commentary

Tomatoes, lysophosphatidic acid, and the small intestine: new pieces in the puzzle of apolipoprotein mimetic peptides?[1]

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In this issue of the *Journal of Lipid Research*, M. Navab and colleagues describe their latest findings on the mechanism of action for apolipoprotein mimetic peptides. Besides having important implications in the design of future therapeutic peptides for the treatment of cardiovascular disease, this paper also adds to the growing evidence for the role of lysophosphatidic acid (LPA) in the pathogenesis of dyslipidemia and atherosclerosis (1).

The story of apolipoprotein mimetic peptides starts with the groundbreaking work of J. Segrest and G. M. Anantharamaiah in the mid-1980s, with the first reports of synthetic peptides, such as 4F, that were designed to be mimetics of apoA-I (2). These peptides, which contain amphipathic helices, were first used as probes for understanding the structure of apoA-I and how it and related proteins interact with phospholipid and cholesterol to form lipoprotein particles. Many years later, the laboratory of A. Fogelman and others showed that the 4F peptide had numerous beneficial biological properties and was effective in reducing atherosclerosis in various animal models as well as in reducing inflammation in many different chronic diseases (3). The discovery of the ABCA1 transporter and the results from clinical trials showing that the infusion of reconstituted HDL can rapidly reduce atherosclerotic plaques suggested a possible anti-atherogenic mechanism for the 4F peptide in promoting the efflux of excess cellular cholesterol (4). Other apolipoprotein mimetic peptides that specifically efflux cholesterol by ABCA1 were subsequently developed (5–7) and also shown to reduce atherosclerosis in animal models (8, 9). However, the plasma levels needed for these peptides to reduce atherosclerosis were much higher than 4F and comparable to the amount of apoA-I used in the reconstituted HDL clinical trials (4). These findings, along with the observation that the 4F peptide has great affinity for oxidized lipids (10), suggested that the 4F peptide may have a different mechanism of action. This was further substantiated when the D and L-stereoisomers of the 4F peptide were tested in clinical trials. In a Phase I clinical trial (11), the 4F peptide made with D-amino acids (D4F) was given orally, because it is resistant to proteolysis in the digestive tract, and was shown to improve the anti-inflammatory properties of HDL. In another Phase I trial, the intravenous infusion of the 4F peptide made with L-amino acids (L4F) resulted in much higher peptide plasma levels than oral D4F but showed no effect in improving the anti-inflammatory function of HDL (12). These puzzling findings prompted the investigators to go back to their animal models, which led to the discovery that the small intestine appeared to be the site of action of the 4F peptide (13). In this study, in which the 4F peptide was given either orally or injected subcutaneously, the plasma levels of the peptide did not appear to correlate with its anti-atherogenic effect. It was the amount of peptide that reached the small intestine that seemed to confer the atheroprotective benefit of the 4F peptide (13).

The unexpected finding that the site of action of the 4F peptide was the small intestine prompted the Fogelman laboratory to develop a low-cost method for the delivery of the peptide in the diet. This was accomplished with the production of transgenic tomatoes expressing a related peptide called 6F (14). The 6F peptide has six phenylalanine residues, which is two more than the 4F peptide and hence the origin of its name. It has been previously shown that the number of highly hydrophobic residues like phenylalanine in the hydrophobic face of these peptides is an important determinant in their ability to bind to lipids with high affinity (15). When LDLr-KO mice were fed transgenic tomatoes containing the 6F peptide, but not control tomatoes, they were protected against the development of atherosclerosis from a high-fat diet (14). Another interesting finding from this study was that the level of LPA was

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Abbreviations: D4F, the 4F peptide made with D-amino acids; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; L4F, the 4F peptide made with L-amino acids; LPA, lysophosphatidic acid; PA, phosphatidic acid; SAA, serum amyloid A.

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lower in the both the small intestine and plasma of mice fed the 6F transgenic tomatoes. In the current study, they go on to further examine the effect of the 6F peptide in modulating lipid metabolism and in particular LPA, which has recently emerged as an important bioactive signaling lipid in numerous biological processes, including atherosclerosis (1, 16).

Although a significant amount of effort was made to complete the current study, the experimental approach was relatively straightforward. Two main diet interventions, namely a high-fat Western diet and an LPA-enriched chow diet, were fed to LDLr-KO mice and compared with a standard mouse chow diet. Both diets were also tested in the presence and absence of the 6F peptide by supplementing the diets with a freeze-dried powder made with either transgenic tomatoes expressing 6F or control tomatoes. The main outcome parameters were the following: intestinal tissue levels of LPA, plasma LPA, plasma lipids [total cholesterol, triglycerides, HDL-cholesterol (HDL-C)], serum amyloid A (SAA), and intestinal gene expression. Even though the Western diet contained slightly less LPA and phosphatidic acid (PA), a LPA precursor, than the standard chow diet, mice on Western diet had significantly higher levels of unsaturated LPA and PA, but not saturated LPA and PA, in both the small intestine and plasma. When 6F was added to the Western diet, the level of unsaturated PA levels returned to normal and it also decreased the high levels of plasma total cholesterol, triglycerides, and SAA induced by the Western diet and also restored HDL-C to near normal levels. Addition of 6F to the Western diet also reversed the gene expression changes from the high-fat diet. The fact that the Western diet had a lower content of unsaturated LPA and PA than the standard chow diet suggests that the increased tissue and plasma levels of unsaturated LPA and PA in the mice on the Western diet may be from increased endogenous production, although genes related to the synthesis and catabolism of LPA were not observed to significantly change, at least in the small intestine. The other possibility is that the high fat content of the Western diet markedly enhances the intestinal absorption of PA and LPA and or prevents their catabolism in the enterocyte.

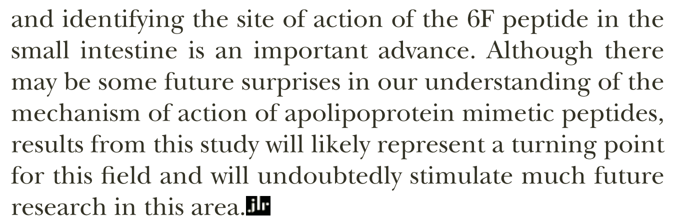
To demonstrate a possible role of LPA in causing the dyslipidemia and inflammation produced by the Western diet, mice were fed a standard chow diet supplemented with either saturated (18:0) or unsaturated (18:2, 20:4) LPA or the corresponding PA precursors. It is important to note that the level of LPA and PA added in these studies was almost 1,000-fold higher than what is present in the standard chow diet. Despite the relatively high dose of added LPA and PA, the plasma levels of LPA and PA in these mice were similar to what was observed when mice were fed the Western diet. Interestingly, the addition of unsaturated LPA or PA, but not saturated LPA or PA, to the diet markedly increased total cholesterol, triglycerides, LDL-cholesterol (LDL-C), and SAA, and decreased HDL-C, in much the same way as did the Western diet. If LPA in the chow was oxidized in vitro prior to feeding the mice, no significant changes were observed in its ability to alter serum lipids or SAA, suggesting that the oxidation of the unsaturated fatty acids in LPA is unlikely responsible for the observed changes. Adding unsaturated LPA to the diet also caused a similar gene expression changes in the intestine as did the Western diet, which was reversed along with the lipid and SAA changes, when 6F was added to the LPA-supplemented diet. Subcutaneous injection of LPA 20:4 or 18:2 into mice was less effective than the same oral dose of LPA 20:4 or 18:2 in causing plasma lipid and SAA changes, which suggests that intestinal exposure of unsatured LPA in some way triggers the observed changes. Finally, the lipid and SAA changes in plasma closely correlated with LPA 20:4 plasma levels, which were elevated when mice were fed either LPA 18:2 or LPA 20:4 but not LPA 18:0, most likely due to the inability of mice to form long chain polyunsaturated fatty acids from saturated (18:0) fatty acids.

Like all good science, this study raises many interesting new questions. One key question is how does LPA cause dyslipidemia and stimulate inflammation? LPA was first described over 25 years ago as a signaling molecule when it was shown to be a growth factor in conditioned media from several different tumors (17). We now know that LPA has a wide variety of effects on many different disease processes (17, 18), including atherosclerosis (16). Like other potent bioactive signaling lipids, it functions as an autacoid. In other words, it is typically produced locally in response to danger signals, such as inflammation or vascular injury, and exerts most of its biological effects at its site of production. It can be made intracellularly and also in the extracellular compartment, such as the plasma (16). It has a relatively short half-life of less than a few minutes due to catabolism by lipid phosphate phosphatases (19). Most extracellular LPA is bound to albumin, which, like S1P, may inhibit its cell signaling activity by reducing its bioavailability. The rate limiting step in the extracellular production of LPA is mediated by autotaxin, a secretory enzyme with lysophospholipase D activity (16). Autotaxin acts on lysophospholipids produced by lecithin:cholesterol acyltranferase, endothelial lipase or by one of several secretory type phospholipaseA2 enzymes (16). Lp-PLA2, also called PAF-acetyl hydrolase, can also contribute to the generation of LPA by the deacylation of oxidized phospholipids on lipoproteins (18). One of the main tissue sources of autotaxin are adipocytes (20), so perhaps an increase in adipocyte cell number or fat content from the feeding of the Western diet in this study could account for the observed increase in plasma LPA levels. Hyperlipidemia itself has also been described to result in increased LPA production by autotaxin (20). At least seven different G protein-coupled receptors on cell membranes have been described to mediate cell signaling by LPA (18). In general, LPA containing unsaturated fatty acids are more potent in their biological activity, which is consistent with the result found in this study showing that LPA 18:0 was relatively inert. Intracellular LPA can also generate cell signaling events by the stimulation of nuclear membrane receptors (19). For example, intracellular LPA can activate the nuclear receptor PPAR-γ (16), which is a well-known transcription factor involved in lipid metabolism and atherosclerosis. Another

interesting and potentially relevant example of cell signaling by LPA is the report that LPA can also enhance the secretion of apoB (21), which could account for the increased LDL-C observed in this study. In addition to altering lipoprotein metabolism, LPA has also been described to be enriched in atherosclerotic plaque where it appears to have several pro-atherogenic effects (16). Intravenous injection of LPA 20:4 but not LPA18:0 has also been shown to increase atherosclerosis in apoE-KO mice, although it had no effect on plasma lipids (22). Blocking LPA (1) and LPA (3) receptors, two G protein-coupled receptors, with a small molecule antagonist abrogated the effect of LPA administration on atherosclerosis. Thus, the results of this study are consistent with the emerging evidence for the importance of LPA in lipid metabolism and atherosclerosis, although more work is clearly needed to fully explain the experimental findings from this study.

A second perhaps less profound but nevertheless important question in terms of developing apolipoprotein mimetic peptides into a therapy is how does the 6F peptide interact with LPA and affect its biological activity? It is known that the 4F peptide by surface plasmon resonance studies can bind LPA with high affinity (23), but this has not yet been reported for 6F. If 6F can indeed bind LPA, then a potentially facile explanation would be that 6F sequesters LPA in the diet and thus prevents LPA absorption. As already discussed, however, the standard chow diet has higher levels of LPA than the Western diet, so it is likely that the increased LPA in the small intestine and plasma may instead be from increased endogenous production stimulated in some way by the Western diet. 6F did blunt the effect of adding LPA to the chow diet, which is consistent with a direct interaction of the peptide and LPA, but the physiological significance of this part of the study is not clear given the extremely high levels of LPA that were used. Because the small intestine is a major site of lipoprotein production, some of the observed lipid and lipoprotein changes could also be due to a direct effect of 6F on enterocytes in the small intestine. Whether the 6F peptide treatment in some way also affects the liver production or catabolism of lipoproteins is not known. The increased levels of LPA in plasma could be a consequence of increased intestinal secretion, but could also be from another source such as increased extracellular production or from release by another tissue. For example, it has previously been shown that 4F and related peptides can prevent the oxidation of LDL (1), which is also a possible source of extracellular LPA. If sufficient amount of 6F enters the plasma, it could have a direct effect on inhibiting lipoprotein oxidation. Alternatively, 6F could potentially alter the susceptibility of lipoproteins to oxidation while they are being formed and secreted by the small intestine. Some of these unresolved questions could be addressed in the future by performing structure-function studies on apolipoprotein mimetic peptides to better understand what structural motifs are necessary for the observed biological changes in either enterocyte cell culture systems or mouse models.

In summary, this interesting study by Navab and colleagues showing the role of LPA in dyslipidemia and inflammation and identifying the site of action of the 6F peptide in the small intestine is an important advance. Although there may be some future surprises in our understanding of the mechanism of action of apolipoprotein mimetic peptides, results from this study will likely represent a turning point for this field and will undoubtedly stimulate much future research in this area.



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