Long-Term Treatment with the Apolipoprotein A1 Mimetic Peptide Increases Antioxidants and Vascular Repair in Type I Diabetic Rats

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ABSTRACT

Apolipoprotein A1 mimetic peptide (D-4F), synthesized from D-amino acid, enhances the ability of high-density lipoprotein to protect low-density lipoprotein (LDL) against oxidation in atherosclerotic disease. Using a rat model of type I diabetes, we investigated whether chronic use of D-4F would lead to up-regulation of heme oxygenase (HO)-1, endothelial cell marker (CD31), and thrombomodulin (TM) expression and increase the number of endothelial progenitor cells (EPCs). Sprague-Dawley rats were rendered diabetic with streptozotocin (STZ) and either D-4F or vehicle was administered, by i.p. injection, daily for 6 weeks (100 mg/kg b.wt.). HO activity was measured in liver, kidney, heart, and aorta. After 6 weeks of D-4F treatment, HO activity significantly increased in the heart and aorta by 29 and 31% (p < 0.05 and p < 0.49), respectively. Long-term D-4F treatment also caused a significant increase in TM and CD31 expression. D-4F administration increased antioxidant capacity, as reflected by the decrease in oxidized protein and oxidized LDL, and enhanced EPC function and/or repair, as evidenced by the increase in EPC endothelial nitric-oxide synthase (eNOS) and prevention of vascular TM and CD31 loss. In conclusion, HO-1 and eNOS are relevant targets for D-4F and may contribute to the D-4F-mediated increase in TM and CD31, the antioxidant and anti-inflammatory properties, and confers robust vascular protection in this animal model of type I diabetes.

The development of the 4F peptides has previously been recorded in detail (Navab et al., 2005b). The major protein in high-density lipoprotein is apolipoprotein A-I (apoA-I). It contains 243 amino acids. Based on the ability to form helixes similar to those in human apoA-I, Anantharamaiah and Segrest searched for peptides smaller than apoA-I. They found that an 18-amino acid peptide with the sequence DWLKAFYDKVAEKLKEAF, which does not have any sequence homology with apoA-I, formed a class A amphipathic helix similar to those found in apoA-I. They named this peptide 18A (Anantharamaiah et al., 1985). The lipid binding characteristics of 18A were improved by blocking the terminal charges (Ac-18A-NH₂), and this modified improved peptide was named 2F to denote that it contains two phenylalanine residues, one each at positions 6 and 18 (Venkatachalapathi et al., 1993). Unfortunately, despite the ability to bind lipids similar to human apoA-I, 2F did not reduce lesions in a mouse model of atherosclerosis (Datta et al., 2001). It was found that the best predictor of anti-inflammatory and antiatherosclerosis activity was the ability of peptides to inhibit the induction of monocyte chemotactic activity in a culture of human aortic cells (Datta et al., 2001). Two peptides that were particularly potent in this assay were tested in mouse models of atherosclerosis, 5F and 4F, which contained five and four phenylalanine residues on the hydrophobic face of the peptides, respectively. The peptide 5F (Ac-DWLKAFYDKVFKEKF-NH₂) when injected into mice given an atherogenic diet significantly inhibited the development of atherosclerotic lesions (Datta et al., 2001). This study was supported by National Institutes of Health Grants DK068134, HL55601, and HL34300 (to N.G.A.). Article, publication date, and citation information can be found at http://jpet.aspetjournals.org. doi:10.1124/jpet.107.119479.

ABBREVIATIONS: apoA-I, apolipoprotein A-I; D-4F, apolipoprotein A1 mimetic peptide; EC, endothelial cell; CD31, endothelial cell marker; TM, thrombomodulin; EPC, endothelial progenitor cell; HO, heme oxygenase; eNOS, endothelial nitric-oxide synthase; STZ, streptozotocin; LDL, low-density lipoprotein; ELISA, enzyme-linked immunosorbent assay; CEC, circulating endothelial cell; FACS, fluorescence-activated cell sorting.
lesion formation (Garber et al., 2001). The 4F peptide (Ac-DWPKAFYDKVAEKFKEAF-NH₂) when synthesized from all D-amino acids (D-4F) was found to be suitable for oral administration to mice models of atherosclerosis and significantly inhibited lesion formation in young mice (Navab et al., 2002). When D-4F was given orally together with pravastatin, there was substantial synergy, and regression of lesions was also found (Navab et al., 2005a).

D-4F re-establishes an antioxidant and anti-inflammatory phenotype through restoration of the balance between nitric oxide and superoxide (O²⁻) production (Ou et al., 2003, 2005), which results in an improvement in vascular function (Ou et al., 2005; Rodella et al., 2006). Thus, D-4F decreases endothelial cell (EC) sloughing and apoptosis and restores vascular EC function (Rodella et al., 2006), although a D-4F effect causing an increase in vascular repair has not been excluded.

Endothelial cell dysfunction, demonstrated by the reduced expression of CD31⁺ and/or thrombomodulin (TM) (Sandusky et al., 2002), has been reported within atherosclerotic blood vessels. A CD31⁺ gene abnormality has also been implicated in the pathogenesis of both atherosclerosis and myocardial infarction. Furthermore, a reduction in plasma TM has also been associated with an increased risk of myocardial infarction (Morange et al., 2004). Conversely, increased expression of TM has been shown to limit thrombus formation as well as neointimal growth (Waugh et al., 2000). Diabetes mellitus is a major risk factor in the development of atherosclerotic heart disease. The hyperglycemia-mediated generation of reactive oxygen species and advanced glycosylation end products accelerate the formation of atherosclerotic lesions (Aronson and Rayfield, 2002), contributing to the pathogenesis of multiple vascular complications (Aronson and Rayfield, 2002; Da Ros et al., 2004; Rodella et al., 2006).

Type 1 diabetes has also been shown to reduce both the number and function of bone marrow-derived endothelial progenitor cells (EPCs) (Loomans et al., 2004). This could potentially contribute to the formation of atherosclerotic disease. There is growing evidence to suggest that proper vascular function relies not only on mature ECs but also on EPCs (Asahara et al., 1997). EPCs have been shown to contribute to vascular remodeling in atherosclerosis (Sata et al., 2002) and other cardiovascular diseases (Raffi and Lyden, 2003). More recently, high-density lipoprotein has been shown to provide vascular protection by increasing EPC in apolipoprotein E-deficient mice (Werner et al., 2005).

The recognition that HO-1 is strongly induced by its substrate heme and by oxidant stress, in conjunction with the robust ability of HO-1 to protect against oxidative insult in cardiovascular disease, suggests that HO-1 may be a target for pharmacological drugs in the alleviation of vascular diseases. The antioxidant effects of HO-1 arise from its capacity to degrade the heme moiety from destabilized heme proteins (Nath et al., 2000) and to generate biliverdin and bilirubin, which are products of HO, that possess potent antioxidant properties. CO, an HO product as well, is not an antioxidant (Wiesel et al., 2000) but can cause the induction of antioxidant genes, decrease O²⁻ levels, and increase glutathione in reduced form levels (Abraham and Kappas, 2005). HO-1-derived bilirubin has also been shown to display cytoprotective properties in the cardiovascular system (Clark et al., 2000). Numerous reports indicate that higher serum bilirubin levels are associated with a decrease in the risk for coronary artery disease in humans (Vitek et al., 2002). We, and others, have previously shown that D-4F has a beneficial effect on vascular function (Rodella et al., 2006); however, the exact mechanism is not known.

The present study explores whether chronic D-4F administration leads to an increase in HO-1 activity specifically relevant to vascular cytoprotection, such as in the heart and aorta. We also investigated the effect of D-4F on the expression of both CD31⁺ and TM, markers for the onset of atherosclerosis and on EPC numbers and function in an animal model of diabetes. We demonstrate, for the first time, that D-4F, by increasing HO-1 and eNOS and decreasing circulating oxidants, protected EPC function and increased the expression of CD31⁺ and TM. These data highlight the chronic effect of daily administration of D-4F in preventing vascular damage, rendering endothelial cells resistant to oxidants in this model of type 1 diabetes.

Materials and Methods

Animal Treatment. Male Sprague-Dawley rats (Charles River Lab, Wilmington, MA), weighing 170 to 190 g, were maintained on standard rat diet and tap water ad libitum. After rats were anesthetized by i.p. injection of sodium pentobarbital (65 mg/kg b.wt.), diabetes was induced by a single injection, via the tail vein, of streptozotocin (STZ, Sigma, St. Louis, MO) (45 mg/kg b.wt.) dissolved in 0.05 M citrate buffer, pH 4.5. Blood glucose levels were elevated (410 ± 35 mg/dl) 2 days after the injection of STZ but were maintained between 240 and 320 mg/dl in all STZ-treated rats for the 6-week duration of the study by the administration of insulin (40–60 U/day/kg neutral protamine Hagedorn). Insulin was essential to assure that ketosis and weight loss were not significant. Glucose monitoring was performed using an automated analyzer (Lifescan Inc., Miligitas, CA). D-4F was given as a daily i.p. injection (100 µg/100 g b.wt.) for 6 weeks, beginning the day after the injection of STZ or sodium citrate buffer (in control rats). Four groups of rats were used: control, STZ alone, STZ plus D-4F, and D-4F alone. The Animal Care and Use Committee of New York Medical College approved all experiments.

Tissue Preparation for Ultrastructural Analysis. Aorta segments were removed and immediately fixed in 2% glutaraldehyde in phosphate buffer, pH 7.4. After 12 h, the specimens were washed in phosphate buffer, stained with uranyl acetate, dehydrated in decreasing acetone concentrations, and embedded in Araldite. Semi-thin (1.5 µm thick) sections were cut by an ultramicrotome and stained with toluidine blue for light microscope observation and to identify the area for the ultrastructural analysis. Sections were then cut and observed by a Philips CM10 transmission electron microscope (New York/New Jersey Scientific, Inc., Middlebush, NJ).

Detection and Quantification of EPCs in Peripheral Blood. Peripheral blood specimens were layered 1:1 onto a Ficoll-Paque Plus (GE Healthcare, Waukesha, WI) centrifuged at room temperature for 35 min at 450g. The mononuclear cell layer was removed and washed three times with phosphate-buffered saline. After the third wash, cells were suspended in 500 µl of phosphate-buffered saline, containing anti-RECA-1 (Novus Biologicals, Littleton, CO) and anti-CD34⁺ fluorescein isothiocyanate-conjugated antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Fluorescein isothiocyanate-conjugated normal mouse IgG (Santa Cruz Biotechnology) was used as a negative control as described previously (Abraham et al., 2003; Rodella et al., 2006).

Effect of Hyperglycemia on EPCs. The ability of bone marrow-derived cells to differentiate was quantified to determine the effects of diabetes and D-4F on EPC formation. Bone marrow hematopoietic colonies were prepared in methylcellulose cultures as described previously (Lutton et al., 1993) and grown in the presence of vascular...
The protective effect of D-4F on the vasculature was demonstrated by the direct quantification of circulating endothelial cells (CECs) in peripheral blood and confirmed by FACS analysis. The number of CECs in peripheral blood was significantly elevated in diabetic rats compared with control animals (50 ± 6 and 4 ± 3 cells/ml peripheral blood). Treatment with D-4F did not affect EC sloughing in control rats. However, in diabetic rats, D-4F attenuated EC sloughing to 20 ± 3 cells/ml peripheral blood (p < 0.003 versus untreated diabetic, n = 13). FACS analysis was used to confirm the increase in CECs in diabetic rats and the reduction in CECs after D-4F treatment (Fig. 3). Long-term treatment with D-4F caused a significant decrease in CECs (p < 0.05 versus untreated diabetic rats), promoting endothelial cell survival.

Effect of D-4F on CD31+ and TM Expression. Since D-4F had a beneficial effect on heart and aortic HO-1, we examined whether D-4F affected CD31+ and TM. Immunohistochemical staining for CD31+ (Fig. 4) and TM (Fig. 5) was conducted in aorta isolated from untreated and D-4F-treated diabetic rats. Staining appeared brown and was localized within the EC cytoplasm. In control animals, strong CD31+ immunoreactivity was seen in the aorta (Fig. 4A). In diabetic animals, CD31+ staining was either weak or absent (Fig. 4B); however, treatment with D-4F restored the pattern to that seen in controls (Fig. 4, C and D). TM staining was strong in the intima of control rats (Fig. 5A), whereas diabetic rats demonstrated moderate to weak staining (Fig. 5B). D-4F treatment restored TM expression in diabetic rats to the level of staining seen in controls (Fig. 5, C and D). Optical density analysis of immunohistochemical staining provided quantification of the changes in both CD31+ (Fig. 4D) and TM (Fig. 5D) expression.
Effect of D-4F on EPC Function. The effect of D-4F on EPC function was assayed in diabetic rats untreated or chronically administered D-4F (Fig. 6). STZ-induced diabetes reduced the formation of EPC colonies from 19.3 ± 1.3 colonies in controls to 8.8 ± 1.3 (p < 0.001). In diabetic rats treated with D-4F, the number of EPC colonies improved to 17.3 ± 1.5 (p < 0.002 versus STZ alone), approaching the level found in control animals.

Effect of D-4F on HO-1 and eNOS in EPCs. Since D-4F increased EPC function, as seen by the restoration in TM and CD31⁺, we examined the effect of D-4F on the levels of HO-1, HO-2, and eNOS in EPC after 10 days of culture. Figure 7A shows the changes in HO-1 (HO-2 is constitutively expressed and was unchanged) and eNOS levels in response to STZ-induced diabetes and administration of D-4F. The effect of STZ-diabetes was to down-regulate HO-1 protein expression. Optical density analysis of eNOS, expressed as a ratio to α-actin, revealed diminished levels of eNOS (0.17 ± 0.04) in STZ-treated rats compared with controls. D-4F treatment produced eNOS expression in diabetic rats (0.30 ± 0.03, p < 0.05) similar to that in controls (Fig. 7B). Treatment with D-4F prevented the loss of HO-1 protein expression to 0.19 ± 0.02 (p < 0.02), a level consistent with controls (Fig. 7C).
We have demonstrated, in this study, that daily administration of D-4F prevents the loss of EPC function and contributes to vascular repair in diabetic rats. Four observations support this conclusion. First, D-4F* selectively increased HO-1 expression in the aorta and heart, an observation not seen in the liver or kidney. Second, the increases in HO-1-derived CO and bilirubin in EPC, following the up-regulation of HO-1 via D-4F administration, paralleled the increases in TM and CD31* expression. HO-1-derived CO and bilirubin have been shown to prevent endothelial cell death and apoptosis both in vitro and in vivo (Ye and Laychock, 1998; Pileggi et al., 2001; Abraham et al., 2003; Rodella et al., 2006). Third, increases in ox-LDL and oxidized proteins were prevented by chronic administration of D-4F. The hyperglycemia-mediated increases in reactive oxygen species generation and O2 production contribute to vascular endothelial cell dysfunction and apoptosis (Turkseven et al., 2005; Rodella et al., 2006) and have been shown to be reversed by the induction of HO-1 (Turkseven et al., 2005; Rodella et al., 2006). Fourth, chronic administration of D-4F changed the diabetic EPC from a naive to a defensive phenotype by producing a robust increase in eNOS and HO-1, as reflected by an increase in EPC function in culture and in vivo. The salutary effect of D-4F was reflected by the increases in TM and CD31* expression. Reduced EC expression of CD31* and TM, an important indicator of endothelial cell death, is associated with the progression of atherosclerotic heart disease (Sandusky et al., 2002), whereas restoration of their expression prevents atherosclerosis and myocardial infarction (Mo range et al., 2004).

Our results are in agreement with previous studies showing that the down-regulation of TM expression in EC occurs in coronary atherosclerosis in humans. Prevention of the diabetes-induced decrease in TM expression may be associated with the progression of atherosclerotic heart disease (Saus Burke et al., 2004). We have demonstrated that an increase in TM and CD31* expression, leading to the repair of the endothelium, may be a contributing factor to the increases in TM and CD31*.

Our results do not distinguish whether D-4F caused an increase in existing EC regeneration within the diabetic aorta or whether the increase was due to new EPC function. Regardless of the mechanism, chronic treatment with D-4F caused restoration of both TM and CD31* and increased vascular repair, which would be considered clinically relevant in diabetes. The increase in TM and CD31* limits neo-intima formation and EC dysfunction. The diminished function of vascular EC that occurs with diabetes (Waugh et al., 2000) is accompanied by a reduction in HO activity and TM expression (Ye and Laychock, 1998). Pileggi et al., 2001; Abraham et al., 2003; Rodella et al., 2006). The induction of HO-1 prevents the loss of EPC function and contributes to vascular repair in diabetic rats. Three observations support this conclusion. First, D-4F selectively increased HO-1 expression in the aorta and heart, an observation not seen in the liver or kidney. Second, the increases in HO-1-derived CO and bilirubin in EPC, following the up-regulation of HO-1 via D-4F administration, paralleled the increased expression of HO-1 in diabetic rats but did not significantly affect HO activity in control rats (Fig. 7D).

**Discussion**

**Effect of D-4F on Serum Oxidative Stress.** The effects of STZ and D-4F on the levels of oxidative stress were assayed using ELISA for oxidized proteins (Fig. 8A) and LDL (Fig. 8B). Oxidized protein (carbonyl) content was elevated in diabetic rats (1.62 ± 0.36 nmol/mg) compared with controls (1.0 ± 0.13 nmol/mg; p < 0.01). D-4F attenuated this increase (p < 0.05 versus untreated diabetic) in carbonyl content (1.33 ± 0.19 nmol/mg). The level of proatherogenic oxidized LDL was elevated in diabetic rats (11.76 ± 0.82 U/l) compared with controls (8.12 ± 1.47 U/l; p < 0.02). D-4F reduced the level of oxidized-LDL (p < 0.05 versus untreated diabetic rats) to 9.18 ± 1.06 U/l, a level consistent with that found in controls. These results suggest that D-4F has a beneficial effect on the vascular system, preventing oxidative stress and restoring EPC function.
increase of HO-1 and eNOS explains the mechanism by which the statins exert antioxidant properties, as seen by the decrease in oxidized LDL. Therefore, the chronic effects of D-4F administration, with the resulting decrease in oxidized LDL and oxidized proteins, may be attributed to the D-4F-mediated increase in both eNOS and HO-1.

HO-1 is induced under a wide variety of conditions associated with oxidative stress and is regarded as a protective response to oxidants. In the present study, we report that HO-1 and eNOS protein levels were restored in isolated mononuclear cells by chronic D-4F treatment. An increase in HO-1 will increase heme degradation and has the associated beneficial effect of increasing CO and bilirubin, which are important regulators of vascular function. Bilirubin is an important antioxidant in humans and an increase in serum levels prevent cardiovascular disease, as has been seen in Gilbert’s disease (Vitek et al., 2002). HO-1 up-regulation also increases the expression of eNOS and superoxide dismutase (Turkseven et al., 2005), which contribute to the reduction in oxidized protein levels in serum, leading to vascular repair. These results are also in agreement with the reported beneficial effect of eNOS on EPC function (Aicher et al., 2003).

HO-1 has been reported to be localized within foam cells that contribute to the formation of atherosclerotic lesions (Nakayama et al., 2001). A decrease in HO activity has been shown to result in the accelerated formation of atherosclerotic lesions in native vessels (Ishikawa et al., 2001a) and vein grafts (Yet et al., 2003). Induction of HO-1 inhibits the formation of oxidized LDL with the resultant prevention of the formation of atherosclerotic lesions (Ishikawa et al., 2001b). The fact that D-4F increases the levels of CO and bilirubin as well as eNOS in EPC suggests that
D-4F has a clinically relevant role in reducing proatherogenic ox-LDL in diabetic rats and may have an anti-inflammatory effect on the vascular system. In conclusion, chronic D-4F treatment resulted in modulating the EPC phenotype, as reflected by the increases in HO-1 and eNOS, which may contribute to the increased levels of aortic CD31* and TM. Therefore, HO-1 and eNOS are considered relevant targets for D-4F. They promote EC cell survival, affording vascular cytoprotection in diabetic animals.

References


