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LIPOXYGENASES AND LIPID SIGNALING IN VASCULAR CELLS IN DIABETES

Rama Natarajan [1] and Jerry L Nadler [2]

[1] Gonda Diabetes Research Center, Beckman Research Institute of City of Hope, Duarte CA; and [2] Division of Endocrinology and Metabolism, University of Virginia School of Medicine, Charlottesville, VA

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. The Lipoxygenase pathway in Atherosclerosis, Restenosis, Hypertension and Diabetes
4. Regulation of Leukocyte-Type 12/15-LO activity and Expression by Hyperglycemia, Growth Factors and Cytokines
5. Lipoxygenase Products have Growth, Chemotactic and Adhesive Effects in VSMC and Endothelial Cells
6. Potential Signal Transduction and Gene Regulation Mechanisms by which LO Products may mediate their Cellular Effects
7. Acknowledgement
8. References

1. ABSTRACT

Evidence suggests that monocyte adhesion to endothelial cells as well as excessive proliferation and migration of vascular smooth muscle cells (VSMC) are key events in the development of atherosclerosis and restenosis after balloon angioplasty. These processes are mainly mediated by growth factors, inflammatory cytokines, chemokines and related factors released by various cells in the vessel wall. The mechanisms of action of these factors are however not very clear. These growth factors and cytokines acting on VSMC and endothelial cells can activate phospholipases with the release of lipids such as arachidonic and linoleic acids. These lipids can be further metabolized by several pathways including the lipoxygenase (LO) pathway. These oxidative pathways can lead to the formation of free radicals and lipid peroxides. LO products have been shown to have potent inflammatory, growth, adhesive and chemoattractant effects in cells. They are also associated with oxidant stress and cellular apoptosis. This chapter reviews the role and mechanisms of action of the LO pathway and its products in the pathogenesis of cardiovascular diseases and diabetic vascular complications. The importance of the leukocyte 12/15-LO in the pathology of these disorders is suggested by studies in which the 12/15-LO null mice displayed attenuated atherosclerosis. Activation of the LO pathway along with associated oxidant stress and signaling pathways may be a common mechanism shared by growth factors and cytokines in leading to increased inflammatory and proliferative disorders, including those associated with atherosclerosis, hypertension and related diabetic vascular complications.

2. INTRODUCTION

Cells respond to extracellular stimuli such as hormones, growth factors or cytokines by inducing various signals, including the activation of several phospholipases which act on membrane phospholipids to release arachidonic acid (1,2) (Figure 1). Arachidonic acid is also the precursor for several eicosanoids with potent biological effects including inflammation, adhesion, chemotaxis and cellular growth (3). The 20-carbon arachidonic acid can be metabolized by three major oxidative pathways; the cyclooxygenase pathway which leads to the formation of prostaglandins; the lipoxygenase (LO) pathway which forms hydroperoxyeicosatetraenoic acids (HPETEs), hydroxyeicosatetraenoic acids (HETEs) and leukotrienes; and thirdly, the cytochrome P-450 monooxygenase pathway which leads to the formation of epoxides such as epoxyeicosatrienoic acids as well as HETEs (4) (Figure 1). The lipoxygenases (LOs) are mainly classified as 5-, 8-, 12- or 15-LO based on their ability to insert molecular oxygen at the corresponding carbon position of arachidonic acid (5-7). The 5-LO pathway leads to the formation of 5(S)-HETE and leukotrienes while the 12- and 15-LOs can form 12(S)- and 15(S)-HETEs. The production of 12(S)- and 15(S)-HETE has been shown in several vascular tissues and cells including cultured vascular smooth muscle cells (VSMC), endothelial cells, as well as in monocytes. Products of the LOs may play important roles in the development of hypertension, atherosclerosis and other vascular disorders. The LO pathway has been shown to modulate aldosterone synthesis in the adrenal and renin in the kidney (8-10). Furthermore, LO products can mediate inflammatory actions of cytokines in pancreatic islets (11,12). This chapter is dedicated to reviewing the regulation and actions of the 12-LO pathway of arachidonic acid metabolism in VSMC and endothelial cells, and in animal models of atherosclerosis, diabetes and insulin resistance.

The mammalian LO enzymes have recently been extensively studied. Three major functionally distinct isoforms of 12-LO have been cloned: platelet, leukocyte and epidermal 12-LO (5-7,13-16). They are encoded by separate genes and vary in tissue distribution. The platelet-type has been cloned from human platelets and the megakaryocytic cell line, HEL (15,16). Human and rabbit 15-LOs and the leukocyte 12-LO have high homology and are classified as 12/15-LOs since they can form both 12(S)-HETE and 15(S)-HETE from arachidonic acid via their

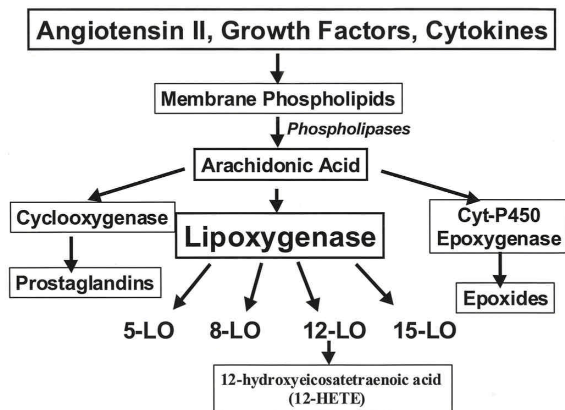

Figure 1. Metabolism of arachidonic acid. The cellular actions of phospholipases can lead to the release of arachidonic acid which is then metabolized by the cyclooxygenase, lipoxygenase and cytochrome P-450 enzymes to various bioactive molecules.

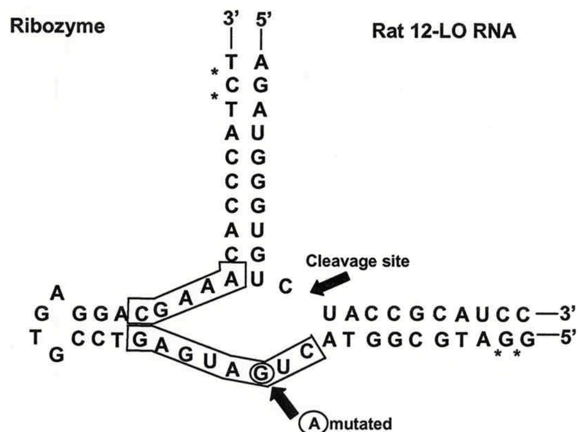

Figure 2. The 12-LO Ribozyme. Sequence of the rat leukocyte-type 12-LO chimeric DNA-RNA hammerhead ribozyme (left) and the complementary sequence of rat 12-LO mRNA (right). The Rz cleaves 3' to the GUC site of 12-LO mRNA (arrow). Ribonucleotides are boxed. Phosphorothioates linkages are marked by asterisks. The MRz has just a G to A mutation as indicated. Reprinted with permission from the American Heart Association: 39).

hydroperoxy precursors, and mainly 13(S)-hydroperoxyocatadecadienoic acid (13-HPODE) from linoleic acid (13,17). 12/15-LO has been detected in porcine leukocytes (13), VSMC (18), endothelial cells (19,20) and in several rat tissues (21-24). The leukocyte 12-LO and human 15-LO also share similarities in substrate specificity, being able to act on fatty acids as well as complex phospholipids (25). The two distinct 12-LO cDNAs, namely platelet- and leukocyte-type, have been cloned from mouse (26).

3. THE LIPOXYGENASE PATHWAY IN ATHEROSCLEROSIS, RESTENOSIS HYPERTENSION AND DIABETES

The LO enzymes and their products namely HETEs (from arachidonic acid) and HODEs (from linoleic acid) have been implicated in the pathogenesis of atherosclerosis. The 12/15-LO enzyme can mediate the oxidative modification of low density lipoprotein (LDL) to oxidized LDL, which is believed to be the atherogenic form of LDL (27-29). Furthermore, 15-LO mRNA and protein are expressed in macrophage-rich areas of rabbit atherosclerotic lesions colocalized with epitopes characteristic for oxidized LDL (30). Atherosclerotic lesions of rabbit and human aorta also show higher levels of 15-LO enzymatic activity than corresponding normal arteries (31). Increased amounts of LO products have been shown to be present in early atherosclerotic lesions (25) but not in later lesions where non-enzymatic oxidation reactions related to lipid peroxidation predominate. Analysis of the lipid oxidation products in human atherosclerotic lesions revealed that the oxidation of polyunsaturated fatty acids therein was mainly mediated by the LO enzyme (32). In another study, Benz et. al. demonstrated that LDL incubated with fibroblasts overexpressing 15-LO had enhanced levels of lipid hydroperoxides, further implicating the LO enzyme in the oxidative modification of LDL (33). Furthermore, angiotensin II (AII) increased macrophage-mediated modification of LDL via the 12/15-LO pathway (34).

Animal models have now demonstrated the key role of the LO pathway in the pathogenesis of atherosclerosis and restenosis. Overexpression of 15-LO in the vascular endothelium could accelerate early atherosclerosis in LDL receptor-deficient mice (35). We also have evidence supporting the presence of a leukocyte-type 12/15-LO mRNA and protein in porcine atherosclerotic lesions which was greatly augmented in diabetic and hyperlipemic pigs displaying accelerated atherosclerosis (36,37). It appears that LO activation may also play a role in neointimal thickening associated with restenosis since there was a marked increase in 12/15-LO expression in balloon injured rat carotid arteries relative to uninjured that was seen in VSMC, endothelial cells and in inflammatory cells (38). Furthermore, pretreatment with a pharmacological LO inhibitor or a molecular inhibitor, namely a ribozyme targeted to rat 12/15-LO (Figure 2), could significantly reduce the rates of neointimal thickening (Figure 3) thereby demonstrating the *in vivo* functional significance of increased 12/15-LO expression (38,39). In contrast, a control catalytically inactive mutant ribozyme (MRz) was not effective (39).

In other studies, a 15-LO inhibitor blocked diet-induced atherosclerosis in rabbits (40). Convincing evidence supporting a pathological role for leukocyte 12/15-LO in atherosclerosis comes from recent reports showing marked decrease in atherosclerosis in apo E -/- mice and LDLR-/- that were cross bred with leukocyte 12/15-LO -/- mice (41,42). In these *in vivo* models, the decrease in atherosclerosis was attributed to reduced LDL oxidation and lipid peroxidation due to the absence of macrophage 12/15-LO (43,44). Furthermore, very recently Funk and coworkers suggested a novel inflammatory link since the macrophages from 12/15-LO -/- mice had a selective defect in lipopolysaccharide -induced interleukin-12 synthesis (45). An interesting new recent genetic study

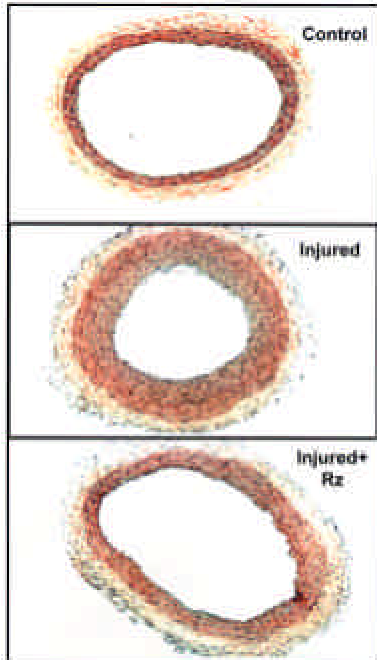

Figure 3. Effect of the 12-LO Rz on neointimal thickening in balloon-injured rat carotid arteries. 10 ug Rz oligonucleotide mixed with lipofectin was instilled into injured arteries for 30 min immediately following injury. 12 days after injury, carotid arteries were fixed and processed as described under Methods. The figure shows representative results of hematoxylin and eosin-stained arterial sections of uninjured right carotid (top), injured-untreated left carotid (middle), and injured-Rz treated left carotid (bottom) from one rat. Reprinted with permission from the American Heart Association: 39).

suggests that 5-LO may be an important pro-atherogenic gene locus (46). Thus, the LOs may be important in the pathology of atherosclerosis by virtue of their capacity to oxidize LDL, their growth and inflammatory properties as well as being in a key atherogenic mouse genetic locus. The relative importance of the different LOs in this regard is not fully clear.

In another study, it was demonstrated that treatment of human aortic endothelial cells with 12(S)-HETE, but not the stereoisomer 12(R)-HETE which is not an LO product, directly induced monocyte binding to the endothelial cells, a key early step in the development of atherosclerosis (47). Furthermore, the 12/15-LO ribozyme blocked high glucose-induced binding of monocytes to endothelial cells (48). Other related studies further supporting the role of the LO pathway in atherosclerosis are those showing that 12(S)-HETE induced transendothelial migration of monocytic cells (49) and certain LO products increased the surface expression of key cell endothelial adhesion molecules such as VCAM-1 via activation of the transcription factor, NF-kB (49). LO products also directly increased migration, cellular hypertrophy and fibronectin synthesis in VSMC (50,51). These studies are discussed further in other sections below.

Increasing evidence has also implicated the LO pathway in the pathogenesis of hypertension and in the vasopressor and renin-inhibitory effects of angiotensin II (AII) (52-55). Inhibition of the LO pathway had a potent hypotensive effect in renovascular hypertensive rats (56) as well as in spontaneously hypertensive (SHR) rats (57). The SHR rats also had increased 12-LO activity compared to the normotensive control rats (58). A novel variant form of platelet-type 12-LO was recently detected in human VSMC prepared from umbilical arteries (59).

Several lines of evidence indicate that the LO pathway may play a role in the cardiovascular complications associated with diabetes. Porcine VSMC cultured under high glucose (HG) conditions to mimic chronic hyperglycemia of diabetes exhibited significantly increased rates of proliferation in response to 10% fetal calf serum when compared to cells cultured under normal glucose (NG) conditions (60). This suggested one potential mechanism for the increased atherosclerotic and hypertensive disorders associated with diabetes. In addition, treatment with a specific LO inhibitor, baicalein, led to an inhibitory effect on the proliferation of the cells cultured in HG towards that seen in NG (unpublished observations). This suggests that the LO pathway may play a role, at least in part, in the accelerated proliferation of VSMC under hyperglycemic conditions.

Other studies implicating HETEs in the pathogenesis of diabetic vascular disease include the observation that vessels from the infants of diabetic mothers had significantly elevated levels of 15-HETE and decreased formation of the vasodilatory prostanoid, prostacyclin (61) and endothelial cells cultured under hyperglycemic conditions produced increased amounts of HETEs (47,62). Furthermore, HG-induced adhesion of monocytes to endothelial cells could be mediated by the LO pathway in endothelial cells (47,48). Products of 12/15-LO also appear to play an important role in minimally modified LDL-induced monocyte binding to endothelial cells (63). 12(S)-HETE could also upregulate the expression of the potent angiogenic growth factor, vascular endothelial growth factor, which has been implicated in the pathogenesis of diabetic retinopathy (64). LO products have potent chemotactic effects in VSMC at concentrations as low as 1pM (50). The hypertrophic effects of 12(S)-HETE in VSMC were markedly enhanced under HG culture conditions similar to the effects of AII (51). Studies in diabetic subjects show that urinary excretion of the 12-LO product, 12(S)-HETE is markedly increased compared to matched non-diabetic controls (65).

Recently, we obtained evidence of increased 12/15-LO activation in a swine model of hyperlipidemia and diabetes-induced accelerated atherosclerosis (36,37). Diabetes (HG) and hyperlipidemia (HL) alone increased both monocyte oxidant stress and 12/15-LO expression in arteries, but the combination of these two risk factors not only led to a marked acceleration in atherosclerosis, but also a synergistic increase in oxidant stress and 12/15-LO activation. Figure 4A shows 12/15-LO immunostaining of abdominal aortic section from four groups of pigs;

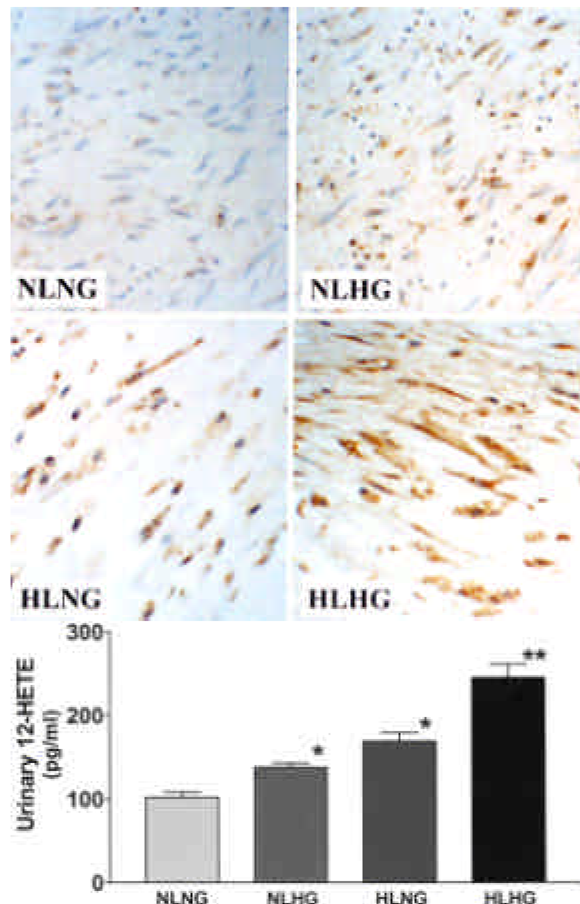

Figure 4 A. 12-LO Immunostaining in abdominal aortic sections from the four groups of swine, NLNG, NLHG, HLNG, HLHG. Paraffin embedded sections were used from same region of abdominal aortas which were removed at necropsy 20 weeks after initiation of the high lipid diet. A polyclonal antibody raised to a peptide from the porcine leukocyte 12-LO was used at 1:400 dilution for the immunostaining. **B.** Concentrations of 12-HETE excreted in the urines of normal versus diabetic and hyperlipemic swine at 20 weeks. Urinary 12-HETE was extracted and quantitated by a specific radioimmunoassay. Results shown are mean ± SEM of 12-HETE data obtained from the urine of two sets of animals run in triplicate. Reprinted with permission from the Springer Verlag: 36).

normolipemic normoglycemic (NLNG); normolipemic hyperglycemic (NLHG); hyperlipemic normoglycemic (HLNG); and finally HLHG (36). In this swine model, the HL pigs alone showed atherosclerosis but this was greatly enhanced in the HLHG group relative to the HLNG (37). Interestingly, it is seen in Figure 4A that a similar trend was also observed with the 12/15-LO immunostaining (seen in VSMC and macrophages) which is evident in the HG and HL animals but markedly enhanced in the HLHG group that has two risk factors (36). Competitive PCR and immunoblotting demonstrated parallel increases in 12/15-LO mRNA and protein in these artery sections. This was associated with a parallel augmentation of oxidant stress and urinary excretion of LO product, 12(S)-HETE (Figure 4B) (36,37). These results suggest an *in vivo* role for leukocyte 12/15-LO in diabetic atherosclerosis.

In addition, we recently demonstrated markedly increased neointimal thickening in balloon injured Zucker fatty insulin resistant rats relative to control lean rats. The fatty rats also had increased 12/15-LO immunostaining in the neointima relative to the lean rats (unpublished observations) (Figure 5). Elevated levels of HETEs have also been observed in other animal models of diabetes (66,67). Therefore, substantial *in vitro* and *in vivo* evidence support the hypothesis that elevated glucose, diabetes and insulin resistance can augment LO pathway activation. Furthermore, factors such as growth factors, cytokines and high glucose, all factors relevant to the pathogenesis of diabetes, can upregulate the activity and expression of 12/15-LO as discussed below. Future studies will determine whether ablation of 12/15-LO expression by either pharmacologic or genetic maneuvers can attenuate the progression of diabetic complications in these animal models.

4. REGULATION OF LEUKOCYTE-TYPE 12/15-LO ACTIVITY AND EXPRESSION BY HYPERGLYCEMIA, GROWTH FACTORS, AND CYTOKINES

Evidence indicates that elevated glucose can enhance 12/15-LO pathway activation and expression. Porcine VSMC (PVSMC) cultured under hyperglycemic conditions proliferated faster and also synthesized significantly greater amounts of cell-associated 12-LO product, 12(S)-HETE, than those cultured under normal glucose (NG, 5.5 mmol/L) conditions (18). In addition, the cells in high glucose (HG, 25 mmol/L) displayed much greater intracellular 12-LO enzyme activity levels. AII (100 nmol/L) treatment caused a further increase in 12-LO activity in NG as well as in HG, but its effects were much more pronounced in the cells cultured in HG (18). Furthermore, we have observed that treatment of VSMC with advanced glycation end products (which also mimic the diabetic milieu) was also effective in increasing 12-LO activity (unpublished observations).

The leukocyte 12-LO is the only 12/15-LO cloned in swine (13). A leukocyte-type 12-LO was identified in VSMC using a combination of molecular and immunological approaches (18,19). The expression was very low under basal normal glucose conditions but markedly induced by treatment of the PVSMC with HG or with AII (18). HG and AII also increased levels of the 12-LO product, 12(S)-HETE in the cells and increased intracellular enzyme activity (18).

Immunoblotting of VSMC with a specific polyclonal peptide antibody to the porcine leukocyte-type 12-LO demonstrated the presence of a protein with a molecular mass of about 72 kD, similar to the reported molecular mass of porcine leukocyte 12-LO (18). Furthermore, using a specific 15-LO antibody, no band was detected around 70 kD. Basal 12-LO protein expression was increased nearly 5-fold in the cells cultured in HG

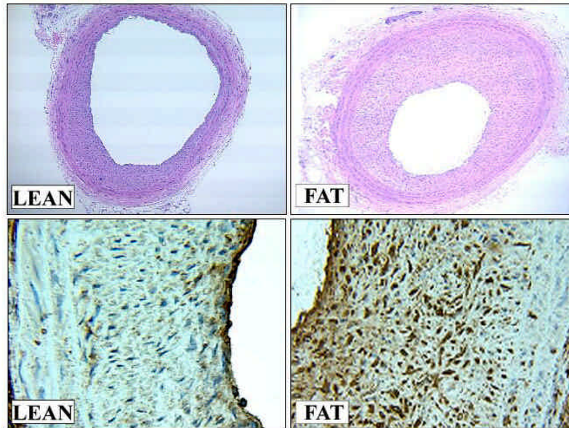

Figure 5. Evidence of increased 12-Lipoxygenase (12-LO) protein expression and neointimal response in obese Zucker rats. The top panels show H&E staining of carotid lesions of lean (left) and fat (right) Zucker rats after 28 days of carotid artery balloon injury. The bottom panels show 12-LO immunostaining with a leukocyte specific 12-LO antibody in lean (left) and fat (right) Zucker rats. Staining of 12-LO is predominantly in VSMC. (Study done in collaboration with Dr. Hong Pei).

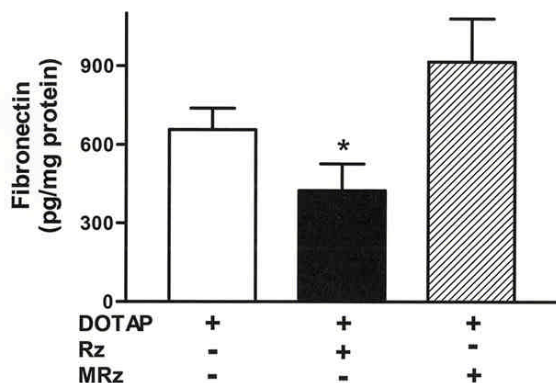

Figure 6. The 12-LO Rz decreases RVSMC fibronectin levels. Fibronectin was quantitated by a specific ELISA. Results are expressed as mean ± SEM of three experiments. *, $p < 0.03$ versus control. Rz, specific ribozyme directed to rat 12-LO; MRz, control mutant. Reprinted with permission from the American Heart Association: 39).

compared to those in NG. Treatment of the cells with AII (100 nM) caused a marked stimulation (2- to 3-fold) in NG as well in HG (18).

12-LO activity and expression could also be potently upregulated by another VSMC growth factor, namely, platelet-derived growth factor-BB (PDGF) (68). One report suggested that the LO pathway may mediate the proliferative effects of not only PDGF but also that of fibroblast growth factor in vascular smooth muscle cells (69).

Cytokines such as interleukins-1, -4 and -8 can also increase 12/15-LO activity and mRNA expression in VSMC (70) and other cells. Evidence suggests that LOs are also expressed in endothelial cells (19,20). Furthermore, treatment with HG increased LO activity and expression, and production of LO and not cyclooxygenase products (62,63). Other studies have shown that 15-LO expression was induced in monocytes and endothelial cells by interleukin-4 or interleukin-13 (20,71-74). It appears that these cytokines can regulate 15-LO transcriptionally or translationally (20,75-77) and a recent study showed that IL-4-stimulated 15-LO transcriptional activation involves both phosphorylation and acetylation of STAT6 as well as acetylation of nuclear histones (78). Several possible pro- versus anti-atherogenic functions may be mediated by various products of 15-LO lipid oxidation (28). Thus, 12/15-LO in vascular and mononuclear cells can be induced by growth factors and cytokines and may contribute to their biological effects such as growth, chemotactic and inflammatory effects, thereby playing a role in the formation of the atherosclerotic plaque.

5. LIPOXYGENASE PRODUCTS HAVE GROWTH, CHEMOTACTIC AND ADHESIVE EFFECTS IN VSMC AND ENDOTHELIAL CELLS

In order to evaluate potential growth-promoting effects of LO products in VSMC, studies were conducted to determine if LO products have hypertrophic effects in these cells (51). AII-induced increases in total cellular protein content of PVSMC were significantly attenuated by a specific LO inhibitor, baicalein, whereas, in contrast, a cyclooxygenase inhibitor, ibuprofen, had no effect. Furthermore, direct addition of the 12-LO product, 12(S)-HETE, increased total cell protein content in cells cultured under NG conditions to nearly the same extent as AII. 15(S)-HETE had no effect under these conditions. However, interestingly, the stimulatory effects of not only AII but also 12(S)-HETE were markedly enhanced in cells cultured under HG (25 mM) conditions with 12(S)-HETE at 10^{-8}M showing a significant response (51).

The study also examined the effects of AII and 12-HETE on the levels of the matrix protein fibronectin as measured by a specific ELISA (51). Neither AII nor 12-HETE had any effect on released fibronectin levels in PVSMC cultured under NG conditions. In contrast, both agents led to significant increases in fibronectin levels in the cells cultured under HG conditions (51). Using rat VSMC, we noted that a specific molecular inhibitor, namely a ribozyme designed to specifically target rat 12/15-LO (Figure 1) could significantly inhibit HG-induced fibronectin production (39) (Figure 6). This hammerhead ribozyme has phosphorothioate residues for increased stability to nucleases and can catalytically cleave its target (39). Interestingly, the rat ribozyme also blocked AII-induced fibronectin expression in rat renal mesangial cells (79). Here we used a novel new "short" ribozyme with propane diol modifications to improve accessibility and efficiency over the original "long" ribozyme (79). Since AII as well as HG culture can increase the formation of LO products in the VSMC, it is attractive to speculate that the enhanced growth promoting and matrix producing effects of the LO products in HG may represent a potential

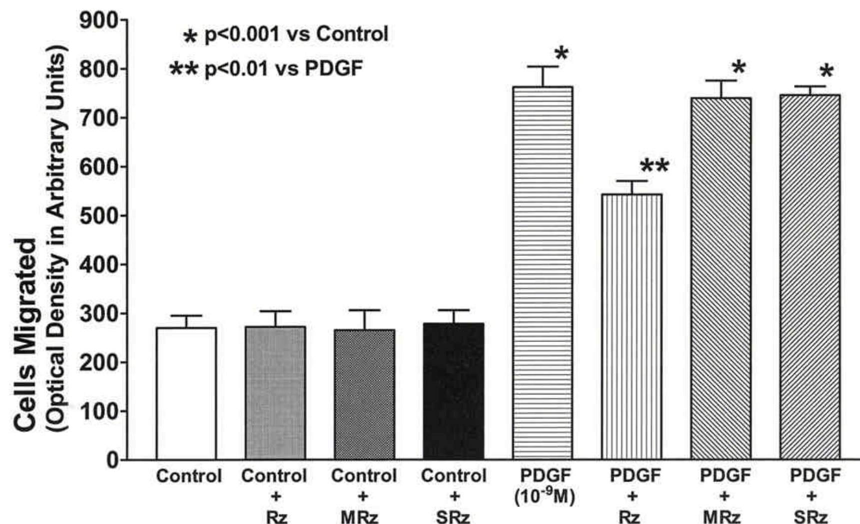

Figure 7. Effect of 12-LO Rz on RVSMC migration. The lower chamber of a Boyden chamber was filled with either medium alone, medium mixed with Rz, MRz or scrambled Rz (SRz) (5 umol/L each), recombinant human PDGF-BB at 1 nmol/L, or PDGF mixed with Rz, MRz, or SRz (5 umol/L). The upper wells received 52 ul cell suspension alone (approximtely 50,000 cells), or mixed with 5 umol/L Rz, MRz, or SRz. The chamber was incubated for 5 hours and migrated cells quantitated. Results are expressed as mean ± SEM. *, p< 0.001 versus control. **, p<0.01 versus PDGF. Reprinted with permission from the American Heart Association: 39).

mechanism for the accelerated growth of the VSMC and for the enhanced hypertrophic effects of AII observed. In support of this it was noted that rat A10 VSMC and cardiac fibroblasts stably expressing mouse 12/15-LO showed increased growth properties (80,81).

Studies indicate that the 12-LO pathway can also mediate the mitogenic effects of AII (82). These studies were performed in a CHO cell overexpressing the AII type 1a receptor, CHO-AT1a. AII was a potent mitogen in these cells and the mitogenic effects were significantly inhibited by the LO blocker, baicalein, but not a cyclooxygenase inhibitor. Interestingly, 12(S)-HETE had direct mitogenic effects in these cells (82). The mitogenic effects of AII were partially and those of 12(S)-HETE fully blocked by pretreatment with pertussis toxin indicating the involvement of G-proteins in the actions of 12(S)-HETE (82), at least in these cells.

Since evidence suggests that LO products have potent chemoattractant effects in VSMC (50), the role of the LO pathway in the chemotactic effects of PDGF were investigated (68). Pharmacological LO inhibitors, CDC and baicalein (10 micromolar each), could significantly inhibit PDGF induced migration of PVSMC as measured in a modified Boyden chamber (68). In contrast, a cyclooxygenase inhibitor, indomethacin (indo) did not alter basal or PDGF-induced migration. Since pharmacological inhibitors can have non-specific effects, we used ribozymes designed to specifically cleave the porcine or rat leukocyte type12/15-LO. We showed that these ribozymes (Rz) were effective *in vitro* and in porcine and rat VSMC in reducing levels of 12/15-LO mRNA and protein (39,83) and could also significantly attenuate the chemotactic effects of PDGF in VSMC (Figure 7). In contrast, the control catalytically inactive ribozyme (MRz) with a point mutation in the catalytic site was without any significant effect. Both the Rz and MRz did not alter basal migration. Since PDGF can upregulate 12-LO activity and expression (68), these results suggest a key function for the 12-LO pathway in the biological effects of this atherogenic growth factor. Recently, in order to achieve more sustained expression, a recombinant adenovirus expressing the Rz targeted to porcine 12-LO was developed (48). This viral vector was effective in attenuating PDGF-induced PVSMC migration. Taken together these results suggest the involvement of LO activation, at least in part, in the chemotactic effects of PDGF. The adenoviral vector expressing the Rz targeted to porcine 12-LO also effectively inhibited HG-stimulated 12(S)-HETE production in porcine aortic endothelial cells (48). In the Rz treated endothelial cells, there was also a significant inhibition of HG-stimulated monocyte adhesion (48). Direct treatment of the endothelial cells with 12(S-HETE) and not the stereoisomer 12(R)-HETE increased the adhesion of monocytes (47).

6. POTENTIAL SIGNAL TRANSDUCTION AND GENE REGULATION MECHANISMS BY WHICH LO PRODUCTS MAY MEDIATE THEIR CELLULAR EFFECTS

There are several signaling mechanisms by which LO products may mediate cellular growth, adhesive, chemotactic and inflammatory effects associated with vascular disease. HETEs can also activate certain isoforms of protein kinase C directly (84,85) or indirectly by incorporating into membrane phospholipids which then generate HETE-containing diacylglycerol species to activate protein kinase C (86). Activation of the leukocyte-

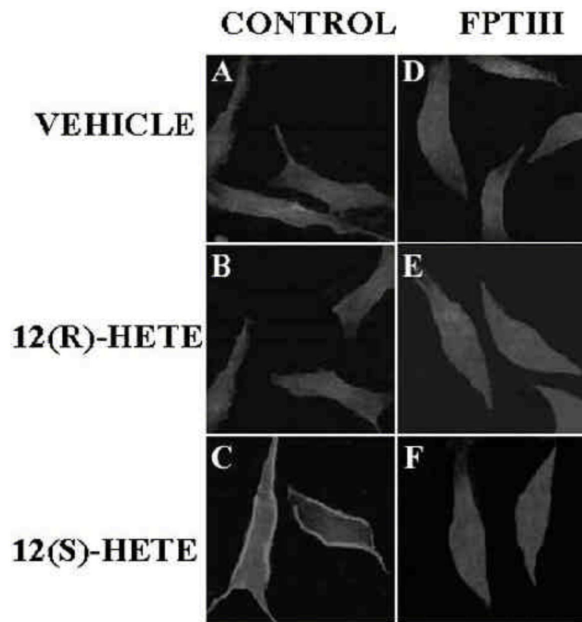

Figure 8. Stimulation of Ras translocation to the cell membrane by 12(S)-HETE. Serum depleted PVSMC were left untreated (Control) or treated with the farnesyl transferase inhibitor (FPT III, 10 uM) overnight and stimulated with either 0.1% ethanol (Vehicle) or 0.1 uM each of 12(S)-HETE or 12(R)-HETE for 10 minutes. Then the cells were stained with Ras antibody and visualized by confocal microscopy. Reprinted with permission from The American Society for Biochemistry and Molecular Biology: 80).

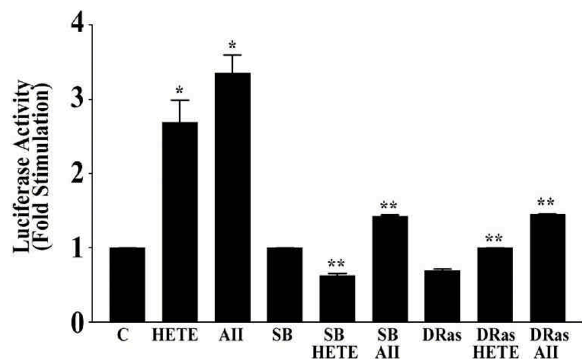

Figure 9. Stimulation of transcription from fibronectin promoter by 12(S)-HETE. PVSMC were transiently transfected with reporter plasmid FN510-Luc, which contains luciferase gene under the control of minimal fibronectin promoter (-510) with three CREB binding sites in the upstream sequences. Then the cells were stimulated with 12(S)-HETE or angiotensin II (AngII) (0.1 umol/L each) in the presence or absence of SB202190 (5 umol/L) for six hours and luciferase activity was determined. In some experiments an expression vector for dominant negative (DN) mutant of Ras (Ras-N17) was also co-transfected. *, p<0.001 vs Control, **, p<0.001 vs 12-HETE or AngII. Reprinted with permission from The American Society for Biochemistry and Molecular Biology: 80).

type of 12-LO and 15-LO may cause structural modification of membrane phospholipids since they can not only oxidize free fatty acids but also those esterified to phospholipids, unlike platelet 12-LO (87). Studies have also shown that LO products can modulate intracellular calcium levels in VSMC (88). Thus, LO inhibitors could attenuate angiotensin II-stimulated increases in cytosolic calcium. The addition of 12(S)-HETE, but not 5- or 15(S)-HETE could specifically restore the initial calcium response to angiotensin II in rat VSMC, suggesting that LO products, such as 12(S)-HETE, may act as mediators of the intracellular signaling actions of angiotensin II and also other growth factors (88). Some studies have suggested a receptor-mediated mechanism of action with high affinity binding sites for 12(S)-HETE in certain cells (89). Such receptors have not yet been identified in VSMC or endothelial cells. However since LO products are increased both intracellularly and extracellularly in response to agonists, it is of interest to determine the mechanisms by which they elicit their cellular effects.

In VSMC, LO products of arachidonic acid (HETEs) and linoleic acid (HODEs) could directly activate mitogen activated protein kinases (MAPKs) (80,90,91). Furthermore, in the CHO-AT1a cells, LO inhibitors could attenuate AII-induced MAPK (ERK1/2) activity, and LO products directly activate ERK1/2 (82). In these CHO-AT1a cells 12(S)-HETE also directly activated another member of the MAPK family, c-Jun aminoterminal kinase (JNK) and furthermore, LO inhibitors blocked AII-induced JNK activation (92) and also activated the p21-activated kinase1 (PAK1) (93). Extensive studies on the signal transduction mechanisms of action of 12(S)-HETE in VSMC were recently published (80). Here it was noted that the LO product, 12(S)-HETE, but not stereoisomer 12(R)-HETE, directly activated Ras, the key upstream activator of the MAPK signaling cascade, thereby providing key information on the mechanism by which these lipids can activate MAPK and downstream signaling (80) (Figure 8). Furthermore, 12(S)-HETE, but not 12(R)-HETE led to a significant increase in the activation of p38MAPK and its key downstream target, cyclic AMP response element binding protein (CREB). 12(S)-HETE also induced CREB DNA binding as assessed by gel shift assays. *In vivo* transreporting assays and evaluation of fibronectin promoter transactivation demonstrated that 12(S)-HETE can lead to transcription from the fibronectin promoter via p38MAPK activation and CREB elements play a key role (80) (Figure 9). Interestingly, AII effects on these parameters were blocked by p38MAPK and also LO inhibitors. P38MAPK inhibitors also blocked 12(S)-HETE and AII-induced hypertrophic effects in VSMC. These studies provide a novel link between LO activation and the p38MAPK in AII effects on VSMC hypertrophy and fibronectin production (80). In support of this, it was noted that overexpression of mouse 12-LO in A10 VSMC cell line increased p38MAPK activation and cellular hypertrophy (80). A similar mechanism of action of 12-LO was found in the human adrenal H295R cell line (94,95). Additionally, it was noted that cardiac cells also synthesize 12-LO products that can increase matrix production, cellular hypertrophy and activate MAPKs (96). Since

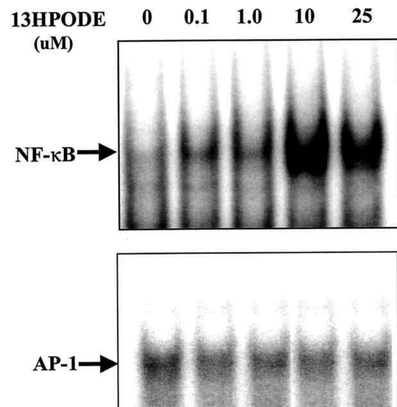

Figure 10. 13-HPODE activates NF-kappaB but not AP-1 in VSMC. Serum-starved PVSMC were treated with vehicle (0.1% ethanol) or 13HPODE for 30 min. Nuclear extracts were subjected to EMSA to evaluate NF-kappaB or AP-1 activation. Reprinted with permission from the American Heart Association, Lippincott Williams & Wilkins: 91.

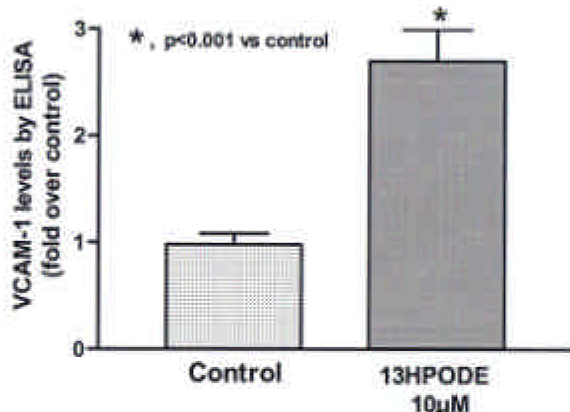

Figure 11. 13-HPODE treatment of VSMC increases the expression of VCAM-1 on the cell surface. Human VSMC were treated with 13-HPODE and the cell surface expression of human VCAM-1 evaluated by flow cytometry using an antibody specific for human VCAM-1.

MAPKs are key components in the signaling pathways initiated by several growth factors and hormones, LO metabolites of arachidonic and linoleic acids may act as novel signal transducers and amplifiers of growth factor and cytokine-induced activation of MAPKs and downstream gene expression. They could therefore play a key role in the aberrant growth and migration of VSMC observed in atherosclerosis, restenosis, hypertension and diabetes.

Recent reports indicate that the 12/15-LO enzyme can lead to the catalytic consumption of the vasodilator, nitric oxide (97). This denotes another key mechanism by which LO activation can mediate the pathogenesis of vascular disease such as atherosclerosis and hypertension by limiting the availability of nitric oxide in the vessel wall. Reactive oxygen species generated during LO pathway activation (98) may mediate growth and inflammatory effects in VSMC and endothelial cells. We recently observed (unpublished) that VSMC derived from 12/15-LO -/- mice grow slower than those derived from genetic control mice and also produce much lesser amounts of the reactive oxygen species, superoxide. LO and its products are also associated with the production of inflammatory genes. Thus the LO product of linoleic acid, 13-HPODE, led to a significant increase in the activation of the redox-sensitive and inflammatory transcription factor, NF-kB in PVSMC (91) (Figure 10). This was associated with increased NF-kB binding to the VCAM-1 promoter and increased promoter transactivation. P38MAPK activation appeared to play an important role in the latter process. 13-HPODE also augmented the cell surface protein expression of the inflammatory adhesive molecule, VCAM-1 (Figure 11) and increased Ras activation in these cells (91). Interestingly, a novel role for HPODE and HPETE as seeding molecules responsible for LDL oxidation by artery wall cells and associated oxidative events related to the pathogenesis of atherosclerosis has been demonstrated (99). Furthermore, transfection of human endothelial cells with antisense to platelet 12-LO (but not sense) eliminated the 12-LO protein and prevented LDL-induced monocyte chemotactic activity (100). In monocytes, 9- and 13-HODE induced the expression of the scavenger receptor, CD36 apparently via activation of the nuclear receptor, peroxisome proliferator activator-gamma (PPAR-gamma) (101). IL-4-induced 12/15-LO was also implicated in this process (102). 12/15-LO -/- mice macrophages had a defect in LPS-induced interleukin-12 synthesis (45). A recent report demonstrated that 12-(S)-HETE could increase COX-2 expression in islet cells (103). Since inflammatory cytokines can increase 12/15-LO activity and expression, it is tempting to suggest that LO can participate in an inflammatory loop with cytokines and other inflammatory genes to amplify or modulate their responses.

In summary, LO enzymes in vascular, inflammatory, renal, cardiac and other cells can form products that have pleiotropic physiological and pathological effects. These include vasoactive, growth promoting, adhesive, chemotactic, oxidative and inflammatory effects which therefore implicate them in the pathogenesis of diseases such as atherosclerosis, hypertension and diabetic complications. There are currently no clinically available safe, selective or optimally bioavailable inhibitors of LO enzymes. Hence, therapeutic modalities, novel ribozyme and related approaches to effectively block these pathways may provide new ways to combat cardiovascular and inflammatory diseases, including those associated with diabetes.

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Send Correspondence to: Rama Natarajan, Ph.D., Gonda Diabetes Research Center, Beckman Research Institute of the City of Hope, 1500, E. Duarte Road, Duarte, CA 91010, Tel: 626-359-8111, ext 62289, Fax: 626-301-8136; E-mail: rnatarajan@coh.org