ASBMB
JOURNAL OF LIPID RESEARCH
JLR

Increased atherosclerosis in diabetic dyslipidemic swine: protection by atorvastatin involves decreased VLDL triglycerides but minimal effects on the lipoprotein profile

Joseph L. Dixon,[1,*,] Siming Shen,[†] James P. Vuchetich,[**] Elzbieta Wysocka,[*] Grace Y. Sun,[†,**] and Michael Sturek[*,§,**]**

Dalton Cardiovascular Research Center,* Department of Biochemistry,† Departments of Physiology and Internal Medicine, School of Medicine,§ Diabetes & Cardiovascular Biology Program,** University of Missouri, Research Park, Columbia, MO

Abstract Male Yucatan swine were allocated to four groups (n = 5–6 pigs per group): low fat (3%) fed control, high fat/2% cholesterol (CH) fed (HF), high fat/CH fed with alloxan-induced diabetes (DF) and DF pigs that were treated with atorvastatin (80 mg/day; DF+A). Pigs were fed two meals per day and daily insulin injections were used in diabetic pigs to maintain plasma glucose between 250 and 350 mg/dl. Diabetic dyslipidemic (DF) pigs exhibited greater coronary atherosclerosis and increased collagen deposition in internal mammary artery compared with normoglycemic hyperlipidemic pigs. Although total and LDL CH concentrations did not differ, triglyceride (TG) were increased in DF pigs and FPLC analysis indicated that the LDL/HDL CH ratio was significantly increased in DF compared with HF pigs. The LDL fraction of DF pigs contained larger, lipid enriched particles resembling IDL. Consumption of the high fat/CH diet caused a moderate increase in the percentage of 14:0 fatty acids in plasma lipids and this was compensated by small-moderate declines in several unsaturated fatty acids. There was a significant increase in phospholipid arachidonic acid in DF compared with HF pigs. Atorvastatin protected diabetic pigs from atherosclerosis and decreased total and VLDL TG, but exerted minimal effects on the FPLC lipoprotein and plasma fatty acid profiles and plasma concentrations of total and LDL CH, vitamin A, vitamin E, and lysophosphatidylcholine. Across all groups the plasma CH concentration was positively correlated with hepatic CH concentration. These findings suggest that atorvastatin's protection against coronary artery atherosclerosis in diabetes may involve effects on plasma VLDL TG concentration. Lack of major effects on other lipid parameters, including the LDL/HDL ratio, suggests that atorvastatin may have yet other anti-atherogenic effects, possibly directly in the vessel wall.—Dixon, J. L., S. Shen, J. P Vuchetich, E. Wysocka, G. Y. Sun, and M. Sturek. **Increased atherosclerosis in diabetic dyslipidemic swine: protection by atorvastatin involves decreased VLDL triglycerides but minimal effects on the lipoprotein profile.** *J. Lipid Res.* **2002.** 43: **1618–1629.**

Supplementary key words Yucatan miniature swine • LDL • HDL • remnants • cholesterol • triglycerides • hypertriglyceridemia • endothelium • vascular smooth muscle • coronary artery • statin • vitamin E • arachidonic acid

The incidence of atherosclerosis is 3–4 times greater in diabetics than non-diabetics at comparable plasma total cholesterol (CH) concentrations (1). Beyond total CH concentration, lipid abnormalities in the plasma of diabetics include elevated triglyceride (TG), decreased HDL CH levels, and the presence of small dense LDL (2–5). Although the altered lipid profile in diabetics is considered atherogenic, it is not known which component is the most detrimental. Epidemiological studies have linked TG-rich and remnant lipoproteins to increased atherosclerosis in non-diabetics (6, 7). There are fewer studies in individuals with diabetes, but these have shown that traditional risk factors, including hypertriglyceridemia and low HDL, may be more detrimental in diabetics (8, 9). Recent studies have identified a host of vessel wall factors and pro-inflammatory molecules that are involved in atherogenesis (10, 11), but their involvement in diabetic atherosclerosis has not been fully investigated.

It is difficult to establish the exact role of risk factors

Abbreviations: CAD, coronary artery disease; CE, cholesteryl esters; CFX, circumflex; CH, cholesterol; DF, high fat/CH fed pigs with alloxan-induced diabetes; DF+A, high fat/CH fed pigs with alloxan-induced diabetes treated with atorvastatin; GC, gas chromatograph; HF or F, high fat/2% cholesterol fed pigs; HPTLC, high performance thin layer chromatography; LAD, left anterior descending; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PL, phospholipids; TG, triglyceride.

[1] To whom correspondence should be addressed.
e-mail: dixonj@missouri.edu

Manuscript received 25 March 2002 and in revised form 1 June 2002.
DOI 10.1194/jlr.M200134-JLR200

Copyright © 2002 by Lipid Research, Inc.
This article is available online at http://www.jlr.org

Downloaded from www.jlr.org by guest, on June 14, 2012

and the exact sequence of events in the development of atherosclerosis in diabetes without an appropriate animal model (12). It is our goal to produce atherosclerosis in coronary arteries of diabetic swine in order to study the atherogenic components in the diabetic milieu and the mechanisms involved in the formation of atherosclerotic lesions. We have chosen the porcine model because pigs possess a human-like cardiovascular system (13, 14) that develops experimental coronary artery disease (CAD) similar to the natural course in human CAD patients (15, 16). Additionally, the pig is a good model to study lipoprotein metabolism associated with hyperlipidemia as pigs carry CH in both LDL and HDL lipoprotein particles (17, 18). Previously, we observed that diabetic hyperlipidemic pigs displayed a dyslipidemic lipoprotein profile that resembled those observed in severely diabetic humans (19). The profile included lipid and apolipoprotein (apo)E-enriched remnant apoB-lipoprotein particles, elevated TG, and a tendency toward lower HDL CH concentrations. These diabetic pigs also displayed both peripheral and coronary artery vascular dysfunction. Although Sudan staining of the carotid bifurcation was increased after 12 weeks in hyperlipidemic diabetic pigs compared with hyperlipidemic, normoglycemic pigs, there was only early atherosclerotic disease in these pigs (19). In the current study, Yucatan miniature swine were made diabetic and fed the high fat/CH diet (two meals per day) for 20 weeks. In this report, we describe the changes in the concentrations of plasma glucose, lipoproteins, and lipids over the course of the study. Atherosclerosis was monitored in coronary arteries of sedated pigs by intravascular ultrasound. We investigated the relationship of disease to plasma lipids and lipoproteins. We also tested hypotheses that changes in plasma vitamin E, lysophosphatidylcholine (LPC), and the fatty acid profile of plasma lipids are associated with increased atherosclerosis in diabetic pigs fed an atherogenic diet. The results show that diabetic high fat/CH-fed pigs exhibited greater coronary atherosclerosis than normoglycemic control pigs fed the same high fat/CH diet. When atorvastatin, a 3-hydroxy-3-methylglutaryl CoA reductase inhibitor, was given in the diet throughout the study, coronary and internal mammary atherosclerosis was strongly inhibited. The major effect of atorvastatin on plasma lipids was blunting the rise in VLDL TG. Atorvastatin did not affect the development of the dyslipidemic lipoprotein profile.

MATERIALS AND METHODS

Animals

All procedures involving animals were approved by the Animal Care and Use Committee of the University of Missouri and complied fully with those approved by the American Veterinary Medical Association Panel on Euthanasia. Male Yucatan miniature swine between 7–12 months of age (sexually mature) were obtained from the Sinclair Research Center (Columbia, MO). Pigs were housed in a temperature controlled room (20–22°C) with a 12-h light/dark cycle. Insulin action was impaired by decreasing plasma insulin levels through destruction of pancreatic β cells with alloxan (19). Anesthesia was induced with the following drugs given intramuscularly (in mg/kg): atropine 0.05, telazol 6.6, and xylazine 2.2; the level of anesthesia was subsequently maintained with isoflurane gas (up to 4%). A vascular access port was implanted into the jugular vein for blood sampling and injection of alloxan monohydrate (175 mg/kg, Aldrich Chemical Co., Inc., Milwaukee, WI) or vehicle as described in Hill et al. (20). During the course of the study, 10 ml of blood was withdrawn from the port or anterior vena cava after an overnight fast for the measurement of total plasma TG and CH levels. For blood glucose measurements, a lancet was used to draw blood from an ear vein and a drop was placed on a Accu-Check ADVANTAGE test strip and glucose was measured with an Accu-Check monitor (Boehringer Mannheim Corp., Indianapolis, IN). Blood samples for glucose measures were drawn 1-2 h postprandially twice weekly during the 20 week study.

Experimental design

Four groups of pigs were planned: control pigs (n = 6) were fed 525 g of a low fat (3% total fat) Minipig chow (Purina Mills, Inc., St. Louis, MO) twice per day (1,050 g total per day). A second group of pigs (HF) (n = 6) was fed twice per day 350 g of a high-fat (21% total fat), high-CH (2%) atherogenic diet (Minipig chow supplemented with (wt.%) CH 2, coconut oil 17.1, corn oil 2.3, and sodium cholate 0.7) (designated high fat/CH) as described (19). A third group consisted of high fat/CH fed pigs with alloxan-induced diabetes (DF) (n = 5). A fourth group consisted of DF pigs that were treated with atorvastatin (DF+A) (n = 6), given in the feed 40 mg twice daily. This dose of atorvastatin is similar to that used in human studies with an aggressive LDL CH lowering design (21). Atorvastatin had no adverse effects on the animals as shown by no change in body weight or liver enzymes (data not shown). Daily insulin injections (insulin mixture consisting of one third of regular (Lilly CP-210P) and two thirds of isophane (Lilly CP-310P) insulin were used to maintain plasma glucose concentration in diabetic pigs at a target range of 250–350 mg/dl. An alloxan-treated group on normal pig chow was not included because a previous study indicated that there were minimal effects of diabetes alone on plasma lipids (22). During the course of the study, we identified a subset of alloxan-treated high fat/CH fed pigs that were initially severely diabetic but over the course of 5 weeks became only mildly diabetic (blood glucose <120 mg/dl) and did not require insulin injections. This group (mildly diabetic fat-fed, MDF, n = 4) was not included in later data analysis. Pigs had free access to water. Supplements for the atherogenic diet were obtained from Research Diets (New Brunswick, NJ). Pig body weights were maintained over the course of the study by adjusting the amount of diet given from the above-cited basal amounts. The mean body weights (kg ± SEM) at the end of the study were not significantly different: C, 47.8 ± 2.0; HF, 51.7 ± 1.0; DF, 56.5 ± 2.3; and DF+A, 47 ± 3.0.

Lipid measures

Blood was taken via the anterior vena cava or vascular access port from overnight fasted pigs before alloxan and/or diet treatment and after 4, 8,12, and 20 weeks of treatment. For total CH or TG levels, plasma was assayed directly by standard enzymatic kit (Sigma, St. Louis, MO). For lipoprotein CH and TG levels, fresh plasma samples (1 ml) were chromatographed by fast protein liquid chromatography (FPLC) on a Superose 6 column (HR 16, Pharmacia) 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 CH (standard enzymatic kit). For lipoprotein analysis, the CH and protein profiles for every pig within a treatment group were averaged and plotted versus fraction number. For VLDL, LDL, and

Downloaded from www.jlr.org by guest, on June 14, 2012

ASBMB JOURNAL OF LIPID RESEARCH

HDL lipid content, fractions corresponding to these lipoproteins were collected and assayed for CH and TG concentration by standard enzymatic assay. Certain plasma lipid concentrations at 12 weeks for C, HF, DF, and DF+A pigs were reported previously in studies directed at investigating calcium metabolism in coronary smooth muscle cells (20, 23, 24). Liver lipids were measured by the HPLC method of Homan and Anderson (25) using a 10 cm × 4.6 mm silica gel column (Spherisorb), a SEDEX 55 evaporative light scattering detector (Richard Scientific, Inc.), and 1,2-di-*O*-hexadecyl-rac-glycerol as the internal standard.

Analysis of plasma fatty acids and LPC

For analysis of plasma fatty acid profile, lipids were extracted from plasma with chloroform-methanol (2:1, v/v) and applied to high performance thin layer chromatography (HPTLC) plates (Whatman silica gel 60). Development with a solvent containing hexane-ether-acetic acid (85:15:2, by vol) resulted in separation of cholesteryl esters (CE), TG, and phospholipids (PL). Fatty acids of TG and PL were converted to their methyl esters by methanolysis with 0.2 N NaOH in methanol and analyzed by gas chromatograph (GC) (Hewlett Packard 5890, St. Louis, MO) using a SP2330 capillary column (Supelco, Bellefonte, PA) (26). For CE, samples were placed in Teflon screw-cap tubes and fatty acids were derivatized using 2 ml of BF3 in methanol (Sigma) at 80°C for 1 h. Cholesteryl heptadecanoate (Sigma) was used as internal standard and was added to samples prior to derivatization. Fatty acid methyl esters (FAME) were purified by HPTLC and subjected to GC analysis. Results are expressed as percent fatty acid distribution (mean ± SD) from each group of samples.

A sensitive radio-enzymatic assay was developed to measure LPC concentration in plasma and lipoprotein samples (27). This assay protocol is based on the conversion of LPC to phosphatidylcholine (PC) by LPC:acylCoA acyltransferase using murine liver microsomes as the enzyme source. After isolation of microsomes, a standard curve was constructed by incubating [^{14}C]20:4 (0.1 μCi in 10 μM), 1-palmitoyl-PC (Sigma), microsomes in the presence of ATP (2.5 mM), $MgCl_2$ (10 mM), CoA (0.1 mM), and 0.32 M sucrose/50 mM Tris buffer (pH 7.4) at 37°C for 30 min. After construction of standard curve, samples were incubated similarly with liver microsomes and labeled PC formed was separated from other lipids by HPTLC using a solvent system containing chloroform-methanol-NH_4OH (65:30:5, by vol). The amount of labeled PC formed was determined by counting radioactivity of the PC band by a Scintillation counter (Beckman LS-5800). The amount of LPC in plasma was calculated based on the standard curve.

Vitamin A and E analysis

Plasma Vitamin E and A were measured after extraction into hexane by a reverse-phase HPLC method (28) using a 250 × 4.6 mm Beckman Ultrasphere C18 (5 μm) column (Beckman Instruments, Inc). Peaks were identified by UV absorbance and quantified using retinyl acetate and α-tocopherol acetate as internal standards.

Intravascular ultrasound

After 20 weeks, pigs were anesthetized as described above and intravascular ultrasound was performed in the left anterior descending (LAD) and circumflex (CFX) arteries. An angioplasty guidewire (0.014 or 0.018 inch diameter) was placed into the LAD and CFX arteries under fluoroscopic guidance. Standard anterior-posterior, right anterior oblique 30, and left anterior oblique 30 angiographic images were obtained to verify placement in LAD and CFX arteries. The IVUS catheter (30 MHz, UltraCross 3.2, Boston Scientific, used on Hewlett Packard Sonos console) 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. Atheroma was defined as any fibrous or soft plaque less echogenic than the adventitia and was easily resolved as distinct from the non-layered appearance of all arteries from control pigs.

Collagen histochemistry

Left internal mammary artery was harvested, placed into a physiological salt solution, and stored on ice 1–2 h before dissection of extraneous matter, such as connective tissue, muscle and fat. The artery was then placed in 10% phosphate buffered formalin until the end of the study (1–4 months). Sections 4–5 mm in length were cut and placed into histology cassettes containing 70%-ethanol-soaked sponges and then placed into 10% phosphate buffered formalin for delivery to the Core Histopathology Laboratory in the College of Veterinary Medicine. Arterial segments were removed from their cassette, paraffin-embedded, cut into sections 1–5 μm thick, mounted on a slide, and stained using Masson's Trichrome technique. Cytoplasmic components are stained pink/red and collagen is stained green/blue. The resulting slides were digitally imaged at 10× or 20× magnification. Four images from each pig were analyzed using Image-Pro 4.0 (Media Cybernetics, Silver Springs, MD). The color segmentation function selected for green/blue color (collagen). An area of interest of 26,700 mm^2 was selected and the amount of collagen as a percent of the total area was determined.

Statistical analyses

Sigmaplot and Sigmastat (Jandel Scientific, Corte Madera, CA) were used for graphics and statistical analyses. Values are expressed as the mean ± SEM or ± SD as indicated in Figure and Table legends. For blood glucose and plasma lipids, one-way ANOVA was performed followed by the Student-Newman-Keuls test for post hoc analysis. Data not having a normal distribution

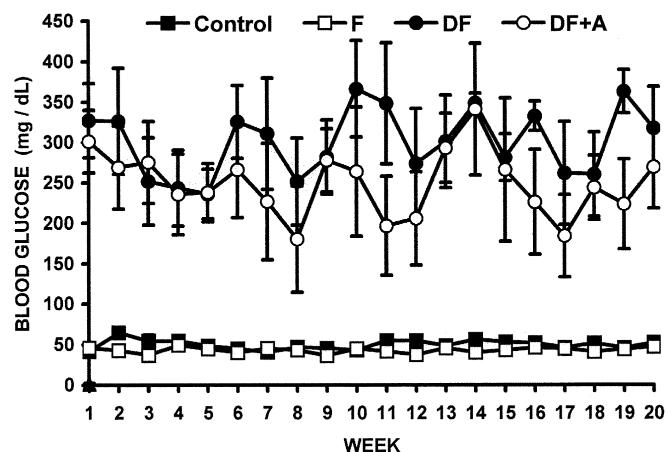

Fig. 1. Plasma blood glucose concentrations over the course of 20 weeks. Experimental groups are: control pigs (n = 6) fed only a low fat (3%) Minipig chow (Purina Mills, Inc., St. Louis, MO). Pigs fed the high fat/2% cholesterol (CH) diet (F, n = 6). High fat/2% CH fed pigs with alloxan-induced diabetes (DF, n = 5). DF pigs that were treated with atorvastatin (80 mg/day) from the start of the study (DF+A, n = 5). Values are means ± SEM. There was no significant difference in glucose concentration between DF and DF+A groups at week 20, or when each of the other weeks were analyzed individually or when the whole data set was analyzed using a repeated measures ANOVA model ($P = 0.305$).

Downloaded from www.jlr.org by guest, on June 14, 2012

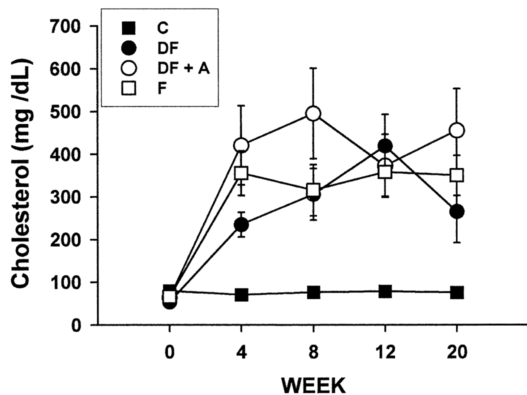

Fig. 2. Total plasma CH concentrations during the course of the 20-week study. Experimental groups are as described in Fig. 1. Values are means ± SEM.

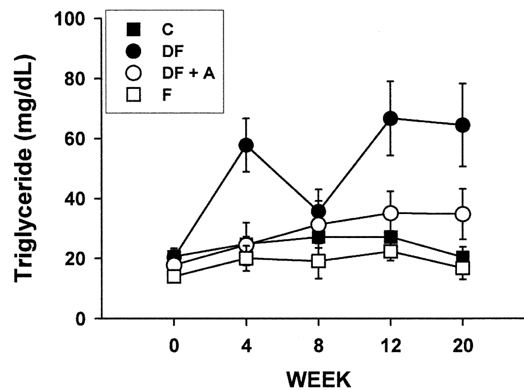

Fig. 3. Total plasma triglyceride (TG) concentrations during the course of the 20-week study. Experimental groups are as described in legend to Fig. 1. Values are means ± SEM.

were analyzed with a Kruskal Wallis one-way ANOVA on ranks test and Dunn's Method for post hoc analysis. Fatty acid and individual week glucose analysis was carried out using Wilcox on Rank Sum test. Significance level chosen was $P < 0.05$.

RESULTS

Post-prandial blood glucose was monitored at least 2 days/week (**Fig. 1**) and averaged 49.1 ± 2.5 and 43.1 ± 2.2 mg/dl in control and HF pigs, respectively, over the course of the study. In alloxan-treated pigs, blood glucose was increased greatly and was maintained by one injection of a mixture consisting of one third regular (Lilly CP-210P) and two thirds isophane (Lilly CP-310P) insulin per day given at the first of two meals of the day. Over the course of the study, blood glucose averaged 316.3 ± 11.3 and 258.3 ± 46.7 mg/dl in diabetic high fat/CH (DF) and DF+A pigs, respectively. Statistical analysis of each day individually and all the data during the course of the study using a repeated measures statistical model showed that plasma glucose was not significantly lower in DF+A pigs compared with DF pigs. The large variability in plasma glucose in certain weeks (Fig. 1) was largely the result of difficulty in managing day to day glucose concentrations with insulin injections.

Figure 2 shows the response of fasting plasma total CH concentration to feeding the atherogenic diet and alloxan-induced diabetes over the course of the study. Total plasma CH concentrations were increased in all groups fed the high fat/CH diet by week 4 and were maintained between 250 and 500 mg/ dl from week 8 to week 20. There were no significant differences between any of the high fat/CH fed groups (**Table 1**). In the presence of a high fat/CH diet, the lack of an effect of atorvastatin on plasma total CH levels was expected as LDL receptor function would already be greatly decreased due to the large influx of dietary CH into tissues (29). A wide variation in individual CH response has also been observed in the CH-fed primate model (29).

Fasting total plasma TG concentrations were elevated in DF pigs by week 4 (**Fig. 3**). Except for week 8, the plasma concentration of TG remained elevated in DF pigs. The fasting plasma concentrations of TG remained very low in control and F pigs. Atorvastatin treatment of diabetic high fat-fed pigs blunted the rise in TG concentration observed in the DF pigs such that plasma TG was 46% and 47% lower in DF+A pigs at 12 and 20 weeks, respectively (Fig. 3).

The mean FPLC CH profile of fasting lipoproteins for each group at week 12 is shown in **Fig. 4**. This time point was chosen as we had previously reported lipid data at this time point (19). When FPLC profiles were performed serially in the same pig throughout 20 weeks of study, the basic lipoprotein lipid profile observed at 12 weeks was

TABLE 1. Fasting total cholesterol concentrations and distribution among FPLC lipoprotein fractions at week 12

Group	Cholesterol				
	Total	VLDL	LDL	HDL	LDL/HDL CH Ratio
			mg/dl		
Control	78.7 ± 3^{a}	0.94 ± 0.5^{a}	34.6 ± 3.5^{a}	32.6 ± 2.0^{a}	1.08 ± 0.1^{a}
High fat (HF)	358.0 ± 57^{b}	3.0 ± 0.6^{a,b}	197.0 ± 42.1^{b}	127.2 ± 11.8^{c}	1.53 ± 0.2^{a}
Diabetic high fat (DF)	419.7 ± 74^{b}	4.8 ± 0.6^{b}	255.6 ± 44.6^{b}	102.8 ± 9.2^{b,c}	2.54 ± 0.4^{b}
Diabetic high fat + atorvastatin (DF+A)	373.7 ± 74^{b}	3.2 ± 1.4^{a,b}	248.5 ± 57.4^{b}	86.7 ± 14.4^{b}	2.86 ± 0.4^{b}

Plasma was assayed directly (Total) or run on an FPLC column, and fractions corresponding to VLDL, LDL, and HDL were collected and assayed for cholesterol concentration by standard enzymatic assay. Values are mean ± SEM. LDL/HDL is LDL CH/HDL CH.

^{a,b,c} Values with different superscripted letters are significantly different ($P < 0.05$).

Downloaded from www.jlr.org by guest, on June 14, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

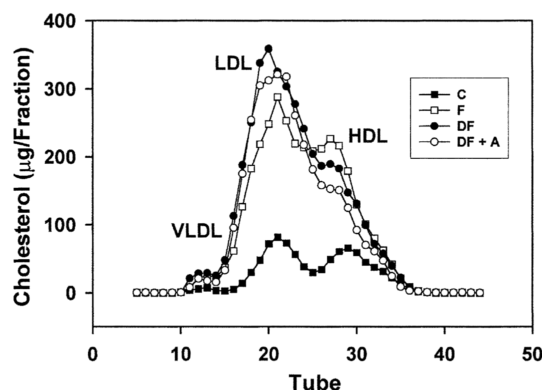

Fig. 4. Fast protein liquid chromatography of plasma from pigs at 12 weeks. Fresh plasma (1 ml) was applied to a Superose 6 column and fractions (2 ml) were collected and assayed for protein (A280) and for CH (standard enzymatic kit) as described in Methods. Values are the means for each group with each sample being individually run on the column. Groups are as described in legend to Fig. 1. In all groups the protein peak for LDL was tube #21. The protein content of this tube did not differ between groups: Control (C), 2.71 ± 0.23; F, 3.14 ± 0.14; DF, 2.83 ± 0.11; and DF+A, 2.89 ± 0.24 mg protein (±SEM)/2 ml fraction. The CH concentrations of tube #21 for the different groups were: C, 82 ± 15; HF, 288 ± 35; DF, 326 ± 38; and DF+A, 322 ± 74 μg CH/2 ml fraction.

maintained at 20 weeks. Control pigs fed the low fat diet have CH distributed almost equally between LDL and HDL fractions. Both LDL and HDL were increased in pigs fed the high fat/CH diet. In both diabetic groups LDL CH was increased compared with HF and a more pronounced left shoulder of the LDL peak was observed. The protein content of the LDL peak fraction was similar among all groups (Fig. 4). The height of the HDL peak was slightly decreased in DF and DF+A groups compared with HF.

Tubes within lipoprotein fractions were pooled and assayed for lipids (Table 1). The increase in LDL CH concentration was highly variable among pigs within each group, with the concentration in the highest pig in a group often being double the concentration of the lowest pig in the same group. As observed in the non-human primate CH-fed animal model (29), HDL CH was substantially increased with high fat/CH feeding. In HF pigs HDL CH was above 125 mg/dl, whereas in both severely diabetic pig groups (DF and DF+A) HDL was below 105 mg/dl. The LDL/HDL ratio was not significantly increased from control in HF pigs but was significantly increased in both diabetic groups.

Table 2 shows the fasting total TG concentrations and distribution of TG among FPLC lipoprotein fractions at week 12. The 3-fold increase in total TG concentration in severely diabetic pigs (DF) was the result of significant increases in both VLDL and LDL TG. These results show that plasma TG responds to insulin deficiency in pigs similar to diabetic human patients. Administration of atorvastatin significantly decreased total plasma TG compared with the DF group. Interestingly, the decrease in TG was specific in the VLDL but not the LDL fraction. There were minimal effects of diet or diabetes on HDL TG content.

The effects of diabetes and atherogenic diet on the fatty acid composition of the major plasma lipids were measured at 20 weeks (**Fig. 5**). It must be noted that the data reflect percent distribution and not mass of CE and PL, which were greatly increased in plasma of pigs fed the high fat/CH diet compared with low fat-fed controls. The fatty acid profiles were different for each class of lipid. Triglyceride (TG) was high in 16:0, 18:1, and 18:2; CE was high in 18:2; and PL was high in 16:0, 18:0 and 18:2. Feeding the high fat/CH diet containing 17.1% coconut oil and 2.3% corn oil increased the content of 14:0 fatty acids 4–5-fold in all lipid fractions. This was compensated by slight-moderate decreases in certain unsaturated fatty acids, e.g., 20:5 and 22:6 in PL. Although not statistically significant, the percent arachidonic acid (AA) in PL of the high fat/CH group (HF) was decreased almost 40% compared with the control group (Fig. 5C). Interestingly, proportion of AA in diabetic pigs (DF) was significantly higher than that in the non-diabetic pigs (HF) fed the same diet. Atorvastatin treatment did not alter the proportion of AA in diabetic pigs. Because the plasma PL concentration increased in all high fat/CH fed groups (legend, Fig. 5), plasma PL AA concentration also increased greatly in these groups. In CE, the increase in 14:0 was accompanied by decreases in 18:2 (12%). The decrease in 18:2 and increases in saturated fatty acids in plasma CE are consistent with changes observed in LDL when an atherogenic diet was fed to cynomologous monkeys (30). Except for the increase in 14:0, there were no obvious changes in other fatty acids in TG due to diabetic or atorvastatin treatment. 20:4 was ex-

TABLE 2. Fasting total triglyceride concentrations and distribution among FPLC lipoprotein fractions at week 12

Group	Triglyceride			
	Total Plasma	VLDL	LDL	HDL
	mg/dl			
Control	27.2 ± 2.0^{a}	13.6 ± 1.9^{b}	8.79 ± 0.22^{a}	2.6 ± 0.5
High fat (HF)	22.3 ± 3.1^{a}	7.04 ± 1.3^{a}	9.83 ± 1.70^{a}	3.6 ± 0.4
Diabetic high fat (DF)	66.7 ± 12.3^{b}	27.8 ± 5.7^{c}	18.5 ± 5.0^{b}	3.2 ± 0.5
Diabetic high fat + atorvastatin (DF+A)	35.1 ± 7.3^{a}	8.77 ± 3.0^{a,b}	18.3 ± 3.8^{b}	3.4 ± 1.2

Plasma was assayed directly (Total) or run on an FPLC column, and fractions corresponding to VLDL, LDL, and HDL were collected and assayed for triglyceride concentration by standard enzymatic assay. Values are mean ± SEM.
$^{a, b, c}$ Values with different superscripted letters are significantly different ($P < 0.05$).

Downloaded from www.jlr.org by guest, on June 14, 2012

ASBMB JOURNAL OF LIPID RESEARCH

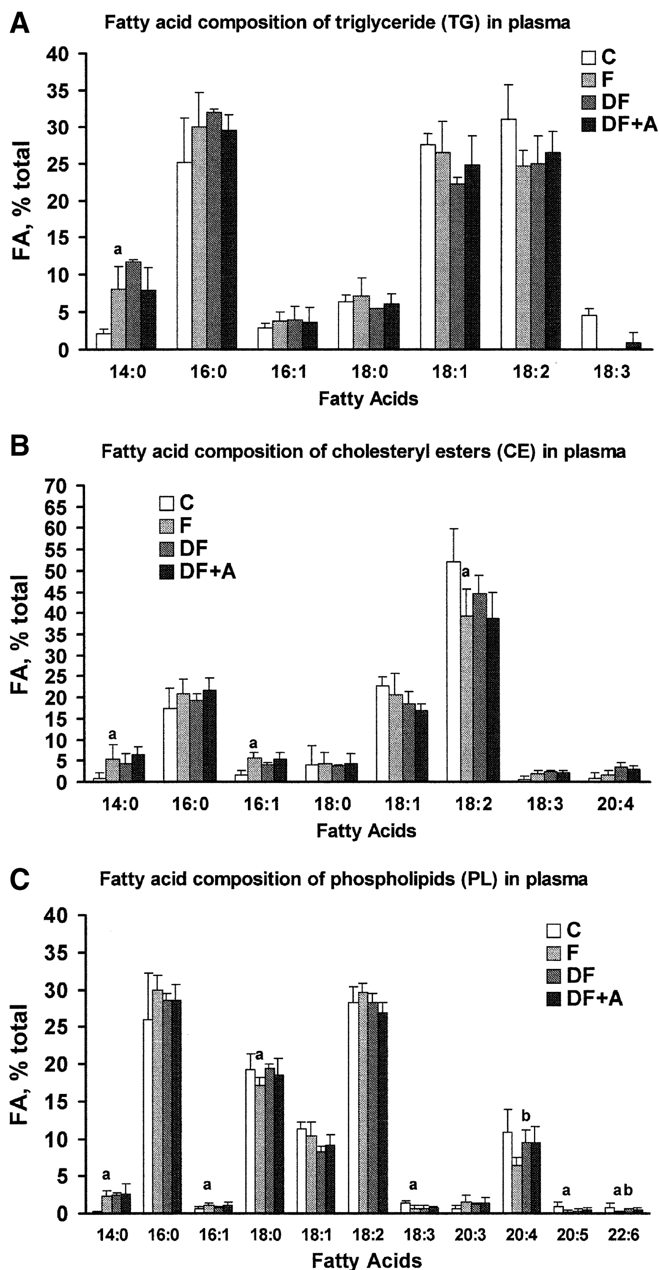

Fig. 5. Effects of diabetes and an atherogenic diet on the fatty acid composition of the major lipids of plasma at 20 weeks. Plasma lipids were extracted and applied to high performance thin layer chromatography (HPTLC) plates. Triglyceride (A), cholesteryl ester (CE) (B), and phospholipid (C) components were separated and analyzed for fatty acid composition by gas liquid chromatography (fatty acid species indicated below each group of bars). Results are expressed as percent fatty acid distribution (mean ± SD). A letter "a" above a F bar indicates a significant difference ($P < 0.05$) comparing F with control. A letter "b" above a DF bar indicates a significant difference comparing DF with F. In no group was DF+A different compared with DF. In CE (B) 20:4 in all high fat/CH-fed groups are greater than control ($P < 0.043$). In TG (A), high fat/CH-fed groups show hardly any 18:3, and TG 20:4 was so low as to be undetectable in a large percentage of plasma samples. Calculated from the fatty acid concentration using C17:0 methyl ester as internal standard, the concentrations of total PL in plasma were increased ($P < 0.05$) in high fat/CH fed pigs: 31.3 ± 3.9, 79.7 ± 13, 75.9 ± 25, and 93.5 ± 42 mg/dl (± SD) in control (C), F, DF, and DF+A groups, respectively.

tremely low in TG in all groups. These results indicate that the inclusion of corn oil in the high fat diet at about 10% total dietary fat prevented more severe declines in the percentage of monounsaturated and polyunsaturated fatty acids in plasma lipids.

Feeding the high fat/CH diet significantly increased the liver concentrations of free CH in HF and DF+A pigs, with more modest and variable increases observed in DF pigs (**Table 3**). The CE concentration was significantly increased in all high fat/CH fed groups with the greatest increase in HF pigs. TG concentrations were increased in livers of pigs fed the high fat/CH diet, but although the mean values were much greater, the variation in HF and DF groups were too high to reach significance. Nevertheless, liver TG in DF+A pigs appeared to be lower than the HF and DF groups.

One common observation in studies of CH-fed animal models is the wide variation in plasma CH concentrations in response to CH feeding (29, 31). As a wide range in values for hepatic free CH concentration was also observed in the current study, we wished to test whether there was an association between hepatic lipids and plasma CH concentration by correlation/regression analysis. **Figure 6** demonstrates that there was a significant relationship between liver free CH (μg/mg cell protein) and plasma total CH concentration ($r = 0.75$, $P < 0.001$, n = 22). There was also a significant positive association between liver CE and plasma CH ($r = 0.69$, $P < 0.001$, n = 22) (data not shown). No relationship was found between liver TG and plasma CH (data not shown).

We were also interested in whether severe diabetes and consumption of the atherogenic diet changed several other important parameters in plasma. There were no significant differences in plasma vitamin A or E concentrations among the treatment groups (**Table 4**). A sensitive assay was used to measure LPC in plasma and LDL. LPC was approximately 1 μM in plasma. Several pigs in the DF group contained undetectable levels of LPC that resulted in a higher standard error for this group. Assays of LPC in LDL isolated by ultracentrifugation showed that LPC was greater in LDL from high fat/CH fed pigs (control compared with all high fat/CH-fed groups, $P < 0.026$). However, LPC concentrations did not differ among HF, DF, and DF+A groups (Table 4).

Relation of atherosclerosis to LDL CH concentration

An IVUS catheter was selectively placed in the left anterior descending and CFX coronary arteries of pigs by percutaneous catheterization of the femoral artery to measure coronary artery atherosclerosis after 20 weeks. Analysis of video segments generated during pullback indicated substantial raised endothelium (mean 28.0 ± 6.02% of segments with atheroma) in DF pigs that was significantly increased ($P < 0.05$) compared with control (1%), HF (2%), and DF+A (5.23 ± 5.23%) groups. The percentage of segments with raised endothelium in individual pigs was plotted against LDL CH (**Fig. 7A**) or LDL/HDL ratio (Fig. 7B) as measured by FPLC. Atherosclerosis was not increased in normoglycemic high fat/CH fed pigs (HF) above C, even when LDL CH concentrations ap-

ASBMB
JOURNAL OF LIPID RESEARCH
Downloaded from www.jlr.org by guest, on June 14, 2012

TABLE 3. Liver cholesterol and triglyceride concentrations

Group	Lipid		
	Cholesterol	Cholesteryl Ester	Triglyceride
		μg/mg protein	
Control	5.06 ± 1.05[a]	0.982 ± 0.199[a]	3.76 ± 0.59[a]
High fat (HF)	14.05 ± 2.56[b]	28.79 ± 6.55[c]	17.13 ± 9.62[a,b]
Diabetic high fat (DF)	8.90 ± 2.21[a,b]	10.59 ± 3.01[b]	18.80 ± 6.79[a,b]
Diabetic high fat + atorvastatin (DF+A)	11.55 ± 1.79[b]	19.37 ± 6.08[b,c]	10.01 ± 1.73[b]

Liver lipids were assayed by HPLC. Data is μg of lipid per mg total cellular protein as determined by the Lowry method. Values are mean ± SEM.

[a,b,c] Values with different superscripted letters are significantly different ($P < 0.05$).

proached 300 mg/dl. Atheroma increased in diabetic pigs (DF) with increasing LDL CH concentration (Fig. 7A) or LDL/HDL ratio (Fig. 7B). When pigs were fed atorvastatin 80 mg per day, atherosclerosis was largely prevented. One pig in the DF+A group showed increased coronary atherosclerosis (21% of segments with atheroma), but also presented with the highest LDL/HDL CH ratio of the study (LDL/HDL = 4.35, Fig. 7B). **Figure 8** shows Masson's trichrome histochemical stain for collagen in left internal mammary artery. Arteries from diabetic dyslipidemic pigs (DF) had 2-fold greater collagen accumulation than control, HF, or DF+A groups ($P < 0.05$); thus, atorvastatin prevented the remodeling of internal mammary artery. These results show increased atherosclerosis in diabetic dyslipidemic pigs (DF) compared with hypercholesterolemic pigs (HF) at comparable levels of LDL CH. This relationship is similar to an observation made by Bierman concerning athersclerosis in human diabetes (32).

DISCUSSION

The major finding of the current study was that, after 20 weeks on a high fat/CH diet, substantially more atherosclerotic disease was observed in coronary and internal mammary arteries of severely diabetic pigs compared with normoglycemic hyperlipidemic pigs at similar plasma CH concentrations. The major differences between these two groups were increased plasma TG, increased 22:4 in PL, a broader IDL/LDL peak, and higher LDL/HDL CH ratios in DF compared with HF pigs. Atorvastatin (80 mg/day) decreased atherosclerosis and decreased the elevation in fasting TG (mainly in VLDL), but had only minimal effects on other lipid parameters in severely diabetic pigs. Therefore, atorvastatin decreased atherosclerosis either through modulation of plasma TG, or, as the increase in TG was only moderate to begin with, atorvastatin may have prevented atherosclerosis through other yet undisclosed mechanisms, possibly through direct interaction within the vessel wall.

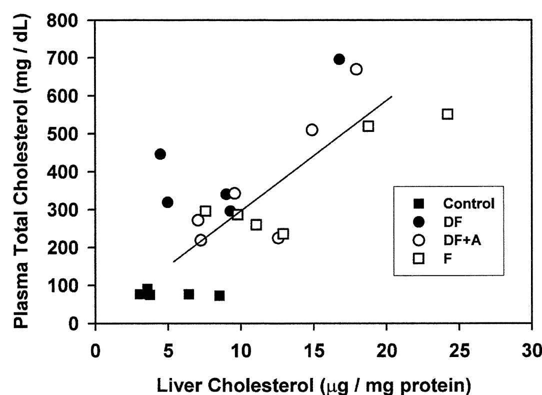

Fig. 6. Correlation of plasma total CH concentration with liver free CH content. The equation for the best fit linear regression line is: $y = 29.1x + 17.6$ ($r = 0.753$, $P < 0.001$), where x is the liver cholesterol concentration in μg/mg protein.

Lipoprotein lipid responses to high dietary fat/CH

A wide range in total CH concentrations was observed among pigs within each group, a common observation in CH fed animal models (29). Plasma total CH was related to the liver free CH concentration (Fig. 6). This observation is consistent with data that hepatic CH availability is an important determinant of VLDL secretion and later plasma CH concentration (33–36). As observed in our previous study (19), the LDL CH peak was higher with a more pronounced left shoulder in the diabetic high fat/CH groups compared with the non-diabetic high fat/CH group (Fig. 4). However, the protein content in LDL was not altered (Fig. 4). Our previous results indicated that the left shoulder contains CH and apoE enriched apoB lipoprotein particles (19), and their location in the FPLC profile (between VLDL and the LDL peak) indicates that they are IDL in size. This contrasts with the very large TG rich particles observed in other diabetic animal models (37). Extremely large lipoprotein particles may be the reason why less atherosclerosis was observed in alloxan-diabetic CH-fed rabbits compared with normoglycemic high CH-fed controls (38). What are the origins of the IDL particles in diabetic high fat/CH fed pigs? The moderate increases in fasting total plasma total CH (250–500 mg/dl) and the trivial amount of apoB-48 observed in fasting IDL by gel electrophoresis (data not shown) support the notion that the IDL in fasting plasma of the high fat-fed pigs are remnants of liver-derived VLDL. The current studies, although performed in diabetic animals, can be compared with another major CH fed animal model of athero-

Downloaded from www.jlr.org by guest, on June 14, 2012

TABLE 4. Fasting plasma concentrations of vitamin A, vitamin E, lysophosphatidylcholine, and LDL lysophosphatidylcholine

Group	Vitamin A	Vitamin E	Plasma LPC	LDL LPC
		μM		
Control	0.84 ± 0.05	3.6 ± 0.67	1.30 ± 0.22	0.30 + 0.05[a]
High fat (HF)	0.72 ± 0.05	4.7 ± 0.57	1.00 ± 0.11	1.04 + 0.46[b]
Diabetic high fat (DF)	0.84 ± 0.14	3.6 ± 0.37	0.72 ± 0.72	1.03 + 0.44[b]
Diabetic high fat + atorvastatin (DF+A)	0.77 ± 0.03	4.6 ± 0.89	1.24 ± 0.25	0.98 + 0.21[b]

LPC, lysophosphatidylcholine. Plasma was assayed as described in Materials and Methods. Values are mean ± SEM.

[a,b]For LDL LPC, high fat/CH-fed pigs were increased compared to control, $P < 0.026$.

sclerosis, the non-human primate. CH-fed pigs are similar to primates in that both show an extensive range in total CH concentrations, size of LDL particles and extent of atherosclerosis in response to an atherogenic diet (31).

Atherosclerosis

A previous 20 week study (22) showed that diabetic pigs fed a conventional, low fat, low CH diet exhibited neither altered lipoprotein levels profiles nor vascular disease. In contrast, diabetic pigs fed a high fat/CH atherogenic diet for 12 weeks exhibited a dyslipidemic lipoprotein profile and developed vascular dysfunction in both coronary and brachial arteries (19). In the latter study, atherosclerosis, as measured by carotid staining, was only slightly enhanced in diabetic high fat-fed pigs after 12 weeks (19). To elicit greater atherosclerotic disease in diabetic pigs, we extended the length of the experiment to 20 weeks. Al-

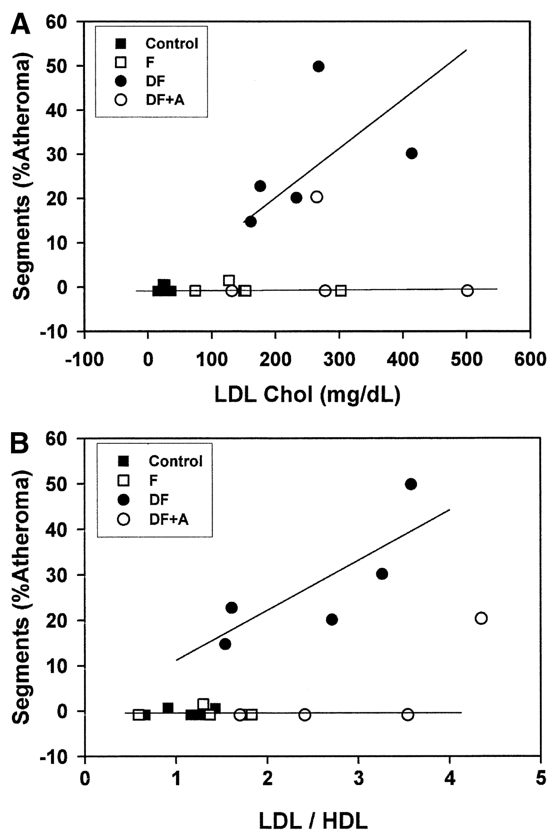

Fig. 7. Relationship of atherosclerosis (percentage of segments with raised endothelium) to LDL CH concentration (A) or LDL/HDL CH ratio (B). Symbols are defined in the figure and each represents an observation from an individual pig. Atheroma in DF pigs (mean 28.0 ± 6.02% of segments with atheroma) was significantly increased ($P < 0.05$) compared with control (1%), F (2%), and DF+A groups (5.23 ± 5.23%).

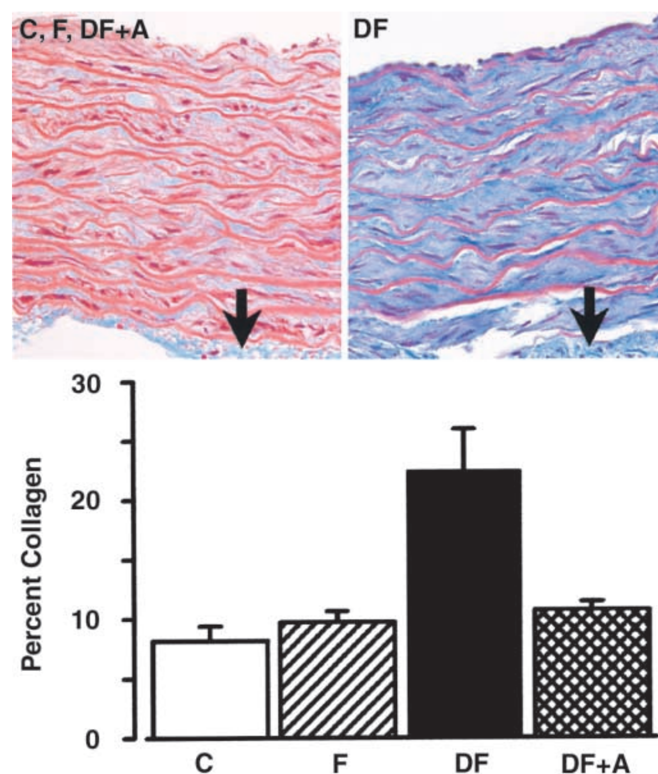

Fig. 8. Collagen is increased in internal mammary artery of diabetic pigs fed an atherogenic diet. Arteries were formalin-fixed, embedded in paraffin, sectioned, and collagen was visualized using Masson's trichrome histochemical stain. Top left: 9% collagen staining in an artery from a DF+A pig, which is also representative of control (C) and F groups. Top right: 63% collagen staining (maximal response) in an artery from a DF pig. Collagen is shown as the blue components and is emphasized in both panels by the arrows pointing to adventitia, which is almost exclusively collagen. Cellular components are pink/red. Single endothelial layer of the intima is shown in the top edge of the artery by the darkened cellular nuclei. Bottom: group data; values are means ± SEM; DF was significantly greater ($P < 0.05$) than control, F, and DF+A.

Downloaded from www.jlr.org by guest, on June 14, 2012

though the mean plasma total CH concentrations did not differ between the high fat/CH groups, IVUS detected 14-fold greater coronary atherosclerosis in severely diabetic pigs (DF) compared with non-diabetic HF pigs. Trichrome staining showed increased collagen deposition in internal mammary arteries of DF pigs, but collagen was not increased in HF or DF+A groups in comparison to control. These data suggest that artery matrix was remodeled in response to dyslipidemia and hyperglycemia, and that atorvastatin inhibited this process. These data strongly suggest that vascular smooth muscle cells have been stimulated to up-regulate collagen production only in the dyslipidemic, diabetic group (DF). Clearly, the diabetic milieu is more atherogenic in pigs as it is in diabetic humans (32).

When severely diabetic high fat/CH pigs were treated with atorvastatin, coronary lesion development was largely prevented. The exception was one pig that not only had the highest LDL/HDL ratio in the DF+A group, but also had the highest LDL/HDL ratio of the entire study. Atorvastatin treatment did not lower total or LDL CH concentrations, nor the LDL/HDL CH ratio, but it did lower plasma total and VLDL TG concentrations (Table 2), suggesting that the drug inhibited VLDL production. This coincides with a report that hepatic VLDL apoB secretion was decreased in miniature pigs after treatment with inhibitors of HMG-CoA Reductase (39). FPLC analysis indicated that atorvastatin did not alter the content of large, lipid enriched apoB particles observed in diabetic high fat/CH fed animals (Fig. 4). Therefore, atorvastatin protected coronary arteries from lesion development with only a modest decrease in TG concentrations and without a major change in total CH concentration or lipoprotein profile.

Increased atherosclerosis in diabetes/ dual effects of low insulin/hyperglycemia and dyslipidemia on coronary artery lesion development

Why is there increased atherosclerosis in the severely diabetic pigs compared with normoglycemic controls? The hallmarks of diabetic dyslipidemia are hypertriglyceridemia, low HDL CH, and small, dense LDL (2–5). The DF group had a higher and broader IDL/LDL peak and a significantly higher LDL/HDL CH ratio compared with the HF group. This difference in lipoprotein profile per se may be the cause of increased atherosclerosis in the DF group. Remnants of TG-rich lipoproteins can enter the subendothelial space of the artery and are toxic to endothelial cells and macrophages (40). Diabetics have delayed removal of postprandial lipoproteins (41–44). The increased residence times of remnants in plasma allow greater trapping and uptake of remnant particles into macrophages and smooth muscle cells. Large LDL produced greater CE accumulation in cells compared with equal molar concentrations of small LDL (45). Remnant particles also increase adhesion of monocytes to the artery wall and can impair endothelium-dependent vasorelaxation of rabbit aortas (46). The large remnant particles observed in diabetic high fat/CH-fed pigs may be an important component that leads to accelerated atherosclerosis. Additional components in the diabetic milieu may enhance atherosclerosis. As virtually no atherosclerotic disease has been observed in diabetic pigs fed low fat diets (22, 47), it appears that both dyslipidemia (high LDL or lipid enriched IDL/LDL) and low insulin/hyperglycemia are required to observe accelerated atherosclerosis in diabetic pigs. Recently, hyperglycemia per se was shown to increase the number of scavenger receptors (CD36) on the surface of artery cells (48). Feeding a high fat/CH diet alone will elicit atherosclerosis in pigs, but this occurs over the course of a much longer time period (49, 50).

Effects of diet and diabetes on plasma fatty acid profile

Since the major source of fat in the high fat/CH diet was coconut oil, which is highly enriched in saturated fat, we hypothesized that the plasma fatty acid profile would change in high fat/CH fed pigs, modifying the binding of lipoproteins to macrophage or smooth muscle cells. Consumption of the high fat/CH diet increased 14:0 in all lipids in plasma, reflecting its high concentration in coconut oil. Small-moderate declines in unsaturated fatty acids, (e.g., 20:4, 20:5, and 22:6 in PL, 18:2 in CE), compensated for the increases in 14:0. With the exception of an increase in 20:4 in PL, the profiles in severely diabetic pigs were, in general, similar to those observed in the other high fat/CH groups. 20:4 is the major polyunsaturated fatty acid in PL and an increase in total plasma PL (Fig. 5) resulted in a 20:4 concentration that was increased in all high fat/CH-fed groups. Inclusion of 2.3% corn oil in the diet may have "buffered" the fatty composition and prevented larger decreases in plasma unsaturated fatty acids.

LPC

LPC has been identified as one of the major oxidized lipids in LDL and has been reported to induce endothelial cell dysfunction. A sensitive and specific radiolabel assay indicated that around 1 μM of LPC was present in pig plasma. Although LPC levels were not different in plasma, a significant increase was found in LDL of high fat /CH-fed pigs. Nevertheless, there were no effects of diabetes or atorvastatin on LDL LPC concentration.

Role of vitamin E

In an effort to discern other factors in the diabetic milieu that might influence coronary artery atherosclerosis, we also measured plasma fat-soluble vitamins A and E. Neither vitamin appeared to be associated with coronary artery lesion development in diabetic dyslipidemic pigs. There were no differences in plasma vitamin E or A concentrations between DF pigs that developed disease and groups (HF and DF+A) that did not develop disease. A recent prospective clinical trial showed little protection by vitamin E in patients with atherosclerosis (51). It must be noted that vitamin E is not protective against some oxidants such as nitric oxide or myeloperoxidase-generated reactive nitrogen (52).

The current studies do not address other forms of oxidized lipids that may be mediators of increased atherosclerosis in diabetes. Other lysophospholipids, such as lyso-

Downloaded from www.jlr.org by guest, on June 14, 2012

phosphatidic acid and sphingosine-1-phosphate, are present in plasma and bind to plasma membrane receptors (53). Phospholipid peroxides, but not LPC, were present in remnant lipoproteins that impaired endothelium-dependent relaxation (46). Oxidized LDL binding to CD36 is mediated through oxidized phospholipids (54). LPC is also produced in the artery wall, most likely by secretory phospholipase A_2 (55). Daugherty et al. (56) showed similar concentrations of LPC in plasma LDL and in LDL isolated from vascular lesions.

Lipoprotein oxidation is thought to occur in the subendothelial space due to cellular production of potent oxidants by myeloperoxidase, nitric oxide synthase, or 15-lipoxygenase (57). Recently, increased 12-lipoxygenase expression was observed in both abdominal aorta and in coronary arteries of diabetic, hyperlipidemic pigs (58). Furthermore, urinary excretion of 12-(*S*)-hydroxyeicosatetraenoic acid (12-HETE), a product of the 12-lipoxygenase pathway, was increased in diabetic, hyperlipidemic pigs. These observations support the hypothesis that there is increased oxidative stress in diabetic, hyperlipidemic pigs, and this may be one of the mechanisms that lead to increased atherosclerosis.

Mechanism for atorvastatin protection of coronary atherosclerotic lesions

Because atorvastatin was so effective in preventing coronary lesion development without affecting the dyslipidemic lipoprotein profile except for VLDL TG, there is the possibility that atorvastatin was acting directly in the artery wall. Indeed, in non-diabetic patients with relatively normal levels of CH, pravastatin decreased cardiovascular mortality, while having minor effects on plasma CH (59). Thus, direct actions of statins on the vascular wall have been proposed to explain this beneficial effect independent of plasma CH lowering (60). We cannot discount that atorvastatin's protective effect is related to its prevention of hypertriglyceridemia in diabetic pigs. However, the absolute concentrations of TG attained in severely diabetic pigs (Fig. 3) are below levels observed in diabetic humans. But an anti-atherogenic effect through prevention of hypertriglyceridemia, especially in the post-prandial period, cannot be entirely ruled out at this time. If atorvastatin prevents atherosclerotic lesion development directly in cells of the coronary vessel wall, what mechanisms are involved?

An intriguing possibility is that atorvastatin may affect diabetes by improving insulin sensitivity. However, similar to studies in human diabetics (61), atorvastatin did not improve glycemic control (Fig. 1) nor insulin sensitivity (Otis, Wamhoff, Sturek, unpublished observations). We (19, 20, 23) and others (62, 63) have suggested that Ca^{2+} metabolism is altered in coronary smooth muscle cells of diabetics. Using cells isolated from the same pigs described in the current paper, Ca^{2+} uptake into sarcoplasmic reticulum (measured in presence of caffeine) and into non-sarcoplasmic reticulum Ca^{2+} stores (measured in presence of ionomycin) was increased in DF smooth muscle cells compared with cells from HF pigs. Atorvastatin treatment reversed increases in Ca^{2+} uptake into both storage sites (20). Alterations in coronary smooth muscle nuclear calcium signaling may be an important component in atherosclerosis development, possibly through induction of cell proliferation (23). Other reports have indicated that HMG-CoA reductase inhibitors affect the vessel wall independent of CH-lowering. Statins may improve endothelial function through increased nitric oxide production (64), decreased leukocyte adherence (65), and altered signaling through blockage of isoprenoid synthesis (66). Additionally, not all statins have identical in vivo effects on the artery wall. Although producing equivalent plasma CH lowering, fluvastatin decreased intimal smooth muscle cell number and collagen content in Watanabe hyperlipidemic rabbits in vivo, whereas pravastatin increased both parameters (67).

In summary, coronary and internal mammary atherosclerosis was increased in diabetic pigs fed a high fat/CH diet in two meals per day compared with control high fat/CH-fed pigs. This study is a significant extension of our previous work (19, 20, 22, 23) and that of Gerrity et al. (47) in several regards. First, we have shown in high fat/CH fed diabetic pigs a strong, direct association of LDL and LDL/HDL ratio with coronary atheroma quantified by the clinically used intravascular ultrasound method. Second, we have clearly shown that accelerated coronary atherosclerosis in diabetes is not due to decreased vitamins E and A or increased LPC, as none of these were changed in diabetic dyslipidemic pigs. Finally, atorvastatin prevented both coronary and internal mammary atherosclerosis without affecting plasma total and LDL CH concentrations, the lipoprotein profile, plasma fatty acids, or hepatic lipids; only VLDL TG were lowered. These observations suggest that atorvastatin prevented atherosclerosis by direct effects at the vessel wall or lowering of VLDL TG.

The authors acknowledge the excellent technical assistance of Jie Fang. We thank Dr. Brian Wamhoff for discussions of the diabetic pig model. We thank Dr. Guy Bouchard of the Sinclair Research Center (Columbia, MO) for breeding the Yucatan miniature pigs. We thank Dr. William Blaner of Columbia University for performing the vitamin A and E analysis. This work was supported by National Institutes of Health Grant HL47586 to J.L.D., American Diabetes Association support and National Institutes of Health Grants HL62552, and RR13223; National Institutes of Health Research Career Development Award (HL 02872) to M.S.; and National Institutes of Health Grants AA06661 and AG18357 to G.Y.S.

REFERENCES

1. Kannel, W. B., and D. L. McGee. 1979. Diabetes and cardiovascular disease: The Framingham study. *J. Am. Med. Assoc.*. **241:** 2035–2038.
2. Steiner, G. 1994. The dyslipoproteinemias of diabetes. *Atherosclerosis.* **110(Suppl.):** S27–S33.
3. Reaven, G. M., and A. Laws. 1994. Insulin resistance, compensatory hyperinsulinaemia, and coronary heart disease. *Diabetologia.* **37:** 948–952.
4. Sniderman, A., C. Michel, and N. Racine. 1992. Heart disease in patients with diabetes-mellitus. *J. Clin. Epidemiol.* **45:** 1357–1370.
5. Howard, B. V., and W. J. Howard. 1994. Dyslipidemia in non-insulin-dependent diabetes mellitus. *Endocr. Rev.* **15:** 263–274.

ASBMB JOURNAL OF LIPID RESEARCH JLR

Downloaded from www.jlr.org by guest, on June 14, 2012

6. Hokanson, J. E., and M. A. Austin. 1996. Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level: a meta-analysis of population-based prospective studies. *J. Cardiovasc. Risk.* **3:** 213–219.
7. Jeppesen, J., H. O. Hein, P. Suadicani, and F. Gyntelberg. 1998. Triglyceride concentration and ischemic heart disease—An eight-year follow-up in the Copenhagen Male Study. *Circulation.* **97:** 1029–1036.
8. Castelli, W. P. 1992. Epidemiology of triglycerides: a view from Framingham. *Am. J. Cardiol.* **70:** 3H–9H.
9. Howard, B. V., D. C. Robbins, M. L. Sievers, E. T. Lee, D. Rhoades, R. B. Devereux, L. D. Cowan, R. S. Gray, T. K. Welty, O. T. Go, and W. J. Howard. 2000. LDL cholesterol as a strong predictor of coronary heart disease in diabetic individuals with insulin resistance and low LDL - The Strong Heart Study. *Arterioscler. Thromb. Vasc. Biol.* **20:** 830–835.
10. Ross, R. 1999. Atherosclerosis–an inflammatory disease. *N. Engl. J. Med.* **340:** 115–126.
11. Lusis, A. J. 2000. Atherosclerosis. *Nature.* **407:** 233–241.
12. Barrett-Connor, E. 1997. Does hyperglycemia really cause coronary heart disease? *Diabetes Care.* **20:** 1620–1623.
13. Douglas, W. R. 1972. Of pigs and men and research: a review of applications and analogies of the pig, sus scrofa, in human medical research. *Space Life Sci.* **3:** 226–234.
14. Lee, K. T. 1986 Swine as animal models in cardiovascular research. *In* Swine in Biomedical Research. M. E. Tumbleson, editor. Plenum Press, New York. 1481–1496.
15. White, F. C., and C. M. Bloor. 1986. The pig as a model for myocardial ischemia. *In* Swine in Biomedical Research. M. E. Tumbleson, editor. Plenum Press, New York. 481–490.
16. Longhurst, J. C., and J. D. Symons. 1993 Function and development of coronary collateral vessels. *In* Collateral Circulation. W. Schaper and J. Schaper, editors. Kluwer Academic Publishers, Boston. 195- 214.
17. Jokinen, M. P., T. B. Clarkson, and R. W. Prichard. 1985. Animal models in atherosclerosis research. *Exp. Mol. Pathol.* **42:** 1–28.
18. Mahley, R. W., K. H. Weisgraber, T. Innerarity, H. B. Brewer, Jr., and G. Assmann. 1975. Swine lipoproteins and atherosclerosis. Changes in the plasma lipoproteins and apoproteins induced by cholesterol feeding. *Biochemistry.* **14:** 2817–2823.
19. Dixon, J. L., J. D. Stoops, J. L. Parker, M. H. Laughlin, G. A. Weisman, and M. Sturek. 1999. Dyslipidemia and vascular dysfunction in diabetic pigs fed an atherogenic diet. *Arterioscler. Thromb. Vasc. Biol.* **19:** 2981–2992.
20. Hill, B. J. F., J. L. Dixon, and M. Sturek. 2001. Effect of atorvastatin on intracellular calcium uptake in coronary smooth muscle cells from diabetic pigs fed an atherogenic diet. *Atherosclerosis.* **159:** 117–124.
21. Karalis, D. G., A. M. Ross, R. M. Vacari, H. Zarren, and R. Scott. 2002. Comparison of efficiency and safety of atorvastatin and simvastatin in patients with dyslipidemia with and without coronary heart disease. *Am. J. Cardiol.* **89:** 667–671.
22. Sturek, M., C. Otis, B. R. Wamhoff, J. L. Dixon, J. R. Turk, and H. K. Reddy. 2001. Dyslipidemia, not hyperglycemia, is the main factor eliciting coronary artery disease in Yucatan swine. *Arterioscler. Thromb. Vasc. Biol.* **21:** 644.
23. Wamhoff, B. R., J. L. Dixon, and M. Sturek. 2002. Atorvastatin treatment prevents alterations in coronary smooth muscle nuclear Ca^{2+} signaling associated with diabetic dyslipidemia. *J. Vasc. Res.* **39:** 208–220.
24. Sturek, M., D. L. Lee, B. R. Wamhoff, L. C. Katwa, H. K. Reddy, D. J. Voelker, and J. L. Dixon. 2001. Increased endothelin-induced Ca^{2+} signaling, tyrosine phosphorylation, and coronary artery disease in diabetic dyslipidemic swine are prevented by atorvastatin. *Arterioscler. Thromb. Vasc. Biol.* **21:** 691.
25. Homan, R., and M. K. Anderson. 1998. Rapid separation and quantitation of combined neutral and polar lipid classes by high-performance liquid chromatography and evaporative light-scattering mass detection. *J. Chromatogr. B.* **708:** 21–26.
26. Sun, G. Y. 1988. Preparation and analysis of acyl and alkenyl groups of glycerophospholipids from brain subcellular membranes. *In* Neuromethods: Lipids and Related Compounds. A. A. Boulton, G. B. Baker, and L. A. Horrocks, editors. Humana Press, Inc., Clifton, New Jersey. 63–81.
27. Corbin, D. R., and G. Y. Sun. 1978. Characterization of the enzymic transfer of arachidonoyl groups to 1-acyl-phosphoglycerides in mouse synaptosome fraction. *J. Neurochem.* **30:** 77–82.
28. Redlich, C. A., J. N. Grauer, A. M. Van Bennekum, S. L. Clever, R. B. Ponn, and W. S. Blaner. 1996. Characterization of carotenoid, vitamin A, and alpha-tocopheral levels in human lung tissue and pulmonary macrophages. *Am. J. Respir. Crit. Care Med.* **154:** 1436–1443.
29. Rudel, L. L., J. S. Parks, C. C. Hedrick, M. Thomas, and K. Williford. 1998. Lipoprotein and cholesterol metabolism in diet-induced coronary artery atherosclerosis in primates. Role of cholesterol and fatty acids. *Prog. Lipid Res.* **37:** 353–370.
30. Tall, A. R., D. M. Small, D. Atkinson, and L. L. Rudel. 1978. Studies on the structure of low density lipoproteins isolated from *Macaca fascicularis* fed an atherogenic diet. *J. Clin. Invest.* **62:** 1354–1363.
31. Marzetta, C. A., and L. L. Rudel. 1986. A species comparison of low density lipoprotein heterogeneity in nonhuman primates fed atherogenic diets. *J. Lipid Res.* **27:** 753–762.
32. Bierman, E. L. 1992. George Lyman Duff Memorial Lecture. Atherogenesis in diabetes. *Arterioscler. Thromb.* **12:** 647–656.
33. Zhang, Z., K. Cianflone, and A. D. Sniderman. 1999. Role of cholesterol ester mass in regulation of secretion of ApoB100 lipoprotein particles by hamster hepatocytes and effects of statins on that relationship. *Arterioscler. Thromb. Vasc. Biol.* **19:** 743–752.
34. Watts, G. F., R. Naoumova, M. H. Cummings, A. M. Umpleby, B. M. Slavin, P. H. Sonksen, and G. R. Thompson. 1995. Direct correlation between cholesterol synthesis and hepatic secretion of apolipoprotein B-100 in normolipidemic subjects. *Metabolism.* **44:** 1052–1057.
35. Cummings, M. H., and G. F. Watts. 1995. Increased hepatic secretion of very-low-density lipoprotein apolipoprotein B-100 in cholesteryl ester storage disease. *Clin. Chem.* **41:** 111–114.
36. Cummings, M. H., G. F. Watts, A. M. Umpleby, T. R. Hennessy, R. Naoumova, B. M. Slavin, G. R. Thompson, and P. H. Sönksen. 1995. Increased hepatic secretion of very-low-density lipoprotein apolipoprotein B-100 in NIDDM. *Diabetologia.* **38:** 959–967.
37. Brecher, P., A. V. Chobanian, D. M. Small, W. Van Sickle, A. Tercyak, A. Lazzari, and J. Baler. 1983. Relationship of an abnormal plasma lipoprotein to protection from atherosclerosis in the cholestrol-fed diabetic rabbit. *J. Clin. Invest.* **72:** 1553–1562.
38. Duff, G. L., and G. C. McMillan. 1949. The effect of alloxan diabetes on experimental cholesterol atherosclerosis in the rabbit. *J. Exp. Med.* **89:** 611–630.
39. Burnett, J. R., L. J. Wilcox, D. E. Telford, S. J. Kleinstiver, P. H. Barrett, R. S. Newton, and M. W. Huff. 1999. The magnitude of decrease in hepatic very low density lipoprotein apolipoprotein B secretion is determined by the extent of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibition in miniature pigs. *Endocrinology.* **140:** 5293–5302.
40. Yu, K. C., and A. D. Cooper. 2001. Postprandial lipoproteins and atherosclerosis. *Front. Biosci.* **6:** D332–D354.
41. Reaven, G. M. 1995. Pathophysiology of insulin resistance in human disease. *Physiol. Rev.* **75:** 473–486.
42. Curtin, A., P. Deegan, D. Owens, P. Collins, A. Johnson, and G. H. Tomkin. 1996. Elevated triglyceride-rich lipoproteins in diabetes. A study of apolipoprotein B-48. *Acta Diabetol.* **33:** 205–210.
43. Mero, N., M. Syvänne, and M. R. Taskinen. 1998. Postprandial lipid metabolism in diabetes. *Atherosclerosis.* **141:** S53–S55.
44. Noutsou, M., and A. Georgopoulos. 1999. Effects of simvastatin on fasting and postprandial triglyceride-rich lipoproteins in patients with type I diabetes mellitus. *J. Diabetes Complications.* **13:** 98–104.
45. St Clair, R. W., P. Greenspan, and M. Leight. 1983. Enhanced cholesterol delivery to cells in culture by low density lipoproteins from hypercholesterolemic monkeys. Correlation of cellular cholesterol accumulation with low density lipoprotein molecular weight. *Arteriosclerosis.* **3:** 77–86.
46. Doi, H., K. Kugiyama, M. Ohgushi, S. Sugiyama, T. Matsumura, Y. Ohta, H. Oka, N. Ogata, A. Hirata, Y. Yamamoto, and H. Yasue. 1999. Membrane active lipids in remnant lipoproteins cause impairment of endothelium-dependent vasorelaxation. *Arterioscler. Thromb. Vasc. Biol.* **19:** 1918–1924.
47. Gerrity, R. G., R. Natarajan, J. L. Nadler, and T. Kimsey. 2001. Diabetes-induced accelerated atherosclerosis in swine. *Diabetes.* **50:** 1654–1665.
48. Griffin, E., A. Re, N. Hamel, C. Fu, H. Bush, T. McCaffrey, and A. S. Asch. 2001. A link between diabetes and atherosclerosis: Glucose regulates expression of CD36 at the level of translation. *Nat. Med.* **7:** 840–846.
49. Bell, F. P., and R. G. Gerrity. 1992. Evidence for an altered lipid metabolic state in circulating blood monocytes under conditions

Downloaded from www.jlr.org by guest, on June 14, 2012

of hyperlipemia in swine and its implications in arterial lipid metabolism. *Arterioscler. Thromb.* **12:** 155–162.
50. Reitman, J. S., R. W. Mahley, and D. L. Fry. 1982. Yucatan miniature swine as a model for diet-induced atherosclerosis. *Atherosclerosis.* **43:** 119–132.
51. Yusuf, S., G. Dagenais, J. Pogue, J. Bosch, and P. Sleight. 2000. Vitamin E supplementation and cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *N. Engl. J. Med.* **342:** 154–160.
52. Heinecke, J. W. 2001. Is the emperor wearing clothes? Clinical trials of vitamin E and the LDL oxidation hypothesis. *Arterioscler. Thromb. Vasc. Biol.* **21:** 1261–1264.
53. Hla, T., M. J. Lee, N. Ancellin, J. H. Paik, and M. J. Kluk. 2001. Lysophospholipids–receptor revelations. *Science.* **294:** 1875–1878.
54. Boullier, A., K. L. Gillotte, S. Hörkko, S. R. Green, P. Friedmann, E. A. Dennis, J. L. Witztum, D. Steinberg, and O. Quehenberger. 2000. The binding of oxidized low density lipoprotein to mouse CD36 is mediated in part by oxidized phospholipids that are associated with both the lipid and protein moieties of the lipoprotein. *J. Biol. Chem.* **275:** 9163–9169.
55. Hurt-Camejo, E., G. Camejo, H. Peilot, K. Öörni, and P. Kovanen. 2001. Phospholipase A(2) in vascular disease. *Circ. Res.* **89:** 298–304.
56. Daugherty, A., B. S. Zweifel, B. E. Sobel, and G. Schonfeld. 1988. Isolation of low density lipoprotein from atherosclerotic vascular tissue of Watanabe heritable hyperlipidemic rabbits. *Arteriosclerosis.* **8:** 768–777.
57. Glass, C. K., and J. L. Witztum. 2001. Atherosclerosis: The road ahead. *Cell.* **104:** 503–516.
58. Natarajan, R., R. G. Gerrity, J. L. Gu, L. Lanting, L. Thomas, and J. L. Nadler. 2002. Role of 12-lipoxygenase and oxidant stress in hyperglycemia-induced acceleration of atherosclerosis in a diabetic pig model. *Diabetologia.* **45:** 125–133.
59. Tonkin, A., P. Aylward, D. Colquhoun, P. Glasziou, P. Harris, S. MacMahon, P. Magnus, D. Newel, P. Nestel, N. Sharpe, D. Hunt, J. Shaw, R. J. Simes, P. Thompson, A. Thomson, M. West, H. White, S. Simes, W. Hague, S. Caleo, J. Hall, A. Martin, S. Mulray, and P. Barter. 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.
60. Koh, K. K. 2000. Effects of statins on vascular wall: vasomotor function, inflammation, and plaque stability. *Cardiovasc. Res.* **47:** 648–657.
61. Pyörälä, K., T. R. Pedersen, J. Kjekshus, O. Faergeman, A. G. Olsson, G. Thorgeirsson, and The Scandinavian Simvastatin Survival Study (4S) Group. 1997. Cholesterol lowering with simvastatin improves prognosis of diabetic patients with coronary heart disease: A subgroup analysis of the Scandinavian Simvastatin Survival Study (4S). *Diabetes Care.* **20:** 614–620.
62. Tam, E. S. L., D. G. Ferguson, D. R. Bielefeld, J. N. Lorenz, R. M. Cohen, and R. Y. K. Pun. 1997. Norepinephrine-mediated calcium signaling is altered in vascular smooth muscle of diabetic rat. *Cell Calcium.* **21:** 143–150.
63. Fleischhacker, E., V. E. Esenabhalu, M. Spitaler, S. Holzmann, F. Skrabal, B. Koidl, G. M. Kostner, and W. F. Graier. 1999. Human diabetes is associated with hyperreactivity of vascular smooth muscle cells due to altered subcellular Ca^{2+} distribution. *Diabetes.* **48:** 1323–1330.
64. Laufs, U., V. La Fata, J. Plutzky, and J. K. Liao. 1998. Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. *Circulation.* **97:** 1129–1135.
65. Scalia, R., M. E. Gooszen, S. P. Jones, M. Hoffmeyer, D. M. Rimmer III, S. D. Trocha, P. L. Huang, M. B. Smith, A. M. Lefer, and D. J. Lefer. 2001. Simvastatin exerts both anti-inflammatory and cardioprotective effects in apolipoprotein E-deficient mice. *Circulation.* **103:** 2598–2603.
66. Tesfamariam, B., B. H. Frohlich, and R. E. Gregg. 1999. Differential effects of pravastatin, simvastatin, and atorvastatin on Ca^{2+} release and vascular reactivity. *J. Cardiovasc. Pharmacol.* **34:** 95–101.
67. Fukumoto, Y., P. Libby, E. Rabkin, C. C. Hill, M. Enomoto, Y. Hirouchi, M. Shiomi, and M. Aikawa. 2001. Statins alter smooth muscle cell accumulation and collagen content in established atheroma of watanabe heritable hyperlipidemic rabbits. *Circulation.* **103:** 993–999.

ASBMB JOURNAL OF LIPID RESEARCH

Downloaded from www.jlr.org by guest, on June 14, 2012