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Journal

Diabetes, 59(12)

ISSN

0012-1797

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Publication Date

2010-12-01

DOI

10.2337/db10-0844

Peer reviewed

Apolipoprotein A-I Mimetic Peptides Prevent Atherosclerosis Development and Reduce Plaque Inflammation in a Murine Model of Diabetes

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OBJECTIVE—To determine the effect of the apolipoprotein A-I (ApoA-I) mimetic peptide, D-4F, on atherosclerosis development in a pre-existing diabetic condition.

RESEARCH DESIGN AND METHODS—We induced hyperglycemia in 6-week-old apoE^{-/-} female mice using streptozotocin. Half of the diabetic apoE^{-/-} mice received D-4F in drinking water. Ten weeks later, plasma lipids, glucose, insulin levels, atherosclerotic lesions, and lesion macrophage content were measured.

RESULTS—Diabetic apoE^{-/-} mice developed ~300% more lesion area, marked dyslipidemia, increased glucose levels, and reduced plasma insulin levels when compared with nondiabetic apoE^{-/-} mice. Atherosclerotic lesions were significantly reduced in the D-4F-treated diabetic apoE^{-/-} mice in whole aorta (1.11 ± 0.73 vs. 0.58 ± 0.44 , percentage of whole aorta, $P < 0.01$) and in aortic roots ($36,038 \pm 18,467 \mu\text{m}^2/\text{section}$ vs. $17,998 \pm 12,491 \mu\text{m}^2/\text{section}$, $P < 0.01$) when compared with diabetic apoE^{-/-} mice that did not receive D-4F. Macrophage content in atherosclerotic lesions from D-4F-treated diabetic apoE^{-/-} mice was significantly reduced when compared with nontreated animals (78.03 ± 26.1 vs. 29.6 ± 15.2 , $P < 0.001$, percentage of whole plaque). There were no differences in glucose, insulin, total cholesterol, HDL cholesterol, and triglyceride levels between the two groups. Arachidonic acid, PGE₂, PGD₂, 15-HETE, 12-HETE, and 13-HODE concentrations were significantly increased in the liver tissue of diabetic apoE^{-/-} mice compared with nondiabetic apoE^{-/-} mice and significantly reduced by D-4F treatment.

CONCLUSIONS—Our results suggest that oral D-4F can prevent atherosclerosis development in pre-existing diabetic mice and this is associated with a reduction in hepatic arachidonic acid and oxidized fatty acid levels. *Diabetes* 59:3223–3228, 2010

Type 1 diabetes is associated with two- to fourfold higher risk of coronary artery disease (CAD) and macrovascular disease (1,2). The excess cardiovascular risk in this population is not entirely explained by traditional risk factors, including hyperglycemia.

Oxidative modification of LDL and the subsequent formation of foam cells are thought to be an initial step in atherogenesis (3). Multiple animal and in vitro studies have supported a role for oxidative processes in all phases of CAD, from foam cell formation to plaque rupture and thrombosis (4–6). Initiation of lipid peroxidation and formation of an array of bioactive fatty acid oxidation products are widely held as critical steps in the atherosclerotic process. It has been suggested that the inflammatory properties of lipoproteins may also be important for the development of the atherosclerotic process in diabetes (7). However, the mechanism by which diabetic dyslipidemia contributes to the development of CAD in type 1 diabetes is not clear.

HDL and apolipoprotein A-I (apoA-I), its major protein, have been efficacious in the treatment of atherosclerosis (8). ApoA-I mimetic peptides 4F (L-4F and D-4F), that form a class A amphipathic helix similar to those found in apoA-I, were found to be efficacious in murine models of atherosclerosis (9) by a mechanism that is independent of plasma cholesterol levels and in part related to its ability to remove oxidized lipids from lipoproteins (9). Moreover, recent studies have shown that apoA-I mimetic peptides increase antioxidants, confer robust vascular protection and improve insulin sensitivity in rodent models of diabetes and obesity (10–12).

In this study, we examined whether oral administration of D-4F can inhibit atherosclerosis development in a pre-existing diabetic condition. Our results show that D-4F is able to decrease atherosclerotic lesion development in diabetic mice, and this is associated with a reduction of hepatic arachidonic acid and hepatic oxidized fatty acids levels.

RESEARCH DESIGN AND METHODS

The Animal Research Committee at University of California Los Angeles approved all the protocols used in these studies. Five-week-old female apoE^{-/-} mice were obtained from Jackson Laboratories (Bar Harbor, ME) and were housed in the Division of Laboratory Animal Medicine at the University of California Los Angeles. The mice were fed normal mouse chow diet and given free access to both food and water throughout the study, except when fasting blood specimens were obtained. After 1 week of acclimatization (at the age of 6 weeks), 40 mice were administered intraperitoneal injections of streptozotocin (STZ), Sigma-Aldrich) at a dosage of 65 mg/kg daily for 5 consecutive days. Control animals ($n = 20$) received vehicle (citrate buffer, pH

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Received 17 June 2010 and accepted 23 August 2010. Published ahead of print at <http://diabetes.diabetesjournals.org> on 8 September 2010. DOI: 10.2337/db10-0844.

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4.5) alone. Nine days after the last STZ injection, fasting (6 h) blood glucose levels were measured using a glucose machine (HemoCue 201 AB, Anghelhom, Sweden), and diabetes was verified on the basis of glucose level >250 mg/dl. By random selection, half of the diabetic mice ($n = 17$) were chosen to receive the apoA-I mimetic peptide, D-4F, in drinking water at a concentration of 0.2 mg/ml for 8 weeks; the remaining mice received regular drinking water. The mice consumed ~5 ml of water daily (from 3 to 5 ml daily). After 8 weeks of treatment, all of the mice (control, diabetic, and diabetic/D-4F-treated) were fasted for 6 h, after which blood and organs were collected.

Metabolic parameters. Plasma lipids were determined by enzymatic colorimetric assays as described previously (13). Plasma glucose and insulin levels were determined as previously described (14).

Atherosclerosis quantification. En face lesions analysis in the entire aorta were performed according to procedures described by Tangirala et al. (15). Briefly, after perfusion-fixation, the aorta was dissected out, opened longitudinally from heart to the iliac arteries, pinned on a black wax pan, and stained with Sudan IV solution. The image of the aorta was captured using a SONY DXC-970MD color video camera, and the image analysis was performed using the Image-Pro plus program (Media Cybernetics, Silver Spring, MD) in a blinded fashion. The area covered by atherosclerotic lesions divided by the area of the entire aorta was calculated for each group, expressed as a percentage, and compared.

Atherosclerotic lesion area in the aortic root was determined as described previously (16). Briefly, the heart and proximal aorta were removed and embedded in optimum cutting temperature compound. Serial 10 μm -thick cryosections from the middle portion of the ventricle to the aortic arch were collected, mounted on precoated slides, and stained with Oil Red O and hematoxylin. The lipid-containing area on each section, centered around the aortic valves, was determined in a blinded fashion, using an ocular piece with a $20 \times 20 \mu\text{m}^2$ grid on a light microscope. The average lesion area per aorta, calculated from 5 to 10 sections of each aorta, was determined.

Macrophage content. Fresh-frozen aortic root sections were stained for CD-68. Briefly, after fixation in ice-cold acetone, sections were blocked in 4% BSA plus 10% goat serum for 3 h at room temperature. Rat anti-mouse CD-68 antibodies (1:100; Serotec) were used with an overnight incubation at 4°C. Goat anti-rat and goat anti-rabbit alkaline phosphatase secondary antibodies (Jackson Immuno Research) were used at 1:200 for 1-h incubation at room temperature. Immunostaining was visualized using Vector Red substrate plus levamisole to inhibit endogenous alkaline phosphatase activity (Vector Laboratories). Immunostaining was quantified in a blinded fashion using an ocular piece with a $20 \times 20 \mu\text{m}^2$ grid on a light microscope, and the area stained was divided by the area of the entire plaque and the ratios obtained were compared between groups.

Hepatic free fatty acid levels

Chemicals. Arachidonic acid (AA); 9-oxo-11a,15S-dihydroxy-prosta-5Z,13E-dien-1-oic acid (prostaglandin E_2 ; PGE_2), 9a,15S-dihydroxy-11-oxo-prosta-5Z,13E-dien-1-oic acid (prostaglandin D_2 ; PGD_2), (\pm)12-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (12-HETE), (\pm)15-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-HETE), (\pm)13-hydroxy-9Z,11E-octadecadienoic acid (13-HODE), (\pm)9-hydroxy-10E,12E-octadecadienoic acid (9-HODE), 9a,15S-dihydroxy-11-oxo-prosta-5Z,13E-dien-1-oic-3,3,4,4-day₄ acid (prostaglandin D_2 -c4; PGD_2 -d₄), 12[S]-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic-5,6,8,9,11,12,14,15-day₈ acid (12(S)-HETE-d₈), 15(S)-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic-5,6,8,9,11,12,14,15-day₈ acid (15(S)-HETE-d₈), 13(S)-hydroxy-9Z,11E-octadecadienoic-9,10,12,13-day₄ acid (13[S]-HODE-d₄) were purchased from Cayman Chemicals (Ann Arbor, MI).

Sample preparation. We performed lipid extraction as described previously with slight modification (17). Briefly, a piece of liver, previously dissected and weighed on dry ice, after the addition of methanol (0.02% BHT), internal standard (15[S]-HETE-d₈), 12[S]-HETE-d₈, 13[S]-HODE-d₄, PGD_2 -d₄), formic acid and water, was homogenized, vortexed, and incubated for 1 h on ice. The homogenate was centrifuged and the supernatant was loaded onto an Oasis HLB (60 mg, 30 μm) solid-phase extraction cartridge (Waters) and equilibrated with 2-ml methanol and 2-ml water, and then washed with 2-ml 0.5% methanol, and eluted with 3-ml 0.5% methanol. The solvent was evaporated under argon and reconstituted with 50 μl of methanol, vortexed, and transferred to an auto sampler vial for liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis.

LC/MS/MS analysis. LC/MS/MS analysis was performed as previously described (18). The transitions monitored were mass-to-charge ratio (m/z): 303.1→259.2 for AA; 351.1→271.2 for PGD_2 ; 351.1 →271.2 for PGE_2 ; 319.1→179.0 for 12-HETE; 319.1→219.0 for 15-HETE; 295.0→194.8 for 13-HODE; 295.0→171.0 for 9-HODE; 327.1→226.1 for 15(S)-HETE-d₈; 327.1→184.0 for 12(S)-HETE-d₈; 299.0 →197.9 for 13(S)-HODE-d₄; 355.0→237.0 for PGD_2 -d₄.

TABLE 1
Characteristics of control apoE^{-/-} mice, diabetic apoE^{-/-} mice, and diabetic apoE^{-/-} mice treated with D-4F

	Controls	Diabetic	Diabetic + D-4F
Total cholesterol (mg/dl)	493 ± 124	993 ± 521*	1,082 ± 417
LDL cholesterol (mg/dl)	467 ± 120	954 ± 526*	1,027 ± 410
HDL cholesterol (mg/dl)	18 ± 4	14 ± 4*	14 ± 3
Triglyceride (mg/dl)	41 ± 17	59 ± 65	62 ± 35
Glucose (mg/dl)	181 ± 32	366 ± 123*	420 ± 89
Insulin (pg/ml)	281 ± 113	132 ± 68*	181 ± 114
Weight (g)	20.1 ± 1.4	16.9 ± 3.7*	16.2 ± 2.6

Values are expressed as average ±SD. Mice were treated with 8 weeks of D-4F treatment in drinking water; 0.2 mg/ml as described in methods. * $P < 0.01$ diabetic vs. control.

Statistical analysis. All data were expressed as mean ± SD. Differences between groups were determined by ANOVA or Wilcoxon/Kruskal-Wallis for nonparametric analysis.

RESULTS

Metabolic parameters were not affected by D-4F treatment. Diabetic apoE^{-/-} mice were found to have a significant reduction in body weight and decreased serum insulin levels and HDL cholesterol levels. The diabetic apoE^{-/-} mice developed significant elevations in plasma total cholesterol, LDL cholesterol, and serum glucose levels compared with nondiabetic apoE^{-/-} mice. Treatment with D-4F in drinking water at 0.2 mg/ml for 8 weeks had no effect on body weight, serum glucose, insulin levels, and lipoprotein cholesterol levels compared with untreated diabetic apoE^{-/-} mice (Table 1).

D-4F treatment reduced atherosclerotic lesions in diabetic apoE^{-/-} mice. Total atherosclerotic lesion area was quantified in control apoE^{-/-} mice ($n = 20$), untreated diabetic apoE^{-/-} mice ($n = 17$), and D-4F-treated diabetic apoE^{-/-} mice ($n = 17$). Diabetic apoE^{-/-} mice were found to have a 300% increase in whole aortic atherosclerotic lesion area compared with nondiabetic apoE^{-/-} mice. Atherosclerotic lesion area was significantly reduced in D-4F-treated diabetic apoE^{-/-} mice (0.58 ± 0.44 vs. 1.11 ± 0.73 , percentage of the whole aorta, $P < 0.01$) (Fig. 1A and B), when compared with untreated diabetic apoE^{-/-} mice. The average lesion area as measured by Oil-Red-O staining of the aortic sinus sections was significantly reduced in D-4F-treated diabetic apoE^{-/-} mice when compared with untreated diabetic apoE^{-/-} mice ($36,038 \pm 18,467$ vs. $17,998 \pm 12,491 \mu\text{m}^2$ /section, $P < 0.01$, $n = 15$ per group) (Fig. 1C and D).

D-4F treatment causes a reduction in the macrophage content of atherosclerotic lesions in diabetic apoE^{-/-} mice. Quantification for macrophages in the aortic sections using CD-68 immunohistochemistry demonstrated a dramatic reduction in macrophage content in D-4F-treated diabetic apoE^{-/-} mice when compared with untreated diabetic apoE^{-/-} mice (78.03 ± 26.1 vs. 29.6 ± 15.2 , expressed as a percentage of the whole plaque, $P < 0.001$) (Fig. 2A and B).

D-4F treatment results in the reduction of arachidonic acid and oxidized fatty acid content in the livers of diabetic apoE^{-/-} mice. Binding and removal of bioactive lipids, including oxidized fatty acids, has been identified as one of the mechanisms by which apoA-I mimetic peptides prevent the development of atherosclerosis (19,20). Therefore, we determined 1) whether the

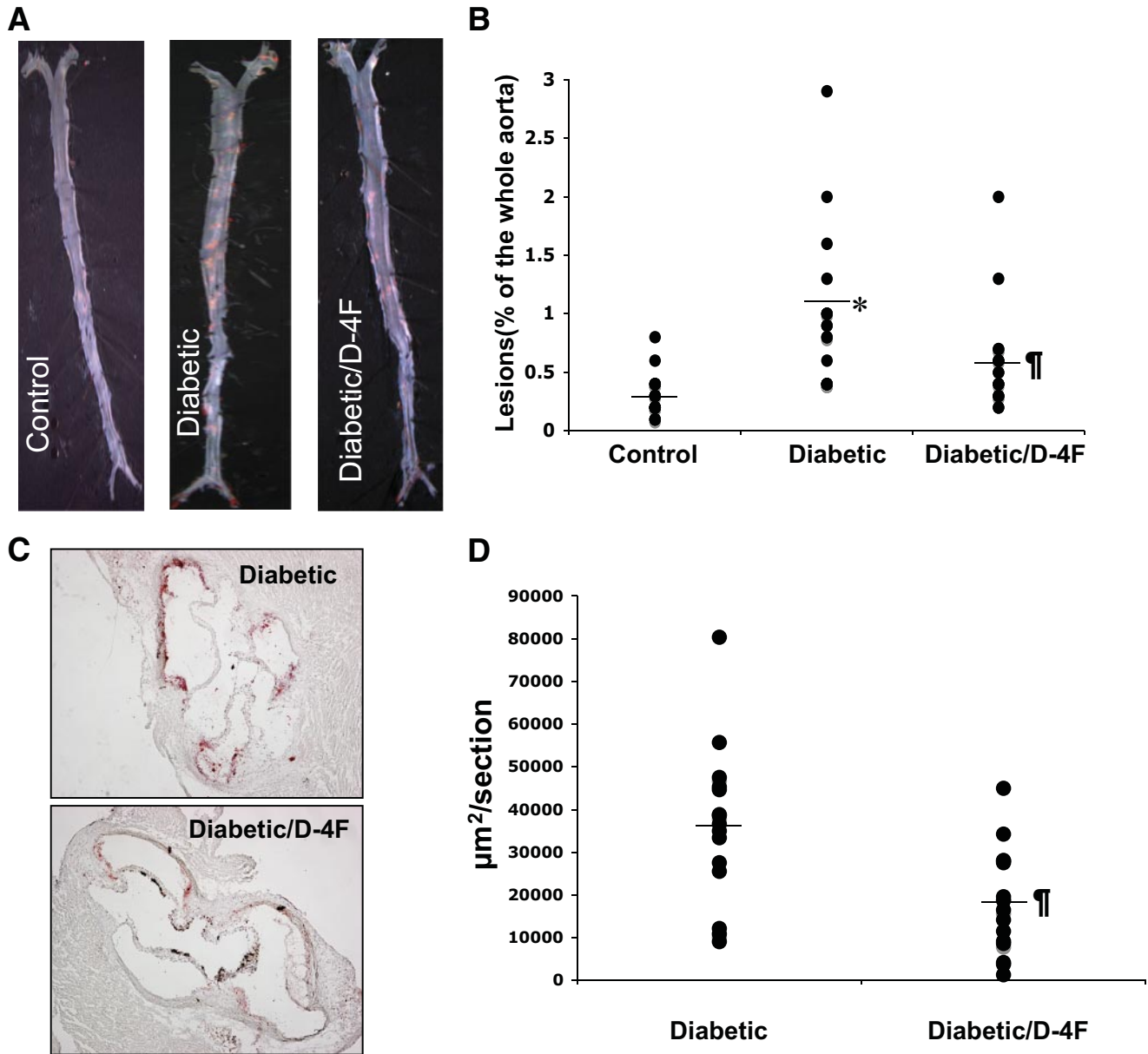


FIG. 1. Decreased atherosclerotic lesion formation and lipid content in the diabetic D4F-treated mice. **A:** Representative images of the whole aorta by en face method in control (apoE^{-/-} mice that were not diabetic), in diabetic apoE^{-/-} mice (diabetic), and in diabetic apoE^{-/-} mice treated with D-4F (diabetic/D-4F). **B:** Quantitative analysis of en face lesions in female apoE^{-/-} mice on a chow diet; $n = 20$ for control; $n = 17$ for diabetic mice; and $n = 17$ for diabetic mice treated with D-4F (diabetic/D-4F). **C:** Representative sections of mouse aortic roots, stained with Oil red O. **D:** Quantitative analysis of lesion area in female diabetic apoE^{-/-} mice (diabetic) and in diabetic apoE^{-/-} mice treated with D-4F (diabetic/D-4F); $n = 15$ for both groups; * $P < 0.01$ diabetic versus control; ¶ $P < 0.01$ diabetic versus diabetic/D-4F. (A high-quality digital representation of this figure is available in the online issue.)

levels of these lipids were elevated in the livers of diabetic apoE^{-/-} mice compared with the livers of control apoE^{-/-} mice, and 2) whether D-4F treatment altered these levels. Liver tissue extracts from control apoE^{-/-}, diabetic apoE^{-/-}, and D-4F-treated diabetic apoE^{-/-} mice ($n = 5$ per group) were analyzed by LC/MS/MS as described in METHODS (20). Arachidonic acid, prostaglandin E₂ (PGE₂), PGD₂, 15-hydroxyeicosatetraenoic acid (15-HETE), 12-HETE, and 13-hydroxyoctadecadienoic acid (13-HODE) were significantly increased in the livers of diabetic apoE^{-/-} mice compared with control apoE^{-/-} mice. The D-4F-treated diabetic apoE^{-/-} mice showed a significant decrease in hepatic tissue levels of these lipids compared with diabetic apoE^{-/-} mice that did not receive D-4F (Fig. 3). The levels of these lipids in the diabetic

apoE^{-/-} mice that were not treated with D-4F compared with those that were treated with D-4F were significantly greater for arachidonic acid ($26,97.5 \pm 1,489.3$ vs. $1,151.0 \pm 452.25$ ng/100 mg of liver, $P < 0.05$), PGD₂ (470 ± 330 vs. 130 ± 110 pg/100 mg of liver, $P = 0.05$), 15-HETE (825 ± 398 vs. 660 ± 95 pg/100 mg of liver, $P = 0.06$), 12-HETE ($1,550 \pm 805$ vs. 805 ± 340 pg/100 mg of liver, $P = 0.05$) and 13-HODE (7.75 ± 3.81 vs. 5.5 ± 1.1 ng/100 mg of liver, $P < 0.05$).

DISCUSSION

The major finding of the present study is that the apoA-I mimetic peptide D-4F prevented the acceleration of atherosclerosis in STZ-induced diabetic apoE^{-/-} mice. We

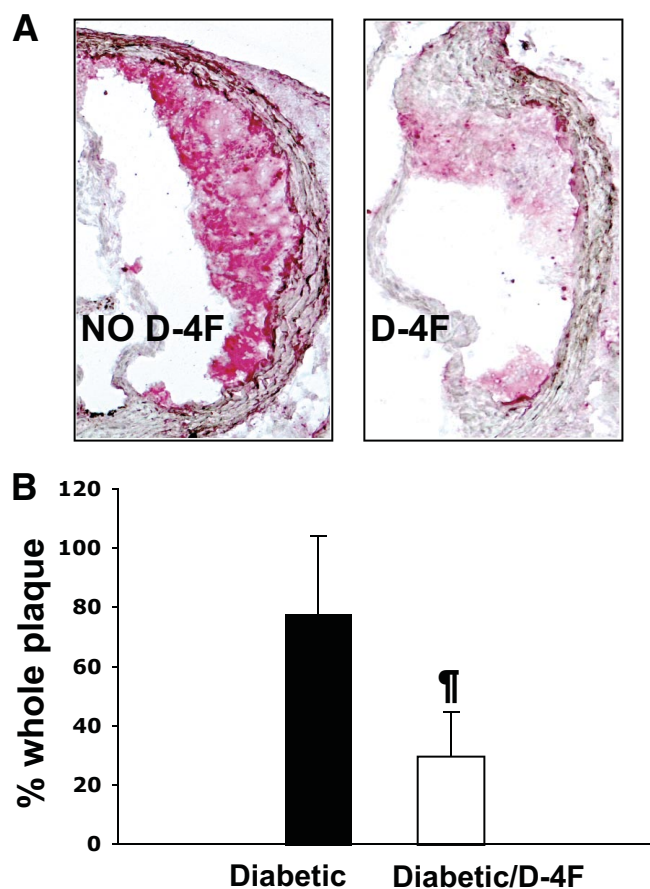


FIG. 2. The D-4F-treated mice demonstrated significantly decreased macrophage content. Sections from mouse aorta were analyzed by immunohistochemistry for the presence of macrophages using CD-68 antibody (as described in RESEARCH DESIGN AND METHODS). **A:** Representative image of macrophage content in atherosclerotic plaques. **B:** Quantitative analysis of macrophage content in diabetic female apoE^{-/-} mice (diabetic) on a chow diet ($n = 10$) and in diabetic female apoE^{-/-} mice treated with D-4F (diabetic/D-4F) ($n = 10$). $\#P < 0.01$ diabetic/D-4F versus diabetic. (A high-quality digital representation of this figure is available in the online issue.)

recognize that diabetic apoE^{-/-} mice may not perfectly mimic human type 1 diabetes; however, this is a well accepted animal model for studying hyperglycemia-induced atherosclerosis. Induction of diabetes by STZ in apoE^{-/-} mice has been previously used to establish a role for both the advanced glycation end products (21) and the renin-angiotensin system (22) in the attenuation of atherosclerosis under conditions of hyperglycemia. Renard et al. (23) used a virally induced pancreatic destruction method to induce diabetes and concluded that diabetic conditions accelerate atherosclerosis similar to the STZ method.

The finding that D-4F drastically reduced atherosclerosis development with no improvement in serum levels of glucose, insulin, or lipoprotein cholesterol levels (Table 1 and Fig. 1) confirms that the effect of the peptide on atherosclerotic lesions is not related to plasma cholesterol levels or alteration in severity of diabetes. D-4F mediated prevention of atherosclerosis development in diabetic apoE^{-/-} mice was associated with a reduction in lipid and macrophage content of the atherosclerotic lesions (Fig. 2), indicating that D-4F treatment significantly altered the structure and composition of the plaque without altering plasma glucose or cholesterol levels.

The antiatherogenic effect of D-4F in the absence of any

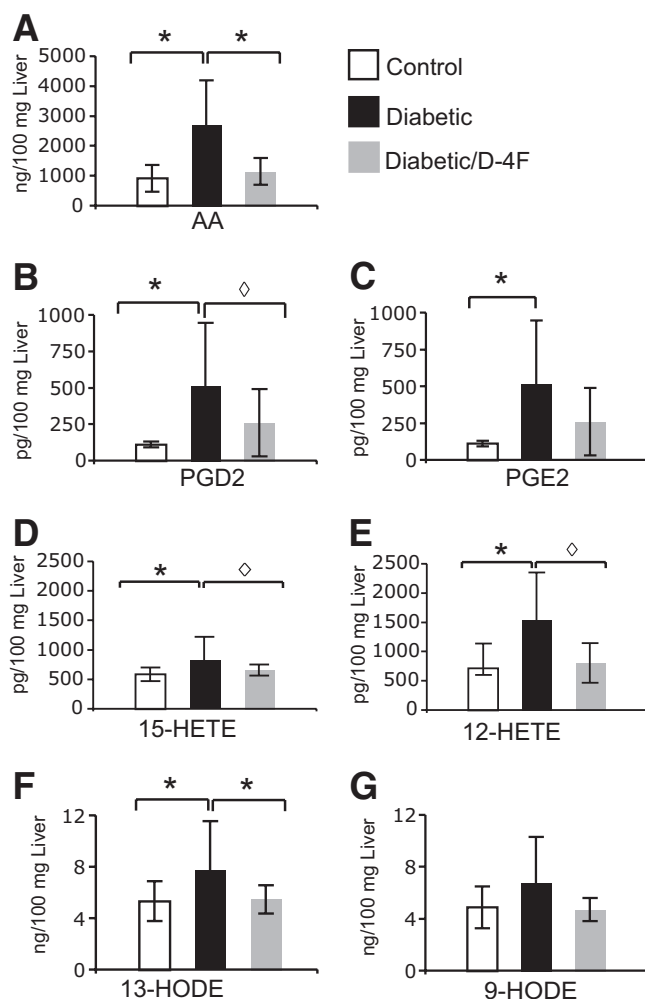


FIG. 3. Effect of D-4F on hepatic lipid levels. As described in METHODS, lipid levels were determined by LC/MS/MS in the livers of apoE^{-/-} mice (control), diabetic apoE^{-/-} mice (diabetic), and diabetic apoE^{-/-} mice treated with D-4F (diabetic/D-4F). **A:** Arachidonic acid (AA); **B:** PGD₂; **C:** PGE₂; **D:** 15-HETE; **E:** 12-HETE; **F:** 13-HODE; **G:** 9-HODE; ($n = 5$ for each group); $*P < 0.05$; $\diamond P \leq 0.06$.

changes in hyperglycemia or plasma lipoprotein or lipid levels suggests that lipoprotein oxidation may have a role in the accelerated atherosclerotic process in apoE^{-/-} diabetic mice. Atherosclerosis is the result of complex interactions between oxidized-lipoproteins, monocytes/macrophages, injured endothelium, and smooth muscle cells. Biologic oxidation products of arachidonic and linoleic acid, including prostaglandins, HETEs, and HODEs, play a role in LDL oxidation, one of the first steps in atherosclerosis (3). LDL oxidation is a complex process influenced by a multitude of oxidation pathways (24) including the lipoxygenase pathway which can generate potent lipid oxidants that include hydroperoxyoctadecadienoic acid (HPODE) and hydroperoxyeicosatetraenoic acid (HPETE) (3,25). A number of previous studies have shown that products from the arachidonic acid and linoleic acid pathways are contained in oxLDL. Sevastian and colleagues noted that a subpopulation of freshly isolated LDL that they described as LDL(-) contains lipid hydroperoxides (26). Parthasarathy (27), Witztum and Steinberg (28), Thomas and Jackson (29), Frei and colleagues (30), and Thomas, Kalyanaraman, and Girotti (31) studied metal ion-dependent LDL oxidation in vitro and hypothe-

sized that LDL must be "seeded" with reactive oxygen species before it can be oxidized. Thomas and Jackson (29) and Parthasarathy (27) suggested a role for lipoxygenases in the seeding of LDL. We previously showed that the seeding molecules present in freshly isolated LDL are derived in part from the cellular metabolism of linoleic acid (HPODE) and arachidonic acid (HPETE) (3). The products resulting from the action of fatty acid hydroperoxides on LDL account for the ability of LDL to induce endothelial cells to bind monocytes and secrete the potent monocyte chemoattractant monocyte chemoattractant protein-1 (MCP-1), which is one of the first steps in the development of atherosclerosis. It has been reported that *in vitro* apoA-I and apoA-I mimetic peptides bound non-oxidized fatty acids such as arachidonic acid and linoleic acid similarly, but the 4F peptides bound oxidized fatty acids derived from arachidonic acid or linoleic acid with a remarkable higher affinity than apoA-I (19). More recently, we reported that plasma oxidized fatty acids levels were significantly reduced within a few hours of L-4F administration in mice (18).

Lipid oxidation products are continuously produced in the tissues and enter the circulation. Because of the amount of plasma required for determining specific plasma-oxidized fatty acid levels, we did not make such measurements in these studies. Instead we determined the hepatic levels of arachidonic acid and oxidized fatty acids. Our results show that the reduction in atherosclerosis with D-4F treatment was associated with a significant reduction of arachidonic acid and oxidized fatty acids in liver.

Liver is the organ with the highest accumulation of oxidized lipids. Previous studies have shown that there is a selective hepatic uptake of oxidized LDL (ox-LDL) and oxidized cholesterol esters (32,33) mediated by the selective binding by scavenger receptors on liver endothelial and Kupfer cells for modified LDL (34). More recently it was shown that there is a selective uptake by hepatocytes of oxidized lipids from ox-LDL-loaded macrophages (35). Shaish et al. (36) reported the organ distribution of ^{125}I -LDL, ^{125}I -HDL, and ^{125}I -oxLDL in apoE^{-/-} mice and demonstrated that the highest accumulation of ox-LDL 24 h after administration was in liver (sixfold more than unoxidized or normal LDL).

Our finding of an increase in arachidonic acid and oxidized fatty acid levels in the livers of diabetic mice is particularly interesting in light of the hypothesis that hyperglycemia may directly contribute to the generation of oxidative stress (37). Enhanced lipid peroxidation in diabetes has been demonstrated in STZ-induced diabetic rats that showed a marked increase in plasma levels and urinary excretion rates of F₂-isoprostanes (a free radical oxidation product derived from the oxidation of arachidonic acid) (38). Enhanced lipid peroxidation has been shown to be one of the early events in the development of type 1 diabetes (39). Arachidonic acid peroxidation products have been proposed as a noninvasive index in type 1 diabetes that may be useful for monitoring pharmacologic interventions aimed at interfering with disease development and progression (40).

The studies detailed here used only female apoE^{-/-} mice. We chose to use female apoE^{-/-} mice because on a chow diet they develop larger atherosclerotic lesions associated with lower cholesterol and triglyceride levels when compared with male apoE^{-/-} mice (41). Addition-

ally, we previously reported that the effect of D-4F was not different in female and male apoE^{-/-} mice (42).

In conclusion, our results suggest that the apoA-I mimetic peptide, D-4F, was effective in preventing the development of accelerated atherosclerosis in mice with pre-existing diabetes. The beneficial effects of D-4F were associated with a significant reduction in hepatic arachidonic acid and oxidized fatty acid levels, which supports the current literature suggesting that diabetes is characterized by glucose-mediated oxidative stress. The relevance of these findings to humans with diabetes remains to be determined.

ACKNOWLEDGMENTS

This work was supported in part by U.S. Public Health Service grants HL-30568, HL-34343, HL-082823, and the Laubisch, Castera, and M.K. Gray Funds at University of California Los Angeles and by an Internal Medicine School of Specialty Fellowship to C.M. from the University of Pisa.

M.N., A.M.F., and S.T.R. are principals in Bruin Pharma, and A.M.F. is an officer in Bruin Pharma.

No other potential conflicts of interest relevant to this article were reported.

C.M. researched data, contributed to discussion, and wrote and reviewed the manuscript. S.I. researched data and contributed to discussion. V.G. and M.N. researched data. A.M.F. reviewed the manuscript. S.T.R. contributed to discussion and reviewed the manuscript.

Parts of this study were presented in oral form at the 70th Scientific Sessions of the American Diabetes Association, Orlando, Florida, 25–29 June 2010.

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