Increased Endothelin-Induced Ca$^{2+}$ Signaling, Tyrosine Phosphorylation, and Coronary Artery Disease in Diabetic Dyslipidemic Swine Are Prevented by Atorvastatin


Department of Medical Pharmacology and Physiology (D.L.L., B.R.W., M.S.), Department of Internal Medicine (H.K.R., D.J.V., M.S.), Dalton Cardiovascular Research Center (J.L.D.), Diabetes and Cardiovascular Biology Program (H.K.R., J.L.D., M.S.), University of Missouri, Columbia, Missouri; and Department of Physiology (L.C.K.), Brody School of Medicine, East Carolina University, Greenville, North Carolina

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ABSTRACT

Endothelin-1 (ET-1) signaling mechanisms have been implicated in the pathogenesis of excess coronary artery disease in diabetic dyslipidemia. We hypothesized that in diabetic dyslipidemia ET-1-induced coronary smooth muscle calcium (Ca$^{2+}$m) and tyrosine phosphorylation would be increased, and the lipid lowering agent, atorvastatin, would inhibit these increases. Male Yucatan miniature swine groups were treated for 20 weeks: normal low-fat fed control, high-fat/cholesterol fed (hyperlipidemic), hyperlipidemic made diabetic with alloxan (diabetic dyslipidemic), and diabetic dyslipidemic treated with atorvastatin (atorvastatin-treated). Blood glucose values were 5-fold greater in diabetic dyslipidemic and atorvastatin-treated versus control and hyperlipidemic. Total and low-density lipoprotein (LDL) plasma cholesterol in hyperlipidemic, diabetic dyslipidemic, and diabetic dyslipidemic treated with atorvastatin (atorvastatin-treated). Attenuation and hypertriglyceridemia were only observed in diabetic dyslipidemic and atorvastatin. In freshly isolated cells, the Ca$^{2+}$m response to ET-1 in diabetic dyslipidemic was greater than in control, hyperlipidemic, and atorvastatin-treated groups. Selective ET-1 receptor antagonists showed in the control group that the ET$\alpha$ subtype inhibits ET$\alpha$ regulation of Ca$^{2+}$m. There was almost a complete switch of receptor subtype regulation of Ca$^{2+}$m from largely ET$\alpha$ in control to an increased inhibitory interaction between ET$\alpha$ and ET$\beta$ in hyperlipidemic and diabetic dyslipidemic groups, such that neither ET$\alpha$ nor ET$\beta$ antagonist alone could block the ET-1-induced Ca$^{2+}$m response. The inhibitory interaction was attenuated in the atorvastatin-treated group. In single cells, basal and ET-1-induced tyrosine phosphorylation in diabetic dyslipidemic were more than 3- and 6-fold greater, respectively, than in control, hyperlipidemic, and atorvastatin-treated. Attenuation by atorvastatin of coronary disease and ET-1-induced Ca$^{2+}$m and tyrosine phosphorylation signaling with no change in cholesterol provides strong evidence for direct actions of atorvastatin and/or triglycerides on the vascular wall.

Coronary artery disease (CAD) is due to many factors, including lipid infiltration into the vascular wall, which elicits growth and migration of coronary smooth muscle (CSM), atherogenesis, and ultimately, formation of flow-limiting stenoses (Lusis, 2000). A less frequent cause of mortality is coronary artery spasm due to CSM and endothelial dysfunction. The augmented vasoconstriction and impaired endothelial-dependent relaxation are early functional events that precede atheroma development (Davis et al., 1996).

Although CAD is increased 3- to 6-fold in patients with diabetes mellitus (Ruderman et al., 1992), the cellular signaling mechanisms accounting for the increased CAD are still largely unknown. It has been proposed that lipids and the diabetic milieu increase endothelin-1 (ET-1) production and release, in turn signaling an increase in CSM growth and vasoreactivity (Hopfner and Gopalakrishnan, 1999). The ET-1 signaling cascade is multifaceted (for review, see Haynes and Webb, 1998); thus, we have focused on ET-1 receptors, tyrosine phosphorylation, and myoplasmic Ca$^{2+}$ (Ca$^{2+}$m) mobilization. The effects of ET-1 are mediated by

**ABBREVIATIONS:** CAD, coronary artery disease; CSM, coronary smooth muscle; ET-1, endothelin-1; Ca$^{2+}$m, myoplasmic Ca$^{2+}$; LDL, low-density lipoprotein; C, control; F, hyperlipidemic; DF, diabetic dyslipidemic; DF-A, atorvastatin-treated; IVUS, intravascular ultrasound; ELISA, enzyme-linked immunosorbent assay; PSS, physiological saline solution; 80K, K$^+$ PSS; FITC, fluorescein isothiocyanate; AOI, area of interest; ANOVA, analysis of variance; PD-145065, Ac-o-Bhg-Leu-Asp-Ile-Ile-Trp; BQ-123, cyclo(o-Asp-Pro-o-Val-Leu-o-Trp); BQ-788, 6-dimethylpiperidine carboxyl-$\gamma$-methyl-Leu-$N_\text{Bz}$-[methoxy carbonyl]-o-Trp-o-Nle-N-[N-[2,6-dimethyl-1-piperidinyl]carbonyl]-4-methyl-L-leucyl-1-[methoxy carbonyl]-o-tropophyl]-o-norleucine, sodium salt.
two major receptors, ET$_A$ and ET$_B$, which are present on endothelial cells and vascular smooth muscle (Cannan et al., 1995). During pathological states, the role and expression of ET-1 receptors are altered in human (Bacon et al., 1995; Dagassan et al., 1996) and porcine (Hasdai et al., 1997; Katwe et al., 1999) coronary arteries. Surprisingly, the role of ET-1 receptor subtypes in regulation of Ca$_{2+}$ in either healthy or diseased CSM has not been determined.

Despite the long-standing, attractive hypothesis that altered Ca$_{2+}$m underlies diabetic vascular disease (Levy et al., 1994), Ca$_{2+}$m regulation has only recently been studied in smooth muscle from peripheral (Fleischhacker et al., 1999) and coronary (Hill et al., 2001; Wamhoff et al., 2002) arteries from diabetic subjects. None of these studies has focused on tyrosine phosphorylation and Ca$_{2+}$m, which seem especially important because of their critical roles in both vascular smooth muscle contraction (Di Salvo et al., 1993) and growth (Yamawaki et al., 1998). Furthermore, because there is a strong dependence of ET-1-induced Ca$_{2+}$m signaling on tyrosine phosphorylation in CSM (Liu and Sturek, 1996), both events would be hypothesized to be increased in accelerated coronary atheroma occurring in diabetic dyslipidemia.

Because of the numerous cellular signaling mechanisms involved and the severity of CAD in diabetic dyslipidemia, it is exceedingly important to provide clinical intervention early in the progression of CAD. Lipid management drugs (statins and fibrates) have become effective pharmacological interventions. Simvastatin effectively decreased cholesterol and myocardial infarction in diabetic patients (Pyorila et al., 1997). Furthermore, gemfibrozil (fibrate) therapy decreased the risk of major cardiovascular events in nondiabetic patients with CAD by increasing high-density lipoprotein cholesterol and decreasing triglycerides without lowering low-density lipoprotein (LDL) cholesterol levels (Rubins et al., 1999). Importantly, in nondiabetic patients with relatively normal levels of cholesterol, pravastatin decreased cardiovascular mortality, while having minor effects on plasma cholesterol (Tonkin et al., 1998). Thus, direct actions of statins on the vascular wall have been proposed to explain this beneficial effect independent of plasma cholesterol lowering (Koh, 2000). Several statins (pravastatin, simvastatin, and atorvastatin) decrease Ca$_{2+}$m release (Tesfamariam et al., 1999), vasoreactivity (Tesfamariam et al., 1999), and proliferation (Negre et al., 1997; Koh, 2000) of vascular smooth muscle upon in vitro treatment; however, there have been no studies on the effect of chronic in vivo treatment with statins on coronary artery Ca$_{2+}$m in diabetic dyslipidemia. We reported that a miniature swine model closely mimics extreme stages of diabetic dyslipidemia in humans (Dixon et al., 2002; Boullion et al., 2003; Otis et al., 2003) and shows increased fatty streaks, coronary vascular dysfunction in vitro (Dixon et al., 1999), and altered Ca$_{2+}$m regulation (Hill et al., 2001; Wamhoff et al., 2002), but we did not determine the association with tyrosine phosphorylation nor clinically relevant measures of CAD.

In the present study, we tested the hypothesis that altered ET-1 receptor regulation, tyrosine phosphorylation, and Ca$_{2+}$m regulation may in part underlie the beneficial, direct actions of statins on the vascular wall, independent of plasma cholesterol. A novel aspect of normal CSM Ca$_{2+}$m regulation independent of CAD is our finding that the ET$_B$ receptor negatively regulates, i.e., attenuates, the Ca$_{2+}$m response to ET-1. We show that in diabetic dyslipidemia both ET-1-induced signaling by tyrosine phosphorylation and the inhibitory interaction between ET$_A$ and ET$_B$ receptor subtypes in regulation of Ca$_{2+}$m were increased, such that neither ET$_A$ nor ET$_B$ antagonist alone could block the ET-1-induced Ca$_{2+}$m response. Atorvastatin inhibited these increases in tyrosine phosphorylation and the inhibitory interaction between ET$_A$ and ET$_B$ receptors. We further provide clinically relevant intravascular ultrasound measures of CAD showing increased coronary atheroma in diabetic dyslipidemia, which was also prevented by atorvastatin.

Materials and Methods

Animals. Procedures involving animals were approved by the Animal Care and Use Committee of the University of Missouri and fulfilled with those approved by the American Veterinary Medical Association Panel on Euthanasia. The animals were treated similarly to previous published methods (Wamhoff et al., 2002; Boullion et al., 2003; Otis et al., 2003). Sexually mature male Yucatan miniature swine between 12 to 16 months of age were obtained from the Sinclair Research Center (Columbia, MO). The animals were anesthetized with the following drugs: 0.05 mg/kg atropine, 6.6 mg/kg telazol, and 2.2 mg/kg xylazine; the level of anesthesia was subsequently maintained with isoflurane gas (up to 4%). A vascular access port was implanted and used for blood sampling and alloxan (125 mg/kg; Aldrich Chemical Co., Inc., Milwaukee, WI) injection (Otis et al., 2003). Blood glucose was measured in samples obtained from an ear vein 1 to 2 h postprandial once per week for the 20 weeks of the study. The blood glucose target range for the diabetic animals was 250 to 350 mg/dl and was maintained by adjusting daily insulin and food dosage (Boullion et al., 2002). Pigs were euthanized by isoflurane overdose and removal of the heart. Diet. The control group (control, C; n = 5) was fed only Minipig chow (Purina Mills, Inc., St. Louis, MO). High-fat, high-cholesterol fed pigs (hyperlipidemic, P; n = 6) were fed an atherosclerotic diet (Minipig chow supplemented with 2% cholesterol by weight, coconut oil, corn oil, and sodium cholate) (Dixon et al., 1999). Diabetic dyslipidemic pigs were alloxan-treated and fed the high-fat/cholesterol atherogenic diet (diabetic dyslipidemic, DF; n = 5). The lipid lowering drug atorvastatin (40 mg twice daily; Parke-Davis Corp.) was given to a diabetic dyslipidemic group (atorvastatin-treated, DF + A; n = 5) with their meal. All groups were fed twice daily and had free access to water. The initial amount of minipig chow was 350 g/feed of the atherogenic diet groups (hyperlipidemic, diabetic dyslipidemic, and atorvastatin-treated) and adjusted to maintain growth of the pig at the level of controls receiving 525 g of Minipig chow/feeding (Boullion et al., 2003).

Lipid Measures (Dixon et al., 1999, 2002). Total cholesterol or triglyceride levels in plasma were assayed directly by standard enzymatic kit (Sigma-Aldrich, St. Louis, MO). For lipoprotein cholesterol and triglyceride levels, fresh plasma samples (1 ml) were chromatographed by fast protein liquid chromatography on a Superox 6 column (HR 16; Pfizer Central Research, Sandwich, Kent, UK) and eluted with (in w/v) 0.9% NaCl, 0.01% Tris, 0.01% EDTA, 0.02% sodium azide, pH 7.6. Fractions (2 ml) were collected and assayed for protein (A$_{280}$) and for cholesterol (standard enzymatic kit). For lipoprotein analysis, the cholesterol and protein profiles for every pig within a treatment group was averaged and plotted versus fraction number. For LDL, lipid content fractions from each pig corresponding to these lipoproteins were collected and assayed for cholesterol and triglyceride concentration by standard enzymatic assay.

Intravascular Ultrasound (IVUS). In vivo measures of coronary morphology were conducted in the Research Animal Angiography Laboratory in the College of Veterinary Medicine. Under isoflurane anesthesia, the right femoral artery was accessed with an 18-gauge needle via percutaneous puncture or after direct exposure.
of the artery. A 0.035-inch J-guide wire was advanced 10 to 15 cm into the artery, and then an introducer and 8F sheath were inserted over the wire into the artery. An 8F Amplatz L 0.75 or 1.5 guiding catheter was inserted through the sheath and advanced to the site of Valsalva or the right coronary artery over a 0.035-inch flexible wire. The guiding catheter was attached to a manifold assembly that allowed continuous blood pressure monitoring, 0.9% saline flush, drug injection, and contrast (Hypaque; Mallinkrodt Medical, St. Louis, MO) injection. An angioplasty guidewire (0.014- or 0.018-inch diameter) was placed into the left anterior descending and circumflex arteries under fluoroscopic guidance. Standard anterior-posterior, right anterior oblique 30, and left anterior oblique 30 angiographic images were obtained to verify placement in left anterior descending and circumflex arteries. The right coronary artery was not interrogated with IVUS to avoid uncertain effects of contrast medium on CSM cells used for functional studies. The IVUS catheter (30 MHz, UltraCross 3.2; Boston Scientific, Boston, MA) was advanced into the right coronary artery (Hewlett Packard, Palo Alto, CA) was advanced over the angioplasty guidewire 30–60 mm distally through the arteries. Precise control of IVUS catheter movement was enabled by an automated pullback device that moved at 0.5 mm/s, thereby obtaining “serial sections” of ultrasound dimensions along the artery to quantify morphological changes indicative of CAD.

We conducted a segmental analysis at 1-mm intervals during the automated pullback to define the presence of CAD as atheroma, thrombus, dysfunction/spasm, and “3-layer” (Hodgson et al., 1993). Plaque calcification, although easily defined as echogenicity greater than adventitia and “shadowing” lateral to the lesion, was not noted in this model representing early stages of atherosclerosis. Thus, we defined atheroma as any fibrous or soft plaque less echogenic than the adventitia, thrombus as a scintillating mass with microchannels and moving in an undulating manner, and dysfunction/spasm as acute, focal narrowing of the lumen without differences in echogenicity. Concentric atheroma was the “3-layer” appearance described as a composite of the thin echogenic layer adjacent to the lumen, echolucent middle layer, and echogenic outer layer of Advanta. These characteristics were easily resolved as distinct from the nonlayered appearance of all arteries from control pigs. Luminal area was defined by fine scintillations from red blood cells and larger scintillations/turbulence upon injection of saline.

**ET-1 Extraction and Quantification by ELISA.** Approximately 30 ml of blood were collected at the time of euthanasia and centrifuged at 10,000g for 10 min at 4°C to separate plasma from cells. Plasma protease inhibitor cocktail was added to the plasma (1 µM/ml plasma) to prevent degradation of ET-1 by proteases. The protease inhibitor contained 1 µM final concentrations of leupeptin, aprotinin, pepstatin, and phenylmethylsulfonylfluoride. Approximately 100 mg of the right, left anterior descending, and left circumflex coronary arteries were frozen at −80°C on the day of tissue harvest for ET-1 tissue assays. Tissue was homogenized in ethanol acid buffer (100 mg of tissue/ml of buffer), and ET-1 was extracted. Tissue extracts were passed through Amersham C18 columns, eluted, evaporated, and dissolved in ELISA assay buffer. ET-1 ELISA assays for plasma and coronary artery tissues were performed per the manufacturer’s guidelines (Endothelin-1 ELISA system; Amersham Biosciences, Piscataway, NJ).

**CSM Cell Isolation.** Segments of the distal right coronary artery were triaged to fail and then removed into tissue culture and stored overnight at 3–5°C in sterile culture type of medium. Experiments were performed on cells acutely isolated by enzymatic dispersion of the cold stored artery (Liu and Sturek, 1996; Hill et al., 2000; Hill et al., 2001; Wamhoff et al., 2002).

**Fura-2 Digital Imaging of Ca2+.** Acutely dispersed cells were incubated 30 min with 2.5 µM Fura-2-ester (Molecular Probes, Inc., Eugene, OR) and Ca2+ responses of single CSM cells were assessed using the InCa2+ calcium imaging system and version 1.2 software (Intracellular Imaging, Inc., Cincinnati, OH) (Hill et al., 2000, 2001). Cells acutely dispersed from cold-stored arteries have identical Ca2+ responses as cells dispersed from arteries harvested within 2 h of euthanizing the pig (Liu and Sturek, 1996; Hill et al., 2000). Data were expressed as a ratio of the emitted light intensity at 340 and 380 nm, rather than absolute Ca2+ as detailed previously (Liu and Sturek, 1996; Hill et al., 2000). Endothelin-1 (Peninsula Labs, San Carlos, CA) was dissolved in 0.01 N acetic acid (Fischer Scientific, Inc., Fair Lawn, NJ) to a final stock concentration of 10−4 M. BQ-123 and BQ-788 (Peptides International, Inc., Louisville, KY) and PD-145065 (Sigma/RBI, Natick, MA) were each dissolved in dimethyl sulfoxide to a final stock concentration of 10−2 M. Cells were bathed in physiological saline solution (PSS) which contained: 2.0 mM CaCl2, 143 mM NaCl, 1 mM MgCl2, 5 mM KCl, 10 mM HEPES, 10 mM glucose, pH 7.4. Drugs were added to PSS to the final concentrations. Membrane depolarization-induced Ca2+ influx was elicited by depolarization with 80 mM K+ (PSS 80K) in which NaCl was replaced equimolar with KCl (Liu and Sturek, 1996; Hill et al., 2000, 2001; Wamhoff et al., 2002).

**Single Cell Digital Imaging of Phosphotyrosine.** The phosphotyrosine immunofluorescence protocol was described in detail previously (Lee and Sturek, 2002). Briefly, to test for ET-1-induced tyrosine phosphorylation, cells were treated for 2 min with ET-1, followed by PSS and 80 mM K+ containing 100 µM sodium orthovanadate to block phosphatase activity. Cells were fixed with 2% paraformaldehyde (2 g/100 ml) and rinsed in phosphate-buffered saline, containing 2.7 mM KCl, 1.5 mM KH2PO4, 137 mM NaCl, and 8.0 mM NaHPO4. Cells were permeabilized with 0.1% Triton X-100 in saline, rinsed three times with saline, and incubated with monoclinal anti-phosphotyrosine antibody conjugated with fluorescein isothiocyanate (FITC; clone PT-66; Sigma-Aldrich) for 45 min. Cells were then incubated with anti-fluorescein Alexa-488 (excitation/ emission 485/535 nm; Molecular Probes) conjugate for 45 min to amplify the FITC signal, thus increasing signal/noise ratio in single cell measurements.

Imaging of phosphotyrosine density was done using a wide-field epifluorescence microscope (Nikon Diaphot, Garden City, NY). Images were collected using volume scan software (Vaytek, Inc., Fairfield, IA) 0.5 µm apart in the z-axis for deconvolution analysis using the nearest neighbor algorithm (Hill et al., 2000; Lee and Sturek, 2002). Camera shutter time was held constant for all images, therefore permitting the comparison of the absolute fluorescence intensity as an index of tyrosine phosphorylation. All digital imaging processing and quantification was done using Image-Pro Plus software (Media Cybernetics, Silver Springs, MD). Briefly, an area imaging processing and quantification was done using Image-Pro Plus software (Media Cybernetics, Silver Springs, MD). Briefly, an area

**Statistics and Data Analysis.** Data are expressed as mean ± standard error, and n represents the number of cells in each group. Comparison of more than two groups was made by two-way analysis of variance (ANOVA). The pairwise multiple comparison procedures were compared by Bonferroni’s method. Treatment groups were considered significantly different if P < 0.05. IVUS data that were not normally distributed were analyzed by Kruskal-Wallis one-way ANOVA on Ranks and Dunn’s all pairwise multiple comparison post hoc test.

**Results.**

**Diabetic Dyslipidemia.** Blood glucose increased more than 5-fold in diabetic dyslipidemic and atorvastatin-treated versus control and hyperlipidemic groups at week 20 (diabetic dyslipidemic 317 ± 81 mg/dl, atorvastatin-treated 269 ± 45 mg/dl, control 52 ± 10 mg/dl, hyperlipidemic 47 ± 7 mg/dl). Total and LDL cholesterol were higher in pigs fed the...
atherogenic diet (hyperlipidemic, diabetic dyslipidemic, and atorvastatin-treated) compared with controls (LDL: hyperlipidemic 197 ± 42 mg/dl, diabetic dyslipidemic 256 ± 45 mg/dl, atorvastatin-treated 249 ± 57 mg/dl, control 35 ± 4 mg/dl). Total triglycerides were increased in the diabetic dyslipidemic (67 ± 12 versus control 22 ± 2 mg/dl, hyperlipidemic 22 ± 3 mg/dl; P < 0.05) and atorvastatin prevented this increase (atorvastatin-treated, 35 ± 7 mg/dl). More detailed analyses of cholesterol fractions and fatty acids in these pigs have been reported (Dixon et al., 2002; Wamhoff et al., 2002).

Peak Ca²⁺m Response to ET-1 Is Potentiated in Diabetic Dyslipidemic Cells. Figure 1A depicts the standard protocol performed in all CSM cells, which included Ca²⁺m measurements at baseline, sustained (plateau) response to 80K, and peak and sustained responses to ET-1. The selective ETB receptor agonist sarafotoxin-6c (S6c) was used to determine the Ca²⁺m response elicited by ETB receptors. Unless otherwise indicated, drugs were administered in PSS. A “responder” was identified as a cell in which Ca²⁺m reached three standard deviations above basal Ca²⁺m during the 2 min ET-1 exposure. We focused on the transient peak Ca²⁺m response because of our previous finding that ET-1-induced nuclear Ca²⁺ transients were directly related to atheroma in these pigs (Wamhoff et al., 2002). There was no difference in basal or 80K-induced Ca²⁺m (n > 30 cells/group).

In contrast, peak Ca²⁺m was 2- to 3-fold greater (P < 0.05) in CSM from diabetic dyslipidemic pigs compared with cells from control, hyperlipidemic and atorvastatin-treated pigs at each dose of ET-1 (Fig. 1B). The Ca²⁺m measures were obtained on CSM cells in PSS without atorvastatin present; thus, the inhibitory effect of atorvastatin on ET-1-induced Ca²⁺m mobilization does not require the continued presence of atorvastatin. The percentage of cells that responded to each dose of ET-1 was not different between each group.

**ET-1 Receptor Subtype Contribution to Peak Ca²⁺m Response.** Selective receptor antagonists, BQ-123 for ETA and BQ-788 for ETB receptors, were used determine the contribution of these receptor subtypes to the regulation of peak Ca²⁺m response to ET-1 (Fig. 2). Cells were preincubated with ET-1 receptor antagonists (each 10⁻⁸ M) separately for 45 min before the application of ET-1 (10⁻⁷ M). BQ-123 attenuated the Ca²⁺m response to ET-1 by 96% in control, 15% in hyperlipidemic, 9% in diabetic dyslipidemic, and 6% in atorvastatin-treated groups, thus indicating that non-ETA receptors largely signal the Ca²⁺m response to ET-1 in CSM cells from hyperlipidemic and diabetic dyslipidemic pigs. In contrast, BQ-788 caused a potentiation (P < 0.05) of the Ca²⁺m response to ET-1 in all groups except atorvastatin-treated. The increases in Ca²⁺m response to ET-1 preincubated with BQ-788 compared with ET-1 alone were 245, 81, and 86% for control, hyperlipidemic, and diabetic dyslipidemic, respectively. The data suggest that ETB receptors normally attenuate the Ca²⁺m response to ET-1 in CSM cells from healthy control pigs by negatively regulating the Ca²⁺m signaling of the ETB receptor and in hyperlipidemia and diabetic dyslipidemia this negative function of ETB receptors is also present, but to a lesser degree (Fig. 2). The ability of ETB receptors to negatively regulate ETFα receptor signaling is specific to the ET-1 receptor family because responses to 3 × 10⁻⁹ M prostaglandin F₂α and 10⁻⁹ M acetylcholine are not affected by BQ-788 (data not shown). In atorvastatin-treated pigs, BQ-788 decreased the Ca²⁺m response to ET-1. The nonselective ETFα/ETB antagonist PD-145065 inhibited the Ca²⁺m response to ET-1 nearly completely in all groups. Most importantly, the Ca²⁺m response to ET-1 alone was greatly decreased in atorvastatin-treated cells (125% of control) compared with hyperlipidemic (156% of control) and diabetic dyslipidemic cells (273% of control) (Fig. 2B).

The selective ETA agonist sarafotoxin-6c provided more evidence that ETα receptor stimulation is not coupled to peak Ca²⁺m responses to ET-1 in CSM. The standard protocol in Fig. 1A accurately depicts the group data because sarafotoxin-6c (10⁻⁸ M) did not significantly increase the peak Ca²⁺m response in any (n = 19–23 cells/group). Although cells did not respond to sarafotoxin-6c, application of ET-1 (10⁻⁸ M) after sarafotoxin-6c elicited a Ca²⁺m response in all groups, thus indicating that cells remained responsive to ET-1 (Fig. 1A). Therefore, the data (Figs. 1A and 2) provide strong evidence that the ETB receptor negatively regulates the Ca²⁺m signaling by the ETA receptor or additional ET-1 receptors in the control, hyperlipidemic, and diabetic dyslipidemic groups. The sustained Ca²⁺m after ET-1 exposure was
examined during minute 15 to 16 of each experiment (Fig. 1A). The steady-state $\text{Ca}^{2+\text{m}}$ was lower in the atorvastatin-treated group at all doses of ET-1 compared with the control, hyperlipidemic, and diabetic dyslipidemic groups ($n = 31–72$ cells/group).

**Basal and ET-1-Induced Tyrosine Phosphorylation.** There was a nearly 3-fold increase in basal tyrosine phosphorylation in diabetic dyslipidemic compared with all other groups (Fig. 3). The ET-1-induced tyrosine phosphorylation was almost 6-fold higher in the diabetic dyslipidemic cells compared with all other groups. Collectively, the data indicate that basal and ET-1-induced tyrosine phosphorylation are significantly higher during diabetic dyslipidemia, and atorvastatin prevented this effect. The subcellular localization of tyrosine phosphorylation is a very important issue. We previously focused on sarcolemmal staining and found in cells from healthy control pigs that cytoplasmic and sarcolemmal PY staining was similar (Lee and Sturek, 2002). The contraction elicited by ET (Figs. 3 and 7; Lee and Sturek, 2002) limits our confidence in higher resolution images to address subcellular localization.

**ET-1 Concentrations.** Figure 4 indicates no difference in the plasma concentrations of ET-1 between groups, but the endothelium-intact coronary artery concentration of ET-1 was increased more than 4-fold in hyperlipidemic, diabetic dyslipidemic, and atorvastatin-treated groups versus control.

**Intravascular Ultrasound.** IVUS permitted us to examine the majority of length of both left anterior descending and circumflex conduit arteries in vivo in a state most closely approximating clinical assessment of atheroma in humans.
This provides a more thorough assessment of atheroma instead of sampling at select sites on the artery using histology. Figure 5 shows virtual absence of atheroma in control and hyperlipidemic pigs, but 26% of arterial segments in diabetic dyslipidemic pigs had atheroma. A major finding was that atorvastatin prevented the increase in atheroma in diabetic dyslipidemia.

**Discussion**

**Major Findings and Overall Experimental Design.** The major findings of this study addressed pharmacotherapeutic mechanisms for the prevention of CAD in diabetic dyslipidemia. First, several ET-1 signaling mechanisms in CSM were increased; ET-1 receptor subtype interaction in regulation of Ca$^{2+}_{m}$ and ET-1-induced tyrosine phosphorylation. Second, these profound increases in cell signaling were associated with greater coronary atheroma in diabetic dyslipidemia compared with hyperlipidemia alone, as evident from clinically relevant intravascular ultrasound measures. Third, chronic treatment with atorvastatin prevented coronary atheroma and the enhanced Ca$^{2+}_{m}$ and tyrosine phosphorylation responses to ET-1, while not decreasing plasma cholesterol in diabetic dyslipidemia. The lack of an effect of atorvastatin on plasma cholesterol concentration is to be expected in cholesterol-fed animal models and most likely results from efficacious suppression of LDL receptor expression by high-dietary cholesterol (Rudel et al., 1998). This aspect of our experimental design allows more confidence in the conclusion that prevention of parallel changes in ET-1 signaling mechanisms and coronary atheroma largely resulted from direct actions of atorvastatin on CSM in the vascular wall and/or plasma triglycerides.

**ET-1 Receptor Regulation of Tyrosine Phosphorylation and Ca$^{2+}_{m}$.** A major purpose of this study was to test the long-standing insightful hypothesis that altered Ca$^{2+}_{m}$ underlies diabetic vascular disease (Levy et al., 1994) because Ca$^{2+}_{m}$ regulation has only recently been studied in vascular smooth muscle after in vivo diabetes (Fleischhacker et al., 1999; Hill et al., 2001; Wamhoff et al., 2002). A coherent picture of ET-1-induced Ca$^{2+}_{m}$ regulation in CSM in a normal/healthy state, hyperlipidemia, and diabetic dyslipidemia is summarized in the model in Fig. 6, which links ET-1 receptor subtypes to tyrosine phosphorylation and Ca$^{2+}$ release from the sarcoplasmic reticulum. To our knowledge, we provide here the first direct measures of Ca$^{2+}_{m}$, which revealed a profound increase in Ca$^{2+}_{m}$ response to a range of ET-1 concentrations in isolated CSM from diabetic dyslipidemic pigs (Fig. 6, large DF letters). This dose-response relationship is a significant extension of our recent article in which a single dose of ET-1 was used (Wamhoff et al., 2002). Indeed, CSM from diabetic dyslipidemic pigs exhibited increased sensitivity of ET-1 receptor stimulation. This increased Ca$^{2+}_{m}$ response was prevented at every concentration of ET-1 by daily atorvastatin treatment during the 20 weeks of in vivo diabetes (Fig. 6, small DF+A letters).
mediated release of nitric oxide from endothelial cells (Haynes and Webb, 1998) and subsequent inhibition of the Ca\(^{2+}\)\(_m\) response because CSM is the only cell type present during our Ca\(^{2+}\)\(_m\) measures (Liu and Sturek, 1996; Hill et al., 2000; Hill et al., 2001; Wamhoff et al., 2002). There was a profound functional switch of receptor subtype regulation of Ca\(^{2+}\)\(_m\) from largely ET\(_A\) in control pigs to an increased inhibitory interaction between ET\(_A\) and ET\(_B\) in all three high-fat/cholesterol fed groups such that neither ET\(_A\) nor ET\(_B\) antagonist alone could block the ET-1-induced Ca\(^{2+}\)\(_m\) response. This increased inhibitory interaction of receptor subtypes resulting in the Ca\(^{2+}\)\(_m\) response to ET-1 is summarized in Fig. 2B as (−)ET\(_A/ET_B\). The inhibitory action of ET\(_B\) on the ET\(_A\) subtype was present in the control, hyperlipidemic, and diabetic dyslipidemic groups [Fig. 6, C, F, DF (−) on dashed line from ET\(_A\) to ET\(_B\)], which resulted in potentiation of the Ca\(^{2+}\)\(_m\) response to ET-1 in the presence of the ET\(_A\) antagonist (Fig. 2A). In addition, an increased inhibitory action of ET\(_A\) on the ET\(_B\) subtype in hyperlipidemic and diabetic dyslipidemic pigs [Fig. 6, F, DF (−) on dashed line from ET\(_A\) to ET\(_B\)] would explain the increased Ca\(^{2+}\)\(_m\) response to ET-1 compared with control pigs in the presence of the ET\(_A\) antagonist (Fig. 2A). Atorvastatin treatment impaired the inhibitory interaction between the ET\(_A\) and ET\(_B\) receptors [Atorvastatin (DF+ATP) and (−) on arrow], as evidenced by the failure of the ET\(_A\) antagonist to potentiate responses to ET-1 in cells from atorvastatin-treated diabetic dyslipidemic pigs (Fig. 2A). These inhibitory interactions are more complex than a simple up-regulation of ET\(_A\) or ET\(_B\) receptors selectively (Dagassan et al., 1996; Hill et al., 2000), but increased interaction of these receptor subtypes is a more parsimonious explanation of our data than suggesting an entirely new receptor subtype for which there is no molecular evidence. Future studies are needed to directly measure ET-1 receptor protein levels to further define the molecules involved in the functional changes in ET-1 receptor signaling we have noted. Since there are no other studies of ET-1 receptor subtypes in functional regulation of CSM Ca\(^{2+}\)\(_m\), the current study is unprecedented. Importantly, our observations are consistent with the general theme that the functions of ET-1 receptors are altered during pathological conditions, such as atherosclerosis and hyperlipidemia.

This study provided novel data on the tyrosine phosphorylation step in Ca\(^{2+}\)\(_m\) regulation in CSM (Figs. 3 and 6). Because ET-1-induced Ca\(^{2+}\)\(_m\) release is strongly dependent on tyrosine phosphorylation (Liu and Sturek, 1996), one explanation for the increased Ca\(^{2+}\)\(_m\) response to ET-1 in diabetic dyslipidemia is our finding that tyrosine phosphorylation was increased in CSM of diabetic dyslipidemic pigs. Increased tyrosine phosphorylation would lead to increased inositol trisphosphate production and activation of the inositol trisphosphate receptor in the sarcoplasmic reticulum, possibly by direct tyrosine phosphorylation (Jayaraman et al., 1996). Conversely, decreased tyrosine phosphorylation may also explain the lower Ca\(^{2+}\)\(_m\) response to ET-1 in atorvastatin-treated cells. Focus on tyrosine phosphorylation and Ca\(^{2+}\)\(_m\) seems especially important because of their critical roles in both vascular smooth muscle contraction and growth; specifically, neointima formation was suppressed in coronary arteries by chronic treatment with a tyrosine kinase inhibitor (Yamawaki et al., 1998). Thus, the dual increases in Ca\(^{2+}\)\(_m\) and tyrosine phosphorylation in response to ET-1 observed in
CSM from diabetic dyslipidemic pigs may be important mechanisms of CAD, and conversely, prevention of these changes strongly argue for novel therapeutic mechanisms of atorvastatin.

Complementary to the observation of increased sensitivity to ET-1 in CSM of diabetic dyslipidemic pigs was the observation that ET-1 concentrations were increased in coronary artery tissue of high-fat/cholesterol fed pigs. This observation lends support to the concept that CSM in diabetic dyslipidemic pigs, subjected to greater ET-1 concentrations and enhanced sensitivity to ET-1, experience increased Ca$^{2+}\_m$ and tyrosine phosphorylation in vivo. Thus, although other receptor signaling systems show down-regulation in the presence of chronic increases in the ligand concentration as a negative feedback mechanism, the ET-1 signaling system shows up-regulation and may account for the severe cardiovascular pathology associated with diabetic dyslipidemia. Plasma ET-1 was not increased. Reports investigating plasma ET-1 levels indicate a significant difference (Lerman et al., 1995), whereas others show no difference in plasma levels of ET-1 during pathological conditions (Kanno et al., 1991). Circulating ET-1 derives from spillover from the vascular wall. In our study, ET-1 either did not spill over significantly into plasma from arteries of hyperlipidemic, diabetic dyslipidemic, and atorvastatin-treated groups or it was cleared quickly from plasma.

Although a small increase in ET-1-mediated contraction in porcine coronary arteries via the ET$_B$ receptor was found during diet-induced hypercholesterolemia (Hasdai et al., 1997), their data are not necessarily at odds with our finding of an increased inhibitory interaction between ET$_A$ and ET$_B$ receptor subtypes because of the vessel specificity discussed above. Hasdai et al. (1997) observed the ET$_B$-mediated contractile increase in coronary microvessels, whereas we studied Ca$^{2+}\_m$ directly in coronary conduit arteries. Furthermore, the association of peak Ca$^{2+}\_m$ with increased tyrosine phosphorylation and atheroma strongly suggest that tyrosine phosphorylation and peak Ca$^{2+}\_m$ influence cell processes other than contraction in conduit CSM, i.e., gene expression, growth, dedifferentiation, and atherosogenesis (Ahmed et al., 1998; Yamawaki et al., 1998; Van Haasteren et al., 1999). Increased ET-1-induced localization of Ca$^{2+}$ in the nuclear region in CSM in diabetic dyslipidemia (Wamhoff et al., 2002), coupled with increased tyrosine phosphorylation (this study), provide powerful stimuli for altering gene expression.

In Vivo Actions of Atorvastatin in Diabetic Dyslipidemia. Intravascular ultrasound showed increased intimal thickening (early atheroma) only in arteries of diabetic dyslipidemic pigs (Fig. 5). This clinically relevant measurement provides a new dimension to other reports that diabetic dyslipidemia elicits more severe CAD than noted in hyperlipidemic or hyperglycemic pigs (Dixon et al., 1999; Suzuki et al., 2001) and is remarkable evidence that the porcine model mimics human epidemiological and clinical data showing that CAD is increased 3- to 6-fold in patients with diabetes (Ruderman et al., 1992). Direct actions of atorvastatin on coronary arteries is very likely because CAD was prevented despite the lack of an effect of atorvastatin on plasma cholesterol and limited effects on triglyceride levels.

Statins have been shown to have positive effects on CSM that are distinct from their lipid lowering functions. Because statins decrease the production of isoprenoid intermediates, which are involved in the post-translational modification of numerous proteins, including G proteins (Casey, 1995), statins are increasingly recognized as having pleiotropic effects via modulation of membrane proteins. Inhibition of isoprenoid synthesis, which would attenuate the synthesis of isopentol trisphosphate and thus intracellular Ca$^{2+}$ release from the sarcoplasmic reticulum (Tesfamariam et al., 1999). The in vitro contractile response to phenylephrine was inhibited in rat aortic rings incubated with atorvastatin and Ca$^{2+}\_m$ responses to angiotensin II were decreased (Tesfamariam et al., 1999). Lovastatin was also shown to inhibit ET-1-induced smooth muscle cell proliferation in vitro (Negre et al., 1997; Koh, 2000). Our study involved animals ingesting atorvastatin tablets for 20 weeks of diabetic dyslipidemia. Although studies have shown an attenuation of agonist-induced Ca$^{2+}\_m$ responses in smooth muscle after treatment in vitro, our results show a more clinically relevant effect on CSM because we used a clinical dose of atorvastatin (40 mg, twice/day) in vivo during diabetes and isolated cells were studied in physiological saline without atorvastatin present. Thus, the continued presence of atorvastatin is not required to elicit adaptations of the cell noted here.

In summary, the data presented here are consistent with the long-held hypothesis that Ca$^{2+}\_m$ underlies vascular disease in diabetes (Levy et al., 1994). The association of ET-1 receptor hypersensitivity and tyrosine phosphorylation-dependent increases in Ca$^{2+}\_m$ with early atheroma development in diabetic dyslipidemia had not been directly measured before this study. Atorvastatin prevented all of the alterations at the intracellular level and inhibited atheroma development in diabetic dyslipidemia.

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Address correspondence to: Dr. Michael Sturek, Department of Medical Pharmacology and Physiology, MA415 Medical Sciences Building, School of Medicine, University of Missouri, Columbia, MO 65212. E-mail: sturekm@missouri.edu