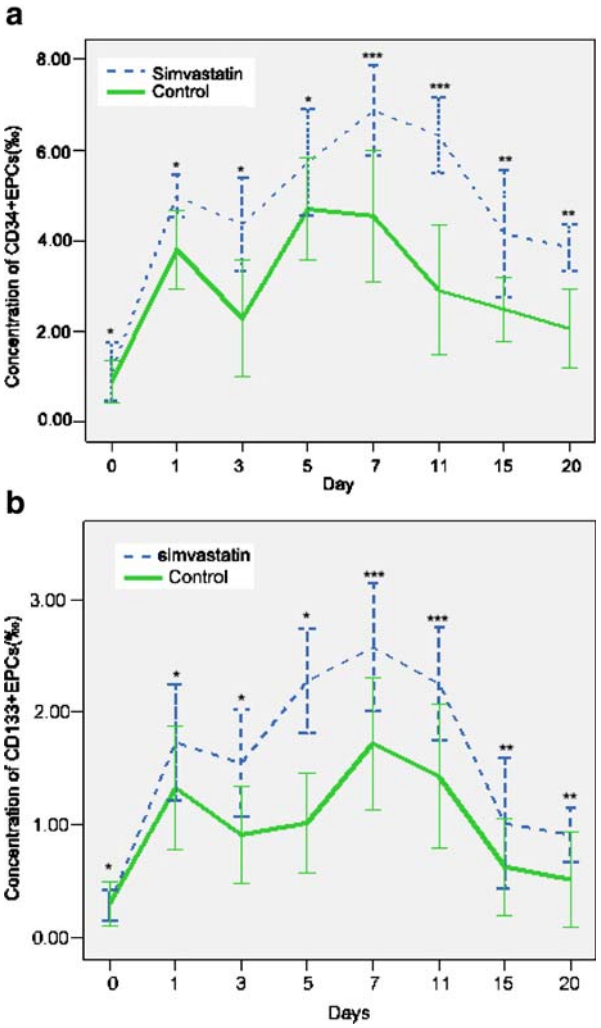

Fig. 1 Mobilization of EPCs after cardiac infarction determined by flow cytometry. Increase in circulating CD34+ EPCs (**A**) and CD133+ EPCs (**B**) after cardiac infarction. Values are expressed as mean±SE. *Real line* represents measurements of the control group, whereas *broken line* represents measurements of the simvastatin group.*$P>0.05$, **$P<0.05$,***$P<0.01$

Results

Mobilization of EPCs After Cardiac Infarction

It is notable that the cardiac infarction alone resulted in a significant increase in circulating EPCs in both groups, indicating that mobilization of these cells is a natural response to myocardial injury. Figure 1A and B shows the time course of circulating CD34+/CD133+ EPCs number. EPCs increased after the onset of cardiac infarction and peaked on day 7 then gradually decreased, but the number on day 20 was still greater than that on day 1. In controls, circulating EPCs on days 1 and 7 were similar and did not differ between the simvastatin and control groups, but the simvastatin group maintained a significantly higher number of circulating EPCs for 2 and 3 weeks after cardiac infarction than control groups (Fig. 1A and B).

Plasma Level of VEGF and AKT After Cardiac Infarction

The results of ELISA indicated that there was no difference in VEGF concentrations between the control group and the simvastatin group before operation. The cardiac infarction led to a significant time-dependent increase of VEGF level and peaked on day 7

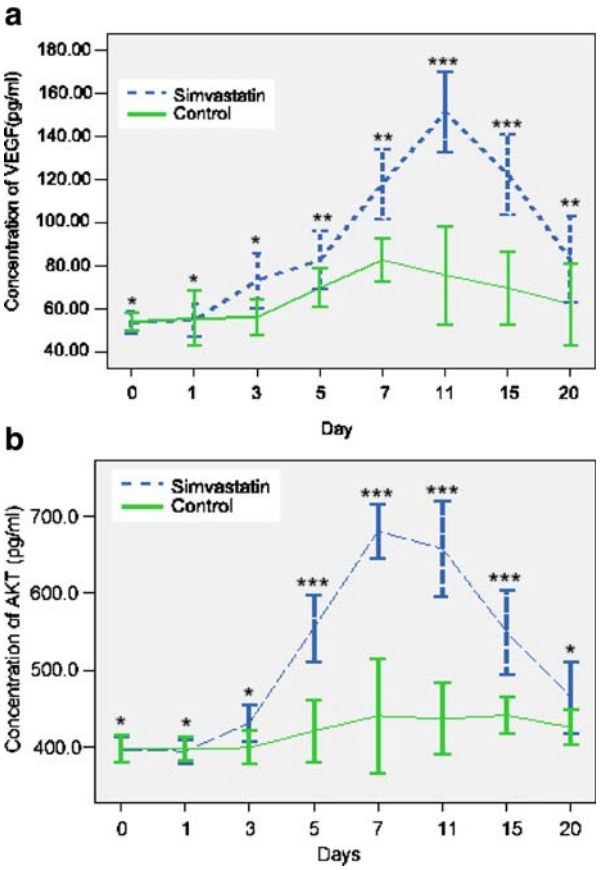

Fig. 2 Plasma level of VEGF and AKT after cardiac infarction detected by ELISA. Increase in plasma level of VEGF (**A**) and AKT (**B**) after cardiac infarction. Values are expressed as mean±SE. *Real line* represents measurements of the control group, whereas *broken line* represents measurements of the simvastatin group. **P*>0.05, ***P*<0.05,****P*<0.01

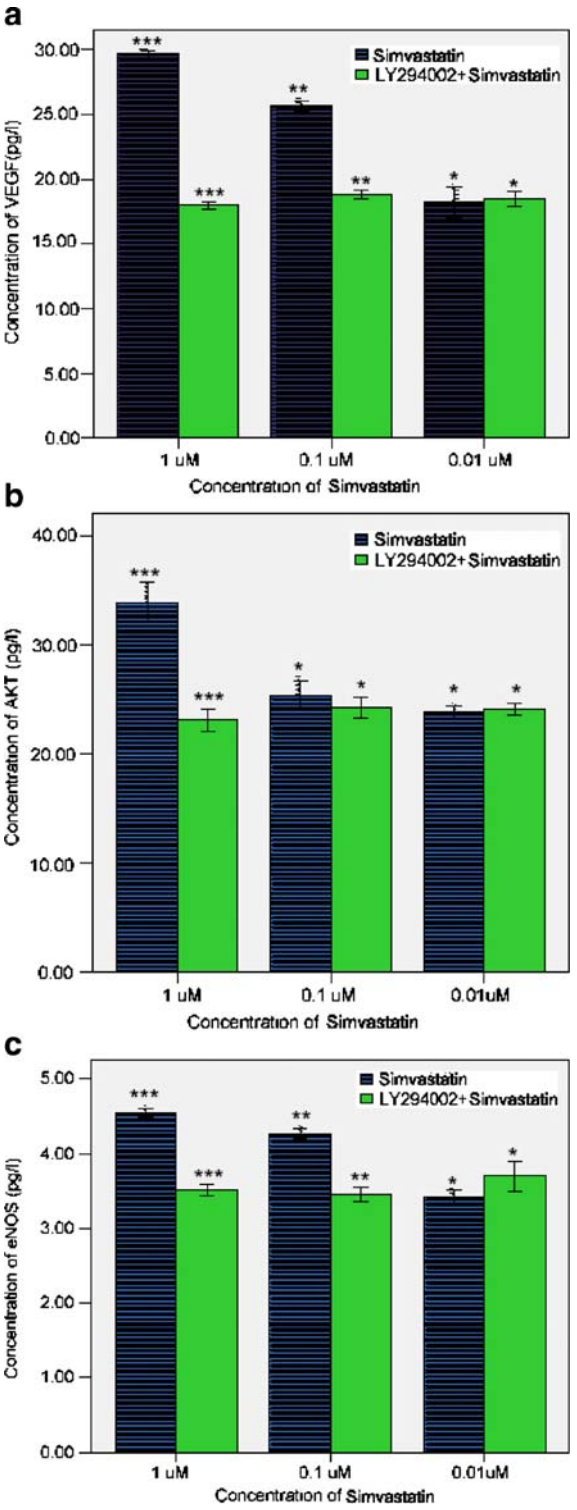

Fig. 3 Effects of simvastatin on function of EPCs detected by ELISA. EPCs were incubated with simvastatin (0.01, 0.1, and 1 μmol/l) in the presence of absence of LY294002 (10 μmol/l). Data are mean±SE. VEGF levels (**A**) in conditioned medium were detected with a VEGF ELISA kit (Quantikine, VEGF immunoassay, R&D Systems). AKT level (**B**) and eNOS level (**C**)in cell lysate were detected with available kit (Quantikine ELISA kit, R&D Systems). **P*>0.05, ***P*<0.05, ****P*<0.01

then gradually decreased. From day 7 to day 20, VEGF levels in the simvastatin groups were significantly higher than that of the control group (Fig. 2A).

There was no significant change in plasma AKT level in the control group before and after operation. From day 7 to day 20 after operation, plasma AKT level in the simvastatin groups were significantly higher than that of the control group (Fig. 2B).

Characterization of EPCs

Attached cells could be observed 4 days after cultured, and cells changed to a fusiform shape 7 days later. EPCs were characterized as adherent cell positive for CD34 (27±12%) and CD133 (31±10.6%) by flow cytometry analysis.

The Effects of Simvastatin on Function of EPC is Mediated Via the PI3-Kinase/Akt Pathway

In cultured EPCs, simvastatin led to a dose-dependent increase in the level of VEGF, AKT, and eNOS; the maximal effect was obtained at 1 μmol/l (Fig. 3A–C). Western blot analysis demonstrated that simvastatin dose-dependently increase the expression of eNOS and phosphorylation of FKHRL1 with maximum effects at μmol/l. Simvastatin induced expression of eNOS, and phosphorylation of FKHRL1 was blocked by the PI3K inhibitor LY 294002 (Fig. 4).

Discussion

The data of the present study establish a novel mechanism of action of statins. Simvastatin could augment EPCs number after cardiac infarction in rats, and the plasma level of VEGF and AKT were also increased. In cultured EPCs, simvastatin increased the concentrations of VEGF, AKT, and eNOS. Western blotting analysis showed that simvastatin phosphorylated eNOS and FKHR, which can be blocked by the PI3K/AKT pathway blocker LY294002, suggesting that simvastatin influenced the functions of EPCs through the PI3K/AKT pathway.

The clinical benefit of statins therapy is primarily attributed to its low-density-lipoprotein-lowering and potentially high-density-lipoprotein-elevating effects. However ,a subgroup analysis of large clinical trials indicates that statins-treated individuals have significantly less cardiovascular diseases than patients with comparable serum cholesterol

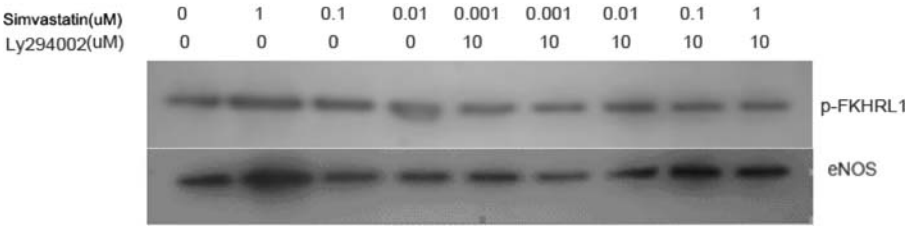

Fig. 4 Effect of simvastatin on phosphorylation of eNOS and FKHRL1 in cultured EPCs. Western blot analysis with the antibodies against phospho-FKHR(Thr-24) and anti-eNOS antibody. Simvastatin induces dose-dependent effects on phosphorylation of eNOS and FKHRL1 and reverse of simvastatin-induced phosphorylation by LY294002

levels [14, 15]. Subsequently, experimental data have shown that statins exhibit pleiotropic effects that can beneficially impact occlusive vascular diseases, including inhibition of smooth muscle proliferation and platelet aggregation, enhancement of endothelial function, and anti-inflammatory actions. Moreover, statins have recently been shown to promote neovascularization and re-endothelialization, which have been documented at least in part from the contribution of EPCs [16–17].

Circulating bone-marrow-derived EPCs importantly contribute to adult neovascularization. EPCs also improve vascularization after myocardial infarction. Clinical studies demonstrated that the number and function of EPCs are adversely affected by classical risk factors for coronary artery disease (CAD) [18–20]. The mechanisms underlying the reduced number of EPCs in patients with CAD are unknown. One conceivable mechanism limiting the number of EPCs might be reduced survival due to the induction of apoptosis. VEGF increases the number of EPCs both in vitro and in vivo. Likewise, statins enhance the number of EPCs in patients with stable CAD. The increase in EPCs number by statins is at least equipotet compared with VEGF [18–20]. VEGF and statins appear to share a common PI3K/Akt signaling pathway to regulate EPCs differentiation levels.

The serine–threonine protein kinase Akt, which is a downstream effector of PI 3′-kinase, is well established to play an important role in endothelial cell biology and angiogenesis by activating anti-apoptotic, pro-survival signaling cascades [21–23]. Akt-dependent phosphorylation leads to the posttranscriptional activation of the endothelial NO synthase via phosphorylation of the amino acid Ser 1177, which has been described as an important anti-apoptotic signaling pathway in endothelial cells [24]. In addition, Akt regulates the activity of a variety of other targets, including the pro-apoptotic protein Bad, caspase-9, and members of the forkhead transcription factor (FoxO) family such as FoxO1 (FKHR), FoxO3a (FKHRL1), and FoxO4 (AFX) [25–26].

The FoxO family is a transcription factor family whose downstream target genes are related to the cell cycle, cell apoptosis, aging, and metabolism. In mammals, activated AKT phosphorylates FoxO protein and decreases their transcriptional activity. However, the effect of the FoxO family on angiogenesis has not been explained clearly. FKHR and FKHRL1 are the major isoforms expressed on the mature epithelial cells. It has been found that in the FKHR and FKHRL1 knocked out epithelial cells, the activity of eNOS is significantly improved, suggesting that eNOS is the target gene of FKHR and FKHRL1.

Taken together, our study demonstrates that simvastatin improved the mobilization of EPCs after cardiac infarction and identified Akt as a target for statins to modify EPCs kinetics. Simvastatin induce Akt-dependent phosphorylation of the forkhead factor FKHRL1, which could be crucial steps in the anti-apoptotic effect. Akt-dependent phosphorylation leads to inhibition of forkhead transcription factor activity and prevents apoptosis signaling .Thus, the downstream pathways and processes following from statin-induced Akt phosphorylation in endothelial cells needed further study.

Reference

1. Laufs, U., La Fata, V., & Plutzky, J. (1998). *Circulation*, *97*, 1129–1135.
2. Wassmann, S., Laufs, U., & Baumer, A. T. (2001). *Hypertension*, *37*, 1450–1457.
3. Martı'nez-Gonza'lez, J., Raposo, B., & Rodriguez, C. (2001). *Arteriosclerosis, Thrombosis, and Vascular Biology*, *21*, 804–809.
4. del Peso, L., Gonzalez-Garcia, M., & Page, C. (1997). *Science*, *278*, 687–689.
5. Vasa, M., Fichtlscherer, S., & Adler, K. (2001). *Circulation*, *103*, 2885–2890.
6. Morales-Ruiz, M., Fulton, D., & Sowa, G. (2000). *Circulation Research*, *86*, 892–896.

7. Llevadot, J., Murasawa, S., & Kureishi, Y. (2001). *Journal of Clinical Investigation*, *108*, 399–405.
8. Dimmeler, S., Aicher, A., & Vasa, M. (2001). *Journal of Clinical Investigation*, *108*, 391–397.
9. Gerber, H. P., McMurtrey, A., & Kowalski, J. (1998). *Journal of Biological Chemistry*, *273*, 30336–30343.
10. Dimmeler, S., Assmus, B., & Hermann, C. (1998). *Circulation Research*, *83*, 334–342.
11. Dimmeler, S., Fleming, I., & Fisslthaler, B. (1999). *Nature*, *399*, 601–605.
12. Fulton, D., Gratton, J. P., & McCabe, T. J. (1999). Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature*, *399*, 597–601.
13. Dimmeler, S., Dernbach, E., & Zeiher, A. M. (2000). *FEBS Letters*, *477*, 258–262.
14. Sacks, F. M., Pfeffer, M. A., & Moye, L. A. (1996). *New England Journal of Medicine*, *335*, 1001–1009.
15. Massy, Z. A., Keene, W. F., & Kasiske, B. L. (1996). Inhibition of the mevalonate pathway: Benefits beyond cholesterol reduction. *Lancet*, *347*, 102–103.
16. Walter, D. H., Rittig, K., & Bahlmann, F. H. (2002). *Circulation*, *105*, 3017–3024.
17. Llevadot, J., Murasawa, K., & Isner, J. M. (2001). *Journal of Clinical Investigation*, *108*, 3999–405.
18. Schatteman, G. C., Hanlon, H. D., Jiao, C., & Dodds, S. G. (2000). *Journal of Clinical Investigation*, *106*, 571–578.
19. Vasa, M., Fichtlscherer, S., & Aicher, A. (2001). *Circulation Research*, *89*, E1–E7.
20. Tepper, O. M., Galiano, R. D., & Capla, J. M. (2002). *Circulation*, *106*, 2781–2786.
21. Asahara, T., Takahashi, T., & Masuda, H. (1999). *EMBO Journal*, *18*, 3964–3972.
22. Kalka, C., Masuda, H., & Takahashi, T. (2000). *Circulation Research*, *86*, 1198–1202.
23. Lefer, A. M., Campbell, B., & Shin, Y. K. (1999). *Circulation*, *100*, 178–184.
24. Kureishi, Y., Luo, Z., & Shiojima, I. (2000). *Natural Medicines*, *6*, 1004–1010.
25. Urbich, C., Dernbach, E., & Zeiher, A. M. (2002). *Circulation Research*, *90*, 737–744.
26. Dimmeler, S., Fisslthaler, B., & Fleming, I. (1999). *Nature*, *399*, 601–605.