JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.
JPET Fast Forward. Published on July 18, 2006 as DOI:10.1124/jpet.106.106823

SIMVASTATIN IMPROVES DIABETES-INDUCED CORONARY ENDOTHELIAL DYSFUNCTION

HUDA E. TAWFIK, AZZA B. EL-REMESSY, SURAPORN MATRAGOON,

GUOCHUAN MA, RUTH B. CALDWELL, R. WILLIAM CALDWELL.

Department of Pharmacology & Toxicology (HET, ABE, SM, GM, RBC, RWC)

and Vascular Biology Center (RBC)

Medical College of Georgia

Augusta GA, 30912

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

Copyright 2006 by the American Society for Pharmacology and Experimental Therapeutics.

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Simvastatin improves coronary endothelial dysfunction

Address for correspondence:
R. William Caldwell, Ph.D.
Pharmacology and Toxicology
Medical College of Georgia
1120 15th Street
CB-3624
Augusta, GA 30912
Phone: (706) 721-3384;
Fax: (706) 721-2347
Email: ***wcaldwel@mail.mcg.edu***

Number of text pages: 36

Number of tables: 1

Number of figures: 9

Number of references: 40

Number of words in the abstract: 250

Number of words in the introduction: 641

Number of words in the discussion: 1157

A list of nonstandard abbreviations used in the paper

U46619: 9,11-Dideoxy-11α, 9α-epoxymethanoprostaglandin $F_2\alpha$

H_2DCFDA: 2', 7'-dichlorofluorescin diacetate

RCAEC: Rat coronary artery endothelial cells

ROS: Reactive oxygen species

eNOS: Endothelial NO synthase

K-H buffer: Krebs-Henseleit buffer

HBSS: Hanks' Balance Salts Solution

DiI AcLDL: DiI-acetylated low-density lipoprotein

EBSS: Earle's balanced salt solution

L-NAME: N_ω-Nitro-L-arginine methyl ester hydrochloride

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

3-Hydroxy-3-methylglutaryl CoA reductase inhibitors decrease cardiovascular morbidity in diabetic patients, but the mechanism is unclear. We studied 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 ^{3}H-Larginine. Supplemental L-arginine (50mg/kg/day-oral) did not improve coronary vasorelaxation to ACh. However, SIM treatment (5mg/kg/day-sc) 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 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 ROS 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.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

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 (Cosentino 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 $O_2\cdot^-$ 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 $O_2\cdot^-$. NADPH oxidase, is considered a major source for the production of $O_2^{\cdot-}$ within the vascular wall and cardiomyocyte. $O_2\cdot^-$ inactivates NO to form peroxynitrite ($ONOO^-$) (Beckman et al., 2001). Peroxynitrite can oxidize the NOS co-factor tetrahydrobiopterin (Milstien and Katusic, 1999) and also reduces cellular transport of L-arginine, eNOS substrate for NO production (Ogonowski et al., 2000). These events uncouple the enzyme, which then preferentially increases $O_2\cdot^-$ production over NO production (Milstien and Katusic, 1999). Thus, therapeutic strategies should aim to increase 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

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

reductase and cholesterol biosynthesis that are used extensively to treat patients with hypercholesterolemia (Borghi et al., 2002). Also, 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 appear 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 isoprenylation of the small G protein RhoA, leading to accumulation of inactive RhoA in the cytoplasm and this is in turn allows upregulation of eNOS expression (Laufs and Liao, 1998; 2000). Additionally, 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 (PI3K/Akt) pathway, as has been reported in cultured human endothelial cells (Kureishi et al., 2000). Also, statins may prolong the availability of NO through an anti-oxidant 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 pleiotrophic effects of statins may protect against micro/macrovascular complications of diabetes.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

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.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

MATERIAL AND METHODS

General procedure

Male Sprague–Dawley rats, weighing 250–300 grams at the beginning of the study, were used. Diabetes was induced by i.v 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, orally), 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 ketoacidosis. This treatment did not affect blood glucose levels.

Preparation of rat coronary arteries

Four and ten 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) containing 5 ml K-H buffer and was mounted onto two tungsten wires with a diameter of 40 μm. The small wires were carefully guided through the

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

lumen of the artery and attached to a force-displacement transducer for isometric force measurements. Subsequently, arteries were allowed to equilibrate in oxygenated (95% O_2 and 5% CO_2) K-H buffer. The composition of the K-H buffer is (in mM) 118.3 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$, and 11.0 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 mmHg. 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 v 5 software and a MacLab/4e data acquisition system (AD Instruments).

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 that were precontracted with U46619 (thromboxane A_2 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 (E_{max}, 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

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

cases, ACh or SNP were 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 hours 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 percent 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%.

L-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 units/kg i.v.), and the heart was excised via a left thoracotomy and placed in 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 minutes. 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 results 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 30-40 minutes before the control measurements. The pH (7.4) and PO_2 (>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 i.a. infusion of acetylcholine

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

(1 μM final conc.) for 2 min. A drop in PP of $\geq$ 40% indicated vascular endothelial cell competence.

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

Specific uptake was determined by subtracting uptake occurring during concurrent presence of L-NMMA (1mM), 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 non-endothelial 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 (RCAEC)

RCAEC were isolated from 12 to 14 week-old Sprague–Dawley rat hearts. Briefly, rat hearts were mounted and perfused retrogradely on a constant-flow Langendorff system with 1 mg/ml of collagenase in Hanks' Balance Salts Solution (HBSS). RCAEC were obtained by sedimentation and were then activated by washing in HBSS and suspended in Medium 199 supplemented with 10% fetal calf serum, 10% newborn calf serum, benzylpenicillin 100 U/ml, and streptomycin 100 mg/ml. Cell suspensions were plated and incubated at 37 °C under 5% CO_2. After 24h incubation, unattached cells were washed off with HBSS and remaining cells were cultured to confluence. Cultured RCAEC were characterized by their typical cobblestone

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

morphology and positive staining to fluorescent probe DiI-acetylated low-density lipoprotein (DiI-AcLDL) (Molecular Probes, Oregon USA) (Marelli-Berg et al., 2000). For our experiments, RCAEC were cultured for 5 days in the growth medium containing either 5.5 mM (normal) or 25 mM (high) D-glucose in the presence or absence of 1 μM simvastatin to investigate the effect of simvastatin on ROS and NO.

Nitric oxide assay

For analysis of NO formation *in vitro*, we used an NO analyzer (Sievers). RCAEC in 24-well plates were incubated in 5.5 mM (normal) or 25 mM (high) D-glucose with and without simvastatin (1 μM). After 5 days, media were processed for the measurement of nitrite (NO^-_2), the stable breakdown product of NO in aqueous solution, by NO-specific analyzer. Media were deproteinized and samples containing NO^-_2 were refluxed in glacial acetic acid containing sodium iodide. Under these conditions, NO^-_2 was quantitatively reduced to NO, which was quantified by a chemiluminescence detector after reaction with ozone in the NO analyzer.

For *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 minutes, centrifuged at 10,000 g for 30 minutes at 4 ^{o}C and frozen at -80 ^{o}C until assay. Briefly, 210 μl of plasma were incubated with nitrate reductase enzyme (10 mU) and NADPH (12.5 mmol/L) for 30 minutes at 37°C. Then the total nitrite in each sample was determined by addition of 200 mU of L-glutamate dehydrogenase, 100 mmol/L NH_4Cl, freshly prepared 4 mmol/L of α-ketoglutarate. The mixture was incubated at 37°C for 10 minutes followed by addition of 250 μl of Greiss reagent and incubation for another 5 minutes at 37°C. The absorbance at 543 nm was recorded and concentrations of NO_2 were calculated from a standard

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

curve constructed using $NaNO_2$ and $NaNO_3$ standards. Nitrite/nitrate level was expressed as µM (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 then reacted with a polyclonal rabbit anti-nitrotyrosine 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. Briefly, the antibody was neutralized by incubation with 10 mM of 3-nitrotyrosine in PBS solution (Cayman Chemical Co.).

Reactive oxygen species assays

Dichlorofluorescein (DCF) assay, DCF is the oxidation product of the reagent 2’, 7’-dichlorofluorescin diacetate (H_2DCFDA; Molecular Probes, Eugene, OR), a marker of cellular oxidation by hydrogen peroxide, peroxynitrite, and hydroxy radicals. H_2DCFDA (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).

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

For cell experiments, RCAEC were plated in 96-well plates and incubated in 5.5 mM (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 H_2DCFDA (10 μM) followed by incubation for 60 minutes at 37°C. DCF fluorescence was detected using morphometry as discussed above.

Chemiluminescence, $O_2\cdot^-$ generation in cultured RCAEC was measured by using a chemiluminescence assay. Cells were plated in 96-well plates in 5.5mM (normal) or 25mM (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 (EBSS) with the same treatment. Then 50 μl of EBSS containing 800 μmol/L of highly sensitive luminol derivative L-012 (Wako Pure Chemical Industries, Osaka, Japan) was added to each well. Chemiluminescence was measured using an automated microplate reader (Lumistar Galaxy; BMG Lab Technologies, Offenburg, Germany).

Western blot

For analysis of eNOS, pooled rat coronary arteries were homogenized in a modified RIPA buffer [20 mM Tris-HCl (pH 7.4), 2.5 mM ethylenediamine tetraacetic acid, 50 mM NaF, 10 mM $Na_4P_2O_7$, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1 mM phenylmethyl sulfonyl fluoride]. Total protein concentrations were measured using Bio-Rad protein assay. Protein samples (40 μg) were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, probed with specific antibody against eNOS and phospho-eNOS (Cell Signaling) followed by secondary antibody and enhanced chemiluminescence (Amersham Pharmacia, San Francisco, CA). The membrane was

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

stripped and re-probed for β-actin antibody to demonstrate equal loading and results were analyzed using densitometry and Image J (NIH web site).

Drugs

Streptozotocin (STZ), acetylcholine chloride (ACh), sodium nitroprusside (SNP), U46619, apocynin, mevalonolactone, L-NAME, and L-arginine were purchased from Sigma Aldrich (St. Louis, MO). Simvastatin was purchased from ULTRA-TECH (New Bombay, India). Ketamine HCl and xylazine were purchased from Butler Animal Health Supply. 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 hours 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 hours, 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 ± SE mean. Maximal relaxation (E_{max}) and half-maximal effective dose (EC_{50}) were calculated from individual dose-response curves. EC_{50} values were derived using Graph-Pad Prism. Statistical comparisons between concentration-response curves of control, diabetics, and diabetics treated groups were analyzed by one-way analysis of variance (ANOVA) with Bonferroni's test for comparison between all pairs of groups. In some

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

experiments, statistical differences were determined by S*tudent* T test. The results were considered significant when $p < 0.05$.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

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 maximal relaxation (E_{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_{max} to ACh (43.5% ± 7.6) and a shift in the concentration response curve to the right with an EC_{50} value of 140 ± 0.3 nM. Simvastatin treatment significantly improved E_{max} relaxation to ACh to 65.8% ± 5.1 and EC_{50} was decreased to 130 ± 0.4 nM (Figure 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 (Figure 1B). Incubation of the coronary arteries with mevalonate (100 µM for two hours) 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-

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

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, diabetic-simvastatin treated) relaxed similarly to SNP (Figure 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 maximum relaxation caused by ACh was significantly less in the vessels from 10 weeks diabetic rats compared to 4 weeks; E_{max} was reduced to 22.3% ± 0.6 compared to 80.7% ± 3.5 in control, and EC_{50} increased to 94 ± 0.93 nM compared to 55.2 ± 2.2 nM of control. Coronary arteries from 10 weeks diabetic simvastatin-treated rats, demonstrated an improvement in E_{max} to 47.1% ± 3.9 and a decrease in EC_{50} to 80 ± 0.2 nM (Figure 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 (Figure 3A). DCF has been used extensively as a marker for both oxidative and nitrative stress. Coronary arteries from 4 weeks diabetic rats

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

demonstrated a significant increase in DCF fluorescence of 23.4% ± 6.0 above control level. Many studies have suggested that $O_2 \cdot^-$ scavenges NO to form $ONOO^-$. We therefore determined the effect of diabetes on $ONOO^-$ formation by nitrotyrosine immunoreactivity in heart sections. Heart sections from 4 weeks diabetic rats demonstrated increase in nitrotyrosine immunoreactivity of 47.0% ± 5.7 above control level (Figure 3B). Simvastatin treatment of diabetic rats suppressed cellular oxidation and nitrotyrosine formation in heart section to control levels (Figure 3A, B).

Simvastatin blocked high glucose- increased ROS formation in RCAEC

In order 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 (Figure 4A).

To evaluate the specific contribution of $O_2^{\cdot -}$, in the high glucose-induced ROS formation in RCAEC, we assessed the effect of high glucose on $O_2^{\cdot -}$ formation via chemiluminescence. High glucose-incubated RCAEC increased $O_2^{\cdot -}$ production to 40.3% ± 1.5 above controls. Pre-treatment with the NADPH oxidase inhibitor apocynin completely blocked the rise in $O_2^{\cdot -}$ in RCAEC. Similarly, simvastatin co-treatment with high glucose substantially blocked the rise in $O_2 \cdot^-$. Simvastatin and apocynin had no effect on RCAEC incubated with normal glucose (Figure 4B).

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Simvastatin restored nitric oxide production

In order to test whether the increased $O_2^{\cdot-}$ 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. Co-treatment with simvastatin (1 μM) restored NO production to control levels. Simvastatin did not affect NO produced by RCAEC in 5.5 mM glucose (Figure 5A). Measurement of plasma NO levels in diabetic rats showed a significantly decrease compared to control. This diabetic effect was prevented by simvastatin treatment (Figure 5B).

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 ^{3}H-Larginine transport across coronary bed in the Langendorff rat heart preparation under basal and ACh stimulated conditions (Figure 6). The specific fractional extraction (uptake) of ^{3}H-Larginine by coronary endothelium across the heart during the basal state was 13.7 ± 1.6% for control versus 8.8 ± 1.5% for 4 weeks diabetic rat hearts. During infusion of acetylcholine to the hearts and at 5 min, fractional extraction of ^{3}H-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 minutes 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

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

concentration-response curves in coronary vessels from 4 weeks diabetic rats treated with orally supplemented L-arginine or both L-arginine and simvastatin. Coronary vessels from diabetic rats demonstrated E_{max} of 44.9% ± 6.4 compared to 79.9% ± 5.2 of control. The EC_{50} was increased to 80 ± 1.6 nM versus 40.9 ± 1.8 nM of 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 (Figure 7A). However, treatment with both L-arginine and simvastatin resulted in a significant increase in ACh maximal relaxation to 66.1% ± 3.0 compared to 44.9% ± 6.4 in vessels from non-treated diabetic rats. The EC_{50} was decreased to 22.6 ± 2.6 nM versus 80 ± 1.6 nM of non-treated diabetics (Figure 7B). There was no significant difference between ACh maximal relaxation in coronary arteries from simvastatin versus simvastatin plus L-arginine treated groups. These results indicate that endothelial-dependent maximum 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

In order to determine whether simvastatin improves NO production through altering eNOS expression, we measured eNOS protein levels from isolated pooled rat coronary arteries of 4 weeks control, diabetic, and simvastatin treated-diabetic rats. Diabetes significantly reduced eNOS expression in coronary vessels. Simvastatin treatment restored eNOS back to control level (Figure 8). These data suggest that simvastatin improved endothelial dysfunction via a mechanism that involves an action of increasing eNOS expression. Similar results were obtained in RCAEC treated with simvastatin (data not shown). Also, we measured phospho-eNOS from

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

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).

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

DISCUSSION

Diabetics have a two to four-fold increase in risk of developing coronary artery disease (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 et al., 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 (Kamata et al., 2005; Chakraphan et al., 2005).

Epidemiological studies have shown that statins significantly reduce mortality and morbidity in CAD (Shepherd et al., 1995; LIPID study, 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 $O_2^{\cdot-}$ to form peroxynitrite ($ONOO^-$) (Wassman et al., 2002). Our data show that diabetes is associated with increased oxidative stress

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

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 statins antioxidant effect in other tissues (Laufs and Liao, 1998; Laufs et al., 2002; Tsubouchia 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 $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ 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-

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

arginine cause eNOS uncoupling and increased 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 (Pieper and Dondlinger, 1997). However, not all studies have demonstrated that L-arginine supplementation increases NO production and vasodilation (Kilbourn and Goldfarb, 1999). Indeed, our results showed that L-arginine treatment caused improvement in diabetic coronary artery- ACh sensitivity by shifting the concentration response curve to the left without affecting the E_{max} value. The combination of simvastatin with L-arginine improved E_{max} responses, but only to the same level as simvastatin treatment alone.

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 marked elevation of vascular and hepatic arginase of STZ diabetic rats and in endothelial cells exposed to ROS *in vitro* and both effects were prevented by simvastatin treatment (Romero et al., 2006). Thus, impaired vasorelaxation seen in the diabetic state may be due to the increased arginase activity leading to decreased L-arginine availability to eNOS. We believe that simvastatin improves endothelial-dependent vasorelaxation by enhancing L-arginine availability

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

to eNOS via decreasing tissue arginase activity and decreasing oxidative stress, thereby preventing oxidation of the L-arginine transporter (Ogonowski et al., 2000).

To explore another mechanism by which simvastatin could improve diabetes-induced endothelial dysfunction, we measured eNOS protein levels in the coronary arteries and NO levels in plasma. Diabetes caused significant reductions in coronary eNOS and plasma NO, which were reversed by simvastatin treatment. The likely mechanism by which statins upregulate eNOS is via inhibiting isoprenylation of RhoA and preventing activation of its down stream target Rho kinase, leading to the upregulation of eNOS mRNA expression (Laufs and Liao, 1998). Our finding that mevalonate reversed the effect of simvastatin to improve diabetic coronary relaxation to ACh supports this hypothesis. Upregulation of eNOS may have an important role in the enhanced endothelial-dependent vasorelaxation we observed with simvastatin treatment. Simvastatin, lovastatin, and pravastatin also have been reported to acutely activate eNOS via inducing its phosphorylation (Kaesemeyer et al., 1999; Harris et al., 2004). However, in our study, simvastatin treatment did not affect eNOS phosphorylation in the coronary arteries (data not shown).

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 endothelium-dependent vasodilation. Exposure of diabetic/simvastatin-treated vessels to mevalonate, which increases levels of isoprenolated, active RhoA and Rac-1, readily reversed their improved vascular function indicating that these factors are primarily involved in the vascular dysfunction.

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

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

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 (Figure 9).

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

REFERENCES

Beckman JA, Goldfine AB, Gordon MB, et al. (2001) Ascorbate restores endothelium-dependent vasodilation impaired by acute hyperglycemia in humans. *Circulation* **103**:1618-1623.

Borghi, C, Dormi A, Veronesi M, Immordino V, and Ambrosioni E (2002) Use of lipid-lowering drugs and blood pressure control in patients with arterial hypertension. *J Clin Hypertens* **4**: 277-285.

Chakraphan D, Sridulyakul P, Thipakorn B, Bunnag S, Huxley VH, and Patumraj S (2005) Attenuation of endothelial dysfunction by exercise training in STZ-induced diabetic rats. *Clin Hemorheol Microcirc* **32**:217-226.

Chicoine LG, Paffet ML, Young TL, and Nelin LD (2004) Arginase inhibition increases nitric oxide production in bovine pulmonary arterial endothelial cells. *Am J Physiol Lung Cell Mol Physiol* **287**: L60-L68.

Cipolla MJ (1999) Elevated glucose potentiates contraction of isolated rat resistance arteries and augments protein kinase C-induced intracellular calcium release. *Metabolism* **48**:1015-1022.

Cosentino F and Luscher TF (1998) Endothelial dysfunction in diabetes mellitus. *J Cardiovasc Pharmacol* **32**: S54-S61.

El-Remessy AB, Abou-Mohamed G, Caldwell RW, and Caldwell RB (2003) High glucose-induced tyrosine nitration in endothelial cells: role of eNOS uncoupling and aldose reductase activation. *Invest Ophthalmol Vis Sci* **44**: 3135-3143.

Gerson RJ, MacDonald J, Alberta AW, Kornbrust J, Majka JA, Stubbs J, and Bokelman DL (1989) Animal safety and toxicology of simvastatin and related hyroxy- ethylglutaryl-coenzyme A reductase inhibitors. *Am J Med* **87**: S28-S38.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Gu K, Cowie CC, and Harris MI (1999) Diabetes and decline in heart disease mortality in U.S. adults. *JAMA* **281**:1291-1297.

Harris MB, Blackstone MA, Sood SG, Li C, Goolsby JM, Venema VJ, Kemp BE, and Venema RC (2004) Acute activation and phosphorylation of endothelial nitric oxide synthase by HMG-CoA reductase inhibitors. *Am J Physiol Heart Circ Physiol* **287**: H560-H566.

Kaesemeyer HY, Caldwell RB, Huang J, and Caldwell RW (1999) Pravastatin sodium activates endothelial nitric oxide synthase independent of its cholesterol-lowering actions. *Am Coll Cardiol* **33**:234-241.

Kamata K, Kobayashi T, Matsumoto T, Kanie N, Oda S, Kaneda A, and Sugiura M (2005) Effects of Chronic Administration of Fruit Extract (*Citrus unshiu* MARC) on Endothelial Dysfunction in Streptozotocin-Induced Diabetic Rats. *Biol Pharm Bull* **28**: 267-270.

Kilbourn RG and Goldfarb RD (1999) Can nitric oxide production be modulated by altering L-arginine levels? *Crit Care Med* **27**:2586-2587.

Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, Lefer DJ, Sessa WC, and Walsh K (2000) The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. *Nat Med* **6**:1004-1010.

Landmesser U, Bahlmann F, Mueller M, Spiekermann S, Kirchhoff N, Schulz S, Manes C, Fischer D, de Groot K, Fliser D, Fauler G, Marz W, Drexler H (2005) Simvastatin versus ezetimibe: pleiotropic and lipid-lowering effects on endothelial function in humans. *Circulation* **10**: 2356-2363.

Laufs U and Liao JK (1998) Post-transcriptional regulation of endothelial nitric oxide synthase mRNA stability by Rho GTPase. *J Biol Chem* **273**: 266-271.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Laufs U and Liao JK (2000) Direct vascular effects of HMG-CoA reductase inhibitors. *Trends Cardiovasc Med* **10**:143-148.

Laufs U, Gertz K, Dirnagl U, Bohm M, Nickenig G, and Endres M (2002) Rosuvastatin, a new HMG-CoA reductase inhibitor, upregulates endothelial nitric oxide synthase and protects from ischemic stroke in mice. *Brain Res* **942**:23-30.

LIPID Study, The Long-Term Intervention with Pravastatin in Ischaemic Disease Group (1998) Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. *N Engl J Med* **339**:1349-1357.

Maguire SM, Nugent AG, and McGurk C, et al (1998) Abnormal vascular responses in human chronic cardiac failure are both endothelium dependent and endothelium independent. *Heart* **80**:141-145.

Marelli-Berg FM, Peek E, Lidington EA, Stauss HJ, and Lechler RI (2000) Isolation of endothelial cells from murine tissue. *J Immunol Methods* **244**:205-215.

Mercuro G, Zoncu S, Saiu F, Sarais C, and Rosano GM (2002) Effect of *atorvastatin* on endothelium-dependent vasodilation in postmenopausal women with average serum cholesterol levels. *Am J Cardiol Oct 1* **90**: 747-750.

Milstien S and Katusic Z (1999) Oxidation of tetrahydrobiopterin by peroxynitrite: implications for vascular endothelial function. *Biochem Biophys Res Comm* **263**:681-684.

Ogonowski AA, Kaesemeyer WH, Jin L, Ganapathy V, Leibach FH, and Caldwell RW (2000) Effects of NO donors and synthase agonists on endothelial cell uptake of L-Arg and superoxide production. *Am J Physiol* **278**: C136-C143.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Omori H, Nagashima H, Tsurumi Y, Takagi A, Ishizuka N, Hagiwara N, Kawana M, and Kasanuki H (2002) Direct in vivo evidence of a vascular statin: a single dose of cerivastatin rapidly increases vascular endothelial responsiveness in healthy normocholesterolaemic subjects. *Br J Clin Pharmacol* **54**:395-399.

Pieper GM, and Dondlinger LA (1997) Plasma and vascular tissue arginine are decreased in diabetes: acute arginine supplementation restores endothelium-dependent relaxation by augmenting cGMP production. *J Pharmacol Exp Ther* **283**:684-691.

Pieper GM, and Peltier BA (1995) Amelioration by L-arginine of a dysfunctional arginine nitric oxide pathway in diabetic endothelium. *J Cardiovasc Pharmacol* **25**:397-403.

Pitkanen OP, Nuutila P, and Raitakari OT, et al. (1998) Coronary flow reserve is reduced in young men with IDDM. *Diabetes* **47**: 248-254.

Romero MJ, Platt DH, Caldwell RB, and Caldwell RW (2006) Does elevated arginase activity contribute to diabetes-induced endothelial dysfunction. (Abstract) *FASEB J Abst* **20**: A1125.

Schwartz GG, Olsson AG, Ezekowitz MD, Ganz P, Oliver MF, Waters D, Zeiher A, Chaitman BR, Leslie S, and Stern T (2001) Effects of atorvastatin on early recurrent ischemic events in acute coronary syndromes: the MIRACL study: a randomized controlled trial. *JAMA* **285**:1711-1718.

Shepherd J, Cobbe S, and Ford I, et al (1995) Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. *N Engl J Med* **333**:1301-1307.

Sobrevia L and Mann GE (1997) Dysfunction of the endothelial nitric oxide signaling pathway in diabetes and hyperglycaemia. *Exp Physiol* **82**:423-452.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Spolarics Z, Bond JS. (1989) Comparison of biochemical properties of liver arginase from streptozocin-induced diabetic and control mice. *Arch Biochem Biophys* **274**:426-433.

Stolk J, Hiltermann TJ, Dijkman JH, Verhoeven AJ (1994) Characteristics of the inhibition of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol. *Am J Respir Cell Mol Biol* **11**:95-102.

Sumi D, Hayashi T, Thankur NK, Jayachandran M, Asai Y, Kano H, Matsui H, Iguchi A (2001) A HMG-CoA reductase inhibitor possesses a potent anti-atherosclerotic effect other than serum lipid lowering effects the relevance of endothelial nitric oxide synthase and superoxide anion scavenging action. *Atherosclerosis* **155**:347-357.

Tan KC, Ai VH, Chow WS, Chau MT, Leong L, and Lam KS (1999) Influence of low density lipoprotein (LDL) subfraction profile and LDL oxidation on endothelium- dependent and independent vasodilation in patients with type 2 diabetes. *J Clin Endocrinol Metab* **84**: 3212-3216.

Tsubouchia H, Inoguchia T, Sontaa T, Satoa N, Sekiguchia N, Kobayashia K, Sumimotob H, Utsumic H, and Nawataa H (2005) Statin attenuates high glucose-induced and diabetes-induced oxidative stress in vitro and in vivo evaluated by electron spin resonance measuremen. *Free Radic Biol & Med* **39**: 444- 452.

Wagner AH, Kohler T, Ruckschloss U, Just I, and Hecker M (2000) Improvement of nitric oxide-dependent vasodilatation by HMG-CoA reductase inhibitors through attenuation of endothelial superoxide anion formation. *Arterioscler Thromb Vasc Biol* **20**: 61-69.

Wassman S, Laufs U, Muller K, Konkol C, Ahlbory K, Baumer AT, Linz W, Bohm M, and Nickenig G (2002) Cellular antioxidant effects of atorvastatin in vitro and in vivo. *Arterioscler Thromb Vasc Biol* **22**:300-305.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Wassman S, Laufs U, Baumer AT, Muller K, Ahlbory K, Linz W, Itter G, Rosen R, Bohm M, and Nickenig G (2001) HMG-CoA reductase inhibitors improve endothelial dysfunction in normocholesterolemic hypertension via reduced production of reactive oxygen species. *Hypertension* **37**:1450-1457.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Footnote

This work was supported by grants NIH 1 RO1 HL70215 and RO1 EY 04618 and AHA (SDG)

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE LEGENDS

Figure 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_{max} compared to diabetic non-treated vessels, while L-NAME (100 μM, 30 min) blocked ACh-mediated relaxation in all groups (A). Pre-treatement with mevalonate (100 μM, 2 hours) blocked simvastatin's protective effects (B). All vessels from different groups relaxed similarly to SNP (C). Values are expressed as means ± SEM, $^*p < 0.05$ *vs.* control.

Figure 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_{max} value from diabetic vessels was significantly lower than control. Simvastatin treatment improved E_{max} value from diabetic vessels. Values are expressed as means ± SEM, $^*p < 0.05$ *vs.* control.

Figure 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 ± SEM, $^*p < 0.05$ *vs.* control.

Figure 4:

Effect of simvastatin on DCF fluorescence (A) and simvastatin or apocynin on $O_2\cdot^-$ formation (B) by RCAEC incubated with normal and high glucose (n = 7 in each group). High glucose

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

increased ROS and $O_2 \cdot^-$ formation by RCAEC, which was prevented by simvastatin or apocynin co-incubation. Values are expressed as means ± SEM, *p < 0.05 *vs.* control.

Figure 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 ± SEM, *p < 0.05 *vs.* control.

Figure 6:

Diabetes reduced ^{3}H-L-arginine uptake by coronary vascular bed in isolated Langendorff rat heart under basal and ACh stimulated condition. Values are expressed as means ± SEM, *p < 0.05 *vs.* basal control, **p < 0.05 *vs.* ACh control, #p < 0.05 *vs.* basal control.

Figure 7:

ACh concentration-response curves of coronary arteries from control (n = 8), diabetic (n = 8), and diabetic L-arginine-treated (n = 4) rats (A) or diabetic L-arginine + simvastatin-treated rats (n = 4) (B). L-arginine had no significant effect on ACh-induced relaxation from diabetic rats. Co-treatment of diabetic rats with simvastatin and L-arginine caused a significant increase in ACh E_{max}. Values are expressed as means ± SEM, *p < 0.05 *vs.* control.

Figure 8:

(A): 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 ± SEM, *p < 0.05 *vs.* control.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 9:

Proposed mechanism by which simvastatin improved diabetes-induced endothelial dysfunction.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Table (1):

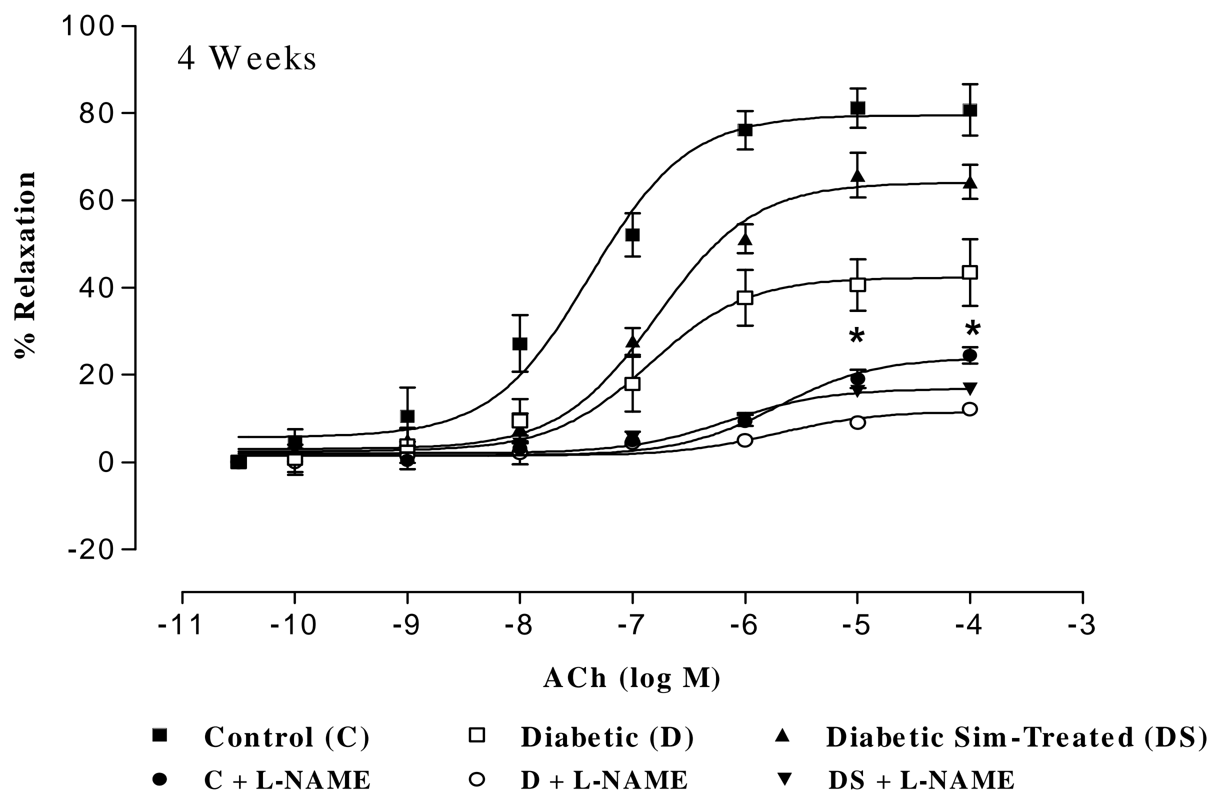
Blood glucose, total cholesterol, and total triglycerides

Group	Blood Glucose (mg/dl)	Total Cholesterol (mg/dl)	Total Triglyceride (mg/dl)
C (control)	106 ± 12	78 ± 4	53 ± 16
D (Diabetic)	506 ± 24 *	121 ± 9 *	391 ± 112 *
DS (Diabetic + Statin)	486 ± 26 *	81 ± 6 †	135 ± 41* †
DSL (Diabetic + Statin + L-arginine)	494 ± 22 *	72 ± 7 †	77 ± 15 †

Effects of diabetes, simvastatin and L-arginine on blood glucose, cholesterol and triglycerides. Simvastatin and L-arginine treatment did not affect blood glucose levels, but simvastatin lowered cholesterol and triglycerides. (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.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

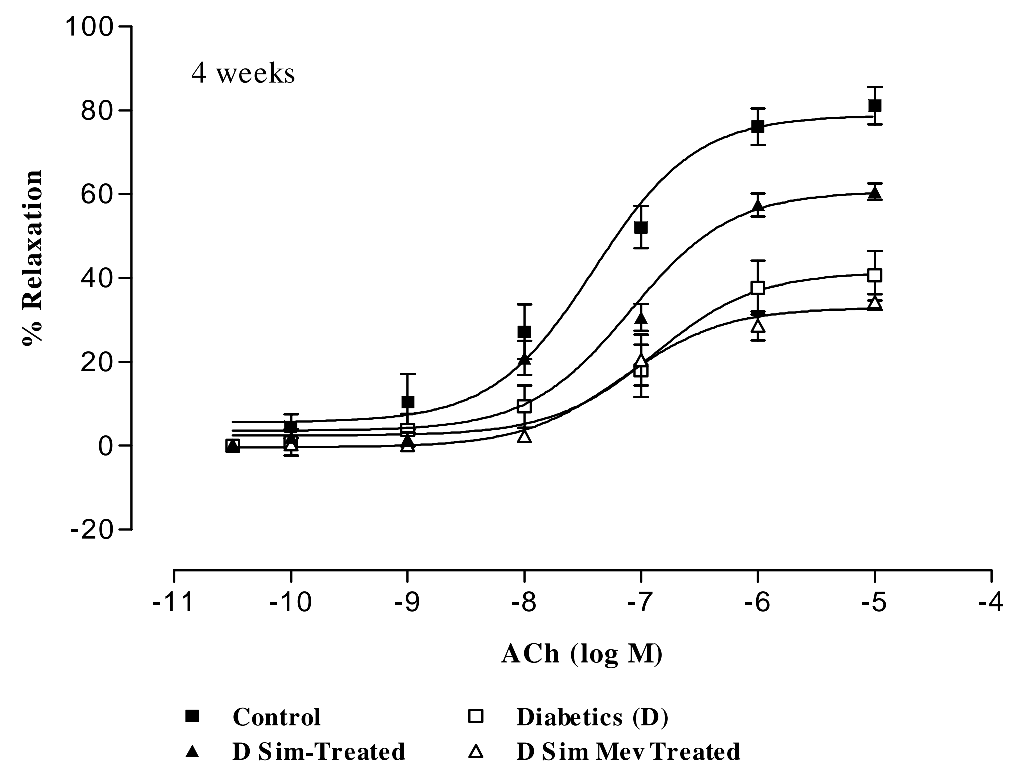
Figure (1A)



JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

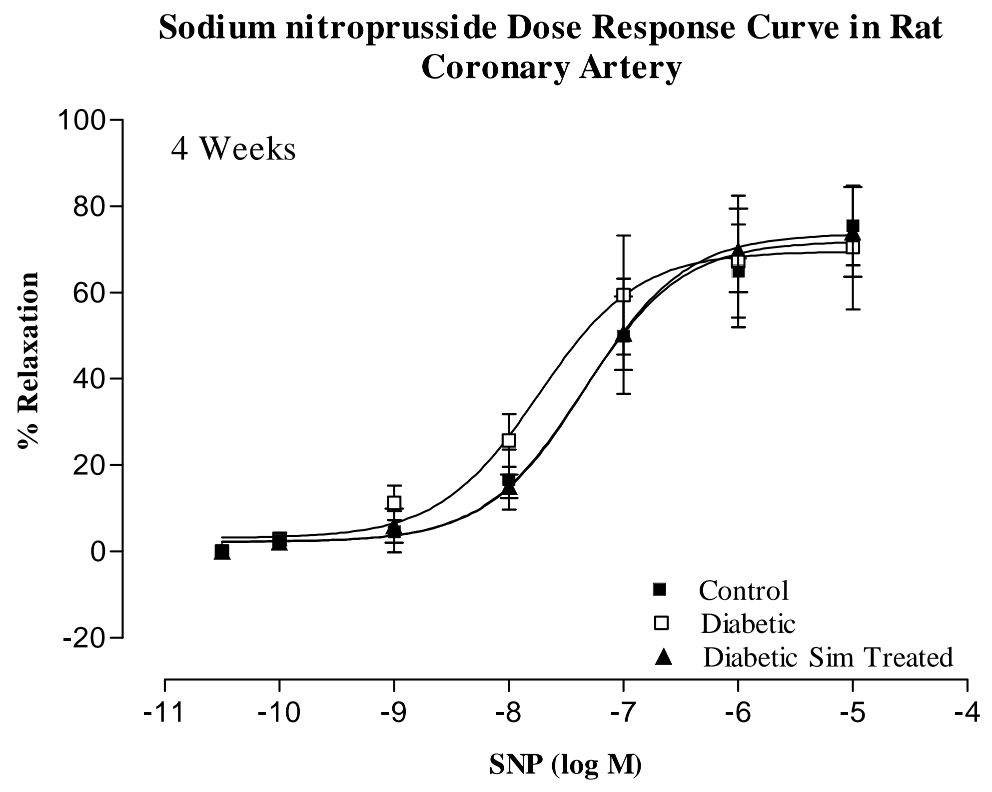
Figure (1B)



JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

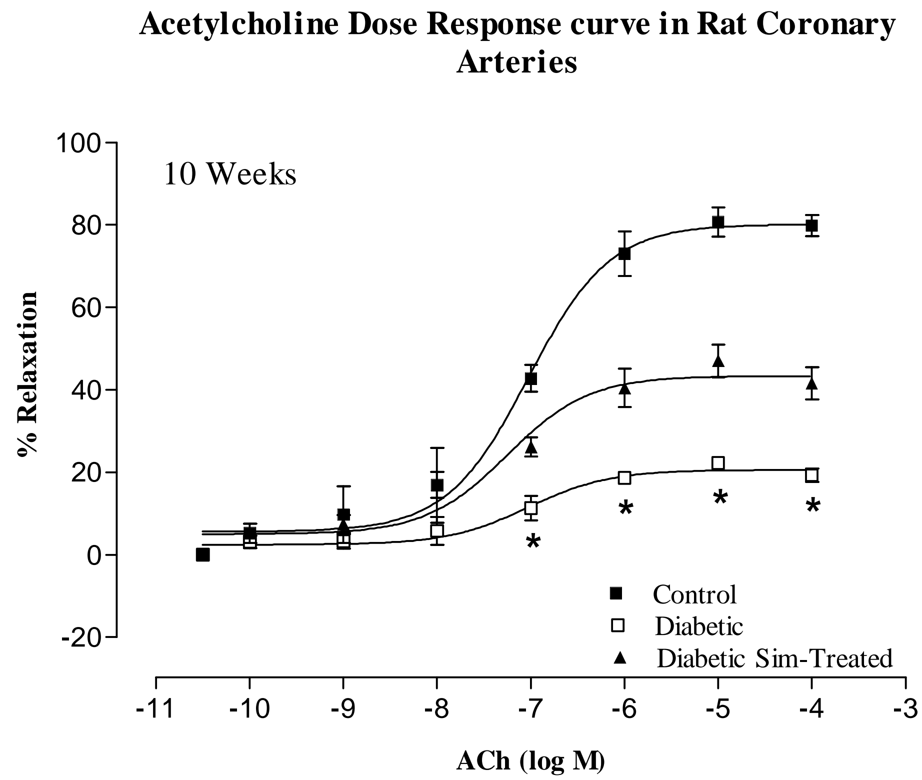
Figure (1C)



JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

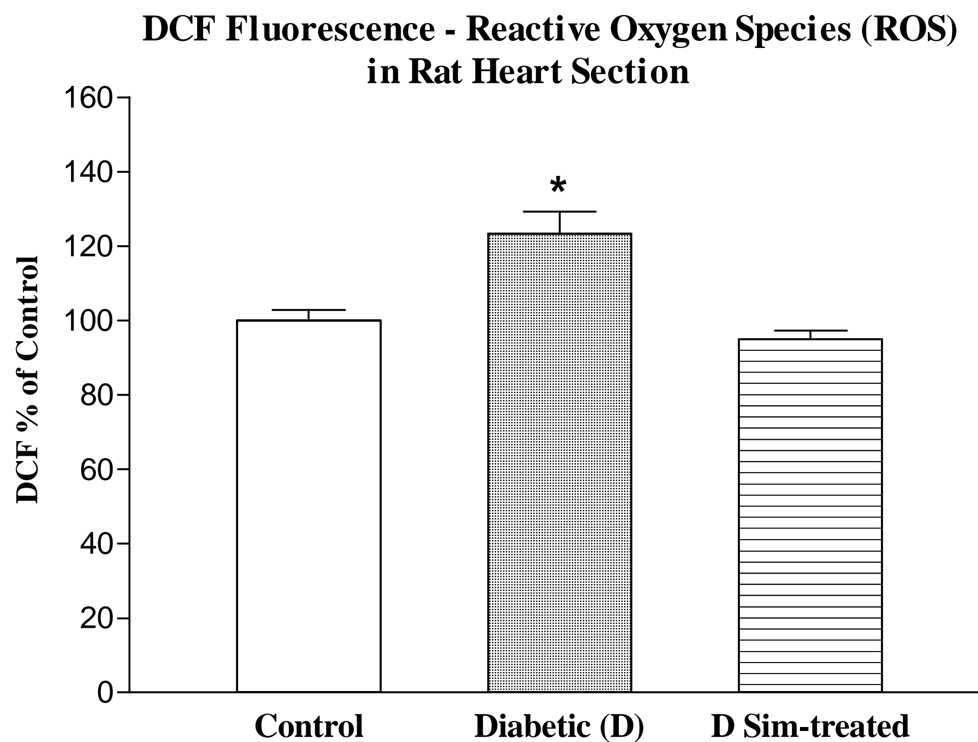
Figure (2)



JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

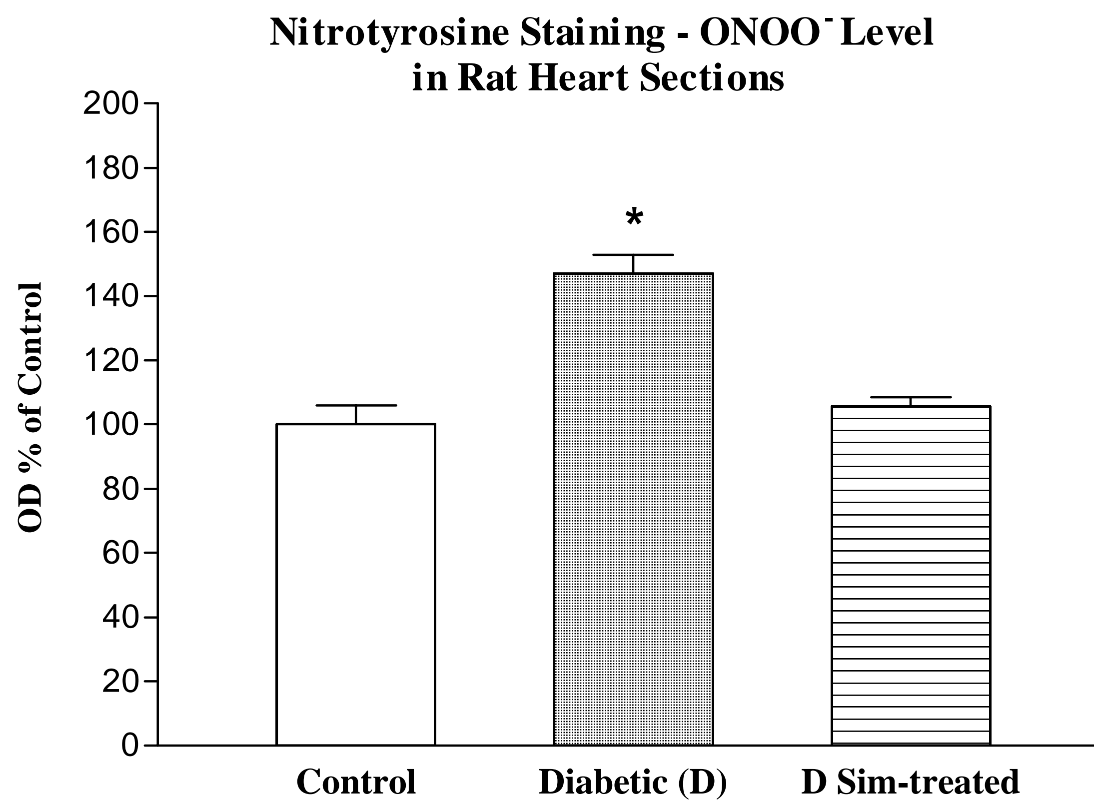
Figure (3A)



JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

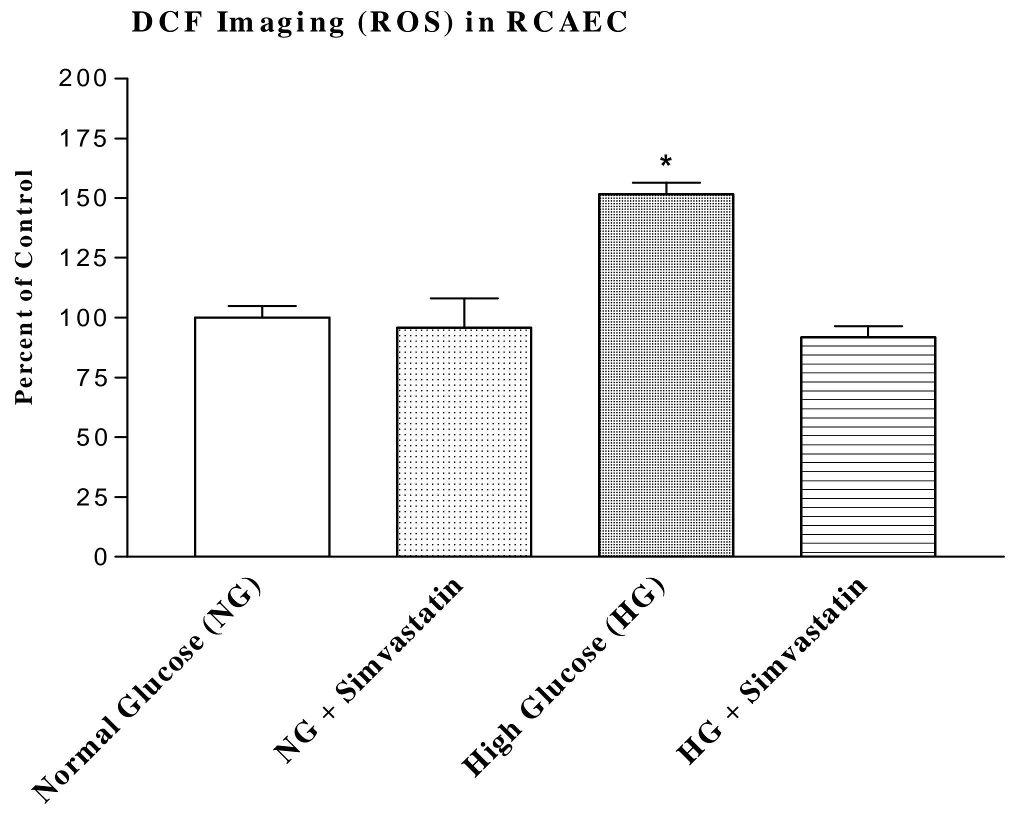
Figure (3B)



JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

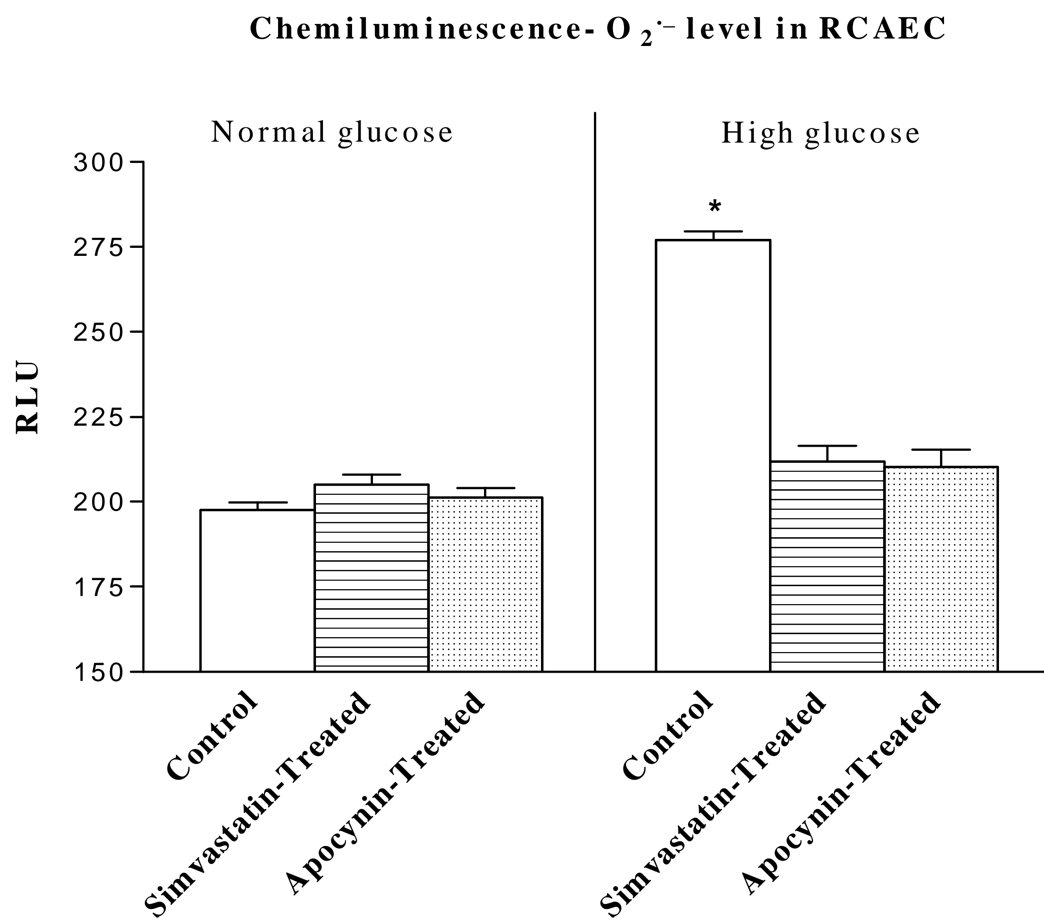
Figure (4A)



JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

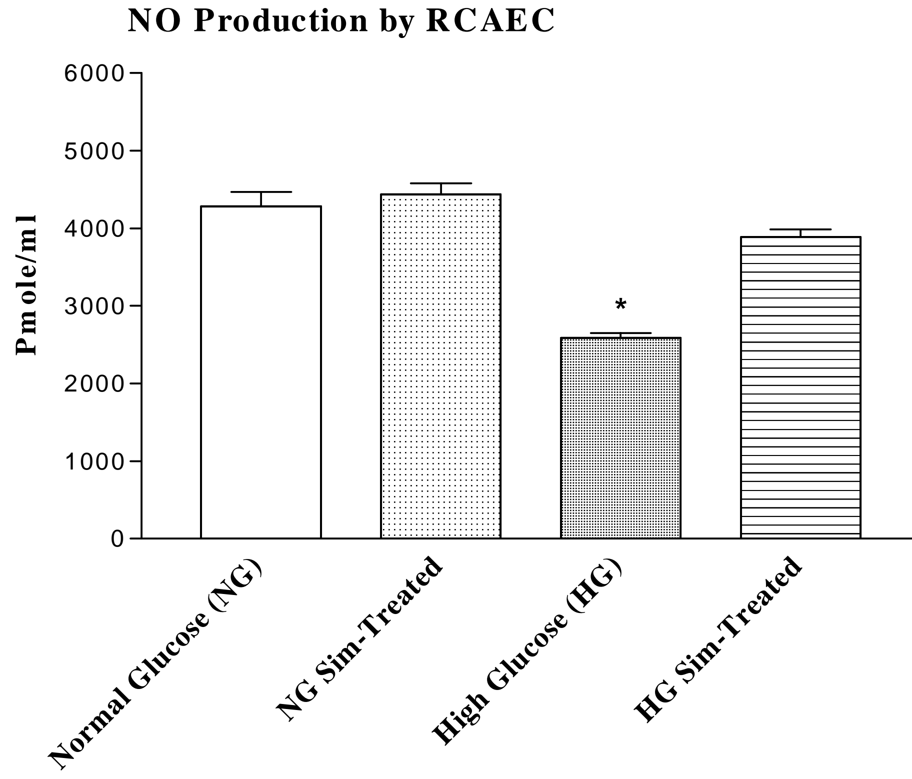
Figure (4B)



JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

Figure (5A)

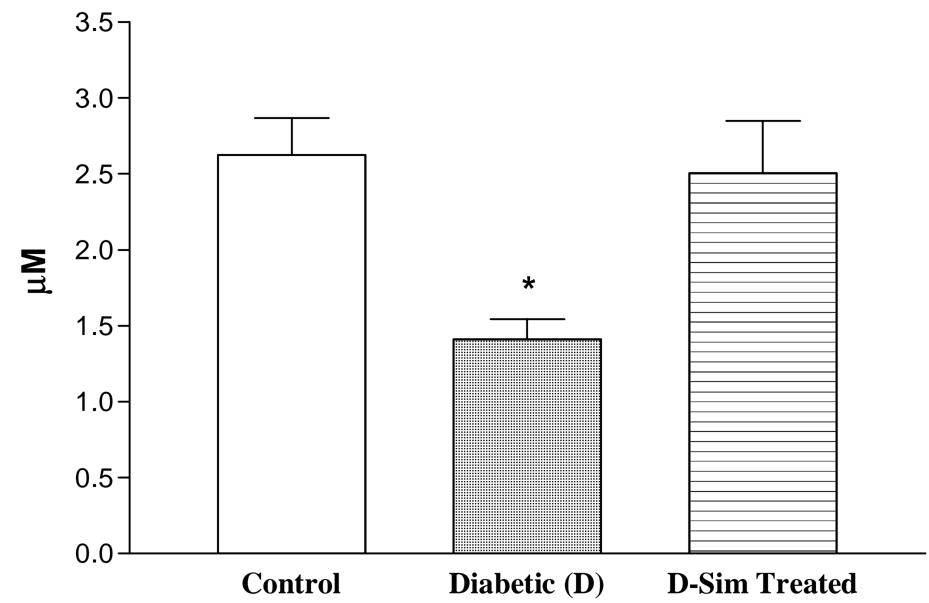


JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

Figure (5B)

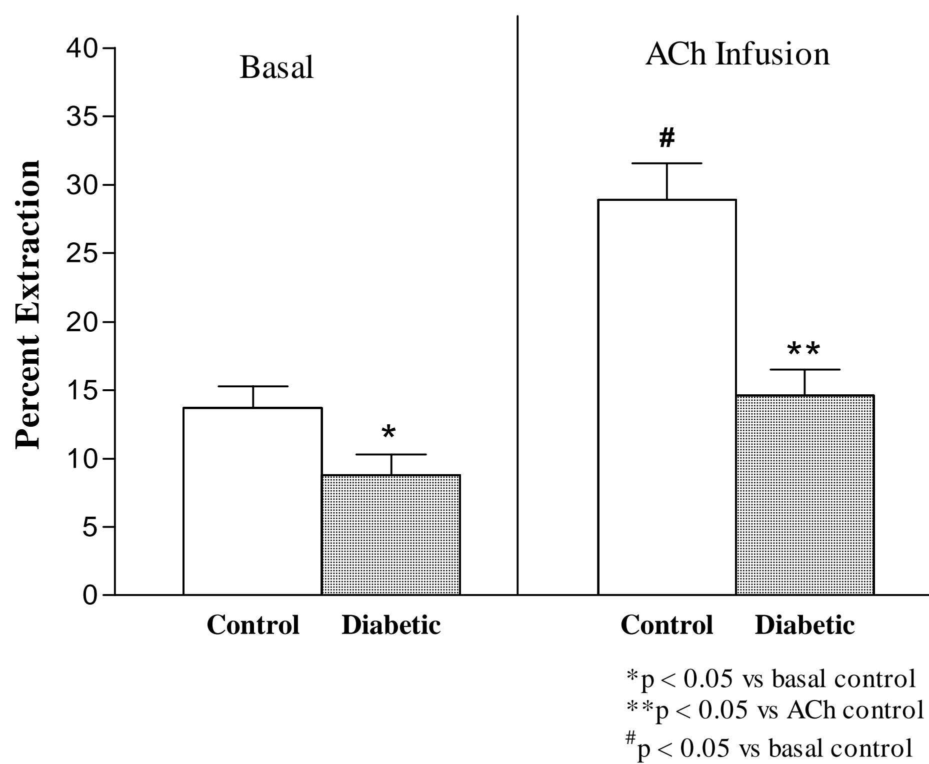
Effect of Simvastatin on Diabetes-Reduced Plasma NO



JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

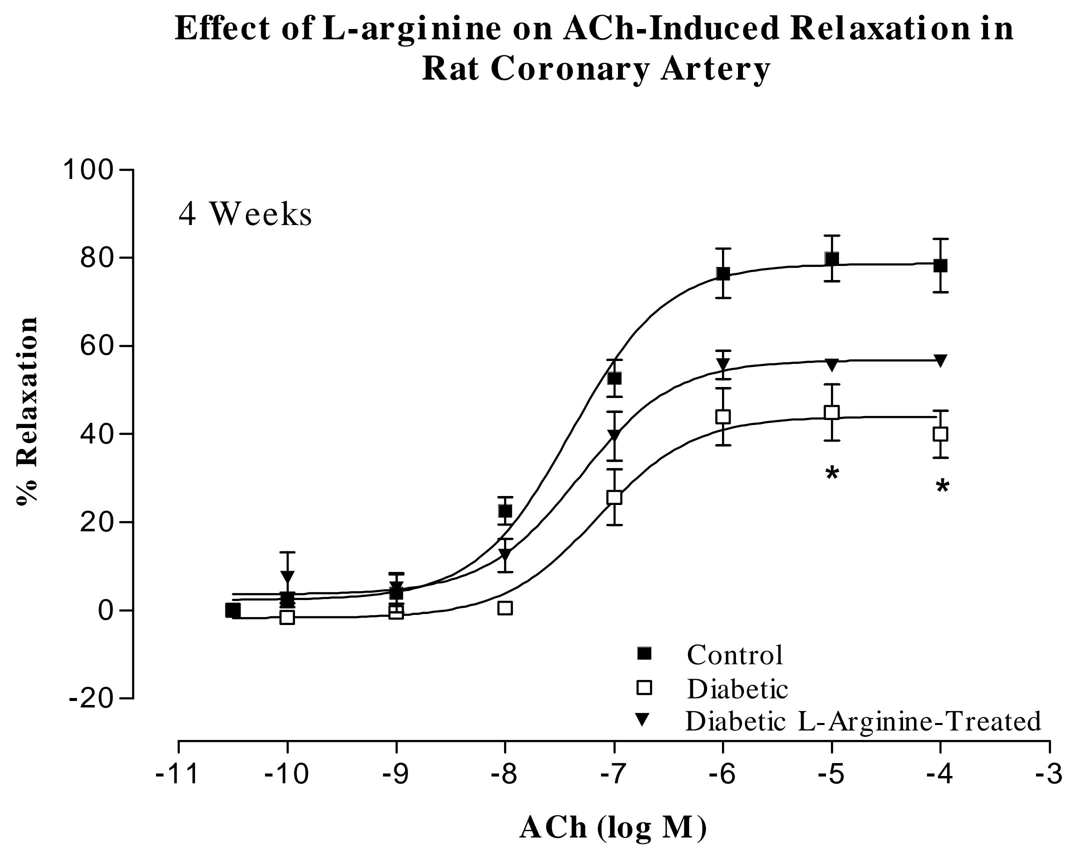
Figure (6)



JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

Figure (7A)



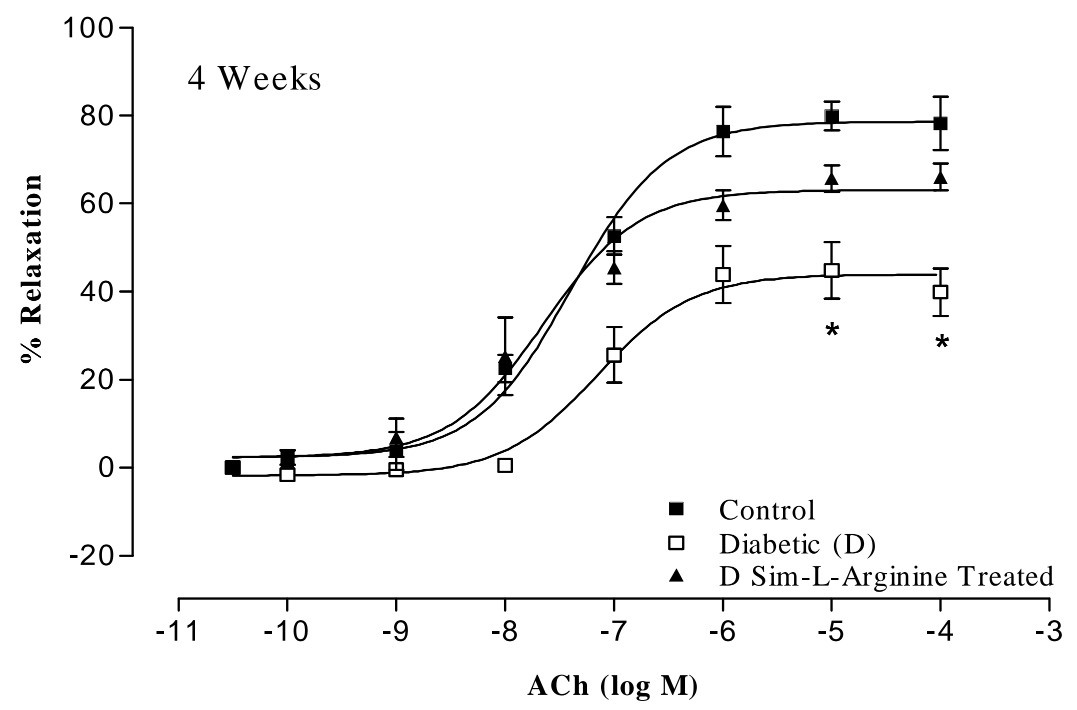
JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

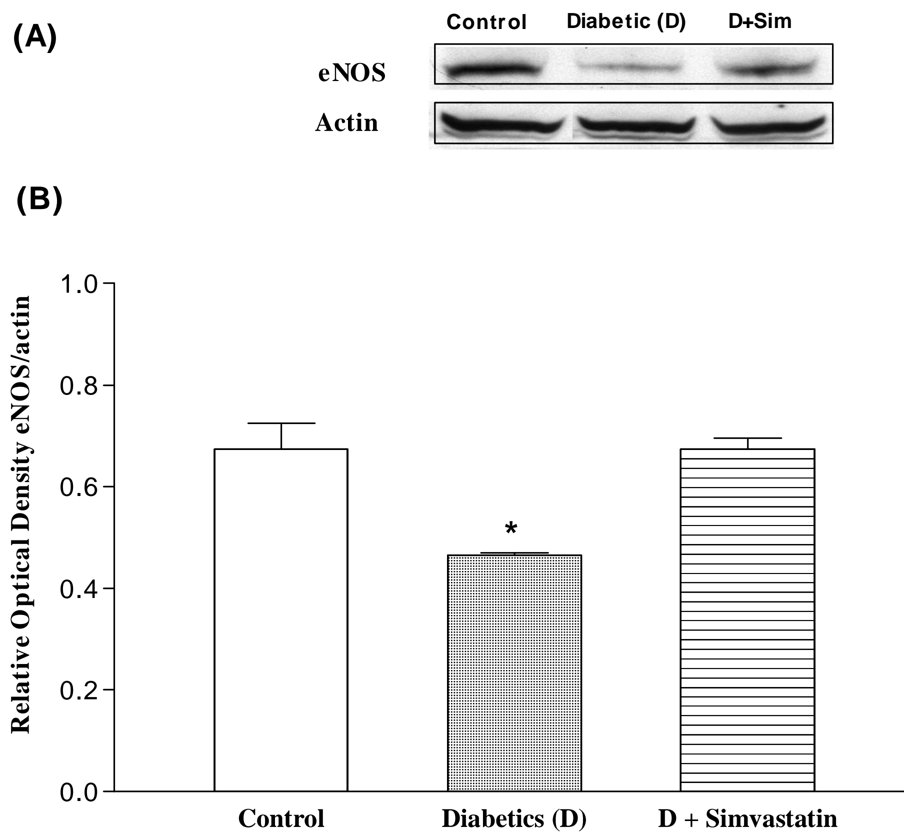
Figure (7B)

Effect of L-arginine and Simvastatin on ACh-Induced Relaxation in Rat Coronary Artery



Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

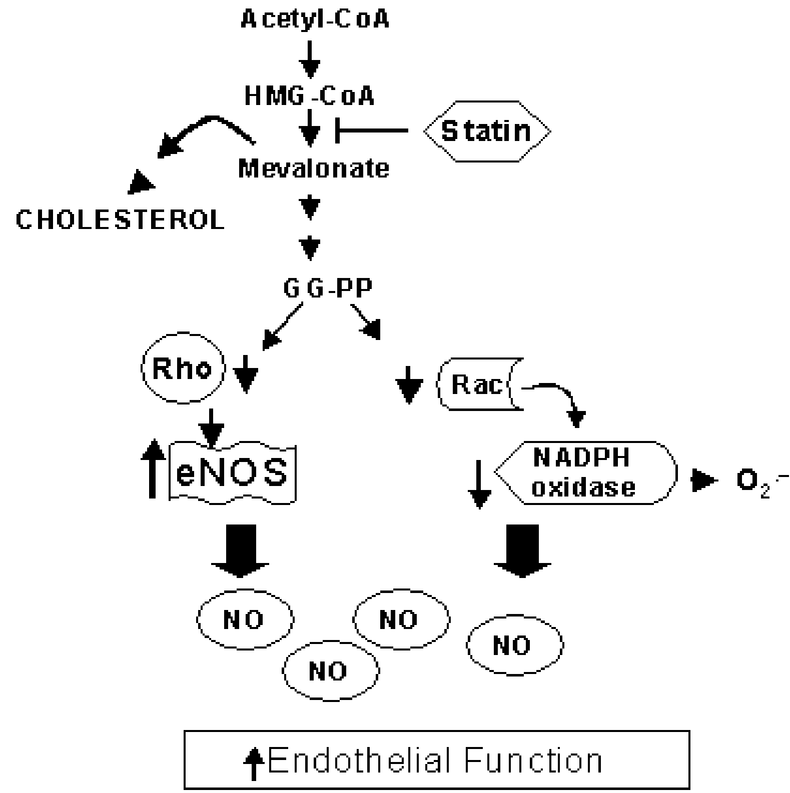
Figure (8)



JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024

Figure (9)



JPET Fast Forward. Published on July 18, 2006 as DOI: 10.1124/jpet.106.106823
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 18, 2024