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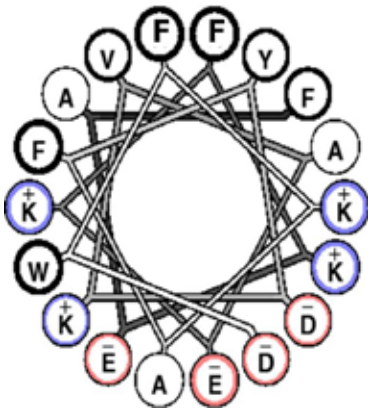
Abstract

Background- *Although there have been many advances in the diagnosis and treatment of coronary artery disease (CAD), CAD remains the major cause of deaths in the U.S. Atherosclerosis, the formation of arterial plaques, is a factor in CAD. Peptide 4F, an apolipoprotein A-I mimetic peptide, has been found to inhibit atherosclerosis in atherosclerosis-susceptible mouse models. Paraoxonase-1 (PON-1) is an enzyme to which many of the anti-oxidative properties of HDL have been credited. Even though peptide 4F is in phase 2 clinical trials, the mechanism in which it operates to increase PON-1 activity and decrease atherosclerosis is not known. We hypothesize that 4F affects PON-1 by increasing PON-1 activity in the plasma or by enhancing plasma levels of the enzyme (or both).* **Methods**-*Female apoE null mice (6 week old) were obtained from Jackson laboratories. Baseline cholesterol levels and PON-1 activity were measured in these animals and animals were divided into 2 groups, group 1 received saline and group 2 received peptide L-4F (50µg/day). Mice were injected intraperitoneally every day for 4 weeks. Animals were euthanized after 2 weeks. Plasma was analyzed for PON-1 activity and PON-1 levels. Livers were analyzed for PON-1 levels by Western blotting and gene expression by quantitative PCR.* **Results**-*There was a significant increase in plasma PON-1 activity in the group treated with L-4F as compared to the group treated with saline ($p<0.05$) and there was an increase of plasma PON-1 levels ($p<0.05$). Hepatic tissue samples showed no change in PON-1 mRNA or protein levels but did exhibit a significant decrease of tubulin levels in the group treated with L-4F compared to the group treated with saline ($p<0.05$), suggesting reduced hepatic inflammation.* **Conclusion**- *Peptide L-4F significantly decreased PON-1 activity and increased PON-1 levels in the plasma but showed no change in paraoxonase-1 levels in the liver. The information obtained from this study will provide insight into potential mechanisms by which peptide 4F decreases atherosclerosis through the regulation of PON-1.*

Introduction

Coronary artery disease (CAD) in humans is the major cause of death in the United States and the leading cause of mortality worldwide. CAD is associated with low levels of high-density lipoprotein (HDL) cholesterol and high levels of low-density lipoprotein (LDL) cholesterol (Parthasarathy, 2008). The major protein component of HDL, apolipoprotein (apo) A-I, is thought to be responsible for the atheroprotective qualities of HDL. ApoA-I has been postulated to possess ten α-helical sequences. The majority of these sequences form class A structures that can be mimicked by several 18-residue peptide analogues. One such peptide, peptide 4F, mimics many of the properties of apo A-I (Figure 1). A close relationship has been observed between paraoxonase-1 (PON-1) deficiency and accelerated progression of atherosclerosis in animals models, such as the apoE null mouse model. The apoE null mouse is a gene knockout of apoE, a ligand for the LDL receptor, and develops atherosclerosis spontaneously.



Paraoxonase-1 is an enzyme synthesized in the liver and secreted into the blood stream, where it is primarily located on HDL and associated with apo ApoA-I (Mackness, 2001) (Figure 2). Many of the anti-oxidative properties of HDL have been credited to PON-1. Previous research has shown that PON-1 destroys lipid hydroperoxides (LOOH), degrades oxidized LDL phospholipids, reduces accumulation of oxidized lipids in LDL, hydrolyzes oxidized LDL-associated compounds, and inhibits both LDL and HDL oxidation (Florentin. 2008). Administration of the apolipoprotein A-I mimetic peptide 4F increased paraoxonase activity and apo A-I, especially in the HDL subfraction containing pre-β HDL (Navab, 2004). Increased levels of PON-1 in the plasma can result either from increased synthesis of the enzyme in the liver or increased efflux from the liver into the plasma. Although peptide 4F is in clinical trial phase 2 studies, the mechanism in which it operates to increase PON-1 activity and decrease atherosclerosis is not known; however, we hypothesize that 4F affects PON-1 by increasing PON-1 activity or by increasing PON-1 levels in the plasma (or both) (Figure 3). To provide insight into the mechanism of peptide 4F's affects on PON-1, we have studied the changes in

Ac-AspTrpPheLysAlaPheTyrAspLysValAlaGluLysPheLysGluAlaPhe-NH_2
D----W----F----K----A----F----Y----D----K----V----A----E----K----F----K----E----A----F

Figure 1. The Class A structure of peptide 4F

PON-1 activity, PON-1 levels, and genetic expression of the enzyme with administration of peptide 4F in apoE null mice.

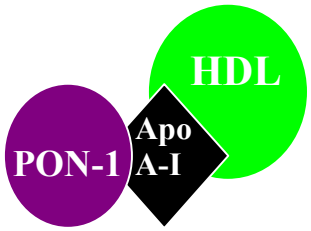


Figure 2. HDL complex with paraoxonase-1

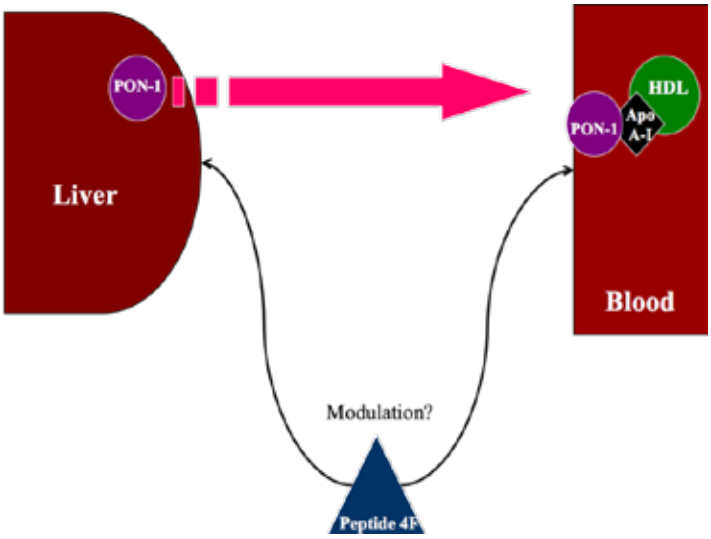


Figure 3. Hypothesized mechanisms of the effect of peptide 4F on PON-1

Methods

Samples

Samples from a previous experiment, with 20 female apoE null mice at 12 weeks of age, were provided by a doctoral candidate, Gaurav Nayyar, for analysis for PON-1 activity and PON-1 levels in the plasma. Animals were separated into 2 groups: vehicle, treated with saline, and peptide, treated with L-4F. Treatment lasted 2 weeks. At 14 weeks of age, animals were euthanized and plasma was collected in heparinized tubes.

Subjects

Female apoE null mice were purchased at 6-weeks of age from Jackson Laboratory (Bar Harbor, ME). They were fed normal chow. Animals were not studied until they had reached at least 7 weeks of age. All procedures were reviewed and approved by the Institutional Animal Use and Care Committee of the University of Alabama at Birmingham.

Peptide synthesis

Peptide L-4F (i.e. Ac-D-W-F-K-A-F-Y-D-K-V-A-E-K-F-K-E-A-F-NH_2) was synthesized by solid-phase peptide synthesis. The purity of synthetic peptides was established by analytical HPLC and mass spectrometry. The peptide was dissolved in sterile saline for injection studies.

Injection Protocols

Animals were anesthetized and blood samples were taken from the retro-orbital sinus under isoflurane anesthesia immediately before injection at 7 weeks of age. Peptide was injected intraperitoneally at a dose of 50 µg/day/mouse (0.5 mg/ml concentration; 100 µl volume). Saline (100 µl) was injected into control animals. Blood samples were collected into heparinized capillary tubes, then placed in microcentrifuge tubes; the plasma was separated by centrifugation.

At the time of euthanasia (11 weeks of age), whole blood samples were obtained under xylazine-ketamine anesthesia from cardiac puncture and placed in heparinized microcentrifuge tubes. Portion of livers were immediate placed in RNAlater (Qiagen; Valencia, CA) and stored at -80°.

Paraoxonase-1 activity

Three µl of whole plasma was mixed with 200 µl of PON buffer (100 mmol/L Tris containing 2 mmol/L CaCl2, pH 8.0) containing paraoxon (1 mmol/L O,O-diethyl-O-p nitrophenylphosphate; Sigma; St. Louis, MO), and the rate of formation of 4-nitrophenol over a period of twenty minutes was determined spectrophotometrically at 405 nm. Blanks were included to correct for the spontaneous hydrolysis of paraoxon. The assay was performed in a 96-well plate (Costar, Fischer; Pittsburg, PA), and readings were taken every 2 minutes. The quantity of 4-nitrophenol formed was calculated using the molar extinction coefficient of 17,100 $mol/L^{-1}cm^{-1}$. One unit of PON activity was defined as 1 nmol of 4-nitrophenol formed per minute (Navab, 2004).

Western Blotting on whole plasma

Five µl of whole plasma was separated on a pre-made 10% Novex gel. Proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane using a semi-dry transfer apparatus. The membrane was incubated with primary antibody anti-PON-1 (gift from Dr. Srinivasa Reddy, UCLA) in 5% milk in phosphate buffered saline (pH 7.4) and 0.05% Tween-20 (PBST) buffer at 4°C overnight with constant shaking. The membrane was then washed and incubated with secondary antibody (CAT 2305; Santa Cruz; CA) for an hour and washed. Western blots were developed with enhanced chemiluminescence (ECL) and exposed to X-ray film. ApoA-I levels were determined after the membrane was stripped at 50°C for 30min. The membrane was then incubated for an hour with primary antibody for A-I (Biotin Anti-mouse apo A-I) in 1% gelatin and TTBS (1% Tween-20 in tris buffered saline). Secondary antibody (Bio-Rad; CA) was then used. Membranes were developed with alkaline phosphatase conjugate substrate (Bio-Rad; CA). Immunoreactivity was quantified by densitometry and Sigma Plot 11.0 software was used for statistical analysis.

Western Blotting of Hepatic tissue

Thirty mg of hepatic tissue was homogenized with a mortar and

pestle in 1.0 ml of tris-buffered saline and protease inhibitor cocktail. Ten µl of the supernatant was separated on a pre-made 10% Novex gel. Proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane using a semi-dry transfer apparatus. The membrane was incubated with primary antibody anti-PON-1 (Srinivasa Reddy; gift, UCLA, CA) in 5% milk phosphate buffered saline (pH 7.4) and 0.05% Tween-20 (PBST) buffer at 4°C overnight with constant shaking. The membrane was then washed and incubated with secondary antibody (CAT 2305; Santa Cruz; CA) for one hour and washed. Western blots were developed with enhanced chemiluminescence (ECL) and exposed to X-ray film. Tubulin levels were determined after the membrane was stripped at 50°C for 30min. The membrane was then incubated for one hour with primary antibody for Tubulin (CAT 5168; Sigma; St. Louis, MO) in 5% milk block. Secondary antibody (CAT 2304; Santa Cruz; CA) was then applied and membranes were developed with ECL. Immunoreactivity was quantified by densitometry and Sigma plot 11.0 software was used for statistical analysis.

Immunohistochemistry

Immunohistochemistry was performed on unstained, unfixed frozen histological sections of livers. Primary PON 1 antibody (N20 Cat SC21146; Santa Cruz; CA) and secondary Donkey Antigoat HRP antibody (Cat-SC-2304; Santa Cruz; CA) were used with a hemotoxin counter-stain. The secondary antibody was biotinylated and contained horseradish peroxidase to observe PON-1 by color.

Quantitative Real-Time PCR

Hepatic tissues (30mg) were placed in RNAlater (Qiagen; CA) and RNA was isolated using the RNA isolation kit according to manufacturer's instruction. Five µg RNA was used and reverse-transcribed into complementary DNA (cDNA) using a SuperScript First Strand Synthesis system (Invitrogen; CA) according to the manufacturer's instructions. A total of 2 µl cDNA was used and amplification was performed in duplicate in a MyiQ Real-Time PCR Detection System (Bio-Rad; CA) using PCR primers for mouse PON1 (forward: GAT TGG CAC TGT GTT CCA C, reverse: ATC ACT GTG GTA GGC ACC TT) (Bradshaw, 2005). Real-Time PCR efficiencies were calculated on average efficiencies received from each sample.

Statistics

Control and experimental groups were compared for significant differences by two-tailed t-tests. Differences were considered significant at $p<0.05$. Regression analysis was considered significant based on R values greater than 0.6 and $p<0.05$.

Results

Cholesterol Levels

An increased cholesterol level is a risk factor for atherosclerotic plaque deposition. ApoE null mice were injected daily for 4weeks with saline or L-4F (50 µg/mouse/day). Peptide 4F significantly affected cholesterol levels by decreasing the amount of cholesterol in plasma of peptide treated apoE null mice (Figure 4; *p=0.002 compared with saline controls).

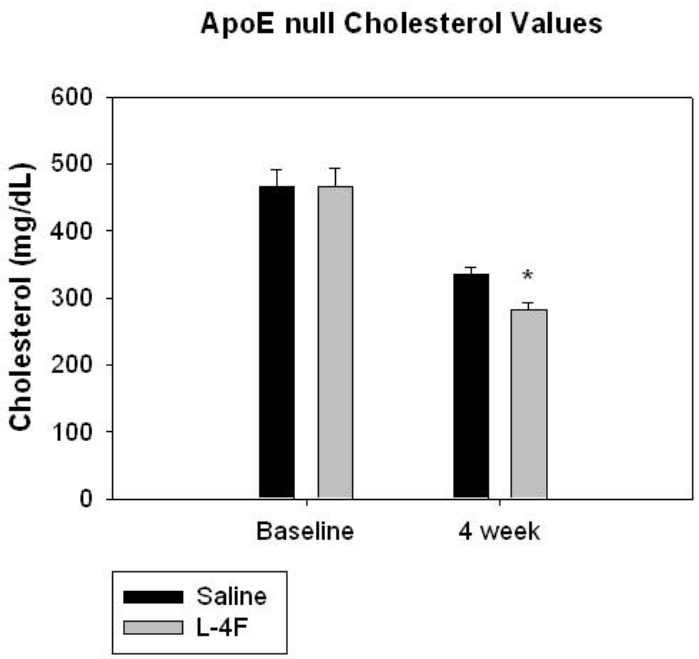


*Figure 4. Cholesterol levels were significantly lower in L-4F treated mice compared to control mice. ApoE- mice were injected daily for 4 weeks. (n=15 in each group; *p=0.002 vs saline).*

PON-1 activity in plasma

The activity of PON-1 in the plasma was measured in both control and experimental groups treated with saline or 4F. PON-1 activity, in the 20 samples provided, showed a significant increase in animals treated with peptide L-4F (Figure 5, *p=0.018). With the 30 apoE null mice, the activity of PON-1 decreased significantly in peptide treated apoE null mice (Figure 6; **p<0.017 between vehicle and L-4F groups at baseline. †p<0.001 between vehicle and L-4F groups at 4weeks.).

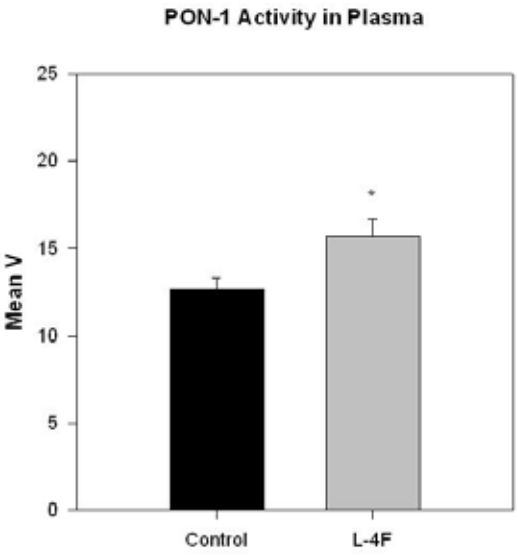


*Figure 5. PON-1 activity in plasma was significantly higher after 2 weeks of administration of the peptide L-4F in 14 week old female apoE- mice. Saline (black bar, n=10) or L-4F (grey bar, n= 10). *p=0.018*

PON-1 levels in plasma

In order to determine if the amount of PON-1 in the plasma correlated with its activity, PON-1 levels were measured through western blotting on 4-20% gels. The peptide-associated increase of PON-1 was barely significant (Figure 7; *p=0.049). PON-1 activity correlation with PON-1 levels in the plasma was measured through regression in both peptide treated and vehicle treated mice (Figure 8). No correlation was detected in peptide treated mice (R=0.140) or control mice (R=0.0525). This was different from the PON-1 activity and PON-1 levels correlation seen previously in the samples provided (Figure 9, R=0.690). Corresponding apoA-I levels were determined to observe if apoA-I levels also increased within the plasma. ApoA-I levels showed no change (Figure 10; p>0.05).

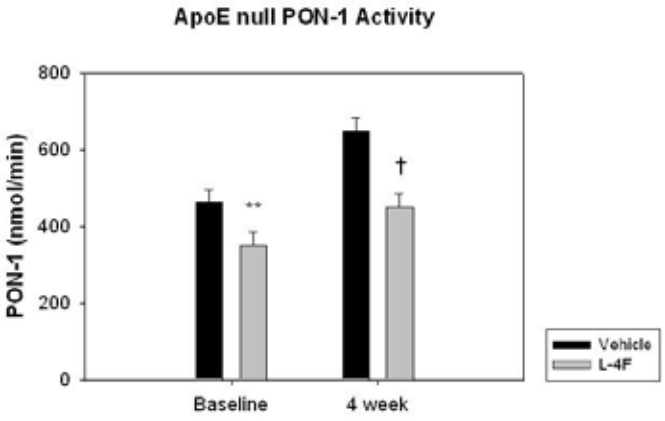


*Figure 6. Chronic administration of the peptide L-4F decreased PON-1 activity. Female apoE- were administered with saline (black bar, n=15) or L-4F (grey bar, n= 15) for 4 weeks. **p<0.017 between vehicle and L-4F groups at baseline (7 weeks old). †p<0.001 between vehicle and L-4F groups at 4weeks (11 weeks of age).*

PON-1 in the liver

To determine if the changes within the amount of PON-1 in the plasma was due to an increased secretion of PON-1 from the liver, histology specific to PON-1 was done (Figure 11). No change in PON-1 within hepatic tissue of both control (saline-treated) and experimental (peptide-treated) mice was seen. PON-1 was found consistently dispersed throughout the hepatic tissues.

Since PON-1 was present in large amounts, and changes in PON-1 levels were not apparent in immunohistochemistry, we decided to blot for PON-1 specifically. Ten µl of protein supernatant (0.5 µg/µl), from 5 animals per group, was separated on 4-20% gels (Figure 12). No significant changes in hepatic PON-1 levels between groups were found (Figure 12; p>0.05). Tubulin was used as a normalizing protein to determine the relative expression of PON-1 within the liver (Figure 13). Blotting for tubulin showed a significant decrease of tubulin levels in the mice treated with peptide 4F (Figure 13; *p=0.002).

Although there was no change in the amount of PON-1 within

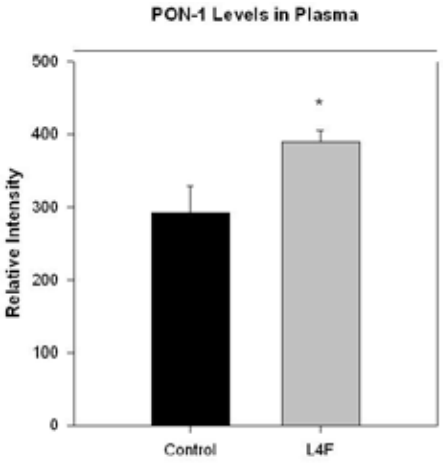
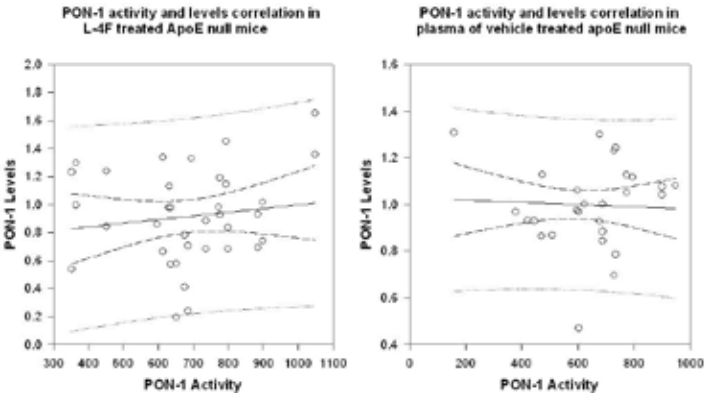
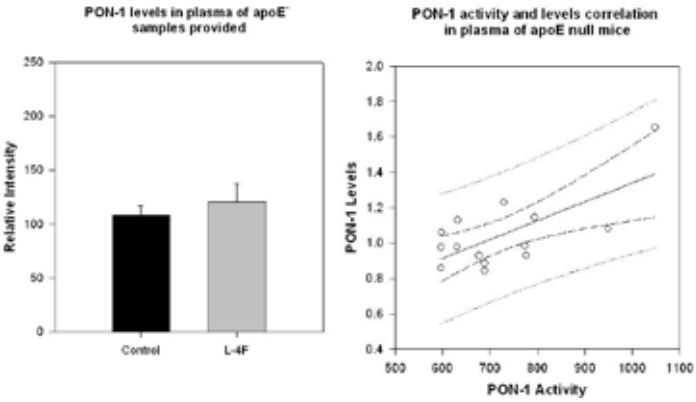


*Figure 7. PON-1 plasma levels increased in peptide L-4F treated apoE- mice. *p=0.049*



A) *B)*

Figure 8. No correlation seen between PON-1 activity and levels in plasma of A) peptide 4F treated apoE- mice R=0.140 or B) vehicle treated apoE- mice R=0.0525



A) *B)*

Figure 9. A) No significant change in PON-1 levels of apoE- mice samples detected B)Correlation seen between PON-1 activity and PON-1 levels in plasma of apoE- mice samples provided R=0.690

the liver, quantitative real time (RT-PCR) was done on PON-1 mRNA levels to decide if peptide 4F affected PON-1 genetic expression. RT-PCR showed fold changes less than 1 (data not shown), representing no detectible change in the genetic expression of PON-1.

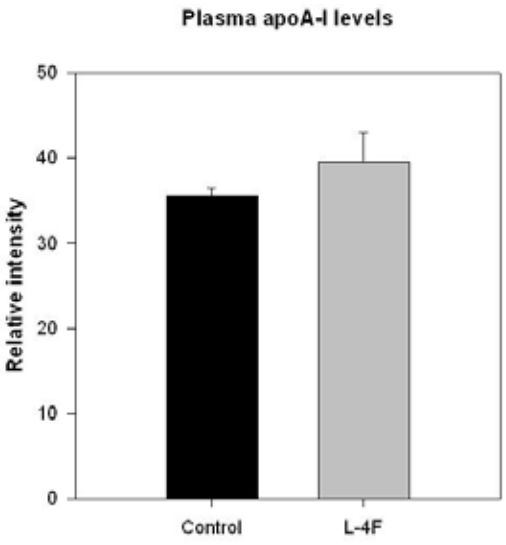
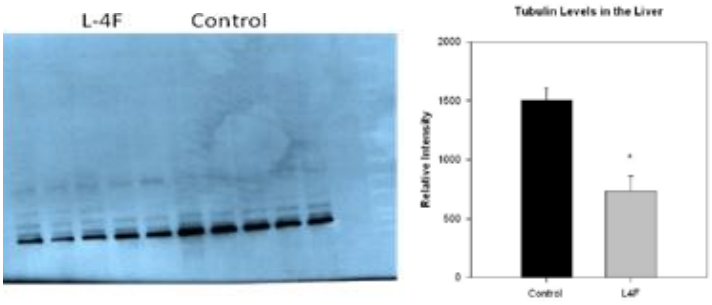


Figure 10. ApoA-1 plasma levels showed no change in peptide L-4F treated apoE- mice at 11 weeks of age.



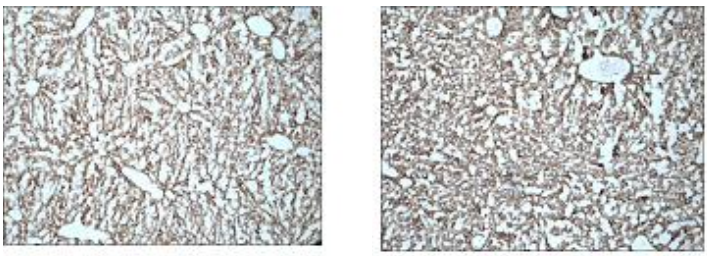
changes plasma PON-1 levels

A) *B)*

*Figure 13. Corresponding tubulin levels in the liver of apoE- mice. A) Tubulin levels are visibly decreased in apoE- mice treated with peptide 4F compared to control at 11 weeks of age. B) Tubulin levels were significantly decreased in peptide treated mice *p=0.002*

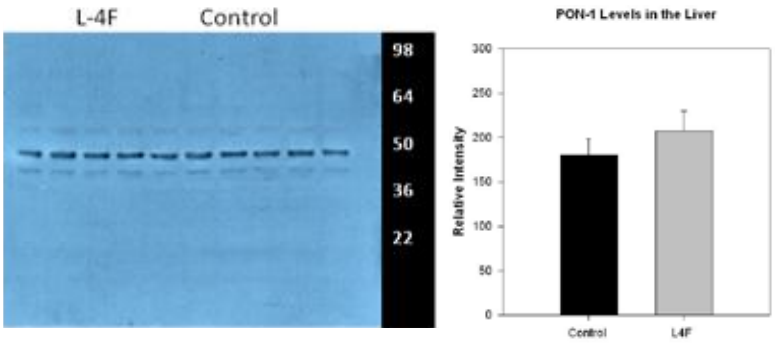
Discussion

Previous studies, including the samples provided, showed that peptide L-4F increased PON-1 activity and reduced atherosclerosis in atherosclerosis susceptible mice models. The general interpretation of this finding was the peptide 4F had antioxidative properties by increasing the activity of antioxidative enzymes. Here, we do not support previous findings by showing that peptide 4F does not increase PON-1 activity within the plasma but without changing apparent hepatic expression or levels. This may be because PON-1 activity was not measured in isolated HDL but in whole plasma samples.



A) *B)*

Figure 11. Immunohistochemical section of hepatic tissue from apoE- mice, using PON-1 antibody A) injected with saline B) injected with L-4F



A) *B)*

Figure 12. PON-1 liver levels A) western blot showed no visible change in PON-1 levels from peptide L-4F treated apoE- mice compared to control mice at 11 weeks of age. B) Quantitave results from western blot.

While decreasing PON-1 activity, peptide L-4F also decreased total cholesterol in these animals. This reduction of cholesterol decreases the accumulation of cholesterol within the artery walls and therefore, reduces plaque burden. Our findings are not consistent with previously reported studies of peptide L-4F in apoE null mice, which showed no change in plasma cholesterol levels.

In order to determine the mechanism in which peptide 4F modulates PON-1 and its activity, we hypothesized that peptide 4F increases PON-1 levels within the plasma and then compensates for this increase within the plasma by secreting PON-1 from the liver and decreasing the amounts of PON-1 within hepatic tissue or by increasing the genetic expression of PON-1 within the liver or both. In the samples provided, we did not find a significant increase of PON-1 levels within the plasma. However, in the apoE null mice treated with peptide, we found a slight increase in PON-1 levels within the plasma. With this finding, we are unsure if the change in PON-1 levels is responsible for the change in PON-1 activity seen in peptide treated mice, or if the peptide increased the activity by remodeling HDL particles or directly activating PON-1. A correlation between PON-1 activity and PON-1 levels was detected in the samples provided by the doctoral candidate. This suggested that peptide L-4F did not activate PON-1 directly but activated PON-1 in the plasma by increasing the PON-1 levels. However, no correlation was detected in this experiment, with 30 apoE null mice, between PON-1 activity and levels in the plasma. These paradoxical results may have been due to group of animals or other factors unknown. ApoA-I levels in the plasma, however, did not change. In order to determine the role

of PON-1 and apoA-I in peptide 4F function, mouse models such as PON-1 null and apoA-I null would need to be used. We hypothesized that an increase in PON-1 levels in the plasma may have been due to an increase of PON-1 secretion from the liver or an increase in genetic expression. No change in PON-1 levels within the liver were detected by immunohistochemistry or immunoblotting in peptide treated mice even though PON-1 levels slightly increased in the plasma, suggesting that, if more PON-1 had been secreted from the liver, the levels were low compared to hepatic PON-1 content, or that the genetic expression of PON-1 within the liver was increased. However, we did not detect increased PON-1 genetic expression, suggesting that peptide 4F does not induce PON-1 genetic expression.

While determining the relative expression of PON-1 levels in the liver, we found in a single experiment that tubulin was decreased in the livers of peptide treated mice. Tubulin, a structural protein commonly used as the normalizing gene in many tissues, has not previously been reported to decrease in atherosclerosis models. Tubulin is used as a normalizing gene to measure the levels of other proteins presented in several tissues including brain and liver. Tubulin levels are usually consistent within tissue levels. In this case, hepatic tissue showed a significant decrease in tubulin levels in mice treated with peptide L-4F. Previous literature has reported increased levels of tubulin as a pro-inflammatory response in the liver (Maurice, 1980). Our data may suggest that peptide 4F decreases inflammatory responses within the liver and therefore tubulin levels in the liver. Future studies using better established inflammation markers within the liver will be needed to confirm a peptide-mediated reduction in hepatic inflammation. If this change in tubulin levels is confirmed, for relative expression of PON-1 other housekeeping genes and proteins, such as β-actin, will be used instead of tubulin.

Conclusion

Peptide L-4F has been shown to act as an anti-inflammatory and anti-oxidant in the plasma in part by increasing the activity of PON-1 in the plasma (Navab, 1980). This increase in PON-1 levels and activity may result in the increased anti-oxidative properties of HDL seen in the plasma of animals treated with peptide L-4F; however, our results did not support this. No highly significant change in hepatic PON-1 levels or genetic expression suggests that peptide 4F does not interact with the PON-1 gene. The mechanism of peptide modulation of PON-1 remains unknown but the results presented here provide insight into the possibilities of peptide 4F activating PON-1 within the plasma through PON-1 levels.

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