Simvastatin Improves Diabetes-Induced Coronary Endothelial Dysfunction

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ABSTRACT

3-Hydroxy-3-methylglutaryl CoA reductase inhibitors decrease cardiovascular morbidity in diabetic patients, but the mechanism is unclear. We studied the actions of simvastatin (SIM) in enhancing NO bioavailability and reducing oxidative stress in coronary vessels from diabetic rats and in rat coronary artery endothelial cells (RCAEC) exposed to high glucose. Coronary arteries isolated from diabetic rats showed decreases in acetylcholine (ACH)-mediated maximal relaxation from 81.0 ± 4.5% in controls to 43.5 ± 7.6% at 4 weeks and 22.3 ± 0.6% at 10 weeks of diabetes. This effect was associated with oxidative stress in coronary vessels as shown by dichlorofluorescein (DCF) imaging and nitrotyrosine labeling. Diabetes also reduced trans-coronary uptake of [3H]-L-arginine. Supplemental L-arginine (50 mg/kg/day p.o.) did not improve coronary vasorelaxation to ACh. However, SIM treatment (5 mg/kg/day subcutaneously) improved maximal ACh relaxation to 65.8 ± 5.1% at 4 weeks and 47.1 ± 3.9% at 10 weeks. Coronary arteries from rats treated with both SIM and L-arginine demonstrated the same maximal relaxation to ACh (66.1 ± 3%) as SIM alone. Mevalonate and L-NAME (N-nitro-L-arginine methyl ester hydrochloride) inhibited the response to ACh in SIM-treated diabetic rats. Coronary arteries from all groups relaxed similarly to sodium nitroprusside. SIM increased endothelial NO synthase protein levels and blocked diabetes-induced increases in DCF and nitrotyrosine labeling in diabetic coronary vessels. SIM treatment restored normal NO levels in media from high-glucose-treated RCAEC and plasma of diabetic rat. Treatment with SIM or the NADPH oxidase inhibitor apocynin also blocked high-glucose-induced increases in reactive oxygen species and superoxide formation in RCAEC. Taken together, these data suggest that SIM improves diabetes-induced coronary dysfunction by reducing oxidative stress and increasing NO bioavailability.

Diabetes mellitus predisposes patients to premature atherosclerotic coronary artery disease (CAD), the leading cause of mortality among patients with diabetes (Gu et al., 1999). Factors such as diabetes-associated hypertension and dyslipidemia may contribute to the severity of vascular dysfunction in diabetes. However, there is evidence that coronary vascular dysfunction is present, even in diabetic subjects without hypertension or dyslipidemia, suggesting that diabetes per se causes this vascular dysfunction (Pitkanen et al., 1998). The vascular endothelium is a target of the diabetic milieu, and endothelial dysfunction is thought to play an important role in diabetic vascular diseases (Cortisentino and Luscher, 1998).

Diminished capacity of NOS to generate NO has been demonstrated when endothelial cells are exposed to elevated glucose levels either in vitro or in vivo (Cipolla, 1999).

An imbalance between the production of O$_2^-$ and NO in the vessel wall has been considered to play an important role in the pathogenesis of diabetes-induced endothelial dysfunction (Tan et al., 1999). Hyperglycemia drives cellular events that increase production of O$_2^-$. NADPH oxidase is considered a major source for the production of O$_2^-$ within the vascular wall and cardiomyocytes. O$_2^-$ inactivates NO to form peroxynitrite (ONOO$^-$) (Beckman et al., 2001). Peroxynitrite can oxidize the NOS cofactor tetrahydrobiopterin (Milstien and Katusic, 1999) and also reduce cellular transport of L-arginine eNOS substrate for NO production (Ogowski et al., 2000). These events uncouple the enzyme, which then preferentially increases O$_2^-$ production over NO production (Milstien and Katusic, 1999). Thus, therapeutic strategies should aim to in-
crease NO and decrease ROS. Therapy for endothelial dysfunction that increases NO and decreases ROS has been shown to ameliorate myocardial ischemia and may even retard plaque progression. Statins are potent inhibitors of HMG-CoA reductase and cholesterol biosynthesis that are used extensively to treat patients with hypercholesterolemia (Borghi et al., 2002). In addition, clinical use of statins markedly decreases the incidence of cardiovascular events in both hypercholesterolemic and normocholesteremic patients within days (Schwartz et al., 2001; Omori et al., 2002). Another means of reducing plasma cholesterol in patients to the same level as that produced by simvastatin has been shown not to improve vascular endothelial dysfunction as has been observed for simvastatin (Landmesser et al., 2005). These findings suggest that statins may indeed improve coronary endothelial function by a mechanism beyond cholesterol reduction.

Experimental data suggest that statins modulate a variety of pathobiological processes besides cholesterol synthesis. The mechanisms for statins’ cardioprotective effects seem to be through their ability to increase endothelial NO production by increasing the expression of eNOS (Laufs and Liao, 1998; Laufs et al., 2002). Statins inhibit iso- pneumonia of the small G protein RhoA, leading to accumulation of inactive RhoA in the cytoplasm, and this is in turn allows up-regulation of eNOS expression (Laufs and Liao, 1998, 2000). In addition, statins may activate eNOS by a mechanism unrelated to HMG-CoA reductase inhibition (Kaesemeyer et al., 1999; Harris et al., 2004). Statins may increase eNOS activity via post-translational activation of the phosphatidylinositol 3-kinase/protein kinase Akt pathway, as has been reported in cultured human endothelial cells (Kureishi et al., 2000). In addition, statins may prolong the availability of NO through an antioxidant capacity. Statins prevent the isoprenylation of the GTP-binding protein Rac, which is essential for the assembly of NADPH oxidase, hence reducing oxidative stress in endothelial cells (Wassman et al., 2002). These pleiotropic effects of statins may protect against micro/macrovascular complications of diabetes.

Endothelial cells from different vascular beds exhibit structural differences and may be affected differentially by hyperglycemia (Sobrevia and Mann, 1997). Several reports indicate that hyperglycemia can impair the function of different vascular beds; however, the exact nature and mechanism of diabetes-induced endothelial dysfunction in coronary vessels is not yet known. Our goal is to examine the effects of simvastatin on diabetes-induced endothelial dysfunction in rat coronary arteries and cultured coronary endothelial cells and to explore the possible mechanisms involved.

Materials and Methods

General Procedure. Male Sprague-Dawley rats, weighing 250 to 300 g at the beginning of the study, were used. Diabetes was induced by intravenous injection of streptozotocin (STZ, 50 mg/kg, dissolved in 0.1 M sodium citrate buffer, pH 4.5). Four groups of diabetic rats received either simvastatin (5 mg/kg/day subcutaneously), L-arginine (50 mg/kg/day p.o.), L-arginine and simvastatin, or no treatment. A group of matched control rats received only the vehicle. All rats were housed in cages and allowed free access to food and water. The concentration of glucose in plasma was determined 2 days after STZ injection and on the day of the sacrifice. Rats were considered diabetic if their blood glucose was greater than 350 mg/dl. Simvastatin and L-arginine did not affect blood glucose level. Diabetic rats kept for 10 weeks received a few small doses of insulin (2–3 U) to prevent ketoadi-osis. This treatment did not affect blood glucose levels.

Preparation of Rat Coronary Arteries. Four and 10 weeks after STZ or vehicle injection, rats were anesthetized with intraperitoneal injection of ketamine HCL (20 mg/kg) and xylazine (4 mg/kg). A thoracotomy was performed, and heart was quickly excised and placed in ice-cold oxygenated Krebs-Henseleit (K-H) buffer. After the right ventricle and anterior wall of the left ventricle were removed under a stereomicroscope, intramyocardial septal arteries were dissected from the septum facing the right ventricular cavity.

Two arteriolar rings from each heart with a length of approximately 2 mm were gently excised from a vascular segment. Each segment was transferred to the chamber of a small vessel myograph (Danish Myo Technology, Winston-Salem, NC) containing 5 ml of K-H buffer and was mounted onto two tungsten wires with a diameter of 40 μm. The small wires were carefully guided through the lumen of the artery and attached to a force-displacement transducer for isometric force measurements. Subsequently, arteries were allowed to equilibrate in oxygenated (95% O2 and 5% CO2) K-H buffer. The composition of the K-H buffer is 118.3 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3, 2.5 mM CaCl2, and 11.0 mM glucose.

After 30 min, arteries were stretched to a circumference equivalent to 90% of the diameter that they would have with an intraluminal pressure of 100 mm Hg. Arteries were incubated for an additional hour, and buffer solution was changed every 15 min. Isometric force was recorded on a computer by use of Chart version 5 software and a MacLab/4e data acquisition system (ADInstruments, Colorado Springs, CO).

Protocol for Acetylcholine Dose-Response Curves. After the equilibration period, the responsiveness of each individual artery was checked by successive vasoconstriction to administration of a submaximally effective concentration of KCl (125 mM). The integrity of the vascular endothelium was tested pharmacologically by acetylcholine-induced relaxation of arteries, which were precontracted with U46619 (thromboxane A2 receptor agonist). Tissues that did not elicit a reproducible and stable contraction with U46619 (1 μM) and relaxed >50% in response to 10 μM acetylcholine were discarded from the study. Preparations were then washed three times with K-H buffer and allowed to relax fully for 30 min before the experimental protocol began.

Coronary arteries were then again precontracted with U46619 at a submaximal dose of 1 μM (Emax, 10 μM). After reaching a plateau of contraction, cumulative concentration-response curves to acetylcholine (ACh, 0.1 nM-100 μM) and sodium nitroprusside (SNP, 0.1 nM-10 μM) were obtained to evaluate endothelium-dependent and endothelium-independent relaxations, respectively. The concentration in the chamber was increased in 1-log steps. In all cases, ACh or SNP was added to yield the next higher concentration only when the response to the earlier dose reached a steady state.

Other experiments on vasorelaxation responses to ACh were performed in coronary vessels with and without prior exposure to either mevalonate (100 μM, the product of HMG reductase) for 2 h or to l-NAME (100 μM, an inhibitor of NOS) for 30 min. Control responses were obtained in vessels from the same animals.

The vasorelaxant responses are expressed as percentage decreases from U46619-induced contraction. The amount of contraction produced by 1 μM U46619 in each ring from its initial resting tension was considered as 100%.

1-Arginine Transport into Rat Coronary Arteries in Isolated Langendorff Rat Heart Preparation. Rats were anesthetized by intraperitoneal injection of ketamine (20 mg/kg) and xylazine (4 mg/kg). A tracheotomy was performed for intubation, and mechanical ventilation was started. The blood was heparinized (250 U/kg intravenous), and the heart was excised via a left thoracotomy and placed in ice-cold (4°C) K-H solution. After cannulation of the
aorta, the heart was immediately flushed with K-H solution and then suspended within the perfusion system. The time between the excision of the heart and the beginning of perfusion was ~2 min. The buffer perfusate was pumped from a reservoir through a filter to the perfusion column. Coronary arteries were perfused via the cannulated aorta with a flow (Q) of 10 ml/min with K-H solution. This resulted in a coronary perfusion pressure (PP) of ~60 mm Hg, measured in the perfusion line. The hearts were maintained at 37°C and were not be paced during the perfusion. Hearts were allowed to stabilize for 30 to 40 min before the control measurements. The pH (7.4) and PO₂ (~550 mm Hg) were controlled before starting the experiment. Competence of vascular endothelial function was assessed by determining the degree of reduction of PP with an intrartrial infusion of acetylcholine (1 μM final concentration) for 2 min. A drop in PP of ≥40% indicated vascular endothelial cell competence.

Uptake of [3H]-arginine (fractional extraction) across the coronary vasculature was assessed by determining the amount of labeled L-arginine that appeared in the coronary venous effluent versus amount delivered. This was determined before, during, and after a 10-min infusion of a known amount of [3H]-arginine (50 mM or 0.11 μCi in 5 μM nonradioactive cold L-arginine) into the coronary artery perfusion circuit via 2nd needle catheter (0.1 ml/min). Samples (~10 ml) were collected before (~5 min), during (9–10 min), and after (~20 min) infusion of [3H]-arginine. Aliquots of effluent were mixed with scintillation fluid and counted for [3H]-arginine content.

Specific uptake was determined by subtracting uptake occurring during concurrent presence of N⁵-methyl-L-arginine acetate salt (1 mM), a specific inhibitor of L-arginine transport function, or after perfusion of the coronary vessels with a 0.1% solution of sodium dodecyl sulfate, a detergent that disrupts vascular endothelium. Nonspecific and nonendothelial uptakes (extraction) were 4.2 ± 0.7 and 4.5 ± 0.5%, respectively, and not different.

Isolation and Characterization of Rat Coronary Artery Endothelial Cells. Rat coronary artery endothelial cells (RCAEC) were isolated from 12- to 14-week-old Sprague-Dawley rat hearts. In brief, rat hearts were mounted and perfused retrogradely on a constant-flow Langendorff system with 1 mg/ml collagenase in Hanks’ balanced salt solution (HBSS). RCAEC were obtained by sedimentation and were then activated by washing in HBSS and suspended in Medium 199 (catalog no. 31100-35; Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum, 1% newborn calf serum, 100 U/ml benzylinicillin, and 100 mg/ml streptomycin. Cell suspensions were plated and incubated at 37°C under 5% CO₂. After a 24-h incubation, unattached cells were washed off with HBSS, and remaining cells were cultured to confluence. Cultured RCAEC were characterized by their typical cobblestone morphology and positive staining to fluorescent probe Dil-acetylated low-density lipoprotein (Invitrogen) (Marelli-Berg et al., 2000). For our experiments, RCAEC were cultured for 5 days in the growth medium containing either 5.5 (normal) or 25 mM (high) D-glucose for 5 days with or without simvastatin (1 μM). Studies were also performed using the NADPH oxidase inhibitor apocynin (30 μM), which was added to the cultures 30 min before the experiment. One hour before measuring superoxide, the medium was replaced with 50 μl of Earle’s balanced salt solution containing the same treatment plus H₂DCFDA (10 μM) followed by incubation for 60 min at 37°C. DCF fluorescence was detected using morphometry as discussed above.

Chemiluminescence O₂⁻ generation in cultured RCAEC was measured by using a chemiluminescence assay. Cells were plated in 96-well plates in 5.5 (normal) or 25 mM (high) D-glucose for 5 days with and without simvastatin (1 μM). The medium was then replaced with Earle’s balanced salt solution containing the same treatment plus H₂DCFDA (10 μM) followed by incubation for 60 min at 37°C. DCF fluorescence was detected using morphometry as discussed above.

Western Blot. For analysis of eNOS formation in vitro, we used an NO analyzer (Sievers; GE Analytical Instruments, Boulder, CO). RCAEC in 24-well plates were incubated in 5.5 (normal) or 25 mM (high) D-glucose with and without simvastatin (1 μM). After 5 days, media were processed for the measurement of nitrite (NO₂⁻), the stable breakdown product of NO in aqueous solution, by NO-specific analyzer. Media were deproteinized, and samples containing NO₂⁻ were refluxed in glacial acetic acid containing sodium iodide. Under these conditions, NO₂⁻ was quantitatively reduced to NO, which was quantified by a chemiluminescence detector after reaction with ozone in the NO analyzer.

In vivo studies, we evaluated plasma NO by measuring total nitrite and nitrate levels using the Griess reaction. Blood samples were collected from the jugular vein of control, diabetic, or diabetic simvastatin-treated rat at the end of the experiment, allowed to clot on ice for 30 min, centrifuged at 10,000g for 30 min at 4°C, and frozen at ~80°C until assay. In brief, 210 μl of plasma were incubated with nitrate reductase enzyme (10 μU) and NADPH (12.5 mM) for 30 min at 37°C. The total nitrite in each sample was then determined by the addition of 200 μl of l-glutamate dehydrogenase, 100 mM NaH₄Cl, and freshly prepared 4 mM α-ketoglutarate. The mixture was incubated at 37°C for 10 min followed by the addition of 250 μl of Griess reagent and incubation for another 5 min at 37°C. The absorbance at 543 nm was recorded, and concentrations of NO₂ were calculated from a standard curve constructed using NaNO₂ and NaNO₃ standards. Nitrate/nitrite level was expressed as micromolars (El-Remessy et al., 2003).

Measurement of Nitrotyrosine in Rat Coronary Blood Vessels. Nitrotyrosine immunoreactivity was measured as an indicator for ONOO⁻ formation by immunostaining. The distribution of nitrotyrosine in coronary blood vessels was analyzed using immunolocalization techniques. Heart sections were fixed with 4% paraformaldehyde and then reacted with a polyclonal rabbit antinitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY) followed by Oregon green-conjugated goat anti-rabbit antibody (Molecular Probes).

Data were analyzed using fluorescence microscopy and Ultra View morphometric software to quantify intensity of immunostaining.

Control experiments to demonstrate specificity of the nitrotyrosine antibody were done by processing the sections in the absence of primary antibody and by neutralizing the primary antibody. In brief, the antibody was neutralized by incubation with 10 mM 3-nitrotyrosine in phosphate-buffered saline solution (Cayman Chemical Co., Ann Arbor, MI).

Reactive Oxygen Species Assays. In dichlorofluorescin (DCF) assay, DCF is the oxidation product of the reagent 2, 7’-dichlorofluorescin diacetate (H₂DCFDA; Molecular Probes), a marker of cellular oxidation by hydrogen peroxide, peroxynitrite, and hydroxyl radicals. H₂DCFDA (10 μM) was directly applied to frozen heart sections of 4-week diabetic and control heart sections. Coronary arteries were photographed, and cellular oxidation in the vascular tissue was assayed by quantifying DCF fluorescence intensity using Ultra-View morphometric software (6 fields/heart, n = 6 in each group).

For cell experiments, RCAEC were plated in 96-well plates and incubated in 5.5 (normal) or 25 mM (high) D-glucose for 5 days with and without simvastatin (1 μM). The medium was then replaced with Earle’s balanced salt solution containing the same treatment plus H₂DCFDA (10 μM) followed by incubation for 60 min at 37°C. DCF fluorescence was detected using morphometry as discussed above.

Chemiluminescence O₂⁻ generation in cultured RCAEC was measured by using a chemiluminescence assay. Cells were plated in 96-well plates in 5.5 (normal) or 25 mM (high) D-glucose for 5 days with and without simvastatin (1 μM). Studies were also performed using the NADPH oxidase inhibitor apocynin (30 μM), which was added to the cultures 30 min before the experiment. One hour before measuring superoxide, the medium was replaced with 50 μl of Earle’s balanced salt solution with the same treatment. Then 50 μl of Earle’s balanced salt solution containing 800 μM of highly sensitive luminol derivative L-012 (Wako Chemical, Richmond, VA) was added to each well. Chemiluminescence was measured using an automated microplate reader (Lumistar Galaxy; BMG Lab Technologies, Offenburg, Germany).
nescence (GE Healthcare, Piscataway, NJ). The membrane was stripped and reprobed for β-actin antibody to demonstrate equal loading, and results were analyzed using densitometry and Image J (National Institutes of Health, Bethesda, MD, web site: http://rsb.info.nih.gov/ij/).

**Drugs.** STZ, ACh, SNP, U46619, apocynin, mevalonolactone, l-NAME, and l-arginine were purchased from Sigma-Aldrich (St. Louis, MO). Simvastatin was purchased from Guidelines Integrated Services (Marama, FL). Ketamine HCl and xylazine were purchased from Butler Animal Health Supply (Chicago, IL). l-NAME, ACh, SNP, and U46619 stock solution (10 mM), and serial dilutions to desired concentrations were made in deionized water; l-arginine was dissolved in deionized water. STZ was dissolved in 0.1 M sodium citrate buffer, pH 4.5. Apocynin was dissolved in Earle’s buffer salt solution before treating the cells. Simvastatin was activated by opening the lactone ring by dissolving in 95% ethanol and 0.1 N NaOH, heating at 50°C for 2 h, and neutralizing with HCl to pH of 7.2. It was then freeze-dried and dissolved in sterile deionized water before injection (Gerson et al., 1989). Mevalonic acid lactone was converted to sodium mevalonic acid by solubilization in 0.1 M sodium hydroxide, heating at 50°C for 2 h, and adjustment of the pH to 7.4 with 0.1 M hydrochloric acid (Wagner et al., 2000).

**Data Analysis.** All values are shown as mean ± S.E.M. Maximal relaxation ($E_{\text{max}}$) and half-maximal effective dose ($EC_{50}$) were calculated from individual dose-response curves. $EC_{50}$ values were derived using Prism (GraphPad Software Inc., San Diego, CA). Statistical comparisons between concentration-response curves of control, diabetics, and diabetes treated groups were analyzed by one-way analysis of variance with Bonferroni’s test for comparison between all pairs of groups. In some experiments, statistical differences were determined by Student’s $t$ test. The results were considered significant when $p < 0.05$.

**Results**

**Blood Chemistries of Rats.** Blood glucose, total cholesterol, and total triglycerides were determined in rats after 4 weeks (Table 1). All diabetic rats exhibited elevated blood glucose values. Total cholesterol and total triglycerides were elevated in the diabetic rats and reduced or blocked by treatment with simvastatin or simvastatin + l-arginine.

**Simvastatin Improves Diabetes Impaired Coronary Endothelial-Dependent Vasorelaxation.** We studied the effect of diabetes on coronary endothelial-dependent vasorelaxation by performing ACh concentration-response curves with coronary arteries from diabetic and age-matched control rats. ACh produced concentration-dependent vasorelaxation in coronary arteries from all groups. ACh produced an $E_{\text{max}}$ of 81.1 ± 4.5% in coronary arteries from control rats with an $EC_{50}$ value of 42 ± 0.15 nM. However, coronary arteries from 4 weeks diabetic rats exhibited decreased $E_{\text{max}}$ to ACh (43.5 ± 7.6%) and a shift in the concentration-response curve.

**TABLE 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood Glucose</th>
<th>Total Cholesterol</th>
<th>Total Triglyceride</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>106 ± 12</td>
<td>78 ± 4</td>
<td>53 ± 16</td>
</tr>
<tr>
<td>D</td>
<td>506 ± 24*</td>
<td>121 ± 9*</td>
<td>391 ± 112*</td>
</tr>
<tr>
<td>DS</td>
<td>486 ± 26*</td>
<td>81 ± 6*</td>
<td>135 ± 41*</td>
</tr>
<tr>
<td>DSL</td>
<td>494 ± 22*</td>
<td>72 ± 7*</td>
<td>77 ± 15*</td>
</tr>
</tbody>
</table>

C, control; D, diabetes; DS, diabetes + simvastatin; DSL, diabetes + simvastatin + l-arginine. Values are means ± S.E.M. * $P < 0.05$, diabetic vs. the time-matched control group. †, $P < 0.05$ vs. diabetic group.

**Fig. 1.** Concentration-response curves for the effect of ACh (A), ACh with or without mevalonate (Mev, B), and SNP (C) on coronary arteries from control, diabetic, and diabetic simvastatin-treated rats at 4 weeks ($n = 8$ in each group). Simvastatin treatment improved ACh $E_{\text{max}}$ compared with diabetic-nontreated vessels, whereas l-NAME (100 μM, 30 min) blocked ACh-mediated relaxation in all groups (A). Pretreatment with mevalonate (100 μM, 2 h) blocked the protective effects of simvastatin (B). All vessels from different groups relaxed similarly to SNP (C). Values are expressed as means ± S.E.M.; *, $P < 0.05$ versus control.
to the right with an EC$_{50}$ value of 140 ± 0.3 nM. Simvastatin treatment significantly improved $E_{\text{max}}$ relaxation to ACh to 65.8 ± 5.1%, and EC$_{50}$ was decreased to 130 ± 0.4 nM (Fig. 1A). Treatment of the vessels with L-NAME (100 μM for 30 min) almost completely blocked the vasorelaxant responses to ACh in control, diabetic, and diabetic simvastatin-treated rats.

To determine whether the protective actions of simvastatin are mediated by the inhibition of HMG-CoA reductase, control experiments were performed using mevalonate (Fig. 1B). Incubation of the coronary arteries with mevalonate (100 μM for 2 h) reduced ACh-mediated relaxation in vessels from diabetic simvastatin-treated rats to the level of the untreated diabetic rats. These data suggest that simvastatin improves endothelial function by inhibiting L-mevalonic acid synthesis and, hence, isoprenylation of Rho GTPases, leading to the accumulation of inactive RhoA and Rac-1 in the cytoplasm, which would enhance eNOS expression and inhibit NADPH oxidase.

There is evidence that vascular smooth muscle sensitivity to NO is reduced in diabetes (Maguire et al., 1998). Therefore, we evaluated endothelial-independent relaxation in rat coronary arteries by analyzing concentration responses to the NO donor SNP. Our results showed that coronary arteries from all groups (control, diabetic, and diabetic-simvastatin-treated) relaxed similarly to SNP (Fig. 1C). These data confirmed that, in our model, diabetes impaired endothelial-dependent relaxation only.

Diabetes-induced coronary endothelial dysfunction correlates with the severity and the duration of the disease. Therefore, we examined the effect of ACh on coronary vessel after long-term diabetes (10 weeks). The maximal relaxation caused by ACh was significantly less in the vessels from 10-week-old diabetic rats compared with 4-week-old rats; $E_{\text{max}}$ was reduced to 22.3 ± 0.6% compared with 80.7 ± 3.5% in control; and EC$_{50}$ was increased to 94 ± 0.93 nM compared

with 55.2 ± 2.2 nM control. Coronary arteries from 10-week-old diabetic simvastatin-treated rats demonstrated an improvement in $E_{\text{max}}$ to 47.1 ± 3.9% and a decrease in EC$_{50}$ to 80 ± 0.2 nM (Fig. 2). These data suggest that simvastatin improved diabetes-induced endothelial dysfunction at short- and long-term duration.

**Simvastatin Blocked Diabetes-Induced Oxidative Stress and Nitrotyrosine Formation in Rat Coronary Vessels.** Diabetes-induced oxidative stress in coronary arteries was analyzed by real-time imaging of DCF fluorescence in fresh-frozen heart sections (Fig. 3A). DCF has been used extensively as a marker for both oxidative and nitrative stress. Coronary arteries from 4-week-old diabetic rats demonstrated a significant increase in DCF fluorescence of 23.4 ± 6.0% above control level. Many studies have suggested that O$_2^.$ scavenges NO to form ONOO$^-$. Therefore, we determined the effect of diabetes on ONOO$^-$ formation by nitrotyrosine immunoreactivity in heart sections. Heart sections from 4-week-old diabetic rats demonstrated increase in nitrotyrosine immunoreactivity of 47.0 ± 5.7% above control level (Fig. 3B). Simvastatin treatment of diabetic rats sup-

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**Fig. 2.** Concentration-response curves for the effect of ACh for coronary arteries from control, diabetic, and diabetic simvastatin-treated rats at 10 weeks ($n = 8$ in each group). ACh $E_{\text{max}}$ value from diabetic vessels was significantly lower than control. Simvastatin treatment improved $E_{\text{max}}$ value from diabetic vessels. Values are expressed as means ± S.E.M.; *, $p < 0.05$ versus control.

**Fig. 3.** Effect of diabetes and simvastatin on DCF fluorescence (A) and nitrotyrosine immunoreactivity (B) in rat heart sections ($n = 6$ in each group). Both DCF fluorescence and nitrotyrosine were increased in diabetic coronary vessels. Simvastatin blocked this effect. Values are expressed as means ± S.E.M.; *, $p < 0.05$ versus control.
pressed cellular oxidation and nitrotyrosine formation in heart section to control levels (Fig. 3, A and B).

Simvastatin Blocked High-Glucose-Increased ROS Formation in RCAEC. To further define the impact of the diabetic milieu on the vascular endothelium, we determined the effect of high glucose on ROS produced by RCAEC using DCF fluorescence imaging. The results showed that incubation with 25 mM glucose for 5 days caused an increase in ROS formation of 51.6 ± 4.9% above controls. Concurrent treatment with simvastatin (1 μM) completely blocked high-glucose-induced ROS formation. Simvastatin treatment of RCAEC incubated in 5.5 mM glucose did not affect ROS level (Fig. 4A).

To evaluate the specific contribution of O$_2^-$ in the high-glucose-induced ROS formation in RCAEC, we assessed the effect of high glucose on O$_2^-$ formation via chemiluminescence. High-glucose-incubated RCAEC increased O$_2^-$ production to 40.3 ± 1.5% above controls. Pretreatment with the NADPH oxidase inhibitor apocynin completely blocked the rise in O$_2^-$ in RCAEC. Likewise, simvastatin cotreatment with high glucose substantially blocked the rise in O$_2^-$. Simvastatin and apocynin had no effect on RCAEC incubated with normal glucose (Fig. 4B).

Simvastatin Restored Nitric Oxide Production. To test whether the increased O$_2^-$ formation in high-glucose-treated RCAEC or diabetic rats reduces NO availability, we determined the effect of high glucose and diabetes on NO formation. Exposure of RCAEC to high glucose (25 mM) for 5 days reduced NO production by 39.5 ± 3.2% below control level. Cotreatment with simvastatin (1 μM) restored NO production to control levels. Simvastatin did not affect NO produced by RCAEC in 5.5 mM glucose (Fig. 5A). Measurement of plasma NO levels in diabetic rats showed a significant decrease compared with control. This diabetic effect was prevented by simvastatin treatment (Fig. 5B).

Fig. 4. Effect of simvastatin on DCF fluorescence (A) and simvastatin or apocynin on O$_2^-$ formation (B) by RCAEC incubated with normal and high glucose (n = 7 in each group). High glucose increased ROS and O$_2^-$ formation by RCAEC, which was prevented by simvastatin or apocynin coinubcation. Values are expressed as means ± S.E.M.; *, p < 0.05 versus control.

Fig. 5. Simvastatin treatment preserved NO production in RCAEC treated with high glucose (A, n = 7 in each group) and prevented diabetes-induced decrease in plasma NO levels in rats (B, n = 6 in each group). Simvastatin did not affect NO produced by RCAEC incubated in normal glucose. Values are expressed as means ± S.E.M.; *, p < 0.05 versus control.
Effect of Supplemental L-Arginine on Coronary Artery Endothelial-Dependent Vasorelaxation. Reduced availability and/or impaired transport of L-arginine are possible mechanisms of decreased NO in diabetic vessels and, hence, could reduce endothelial-dependent relaxation. We tested this concept by determining diabetes effect on [3H]L-arginine transport across coronary bed in the Langendorff rat heart preparation under basal and Ach-stimulated conditions (Fig. 6). The specific fractional extraction (uptake) of [3H]L-arginine by coronary endothelium across the heart during the basal state was 13.7 ± 1.6% for control versus 8.8 ± 1.5% for 4-week-old diabetic rat hearts. During infusion of acetylcholine to the hearts and at 5 min, fractional extraction of [3H]L-arginine was increased to 28.9 ± 2.7% in control and 14.6 ± 1.9% in the diabetic hearts. Fractional extraction in these hearts returned to basal levels within 20 min after infusion of acetylcholine. These data indicate that diabetes for 4 weeks decreases L-arginine transport in the coronary vasculature.

To test whether increasing L-arginine availability could reverse diabetes-induced impairment of endothelial-dependent relaxation, studies were performed to determine Ach concentration-response curves in coronary vessels from 4-week-old diabetic rats treated with orally supplemented L-arginine or both L-arginine and simvastatin. Coronary vessels from diabetic rats demonstrated an \( E_{\text{max}} \) of 44.9 ± 6.4% compared with 79.9 ± 5.2% control. The EC\(_{50}\) was increased to 80 ± 1.6 versus 40.9 ± 1.8 nM control. Coronary vessels from diabetic rats treated with supplemental L-arginine showed no significant improvement in Ach maximal relaxation (56.5 ± 0.7%), but the EC\(_{50}\) was decreased to 47.8 ± 1.4 nM (Fig. 7A). However, treatment with both L-arginine and simvastatin resulted in a significant increase in Ach maximal relaxation to 66.1 ± 3.0% compared with 44.9 ± 6.4% in vessels from nontreated diabetic rats. The EC\(_{50}\) was decreased to 22.6 ± 2.6 versus 80 ± 1.6 nM nontreated diabetics (Fig. 7B). There was no significant difference between Ach maximal relaxation in coronary arteries from simvastatin-treated versus L-arginine-treated groups. These results indicate that endothelial-dependent maximal vasorelaxation is improved by simvastatin, but is not further improved by L-arginine supplement. However, the combination of supplemental L-arginine with simvastatin did improve the sensitivity to Ach as indicated by a significant reduction of the EC\(_{50}\) for the Ach-induced vasorelaxation.

Simvastatin Increased Endothelial Nitric-Oxide Synthase Level in Rat Coronary Arteries. To determine whether simvastatin improves NO production through altering eNOS expression, we measured eNOS protein levels from isolated pooled rat coronary arteries of 4-week-old control, diabetic, and simvastatin-treated diabetic rats. Diabetes significantly reduced eNOS expression in coronary vessels. Simvastatin treatment restored eNOS back to control level (Fig. 8).
Endothelial dysfunction is characterized by decreases in NO-dependent vasorelaxation in response to ACh, which is in agreement with studies of large and resistance arteries of STZ diabetic animals (Chakraphan et al., 2005; Kamata et al., 2005).

Epidemiological studies have shown that statins significantly reduce mortality and morbidity in CAD (Shepherd et al., 1995; LIPID Study Group, 1998). Statin treatment has also been shown to improve endothelial-dependent peripheral artery vasodilation (Mercuro et al., 2002). Here we show for the first time that simvastatin attenuates diabetes-induced coronary endothelial dysfunction and significantly improves ACh-mediated vasorelaxation.

A considerable body of evidence implicates oxidative stress as a critical pathogenic element in diabetic endothelial dysfunction. An important characteristic of endothelial dysfunction is inactivation of NO by combining with O$_2^-$ to form peroxynitrite (ONOO$^-$) (Wassman et al., 2002). Our data show that diabetes is associated with increased oxidative stress in coronary vessels as indicated by increases in DCF fluorescence, a marker for ROS, and in nitrotyrosine levels, a marker of ONOO$^-$. Simvastatin treatment substantially reduced diabetes-induced oxidative stress in coronary vessels. Our results are in good agreement with previous reports of statin-antioxidant effect in other tissues (Laufs and Liao, 1998; Laufs et al., 2002; Taubouchia et al., 2005).

Our finding that simvastatin improved endothelial-dependent vasorelaxation in coronary arteries while reducing oxidative stress prompted us to assess the effect of high glucose on ROS formation by RCAEC. Exposure of RCAEC to high glucose increased ROS and O$_2^-$ levels as shown by DCF fluorescence and chemiluminescence, respectively. Both simvastatin and apocynin treatment blocked these effects. Apocynin specifically blocks activity of NADPH oxidase by interfering with its assembly (Stolk et al., 1994), whereas statins inhibit NADPH oxidase activity by blocking activation of the GTP-binding protein p21 Rac-1 (Sumi et al., 2001; Wassman et al., 2001, 2002). In addition, incubation of coronary vessels from diabetic simvastatin-treated rats with mevalonate completely blocked the simvastatin-induced improvement in relaxation to ACh. These data suggest that simvastatin attenuates diabetes-induced endothelial dysfunction and enhances vascular relaxation by a mechanism involving a decrease in O$_2^-$ formation and, hence, an increase in NO availability. This concept was supported by our finding that simvastatin restores high-glucose- and diabetes-impaired production of NO in RCAEC and plasma, respectively. These findings are consistent with earlier studies that showed increases in NO production from statin-treated endothelial cells from aorta and umbilical vein (Kaesemeyer et al., 1999; Laufs et al., 2002).

Another source of diabetes-induced oxidative stress may involve reduced availability of the NOS substrate l-arginine. We have shown previously that reduced extracellular levels of l-arginine cause eNOS uncoupling and increase formation of O$_2^-$ (Ogonowski et al., 2000; El-Remessy et al., 2003). Markedly reduced serum levels of l-arginine, substrate for NOS, have been observed in diabetic rats (Pieper and Peltier, 1995). Our data indicate that diabetes causes decreases in uptake of $[^{3}H]$l-arginine within the coronary arteries at basal conditions and in response to coronary infusion of acetylcholine, suggesting a decrease in l-arginine supply to eNOS. In accordance with this concept, l-arginine supplementation has been shown to augment endothelium-dependent vasodilation through greater production of NO in some systems.

**Discussion**

Diabetics have a 2- to 4-fold increase in risk of developing CAD. Endothelial dysfunction has been suggested to be an early event in diabetic atherosclerosis and is associated with CAD risk. Endothelial cells from different vascular beds exhibit metabolic and structural differences and may be affected differentially by hyperglycemia (Sobrevia and Mann, 1997). In particular, the specific effects of diabetes on the function of the coronary endothelium are not well defined. In the present study, our aim was to characterize the effects of the diabetic milieu on coronary endothelial cells and define the mechanisms by which statin HMG-CoA reductase inhibitors prevent diabetes-induced coronary endothelial dysfunction.

Endothelial dysfunction is characterized by decreases in NO-dependent vasorelaxation. Our data demonstrated that coronary arteries from STZ diabetic rats show significant reductions in NO-dependent vasorelaxation in response to ACh, which is in agreement with studies of large and resistance arteries of STZ diabetic animals (Chakraphan et al., 2005; Kamata et al., 2005).

**eNOS Expression in Rat Coronary Arteries**

![Western blot analysis of eNOS expression in coronary arteries of control, diabetes, and diabetic simvastatin-treated rats (n = 4 in each group). B, results are qualified by densitometry. Simvastatin increased eNOS expression in diabetic coronary arteries. Values are expressed as means ± S.E.M.; *, p < 0.05 versus control.](image)

These data suggest that simvastatin improved endothelial dysfunction via a mechanism that involves an increase of increasing eNOS expression. Similar results were obtained in RCAEC treated with simvastatin (data not shown). In addition, we measured phospho-eNOS from isolated rat coronary arteries to detect the effect of simvastatin on eNOS activity in this vascular bed. There were no differences between diabetic and diabetic simvastatin-treated groups (data not shown). These data suggest that simvastatin enhances NO availability via increasing eNOS expression and not activity (phosphorylation).
increased arginase activity, leading to decreased L-arginine availability to eNOS, it is important to realize that diabetes causes increased activity of tissue arginase, which metabolizes L-arginine to ornithine (Spolarics and Bond, 1989). Thus, arginase can compete with eNOS for L-arginine and decrease NO production (Chicoine et al., 2004). Recent studies in our laboratory have shown that arginase inhibition in diabetic rats and in endothelial cells exposed to hyperglycemia increases nitric oxide production in bovine pulmonary arterial endothelial cells (Chicoine et al., 2004).

In regard to the potential deficiency in the L-arginine supply to eNOS, it is important to realize that diabetes causes increased activity of tissue arginase, which metabolizes L-arginine to ornithine (Spolarics and Bond, 1989). Thus, arginase can compete with eNOS for L-arginine and decrease NO production (Chicoine et al., 2004). Recent studies in our laboratory have shown that arginase inhibition in diabetic rats and in endothelial cells exposed to hyperglycemia increases nitric oxide production in bovine pulmonary arterial endothelial cells (Chicoine et al., 2004).

In summary, our data showed that simvastatin improves ACh-mediated vasorelaxation in diabetic rat coronary arteries and enhances NO formation in high-glucose-treated RCAEC. These effects were accompanied by decreases in oxidative stress. The antioxidant effect of simvastatin has an important role in reducing ONOO⁻ formation and increasing NO availability. We propose that simvastatin improves diabetes-induced coronary endothelial dysfunction through increased eNOS expression and reduced reactive oxygen species and hence increased NO production and availability (Fig. 9).

Although simvastatin treatment did prevent the rise in levels of total cholesterol that was caused by diabetes in our study, we do not consider this action to be important in the improved endothelial-dependent vasodilation. Exposure of diabetic/simvastatin-treated vessels to mevalonate, which increases levels of isoprenylated active RhoA and Rac-1, readily reversed their improved vascular function, indicating that these factors are primarily involved in the vascular dysfunction.

References


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