

Full Paper

Possible Involvement of Akt Activity in Endothelial Dysfunction in Type 2 Diabetic Mice

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Abstract. We investigated the effects of chronic simvastatin treatment on the impaired endothelium-dependent relaxation seen in aortas from type 2 diabetic mice. Starting at 8 weeks of diabetes, simvastatin (10 mg/kg per day) was administered to diabetic mice for 4 weeks. The significantly elevated systolic blood pressure in diabetic mice was normalized by simvastatin. Aortas from diabetic mice, but not those from simvastatin-treated diabetic mice, showed impaired endothelium-dependent relaxation in response to both clonidine and adrenomedullin. After preincubation with an Akt inhibitor, these relaxations were not significantly different among the three Akt inhibitor-treated groups (controls, diabetics, and simvastatin-treated diabetics). Although clonidine-induced NO_x^- ($\text{NO}_2^- + \text{NO}_3^-$) production was greatly attenuated in our diabetic model, it was normalized by simvastatin treatment. The expression levels of both total Akt protein and clonidine-induced Ser-473-phosphorylated Akt were significantly decreased in diabetic aortas, while chronic simvastatin administration improved these decreased levels. The expression level of clonidine-induced phosphorylated PTEN (phosphatase and tensin homolog deleted on chromosome ten) was significantly increased in diabetic aortas, but chronic simvastatin did not affect it. These results strongly suggest that simvastatin improves the endothelial dysfunction seen in type 2 diabetic mice via increases in Akt and Akt phosphorylation.

Keywords: diabetes, endothelial cell, statin, Akt, phosphatase and tensin homolog deleted on chromosome ten (PTEN)

Introduction

Several epidemiological studies have indicated that the insulin resistance and hyperglycemia associated with type 2 diabetes make important contributions to the development of hypertension and cardiovascular diseases, and moreover impaired endothelium-dependent vasodilation has been described in humans and in animal models of the disease (1–4). We and others have demonstrated that both aortic endothelial dysfunction and hypertension are present in type 2 spontaneously diabetic (db/db^{-/-}) mice and in fructose-fed insulin-resistant mice (5, 6).

In endothelial cells, the main signal-transduction pathway for agonist-stimulated endothelial nitric oxide

synthase (eNOS) activation depends on Ca^{2+} /calmodulin/caveolin-1 (4, 7). In contrast, with other forms of stimuli, such as fluid shear stress (8), estrogen (9), and insulin/IGF-1 (insulin-like growth factor-1) (10), a rise in Ca^{2+} is not required for NO production. Many stimuli, including insulin, vascular endothelial growth factor (VEGF), β -agonists, adrenomedullin, and shear-stress signals, have been reported to regulate NO production by phosphorylation of eNOS, which facilitates the association of the enzyme with calmodulin, thus reducing its inhibitory interaction with caveolin-1 (11–15). There is some evidence that abnormal regulation of the phosphatidylinositol (PI) 3-K/Akt pathway may be one of several factors contributing to vascular dysfunction in diabetes (16). We previously found (17) that addition of a PI3-K or Akt inhibitor had no significant effect on either acetylcholine (ACh)-induced relaxation or NO_x^- /c-GMP production in control mouse aortas, whereas the clonidine- and insulin-induced relaxation responses

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were completely abolished by each of these inhibitors. These observations suggest that clonidine- and insulin-induced vasorelaxations are regulated by the PI3-K/Akt signal pathway (17).

There is a close relationship between insulin-mediated glucose disposal and the incremental increase in blood flow in response to insulin. This normal response is lost in insulin-resistant states, suggesting a resistance to the action by which insulin induces vascular NO production (18). Thus, it may be insulin-resistance, rather than the hyperinsulinemia itself, that is a pathogenic factor for decreased vascular relaxation in diabetes. Interestingly, Jiang et al. found that both vascular insulin-induced phosphorylation and activation of the components of insulin signaling from the receptor level downstream to Akt were blunted in obese insulin-resistant rats (19). It is possible that in type II diabetes, a chronic lack of sensitization of the endothelial component of Akt signaling contributes to a decrease in endothelial function, and hence to the progress of hypertension.

Statins inhibit the activity of 3-hydroxyl-3-methyl coenzyme A (HMG-CoA) reductase, which catalyzes the rate-limiting step in cholesterol biosynthesis (20). Statins are widely prescribed (to lower cholesterol) to hyperlipidemic patients at risk of cardiovascular disease (21). Recently, it has been recognized that the protective effects of these drugs can be extended to myocardial-infarction patients with average plasma cholesterol concentrations and that lipid reduction alone cannot entirely account for the benefits of statin therapy (22). The reported beneficial effects that are independent of lipid-lowering include improvements in endothelial function, which may be due to alterations in endothelial nitric oxide synthase expression and activity as well as to the antioxidant effects of statins (23, 24). Furthermore, the observation that statin-stimulated NO release and phosphorylation can be reduced by PI3-K/Akt inhibitors suggests that acute statin treatment of endothelial cells results in activation of PI3-K/Akt, indicating that statins directly increase NO by activating Akt (25, 26). However, whether the impaired endothelial function and reduced Akt activity that are seen in the aorta in type 2 diabetic mice might be improved by chronic simvastatin treatment has not been investigated in detail.

We recently observed that both the relaxation and NO production induced by clonidine via the Akt pathway are impaired in aortic rings from a nicotinamide + streptozotocin (STZ)-induced type 2 diabetic mouse model (17). In the present study, we examined clonidine- and adrenomedulin-induced relaxations (which are mediated through the Akt pathway) to determine whether the impairments seen in aortas isolated from type 2 diabetic mice might be improved by chronic simvastatin treatment.

Materials and Methods

Reagents

STZ, clonidine hydrochloride, *N*^G-nitro-L-arginine (L-NNA), and (–)adrenomedullin 52 human (AM) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium nitroprusside dehydrate (SNP) was from Wako Chemical Company (Osaka). The particular “Akt inhibitor” we used was manufactured by Calbiochem, Darmstadt, Germany. All drugs were dissolved in saline, except where otherwise noted. All concentrations are expressed as the final molar concentration of the base in the organ bath.

Experimental design

To induce diabetes, Institute of Cancer Research (ICR; Tokyo Animal Laboratories, Tokyo) mice (males, 5-week-old) received an intraperitoneal injection of 1.5 g/kg body weight of nicotinamide dissolved in saline 15 min before an injection via the tail vein of STZ (200 mg/kg) dissolved in a citrate buffer (17). At 12 weeks after nicotinamide-STZ administration, systolic blood pressure was measured in each mouse (see below), and mice were then anesthetized with diethyl ether and euthanized by decapitation. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology, Japan). Mice were randomly allocated to one of three groups as follows: Starting 8 weeks after the nicotinamide + STZ treatment, diabetic mice were fed for 4 weeks on a normal diet either containing or not containing simvastatin (Banyu, Tokyo) (10 mg/kg per day). These mice are referred to as DM-simvastatin and DM groups, respectively. Age-matched control mice (Cont) were fed a normal diet throughout.

Measurement of plasma glucose, insulin, and cholesterol, and blood pressure

Plasma parameters and blood pressure were measured as described previously (25, 26). Briefly, plasma glucose and cholesterol levels were each determined by the use of a commercially available enzyme kit (Wako Chemical Company). Plasma insulin was measured by enzyme immunoassay (Shibayagi, Gunma). Systolic blood pressure and diastolic blood pressure were measured by the tail-cuff method using a blood pressure analyzer (BP-98A; Softron, Tokyo) while the mice were in a constant-temperature box at 37°C.

Measurement of isometric force

Each aorta was separated from the surrounding connective tissue and cut into rings, as previously described (5). For the relaxation studies, rings were precontracted with an equieffective concentration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) ($10^{-6} - 3 \times 10^{-6}$ M). When the $PGF_{2\alpha}$ -induced contraction had reached a plateau level, clonidine ($10^{-9} - 10^{-5}$ M), adrenomedullin ($10^{-10} - 3 \times 10^{-8}$ M), or SNP ($10^{-10} - 10^{-5}$ M) was added in a cumulative manner. When the effects of an Akt inhibitor (7×10^{-7} M) or a NOS inhibitor (L-NNA) (10^{-5} M) on the response to a given relaxant agent were to be examined, the appropriate inhibitor was added to the bath 30 min before the application of $PGF_{2\alpha}$.

Measurement of NO_2^- and NO_3^-

The concentrations of nitrite and nitrate in the effluent from each tissue were sampled and assayed by the method described previously (ENO-20; Eicom, Kyoto) (17). Each aorta was cut into transverse rings 5 mm in length. These were placed in 0.5 ml Krebs-Henseleit-solution at 37°C. Samples were collected on two occasions as follows: for one 20-min period before and one after application of 10^{-6} M clonidine. The amount of NO_x^- was calculated as follows: agonist-stimulated NO_x^- (10^{-7} mol \cdot min $^{-1}$ \cdot g $^{-1}$) = sample / 20 (min) \cdot g (weight of the frozen aorta). The concentrations of NO_2^- and NO_3^- in the Krebs-Henseleit-solution and the reliability of the reduction column were examined in each experiment.

Measurement of the protein expressions of Akt, and of phospho-Akt and phospho-PTEN (phosphatase and tensin homolog deleted on chromosome ten) (by Western blotting)

Aortas (3 pooled vessels, total protein 200 μ g) were homogenized in ice-cold lysis buffer, as previously described (17, 27, 28). Samples (20 μ g/lane) were resolved by electrophoresis on 7.5% SDS-PAGE gels

and then transferred onto PVDF membranes. The membrane was incubated with anti-Akt antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA), anti-phospho-Akt (Ser473) antibody (1:1000, Cell Signaling Technology), anti-phospho-PTEN (Ser380) antibody (1:1000, Cell Signaling Technology), or β -actin (1:5000, Sigma) in blocking solution. Horseradish-peroxidase-conjugated, anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA) was used at a 1:4000 dilution in Tween PBS, followed by detection using SuperSignal (Pierce, Rockford, IL, USA). To normalize the data, we used β -actin as a housekeeping protein. The optical densities of the bands on the film were quantified using densitometry, with correction for the optical density of the corresponding β -actin band.

Statistical analyses

Data are each expressed as the mean \pm S.E.M. When appropriate, statistical differences were assessed by Dunnett's test for multiple comparisons after one-way analysis of variance. Statistical comparisons between concentration-response curves were made by using one-way ANOVA, with Bonferroni's correction for multiple comparisons being performed post hoc. In each test, $P < 0.05$ was regarded as significant.

Results

Plasma glucose, insulin, and cholesterol, and systolic blood pressure and body weight

In the fairly recently devised experimental model employed here (adult mice or rats given STZ and partially protected with a suitable dose of nicotinamide) (29–32), the diabetic syndrome shares a number of features with human type 2 diabetes. It is characterized by stable moderate hyperglycemia, glucose intolerance, altered but significant glucose-stimulated insulin secretion, altered in vivo and in vitro responsiveness to tolbutamide, and a reduction in pancreatic β -cell mass

Table 1. Plasma glucose, plasma insulin, systolic blood pressure, diastolic blood pressure, plasma total cholesterol, and body weight in controls, nicotinamide + STZ-induced diabetic, and simvastatin-treated diabetic mice

Parameters	Cont (n = 12)	DM (n = 12)	DM-simvastatin (n = 12)
Glucose (mg/dl)	151.6 \pm 6.5	563.6 \pm 29.8 ^{###}	627.8 \pm 22.2 ^{##}
Insulin (pg/ml)	771.8 \pm 96.8	850.5 \pm 65.7	706.9 \pm 24.7
Systolic blood pressure (mmHg)	106.0 \pm 0.9	131.8 \pm 1.4 ^{###}	110.3 \pm 2.0*
Diastolic blood pressure (mmHg)	69.9 \pm 0.9	81.1 \pm 1.7 ^{###}	70.6 \pm 2.0*
Total cholesterol (mg/dl)	109.4 \pm 6.0	133.2 \pm 5.2 [#]	133.5 \pm 6.2 [#]
Body weight (g)	47.3 \pm 0.7	46.5 \pm 1.0	47.0 \pm 1.1

Values are means \pm S.E.M. Numbers of determinations are shown in parentheses. [#] $P < 0.01$, ^{##} $P < 0.001$ vs controls.

* $P < 0.001$ vs DM (diabetic mice).

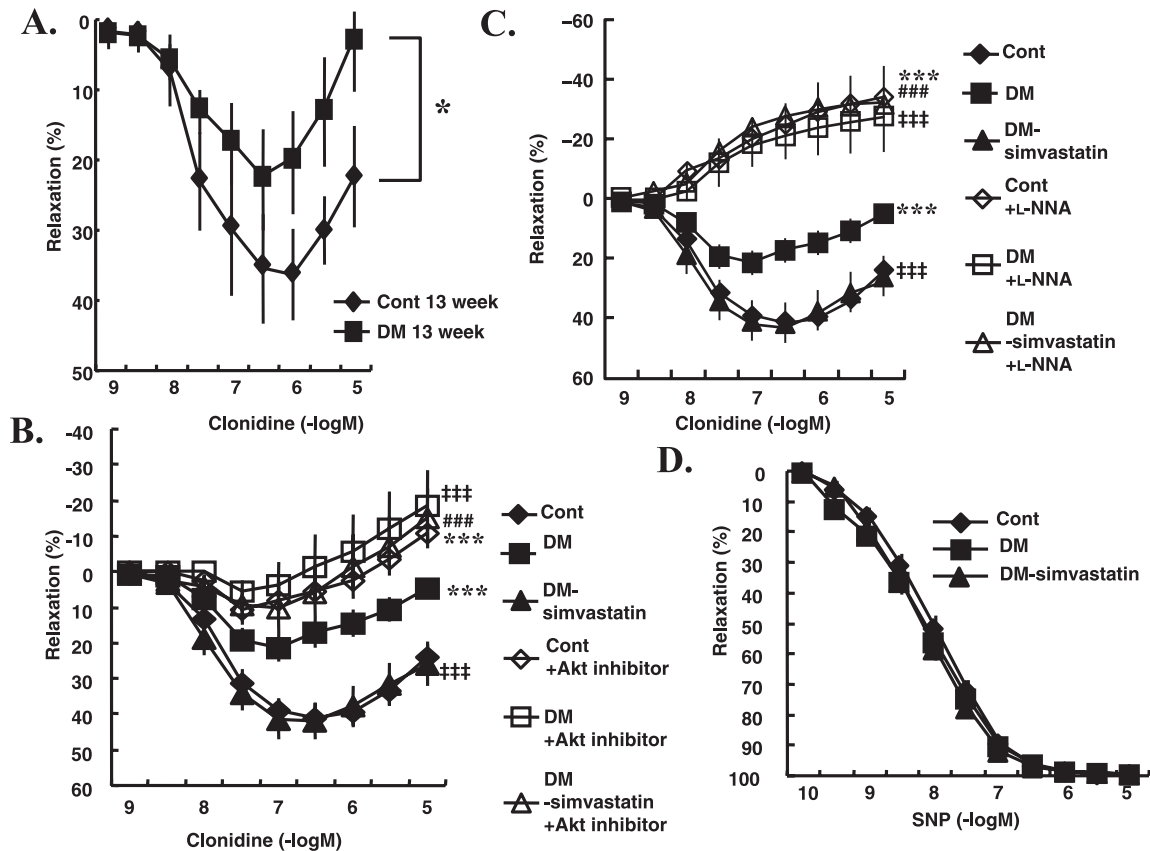


Fig. 1. Concentration-response curves for agonist-induced relaxation of aortic rings. A: Concentration-response curves for clonidine-induced relaxation in isolated thoracic aortic rings from 13-week-old control and diabetic (8 weeks after administration of nicotinamide + STZ) mice. B, C, and D: Concentration-response curves for clonidine-induced (B, C) and SNP-induced (D) relaxations in isolated thoracic aortic rings from control, diabetic, and simvastatin-treated diabetic mice. Each data-point represents the mean \pm S.E.M. of 5–8 observations. A: * P <0.05. B, C: *** P <0.001 vs control, ### P <0.001 vs DM, #### P <0.001 vs DM-simvastatin.

(30, 32). As shown in Table 1, the nonfasting plasma glucose level and the plasma total cholesterol level were significantly elevated in nicotinamide + STZ-induced diabetic mice (versus age-matched controls). These increases were not affected by chronic (4 week) administration of simvastatin. Although systolic blood pressure was significantly higher in diabetic mice than in control mice, simvastatin treatment significantly reduced it (back to the control level). Plasma insulin and body weight were not different among the three groups.

Vascular reactivity in aorta

When the $\text{PGF}_{2\alpha}$ (10^{-6} – 3×10^{-6} M)-induced contraction had reached a plateau, clonidine (10^{-9} – 10^{-5} M), SNP (10^{-10} – 10^{-5} M), or adrenomedullin (10^{-9} – 3×10^{-7} M) was cumulatively added. The clonidine-induced relaxation was significantly weaker (versus the controls) in aortic rings from diabetic mice at 8 weeks after administration of nicotinamide + STZ (Fig. 1A). This attenuated relaxation was significantly improved by

chronic simvastatin treatment (closed symbols in Fig. 1B). The aortic relaxation induced by clonidine was almost completely abolished by preincubation with an Akt-inhibitor at 7×10^{-7} M or L-NNA (NOS inhibitor) at 10^{-5} M (open symbols in Fig. 1, B and C). The relaxation induced by SNP did not differ significantly among the three groups (Fig. 1D). The relaxation induced by adrenomedullin was significantly weaker in rings from diabetic mice (versus the controls), and this impaired relaxation response was recovered by chronic simvastatin treatment (closed symbols in Fig. 2). In the presence of the Akt inhibitor (7×10^{-7} M), the aortic relaxation induced by adrenomedullin was not significantly different among the three groups (open symbols in Fig. 2). However, in simvastatin-treated diabetic mice, the aortic relaxation induced by adrenomedullin was significantly weaker in the presence of the Akt inhibitor than in its absence (Fig. 2).

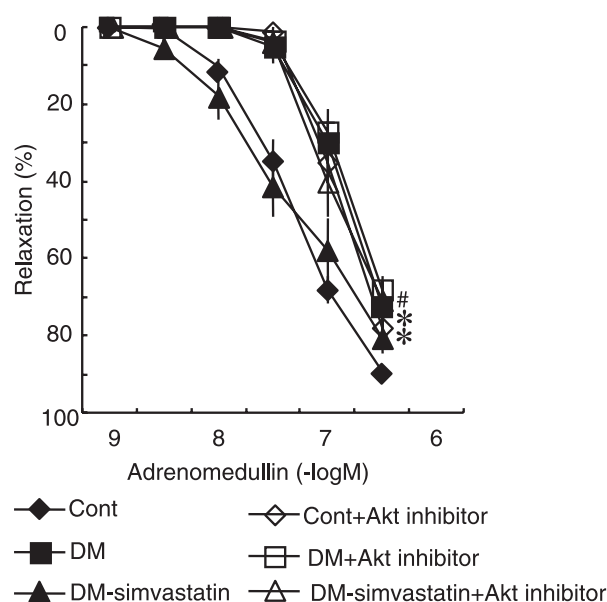


Fig. 2. Concentration-response curves for adrenomedullin-induced relaxation in isolated thoracic aortic rings from control, diabetic, and simvastatin-treated diabetic mice, together with effects of an Akt inhibitor. Each data-point represents the mean \pm S.E.M. of 5–7 observations. * $P < 0.05$ vs control, # $P < 0.05$ vs DM-simvastatin.

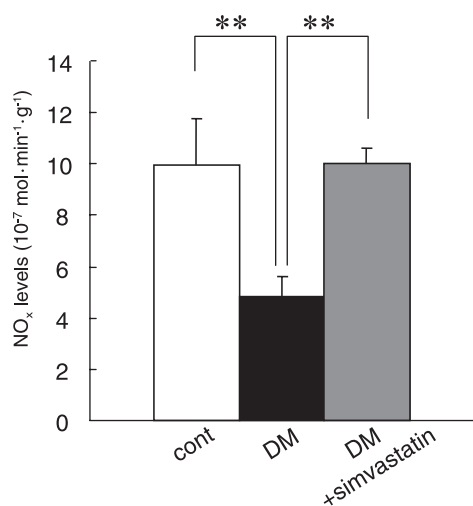


Fig. 3. Release of NO_2^- plus NO_3^- (NO_x^-) from clonidine (10^{-6} M)-stimulated aortic rings in KHS. Each column represents the mean \pm S.E.M. of 8 observations. ** $P < 0.01$.

Measurement of NO_x^- production

Clonidine increased the NO_x^- ($\text{NO}_2^- + \text{NO}_3^-$) level in the perfusate from aortic rings, but the increase was significantly smaller in the diabetics (versus the controls) (Fig. 3). This decrease in NO_x^- production was significantly recovered by chronic simvastatin treatment.

Expressions of Akt protein and phosphorylated Akt and PTEN, and effects of clonidine

Use of anti-Akt antibody allowed detection of an immunoreactive protein with a molecular weight of 60 kDa. The expression of this Akt protein was significantly decreased in aortas from diabetic mice (versus the controls), and this reduction was significantly recovered by chronic simvastatin treatment (Fig. 4: A and B). We next evaluated clonidine-induced Akt phosphorylation (clonidine-stimulated aorta / clonidine-stimulated aorta with Akt inhibitor). Clonidine-stimulated Akt phosphorylation was significantly weaker in aortas from diabetic mice (versus the controls), and this reduction was significantly improved by chronic simvastatin treatment (Fig. 4: C and D).

Use of anti-phospho-PTEN antibody allowed detection of an immunoreactive protein with a molecular weight of 74 kDa. The expression of phosphor-PTEN protein induced by clonidine was significantly increased in aortas from diabetic mice (versus the controls) (Fig. 5). This enhancement was not affected by chronic simvastatin treatment.

Discussion

The novel findings obtained in the present study were that in type 2 diabetic mice, chronic simvastatin treatment had the following effects: 1) normalized the elevated blood pressure without lowering the plasma cholesterol level, 2) improved the impaired endothelium-dependent aortic vasorelaxation responses to clonidine and adrenomedullin and restored clonidine-induced NO production, 3) improved the decreased expression of Akt protein and Akt activity, but 4) did not affect the clonidine-induced increase in phosphorylated PTEN.

When simvastatin was administered for 4 weeks to our nicotinamide + STZ-induced diabetic mice, there was no significant effect on the plasma glucose, insulin, or cholesterol levels. Thus, its beneficial effects are clearly unrelated to a correction of the hyperglycemia and/or hypercholesterolemia in these animals. In the present study, such short-term treatment with a statin did not lower plasma total cholesterol, as is usually seen upon long-term treatment in humans (33). This result is consistent with the previous finding of Sparrow et al. (34) that in mice, plasma cholesterol levels were not affected by a six-week dosing with 10 or 100 mg/kg of simvastatin.

The high blood pressure seen in our diabetic mice was improved by chronic simvastatin treatment, a result consistent with the reported blood pressure-lowering effect of statins (35). It has been reported that mice lacking the gene for endothelial nitric oxide synthase

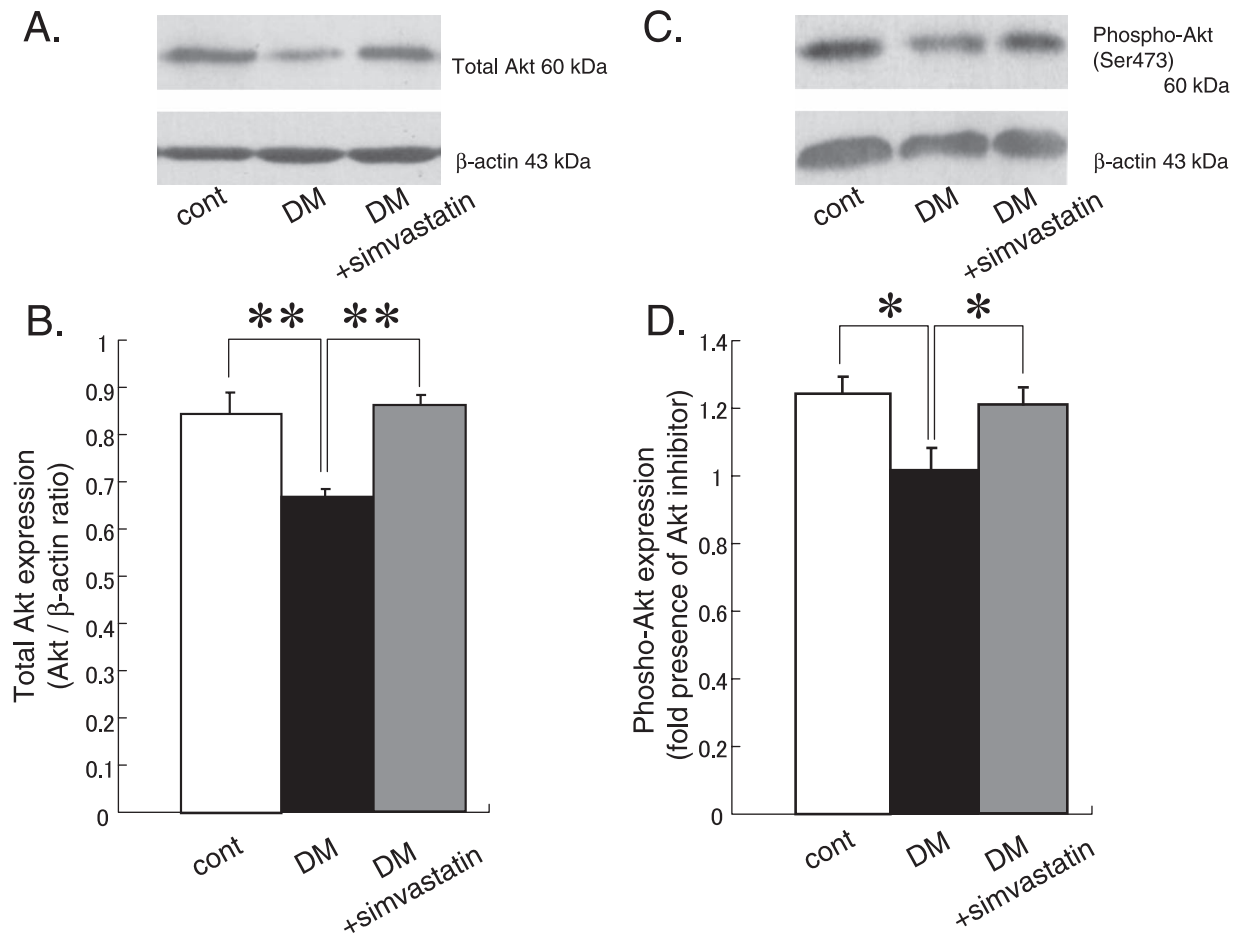


Fig. 4. Protein expression and phosphorylation of Akt in aortas from control, diabetic, and simvastatin-treated diabetic mice. A: Expression of total Akt assayed by Western blotting. B: Quantitative analysis of total Akt expression by scanning densitometry. C and D: Protein expression of phosphorylated Akt in aortas from control, diabetic, and simvastatin-treated diabetic mice. C: Expression of clonidine-induced phospho-Akt-Ser473 assayed by Western blotting. D: Quantitative analysis by scanning densitometry of clonidine-induced phospho-Akt-Ser473 (clonidine-stimulated aorta / clonidine-stimulated aorta in presence of Akt inhibitor). Values are each the mean \pm S.E.M of 10 determinations. * P <0.05, ** P <0.01.

show hypertension (36), suggesting that the consequential impairment in endothelium-dependent vascular relaxation may be involved in the pathophysiology of their hypertension. Clonidine-induced endothelium-dependent vascular relaxation was impaired in our type 2 diabetic mice, and this endothelial dysfunction was significantly recovered by the chronic administration of simvastatin. Hence, we propose that the anti-hypertensive effect of simvastatin may be due to an improvement in endothelial dysfunction in type 2 diabetes.

The most important finding made in the present study was that the chronic simvastatin treatment improved the endothelial dysfunction seen in our type 2 diabetic mice. To examine the endothelium-dependent and -independent relaxations of the isolated aorta, we used clonidine, adrenomedullin, and SNP (Fig. 1: B and C, and Fig. 2).

One well-documented pathway downstream of activated G-protein coupled receptors (GPCRs) includes dissociation of the $G_{\alpha\beta\gamma}$ trimer to yield the G_{α} monomer and $G_{\beta\gamma}$ dimer, and the involvement of the latter proteins in signal-transduction events downstream of α -adrenoceptors. $G_{\beta\gamma}$ dimers can initiate intracellular signal transduction events, and PI3-K has been identified as a major effector of $G_{\beta\gamma}$ in various cell and tissue preparations (37, 38). There are known to be functional α_2 -adrenoceptors and insulin receptors on the endothelium, and stimulation of NO production via each receptor has been shown to inhibit the contractile effects of α -adrenergic agonists and catecholamines in vascular smooth muscle (16, 39). In our previous study on the mouse aorta, we found that when a PI3-K or Akt inhibitor was applied to control aortas, there was no significant difference in the ACh-induced relaxation

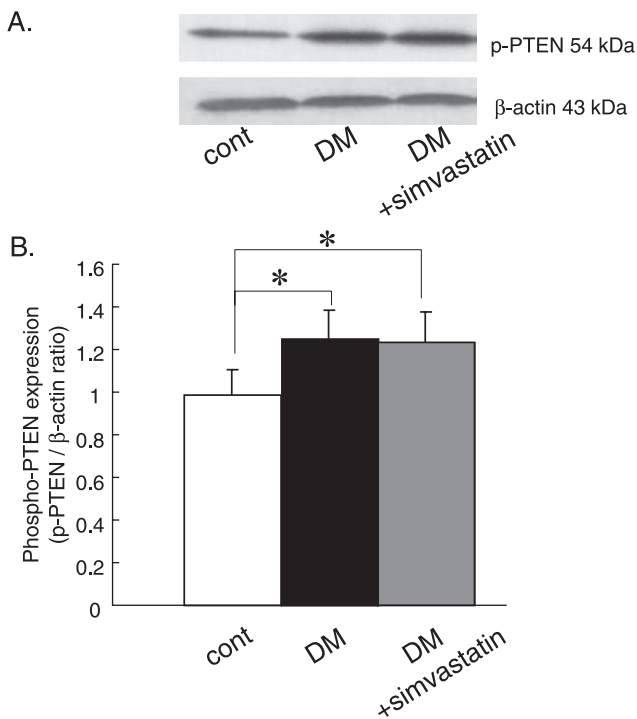


Fig. 5. Protein expression of phosphorylated PTEN in aortas from control, diabetic, and simvastatin-treated diabetic mice. A: Expression of clonidine-induced phospho-PTEN assayed by Western blotting. B: Quantitative analysis of phospho-PTEN expression by scanning densitometry. Values are each the mean \pm S.E.M of 10 determinations. * $P < 0.05$.

or in ACh-induced $\text{NO}_x^-/\text{cGMP}$ production, whereas the clonidine-induced and insulin-induced relaxation responses were completely abolished by each inhibitor (17). Those results indicated that the endothelium-dependent relaxation induced by clonidine, but not that induced by ACh, is regulated by the PI3-K/Akt signal pathway. In addition, adrenomedullin reportedly increases NO production via the PI3-K/Akt/eNOS pathway in the rat aorta (14), and since in the present study the adrenomedullin-induced relaxation was significantly decreased by an Akt inhibitor and by L-NNA, this relaxation is presumably also regulated by the PI3-K/Akt/eNOS signal pathway. Furthermore, the endothelium-dependent aortic relaxations to clonidine and adrenomedullin were weaker in our diabetic group than in the controls, a dysfunction that was improved by 4-week simvastatin treatment. Moreover, the decreased clonidine-induced NO_x^- level was normalized by such a simvastatin treatment. In addition, both Akt expression and the clonidine-induced Akt phosphorylation was decreased in our diabetic mice, and this decrease was normalized by chronic simvastatin treatment, suggesting that the impairment of the endothelium seen in the type 2 diabetic state may be related to decreases in Akt protein

expression and activity. Recently it has been reported that in addition to insulin, VEGF, β -agonists, and shear-stress signals, HMG-CoA reductase inhibitors can directly activate PI3-K/Akt and increase NO production (25, 26). We suspect that direct Akt activation by simvastatin led to the normalization of Akt expression and phosphorylation we observed here. However, the precise mechanisms by which simvastatin acts on the aorta to cause an enhancement in Akt activities will require further investigation.

That the PI3-K/Akt pathway plays a crucial role in cell survival is supported by the observation that the tumor suppressor PTEN, which is inactivated in a number of human cancers, possesses 3'-phosphoinositide-phosphatase activity, and thereby inactivates the PI3-K/Akt pathway (40). Akt phosphorylation is regulated by PIP3, which recruits PDKs and Akt to the plasma membrane, allowing Akt regulatory residues to be more accessible to PDKs and to be phosphorylated (41, 42). PIP3 levels are tightly regulated by PI-3K and phosphatases, such as PTEN, which has been shown to antagonize PI3-K/Akt signaling by dephosphorylating PIP3 (43, 44). A very interesting finding made in the present study was that the clonidine-induced level of phosphorylated PTEN was significantly greater in aortas from diabetic mice than in the controls. To our knowledge, this is the first report of an increase in PTEN activity in the diabetic state. This finding strongly suggests that PTEN is also involved in the decreased activity of the PI3-K/Akt pathway, a decrease that may lead to reductions in both eNOS activity and endothelium-dependent relaxation. Four-week administration of simvastatin to our type 2 diabetic mice did not cause a recovery in PTEN phosphorylation, but it did lead to a recovery in endothelial function, suggesting that the improvement in endothelial dysfunction brought about by simvastatin is due to increased Akt expression and phosphorylation, and not directly to Akt dephosphorylation.

In conclusion, the present data shows that in a type 2 diabetic mouse model, 4-week simvastatin treatment improves blood pressure, as well as clonidine- and adrenomedullin-mediated vasorelaxations and NO production in the aorta. These effects of simvastatin may be accompanied by increases in Akt protein expression and phosphorylation. We propose that simvastatin may improve at least some diabetes-related cardiovascular diseases through increases in Akt expression and Akt activity without lowering the plasma cholesterol level.

Acknowledgments

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