Statins restore ischemic limb blood flow in diabetic microangiopathy via eNOS/NO upregulation but not via PDGF-BB expression

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1 Division of Pathophysiological and Experimental Pathology, Department of Pathology, Graduate School of Medical Science, Kyushu University, Fukuoka; 2 Department of Gene Therapy, Graduate School of Medicine, Chiba University, Chiba; and 3 Department of General Surgical Science, Graduate School of Medicine, Gunma University, Gunma, Japan

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Fujii T, Onimaru M, Yonemitsu Y, Kuwano H, Sueishi K. Statins restore ischemic limb blood flow in diabetic microangiopathy via eNOS/NO upregulation but not via PDGF-BB expression. Am J Physiol Heart Circ Physiol 2008;294:H2785–H2791. First published April 25, 2008; doi:10.1152/ajpheart.00149.2008. — 3-Hydroxy-3-methyl-glutaryl CoA reductase inhibitors or statins are potent inhibitors of cholesterol biosynthesis that have become more widely used in greater numbers of patients with hypercholesterolemia (13, 31). Furthermore, recent studies suggest that statins have pleiotropic effects in a manner independent of their lipid-lowering effect and can protect the vasculature in a manner independent of their lipid-lowering effect. The effectiveness of statins in reducing the risk of coronary events has been shown even in patients with diabetes, and their effects on diabetic complications have been reported. Using a model of severe hindlimb ischemia in streptozotocin-induced diabetic mice (STZ-DM), we investigated the effects and mechanisms of statin therapy in diabetic angiopathy in ischemic hindlimbs. As a result, STZ-DM mice frequently lost their hindlimbs after induced ischemia, whereas non-DM mice did not. Supplementation with statins significantly prevented autoamputation. We previously showed that diabetic vascular complications are caused by impaired expression of PDGF-BB, but statin therapy did not enhance PDGF-BB expression. Statins helped enhance endogenous endothelial nitric oxide (NO) synthase (eNOS) expression. Furthermore, the inhibition of NO synthesis by the administration of Nω-nitro-L-arginine methyl ester impaired the ability of statins to prevent STZ-DM mouse limb autoamputation, indicating that the therapeutic effect of statins in hindlimb ischemia in STZ-DM mice occurs via the eNOS/NO pathway. A combination therapy of statins and PDGF-BB gene supplementation was more effective for diabetic angiopathy than either therapy alone. In conclusion, these findings indicate that statin therapy would be useful for preventing intractable diabetic foot disease in patients with diabetic angiopathy.

Diabetes mellitus (DM) is characterized by a chronic state of hyperglycemia and is increasing to epidemic proportions throughout the world. The morbidity and mortality associated with diabetes is primarily due to macro- and microangiopathy occurring in multiple organs (2, 20). The diabetic foot is an intractable disease categorized by DM-related vascular complications, and patients with it have a much higher risk of gangrene and the need for consequent amputation of the lower extremities (20). Collateral vessel development is insufficient to compensate with the reduced blood flow through occluded arteries in patients with peripheral vascular disease, especially DM (10). Furthermore, surgical and catheter interventions are usually difficult to treat limb ischemia of DM patients with diabetes benefit more than patients without in both primary and secondary prevention (6).

Recent studies have also revealed the modulatory effects of statins in diabetic microangiopathy (7). The effectiveness of statins appears to involve restoring or improving endothelial function through the attenuation of high glucose-induced or diabetes-induced oxidative stress, thereby increasing the bioavailability of nitric oxide (NO) or inhibiting inflammatory responses (7).

On the other hand, using a model of severe hindlimb ischemia in streptozotocin-induced diabetic mice (STZ-DM), we previously showed that the diabetic foot is a disease involving the disturbance of PDGF-BB expression but not of the disturbance of the responses of angiogenic factors (32). Screening of angiogenesis-related factors revealed that the expression of PDGF-BB was impaired in STZ-DM mice at baseline as well as over a time course after limb ischemia. Increased expression of PDGF-BB prevented autoamputation in the STZ-DM mice (32).

In this study, we investigated the effect of and the mechanism underlying statin therapy in hindlimb ischemia under chronic hyperglycemia using STZ-DM. We here demonstrate that statins show significant therapeutic effects in hindlimb ischemia in STZ-DM mice via the endothelial NO synthase (eNOS)/NO pathway but not via PDGF-BB expression. Our results suggest that statin therapy would be useful for preventing intractable diabetic foot disease in patients with diabetic angiopathy.

Materials and Methods

Cells and reagents. Human umbilical vascular endothelial cells (HUVECs) were purchased from Kurabo, Tokyo, Japan. Two intracellular signal inhibitors were used as previously described (11, 12, 13, 31).

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was expressed as the ratio of the left (ischemic) to the right (nonischemic) limb blood flow.

Statistical analysis. All data except for those of limb survival were expressed as means ± SE and were analyzed by one-way ANOVA with Fisher adjustment. For the survival analysis, the survival rate expressed by the limb salvage score was analyzed using Kaplan-Meier’s method (11, 18, 23, 32, 33). The statistical significance of the survival experiments was determined using the log-rank test. \( P < 0.05 \) was considered statistically significant in all analyses.

RESULTS

Effects of statins on serum glucose, LDL cholesterol, and AGE in DM mice. Serum levels of glucose, LDL cholesterol (LDL), and AGE were measured in serum samples of a relevant model for Type 1 diabetes, STZ-DM mice, and a well-accepted model of Type 2 diabetes, namely ob/ob mice that are leptin-deficient C57BL/6 (4). Serum concentrations of glucose, LDL, and AGE were increased in the DM mice. Statin treatment did not affect the increases in glucose and AGE, whereas the increased LDL concentration was relatively decreased (Table 1). In the following experiments, STZ-DM mice were used after we confirmed a significant upregulation of the serum glucose.

Therapeutic effects of statins in hindlimb ischemia in STZ-DM mice. The STZ-DM mice frequently lost their hindlimbs at various levels after surgically induced severe limb ischemia, although the non-DM mice did not. Quantitative analysis of the degree of autoamputation using the limb salvage score (11, 18, 23, 32, 33) demonstrated impaired limb survival in the STZ-DM mice. Administration of statins (pravastatin and pitavastatin) by intraperitoneal injection daily on days 14 to 28 resulted in the significant prevention of autoamputation in the STZ-DM mice (Fig. 1), indicating that the statin therapy effectively restored tolerance against hindlimb ischemia in STZ-DM mice.

ENO/NO is important for the therapeutic effects of statins, but endogenous PDGF-BB expression is not. We previously showed that the diabetic foot is a disease involving the disturbance of PDGF-BB expression (32). To explain the mechanism underlying the therapeutic effect of statins on the tolerance of limb ischemia in STZ-DM mice, we measured the expression of murine (m)PDGF-BB protein in thigh muscles of STZ-DM. Downregulated expression of PDGF-BB was evident in both the STZ-DM and statin therapy did not influence the impaired expression of PDGF-BB (Fig. 2A). In addition, the expression of mPDGF-BB protein was not influenced by statin therapy.

### Table 1. Serum LDL cholesterol, AGE, and glucose concentration are significantly increased in C57BL/6J strain-based 10-week-old Type 1 (STZ-DM) and Type 2 (Lep\(^{ob}/\)Lep\(^{ob}\); ob/ob) mice

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<th>STZ-DM (Type1)</th>
<th>ob/ob (Type2)</th>
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<tbody>
<tr>
<td></td>
<td>Non-DM</td>
<td>Nontreatment</td>
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<tr>
<td>n</td>
<td>6</td>
<td>6</td>
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<tr>
<td>Glucose, mg/dl</td>
<td>113±4.2</td>
<td>621±6.6*</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dl</td>
<td>11.0±0.47</td>
<td>31.5±2.77*</td>
</tr>
<tr>
<td>AGE, µM</td>
<td>1.0±0.12</td>
<td>1.6±0.17*</td>
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Data are means ± SE; n, animals/group. These increased glucose and advanced glycation end product (AGE) levels were not influenced by treatment with statins, whereas the increased LDL concentration was relatively decreased by statins administration. STZ-DM, streptozotocin-induced diabetes mice; PRA, pravastatin, hydrophilic statin; PTA, pitavastatin, lipophilic statin; *\( P < 0.01 \) vs. non-DM.
even in ob/ob mice, a model of Type 2 diabetes. To confirm the modulatory effect of statins on PDGF-BB expression, we examined the induction of PDGF-BB via statins using cultured HUVECs. As shown in Fig. 2B, the statins did not stimulate the production or secretion of PDGF-BB in the culture medium of HUVECs. These results suggest that the effectiveness of statins in hindlimb ischemia in DM mice may be independent of the endogenous expression of PDGF-BB.

We previously reported that PDGF-B gene expression was downregulated in the limb muscles of STZ-DM mice among the factors tested (VEGF-A and -C, hepatocyte growth factor, FGF-2, PDGF-A and -B, and angiopoietin-1 and -2), as well as their receptors (tie-2, flk-1, fibroblast growth factor receptor 1, flt-4, and platelet-derived growth factor receptor-A and -B) (32). Recent important studies indicated that statins activated and upregulated the expression of endogenous eNOS, which has angiogenic potency (1, 21). We next investigated the expression of eNOS and whether or not eNOS/NO is important for the ability of statins to prevent the impairment of limb survival. Statin therapy significantly restored or increased the expression of eNOS in thigh muscles of both STZ-DM and ob/ob DM mice (Fig. 3A). As in previous studies (17, 19, 22, 24, 34), statin stimuli induced the upregulation of endogenous eNOS in the lysate of the cultured cells (HUVECs). Furthermore, the daily oral administration of Nω-nitro-L-arginine methyl ester, an inhibitor of NO synthesis, prevented the autoamputation rate for ischemic limb amputation of STZ-DM mice (Fig. 4), indicating that the therapeutic effect of statins on
tolerance against limb ischemia in the diabetic foot might occur at least partly via the eNOS/NO pathway.

Combination therapy of statins and PDGF-BB gene supplementation for diabetic angiopathy. These results may suggest that the therapeutic effect of statins occurs via eNOS/NO but not via PDGF-BB expression in diabetic vascular dysfunction. As a final assessment, to confirm whether or not statin therapy is independent of the endogenous expression of PDGF-BB, we administered a combination therapy of statins and PDGF-BB gene supplementation and found that this combination is more effective for diabetic angiopathy than either treatment alone. A supplementation study on the plasmid-based intramuscular gene transfer of human PDGF-B (pCEP4-hPDGFB) was previously described (32). In the present study we assessed the recovery of blood flow evaluated by LDPI, because the PDGF-B gene transfer resulted in the complete prevention of autoamputation in STZ-DM mice. As shown in Fig. 5, both statin therapy and the preinjection of pCEP4-hPDGFB significantly improved the disturbed blood perfusion in STZ-DM mice; furthermore, the combination therapy had an effect on
diabetic angiopathy that was superior to that by either therapy alone, indicating that the statin therapy might work independently from PDGF-BB expression and that the combination therapy was sufficient to restore the tolerance against hindlimb ischemia in STZ-DM mice.

**DISCUSSION**

The key observations made in this study are summarized as follows: 1) the administration of statin was effective for preventing autoamputation of the ischemic limb of STZ-DM mice; 2) statin therapy could not restore the disturbed expression of PDGF-BB in DM mice, suggesting that the effect of statins is independent of PDGF-BB expression; 3) statins could restore or increase eNOS expression in the limb of DM mice; 4) supplementation with the NO inhibitor inhibited the ability of statins to prevent autoamputation, indicating that the therapeutic effect of statins in hindlimb ischemia in STZ-DM mice might occur via the eNOS/NO pathway; and 5) the combination therapy of statins and PDGF-BB supplementation was more effective than either therapy alone. These findings suggest that the statin therapy is effective against the formation of hyperglycemia-related vascular complications, and they imply that statin therapy improves limb survival and perfusion via a mechanism involving eNOS/NO pathway in Type 1 DM, and probably in Type 2 DM microangiopathy.

Over the last several years, it has been already demonstrated that statin treatment improved and promoted angiogenesis in a hindlimb ischemia of non-DM mouse model (16, 26). In the case of DM mouse model, we previously demonstrated that the disturbed tolerance against severe limb ischemia under hyperglycemia was due to the disturbance of PDGF-BB expression and not to the angiogenic responses and that the supplementation of PDGF-B gene expression was sufficient to prevent autoamputation due to limb ischemia in STZ-DM mice (32). The reduction of PDGF-BB expression, which was not dependent on the level of hyperglycemia (32), was critical to inducing functional and morphological vascular change, which is the dissociation of pericytes from the capillaries in muscles of STZ-DM mice, indicating that impaired PDGF-BB expression disturbed vessel maturation. In the present study, statin administration did not influence PDGF-BB expression, which is concerned with vessel maturation. We postulated that the mechanism underlying the therapeutic effect of statins might be due to improved endothelial function because of the lower PDGF-BB expression. Many diabetic vascular complications involve endothelial cell dysfunction characterized by reduced NO-dependent phenomena, including vasodilation and protection against leukocyte-endothelial interactions. Several studies have shown that hyperglycemia impairs NO production and have demonstrated impaired endothelium-dependent vasorelaxation in diabetic humans and in experimental diabetic animals (5, 14, 15, 30). A hallmark of endothelial dysfunction is reduced bioavailability of NO, which could be caused by reduced expression of eNOS, impairment of eNOS activation, or increased inactivation of NO by oxidative stress. Upregulation of the activity or expression of eNOS is considered to be effective in diabetic angiopathy, and statins can increase eNOS expression and activation in addition to their lipid-lowering effect (8, 17, 19, 22, 24, 27, 34). Furthermore, statins have also been reported to promote angiogenesis via eNOS (16, 26). In our current study, statin therapy was effective for preventing autoamputation of the ischemic limb under hyperglycemia at least partly via eNOS, and those previous reports essentially support our findings that statins could restore or increase the eNOS expression in the ischemic limb of DM mice.

In addition to eNOS/NO dependent pathway, it has been revealed that statins have additional effects of growing interest that include the ability to recruit endothelial progenitor cells (3, 8, 9) or activate the protein kinase Akt, which leads to angiogenesis and prevents apoptosis in endothelial cells (9,16). As
shown Fig. 4, our current findings indicate that the therapeutic effects of statins on tolerance against limb ischemia in the diabetic foot might occur at least partially via the eNOS/NO pathway. Therefore, there is a possibility that the clinical benefits of statin therapy might be caused partly by these NO-independent pathways; however, further extensive studies should be carried out to determine this hypothesis.

Although statins have the favorable effect of restoring blood flow in the ischemic limb of DM mice (16, 26), they prevented autoamputation of the ischemic limb of STZ-DM mice to some extent, but not completely. In turn, this result also suggests that the therapeutic effect of statins is due to improved endothelial function and is independent of the restoration of the impairment in PDGF-BB expression, which was sufficient to prevent autoamputation due to limb ischemia in STZ-DM mice. Furthermore, the results of the combination therapy of statins and PDGF-BB supplementation confirmed that the favorable effect of statin therapy on the diabetic foot occurs by mechanisms other than the upregulation of PDGF-BB expression.

Some clinical trials have suggested that the vascular effects of hydrophilic statins are similar in extent to those of lipophilic statins, but it is still controversial whether lipophilic or hydrophilic statins have more clinical benefits (23a, 25). An important advance of our current study was to determine that there are clearly differences between lipophilic and hydrophilic statins, although both types are beneficial to ischemic limbs under hyperglycemia. Limb survival was similar in mice receiving lipophilic and hydrophilic statins; however, eNOS expression was more upregulated by the administration of lipophilic statins than by that of hydrophilic statins (Fig. 3). Therefore, the present study indicates that clinically lipophilic statins may be more useful for ischemic diabetic foot.

In conclusion, we demonstrated that statin therapy restored the disturbed blood flow of severe limb ischemia under hyperglycemia by increasing eNOS/NO expression and not by increasing PDGF-BB expression and that the inhibition of NO reduced the ability of statins to prevent autoamputation due to increasing PDGF-BB expression and that the inhibition of NO expression and not by increasing eNOS expression.

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