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

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Submitted 12 February 2008; accepted in final form 11 April 2008

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 294: H2785–H2791, 2008. 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 it has been suggested that patients with diabetes benefit more than patients without diabetes 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 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 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).
data variability due to ambient light and temperature, the LDPI index
(LDI) UK) as previously described (11, 12, 18, 23, 32, 33). To minimize
the noise, control background subtraction was performed. The Doppler perfusion image (LDPI) analyzer (Moor Instruments, Devon,
UK) was used to confirm eNOS and PDGF-BB expression. For statin
therapy, pravastatin and pitavastatin (0.2 mg/g body wt; injected
volume, 0.02 ml/g body wt) were administered by intraperitoneal injection during days 14 to 28. To inhibit NO synthesis, Nnitro-L-
arginine methyl ester (1 mg/ml in drinking water; Sigma) was orally
administered during days 14 to 28.
Marine severe hindlimb ischemia. Details of the surgical treatment
and evaluation of limb prognosis have been described previously (11,
12, 18, 23, 32, 33); specifically, the excision of both the left femoral
terum and vein and their branches from the inguinal ligament up to
and including the saphenous-popliteal bifurcation was performed.
Gene transfer vectors. The plasmid-based gene of human PDGF-BB
was prepared as previously described (32). Human full-length cDNA of
PDGF-B (GenBank: No. BC029822) was amplified by PCR using
specific primers (forward: 5’-AAGGGTACCATGAACTCGTGCTCG-
GAGCTC-3’ and reverse: 5’-TTCTTCGAGCTAGCTCCAGGTCGT-
TCCCTG-3’) and subcloned into a TA cloning vector (Invitrogen, San
Diego, CA). The whole sequence was then determined using the CEQ
2000 Sequence Detection System (Beckman Coulter, Fullerton, CA).
The amplicon was transferred to the Km/Rho sites of the mammalian
expression vector pCEP4 (Invitrogen).
ELISA. The protein contents in the limb muscels and culture
medium were determined using Quantikine Immunoassay systems for
human eNOS (available for both humans and mice: R&D Systems,
Minneapolis, MN), murine PDGF-BB (R&D Systems) and human
PDGF-BB (specific for humans: R&D Systems) according to the
manufacturer’s instructions, as previously described (11, 12, 18, 23,
32, 33).
Biochemical analysis. Serum levels of LDL cholesterol and ad-
anced glycation end product (AGE) were measured in serum samples
when all animals were euthanized. Concentrations of LDL cholesterol
and AGE were determined by an automatic analyzer (Research Test-
ing Department, SRL, Hachioji-shi, Tokyo, Japan).
Laser-Doppler perfusion images. Measurements of the ischemic
(left)/normal (right) limb blood flow ratio were made using a laser-
Doppler perfusion image (LDPI) analyzer (Moor Instruments, Devon,
UK) as previously described (11, 12, 18, 23, 32, 33). To minimize
data variability due to ambient light and temperature, the LDPI index
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 38 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.

| 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°°/Lep°°); ob/ob) mice |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | STZ-DM (Type 1) |                 |                 |                 |
|                 | Non-DM PRA PTA  |                 |                 |                 |
|                 | 6               | 6               | 6               |                 |
| Glucose, mg/dl  | 113±4.2        | 621±6.6*        | 622±17.9*       | 598±14.2*       |
| LDL cholesterol, mg/dl | 11.0±0.47 | 31.5±2.77* | 25.2±2.59* | 24.9±3.87* |
| AGE, μM/l      | 1.0±0.12       | 1.6±0.17*       | 1.7±0.23*       | 1.8±0.14*       |

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, fli-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

![Graph A](image1.png)

![Graph B](image2.png)

Fig. 3. A: endothelial nitric oxide (NO) synthase (eNOS) protein expression in the thigh muscles of non-DM or DM mice assessed by ELISA. Statin therapy significantly restored or increased the expression of eNOS in both STZ-DM (right) and ob/ob (left) DM mice (n = 6 animals/group). *P < 0.01; #P < 0.05 vs. non-DM. B: eNOS expression was increased by statin stimulation in HUVECs. Twenty-four hours after preincubation with 5% FBS, HUVECs were stimulated with statins (0.01 or 0.1 mM, respectively). Twenty-four hours later, the lysate of the cultured HUVECs was subjected to ELISA. *P < 0.01; #P < 0.05.

Fig. 4. Limb prognosis curve according to the limb salvage score after the daily oral administration of Nω-nitro-l-arginine methyl ester (l-NAME), an inhibitor of NO synthesis in STZ-DM mice. These curves were obtained using Kaplan-Meyer’s method, and the data were analyzed using the log-rank test. Administration of l-NAME prevented the therapeutic effect for ischemic limb autoamputation in STZ-DM mice by treatment with either statin: PRA (left) and PTA (right). #P < 0.05 vs. others.
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 leucocyte-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 partly 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 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 vasculature 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 limb ischemia in STZ-DM mice. Therefore, statin therapy is expected to be useful for preventing intractable diabetic foot disease in patients with diabetic angiopathy via eNOS/NO.

ACKNOWLEDGMENTS

We thank Chie Arimatsu for assistance with the animal experiments.

GRANTS

This work was supported in part by a Japanese Ministry of Education, Culture, Sports, Science, and Technology of Japan grant-in-aid (to Y. Yonemitsu and K. Sueishi) and by the National Institute of Biomedical Innovation (Japan) Program for Promotion of Fundamental Studies in Health Sciences No. 210210002015001. The study was supported in part by a Grant-in-Aid for Scientific Research (C) (No. 2159165) from the Japan Society for the Promotion of Science. The authors declare that they have no conflict of interest.

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