Ribozyme-Mediated Inhibition of Rat Leukocyte-Type 12-Lipoxygenase Prevents Intimal Hyperplasia in Balloon-Injured Rat Carotid Arteries

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Background—12-Lipoxygenase (12-LO) products of arachidonate metabolism have growth and chemotactic effects in vascular smooth muscle cells. We have also recently demonstrated increased 12-LO mRNA and protein expression in the neointima of balloon-injured rat carotid arteries. In this study, we evaluated whether 12-LO activation plays a role in neointimal thickening in this rat model by using a specific ribozyme (Rz) directed to rat 12-LO.

Methods and Results—We designed a chimeric DNA-RNA hammerhead Rz to cleave rat leukocyte-type 12-LO mRNA. This Rz dose-dependently cleaved a 166-nucleotide target 12-LO mRNA substrate in vitro and reduced 12-LO mRNA and protein expression in rat vascular smooth muscle cells. A control mutant Rz (MRz) with a point mutation in the catalytic site was inactive. To test the in vivo efficacy of the 12-LO Rz, the left common carotid arteries of rats were injured with a balloon catheter. The distal half of the injured arteries was treated with Rz or MRz mixed with lipofectin. The proximal half received only lipofectin. Twelve days after injury, intima-to-media ratios were significantly lower in the Rz-treated sections than in untreated sections from the same rat (0.742±0.16 versus 1.749±0.12, \(P<0.001\)). In contrast, the MRz had no significant effect.

Conclusions—These results indicate the important role of the leukocyte-type 12-LO pathway in restenosis in response to injury. (Circulation. 2001;103:1446-1452.)

Key Words: restenosis ■ lipids ■ vasculature ■ growth substances

Neointimal thickening and restenosis occur in nearly 40% of patients who undergo PTCA.\(^1\) Vascular smooth muscle (VSMC) migration and proliferation are key events in the development of atherosclerosis and restenosis after angioplasty.\(^2,3\) Therefore, the targeting of factors mediating VSMC growth and chemotaxis would be an effective route to reduce the rates of restenosis. The mechanisms responsible for neointimal thickening are not fully understood. Injury-induced release of growth factors and cytokines can activate phospholipases and the release of arachidonic acid, which in turn can be metabolized by pathways such as the lipoxygenase (LO) pathway.\(^4,5\) 12-LO activation can lead to the formation of products such as 12-hydroxyeicosatetraenoic acid (12-HETE) from arachidonic acid.\(^6\) These products have potent cellular effects.\(^6,7\) There are 2 major types of 12-LO: platelet type and leukocyte type.\(^8\) VSMCs, endothelial cells, and leukocytes express a leukocyte-type 12-LO.\(^9-11\) Rat tissues mainly express a leukocyte-type 12-LO, which also possesses some 15-LO activity.\(^12\)

Cytokines such as interleukin (IL)-4 and IL-13 can induce 15-LO expression in monocytes.\(^13,14\) VSMC growth and chemotactic factors, such as angiotensin II and platelet-derived growth factor (PDGF)-BB, are potent inducers of 12-LO activity and expression in VSMCs.\(^9,15\) Furthermore, the growth-promoting effects of angiotensin II as well as the chemotactic effects of PDGF may be mediated, at least in part, by 12-LO activation.\(^15,16\) IL-1\(\beta\), IL-4, and IL-8 can also induce 12-LO in porcine VSMCs.\(^17\) Thus, LO activation in the component cells of blood vessels by atherogenic and inflammatory stimuli can play a key role in the pathogenesis of atherosclerosis and restenosis. Hence, we hypothesized that 12-LO could serve as a key common target for therapeutic intervention.

We very recently demonstrated a significant upregulation of leukocyte-type 12-LO expression in inflammatory cells and VSMCs of the neointima in a rat carotid artery model of balloon injury and restenosis.\(^18\) Furthermore, phenidone, a nonselective LO inhibitor, can attenuate the extent of neointimal thickening.\(^18\) However, specific involvement of the 12-LO pathway cannot be firmly established with these pharmacological nonspecific inhibitors. No specific leukocyte 12-LO inhibitors are commercially available for in vivo...
Methods

Designing Rzs Targeting Rat Leukocyte-Type 12-LO

A hammerhead Rz was designed to cleave the rat leukocyte-type 12-LO mRNA. The sequence of rat leukocyte-type 12-LO cDNA has been previously reported.12 Our Rz was designed to cleave 12-LO mRNA at the 3’ end of the GUC triplet located at position 9 bases downstream from the AUG start site of translation (Figure 1). The Rz contained a chimeric DNA-RNA sequence, in which the RNA bases of the substrate complementary arms and the stem loop were replaced with deoxyribonucleotides; ribonucleotides were present in the core center area. Such substitutions would prevent exoribonuclease activity and reduce the number of endonuclelease targets within the Rz.22,23 To further improve stability, the Rz contained 2 phosphorothioate linkages at both the 5’ and 3’ ends (Figure 1).

As a control, a catalytically inactive mutant Rz (MRz) was generated by a single base substitution at core center position 5 (G to A) according to the uniform numbering system,26 and a scrambled Rz (SRz) was generated by randomly changing nucleotide sequences at 5’ and 3’ complementary arms. In addition, a fluorescein-conjugated Rz (FRz) was made by replacing 2 phosphorothioate linkages at the 5’ terminal with 5’-propanediol fluorescein and removing 1 nucleotide at both substrate-binding arms. All the oligonucleotides were chemically synthesized and purified at the DNA Synthesis Core at the City of Hope.

In Vitro Assay of Rz Activity

A rat leukocyte 12-LO mRNA target substrate corresponding to the 12-LO Rz cleavage site was synthesized by in vitro transcription of the 12-LO cDNA/pCDNA plasmid with T7 RNA polymerase and labeled with [α-32P]UTP according to manufacturer’s instructions (Promega Corp). The purified 166-nt RNA transcript containing 5’ flanking sequences of 12-LO mRNA and a partial sequence of the plasmid served as an in vitro substrate for the Rz.

In vitro cleavage reactions were performed as described previously.12,22 The 166-nt 12-LO mRNA substrate was mixed with Rz and incubated for 14 hours at 37°C. The reactions were stopped by the addition of 80% formamide loading buffer, and products were resolved by electrophoresis and detected by autoradiography.

Transfection of Rz Into Cells

Rzs or control oligonucleotides were delivered into rat aortic VSMCs (RVSMS) by cationic liposome-mediated transfection. RVSMS were prepared from rat thoracic aortas (generous gift from M. Hori and Dr M. Tuck, Department of Veterans Affairs, Sepulveda, Calif). They were maintained in DME containing high glucose and 10% FCS and transfected with the cationic lipid N-[1-(2,3-dioleoyloxy)propyl]-N,N,N,N-tetramethylammonium salts (DOTAP, Boehringer-Mannheim) either alone or complexed with Rz or MRz for 6 hours. Cells were plated in 6- or 10-cm culture dishes to yield ~80% confluence at the time of transfection.

To determine the efficiency of cellular uptake and optional delivery conditions to RVSMS, transfections were performed in 10-cm dishes with several ratios of the fluorescence-modified FRz and DOTAP mixture. Cells were washed with OPTI-MEM medium (GIBCO-BRL), incubated in 8 mL OPTI-MEM for 1 hour, and transfected for 6 hours with 7 μmol/L DOTAP alone or complexed to FRz (1 to 2 μmol/L). The transfection medium was then replaced with DME containing 0.4% FBS and 0.2% BSA, and cells were harvested 4, 16, and 24 hours after transfection, trypsinized, resuspended in 1 mL medium, and analyzed by flow cytometry. The fluorescence of individual cells was plotted against cell numbers to evaluate FRz uptake.

For the detection of 12-LO mRNA, protein, or other studies after Rz or MRz treatments, RVSMS were incubated with 5 μL OPTI-MEM for 1 hour and transfected with either 3.5 to 7.0 μmol/L DOTAP alone or complexed with either 0.5 to 1.0 μmol/L Rz or MRz in HEPEs buffer for 6 hours. The transfection protocol was repeated after 24 hours, and cells were harvested 24 hours later.

Detection of 12-LO mRNA by Reverse Transcriptase–Competitive PCR

Total RNA and cDNA were prepared as described.18 A 12-LO competitor DNA served as an internal standard for polymerase chain reaction (PCR) with use of the same primers as for 12-LO cDNA.18

For competitive PCR reactions, sample cDNAs corresponding to 5 ng sample RNA were coamplified with a constant amount of competitor DNA in the presence of [α-32P]dCTP and AmpliTaq Gold (Perkin Elmer) and rat 12-LO primers (sense primer at 324 to 339, antisense primer at 718 to 735), PCR conditions were as follows: 95°C for 10 minutes, 94°C for 30 seconds, 68°C for 1 minute, and 72°C for 30 seconds for 36 to 40 cycles and then 72°C for 7 minutes (in a Perkin Elmer PCR System 9600). The PCR products were separated by 5% polyacrylamide gel electrophoresis, and radioactive bands were quantified on a phosphorimagery by using ImageQuant software. The ratio of signal in the 12-LO cDNA band versus competitor band was used to compare the levels of 12-LO mRNA.

RVSMS migration was assayed by using a 48-well microchemotaxis modified Boyden chamber as described.18 Serum-starved RVSMS (transfected with oligonucleotides as described earlier) were trypsinized and suspended in DME containing 0.2% BSA and 0.4% FCS. The lower wells of the Boyden chamber were filled with medium alone, medium mixed with 5 μmol/L Rz, MRz, or SRz, 1

Figure 1. 12-LO Rz: sequence of rat leukocyte-type 12-LO chimeric DNA-RNA hammerhead Rz (left) and complementary sequence of rat 12-LO mRNA (right). Rz cleaves 3’ to GUC site of 12-LO mRNA (arrow). Ribonucleotides are boxed. Phosphorothioate linkages are marked by asterisks. MRz has just a G-to-A mutation as indicated.
nmol/L PDGF alone, or PDGF mixed with 5 μmol/L Rz, MRz, or SRz. The upper wells received cell suspension alone or cell suspension mixed with 5 μmol/L Rz, MRz, or SRz. The chamber was incubated 5 hours, and migrated cells were stained with Mayer’s hematoxylin and quantified densitometrically.

**Animals and In Vivo Experiments**
All animal studies were conducted in accordance with guidelines of the Research Animal Care Committee of the City of Hope Medical Center. The left common carotid artery of male Sprague-Dawley rats was subjected to balloon angioplasty with use of a 1.8F PTCA balloon catheter. Local delivery of Rz or control oligonucleotides was performed by use of the dwell technique immediately after injury. The neck portion (distal) of the common carotid artery was temporarily isolated with microvascular clips as described. An Rz was performed by use of the dwell technique immediately after (frozen) as described. An Rz complex was introduced into the distal portion of the injured artery and maintained for 30 minutes. The proximal injured artery did not receive Rz and serves as an internal control. At the end of the incubation, the Rz complex was suctioned off, and the surgical wound was closed. Each animal served as its own control, because the oligonucleotide-treated and untreated sections were from the same injured left artery; the right artery served as the uninjured control.

The animals were maintained for 12 days after initial surgery, and the injured-treated (neck portion of left common carotid), injured-untreated (chest portion of left carotid), and uninjured (right carotid) sections were harvested for histology (fixed) or biochemical analysis (frozen) as described.

**Data Analyses**
All RVSMC experiments are expressed as mean±SEM of multiple experiments. For the in vivo rat experiments, intima-to-media ratios of treated versus untreated sections were compared by paired analyses. Paired Student t tests were used to compare 2 groups; ANOVA with the Dunnett post test was used for multiple groups with the use of PRISM software (GraphPad). Statistical significance was detected at the 0.05 level.

**Results**

**In Vitro Cleavage Activity of 12-LO Rz**
12-LO Rz was designed to form a complex with its substrate at the corresponding cleavage site (Figure 1). The internally labeled, transcribed, 166-nt RNA served as an in vitro substrate for the Rz. The substrate amount was kept constant (9 nmol/L), whereas Rz was varied starting at 0.6 nmol/L. Substrate-to-Rz ratios were from 1:0.07 to 1:70. Cleavage resulted in 2 fragments of 93 and 73 bases, which was consistent with the predicted size (Figure 2). The fragments were detected even at 1:0.07 substrate-to-Rz ratios.

**5′-Fluorescein–Conjugated Rz Uptake by RVSMCs**
Catalytic liposomes are efficient tools for introducing DNA or RNA into cells without apparent toxicity. To determine the optimum conditions of Rz delivery to cells, we used cationic lipid DOTAP as a vehicle to deliver a fluorescently tagged FRz that allows detection of intracellular uptake by flow cytometry. Figure 3 shows a marked increase in relative fluorescence units from 5 in control cells to 200 and 400 in FRz-transfected cells. Fluorescence intensity was markedly increased even by 4 hours, further increased by 16 hours, and leveled off by 24 hours. Furthermore, 2 μmol/L FRz led to greater fluorescence than did 1 μmol/L. Thus, the Rz can be efficiently delivered into RVSMCs by this technique.

**Effect of 12-LO Rz on 12-LO mRNA and Protein Levels in RVSMCs**
We next evaluated the ex vivo efficacy of the 12-LO Rz in inhibiting rat 12-LO mRNA and protein expression. RVSMCs were treated with the Rz or MRz in DOTAP as described in Methods. Total RNA was subjected to reverse transcriptase–competitive PCR to detect 12-LO mRNA levels, and total protein was subjected to immunoblotting to detect 12-LO as described. Figure 4A shows a representative autoradiogram. The upper band is 12-LO cDNA (312 bp), and the lower band is the competitor (281 bp). The bar graph shows that the Rz (0.5 μmol/L) led to >85% inhibition of 12-LO mRNA levels. In contrast, MRz was ineffective under these conditions. The Western blot in Figure 4B shows that the Rz was also highly effective in reducing 12-LO protein levels. The Rz also correspondingly decreased 12-HETE levels (Rz 55±14% of control DOTAP alone, P<0.05; MRz 93±2% of control, P>0.05).

**Effect of 12-LO Rz on RVSMC Migration**
The potent VSMC chemotactic agent PDGF-BB is a strong inducer of 12-LO activity and expression in VSMCs. To determine the functional significance, we tested whether
12-LO Rz could reduce PDGF-induced migration of RVSMCs. Figure 5 shows that PDGF led to a 3-fold increase in RVSMC migration. Rz, MRz, and SRz did not significantly alter basal migration. However, 12-LO Rz significantly attenuated the effects of PDGF. In contrast, MRz or SRz was ineffective. Furthermore, 10 nmol/L of 12-HETE, but not 15-HETE, could restore the effects of PDGF (PDGF 3.3±0.12-fold over control; PDGF 1 Rz 2.1±0.1-fold over control; PDGF+Rz 3.1±0.08-fold over control, P<0.01 versus PDGF+Rz; and PDGF+Rz+15-HETE 2.4±0.11-fold over control). Thus, PDGF-induced migration is mediated, at least in part, by 12-LO activation.

Effect of 12-LO Rz on RVSMC Fibronectin

Because evidence suggests that 12-LO products, such as 12-HETE and high glucose, can increase cell-associated levels of fibronectin, the key matrix protein, in VSMCs, we examined fibronectin levels in RVSMCs that had been pretreated with rat 12-LO Rz. RVSMCs were transfected for 4 hours with 0.5 mmol/L each Rz or MRz. The medium was then replaced with high-glucose DME containing 0.2% BSA and 0.4% FCS, and the cells were incubated for 18 hours. The cells were lysed and saved for assay of cellular fibronectin by a specific ELISA as described. Figure 6 shows that the 12-LO Rz led to a partial, but significant, attenuation of cellular fibronectin. Thus, 12-LO activation may mediate, at least in part, changes in fibronectin under high-glucose conditions.

In Vivo Efficacy of Rat 12-LO Rz

We recently showed that 12-LO expression was increased in the neointima in a rat carotid artery model of restenosis. To determine whether 12-LO activation may play a role in neointimal thickening, we tested the effects of 12-LO Rz in this rat model. As important controls, we also compared the effects of the MRz and SRz.

Figure 7A shows that treatment of injured arteries with 10 µg Rz markedly reduced the extent of neointimal thickening in the Rz-treated section (bottom of panel A) relative to the untreated section (middle of panel A). The uninjured right carotid is seen at the top of panel A. Immunostaining with a leukocyte-type 12-LO antibody as described shows that this effect is accompanied by markedly reduced 12-LO expression (Figure 7B). In contrast, MRz (10 µg) (Figure 8) or SRZ (10 µg) (Figure 9) did not alter the extent of neointimal thickening. Hence, the effects of the Rz were specific. Cumulative data from several rats (n=10 for Rz, n=8 for MRz, and n=5 for SRz) are shown in Figure 10. 12-LO Rz had significant inhibitory effects on intima-to-media ratios (1.749±0.12 for untreated versus 0.742±0.16 for treated,

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**Figure 4.** 12-LO Rz reduces 12-LO mRNA and protein levels in RVSMCs. A, Representative autoradiogram of competitive reverse transcriptase-PCR products from RVSMCs treated with DOTAP alone, DOTAP with Rz, and DOTAP with MRz (0.5 µmol/L each). Upper band is 12-LO, and lower band is competitor. Cont indicates control. Bar graph below with phosphorimager quantification from 4 experiments reveals significant reduction in 12-LO mRNA levels by Rz (P<0.0001), but not by MRz. B, Immunoblot of proteins from experiment similar to that in panel A (except that oligonucleotides were used at 1 µmol/L each) probed with leukocyte-type 12-LO antibody (1:400). Results shown are representative of 2 experiments with similar results.

**Figure 5.** Effect of 12-LO Rz on RVSMC migration. Lower chamber of a Boyden chamber was filled with either medium alone or medium mixed with Rz, MRz, or SRz (5 µmol/L), recombinant human PDGF-BB at 1 nmol/L, or PDGF mixed with Rz, MRz, or SRz (5 µmol/L). Upper wells received 52 µL cell suspension alone (~50 000 cells) or suspension mixed with 5 µmol/L Rz, MRz, or SRz. Chamber was incubated for 5 hours, and migrated cells were quantified. Results are expressed as mean±SEM from 3 experiments run in triplicate. *P<0.001 vs control; **P<0.01 vs PDGF.

**Figure 6.** Effect of 12-LO Rz on RVSMC fibronectin levels. Fibronectin was quantified by ELISA. Results are expressed as mean±SEM of 3 experiments. *P<0.03 vs control.
Discussion

In the present study, we have shown that a specific Rz was effective in cleaving 12-LO mRNA in vitro and decreasing 12-LO mRNA and protein expression in RVSMCs. To enhance Rz stability within cells, we used a chimeric DNA-RNA Rz with phosphorothioate modifications at the 5' and 3' ends. This is consistent with other studies. We also showed for the first time that this Rz was effective in vivo, inasmuch as it significantly reduced neointimal thickening in a rat model. In contrast, the controls, MRz and SRz, were ineffective. Our recent study demonstrated significant up-regulation of 12-LO expression in the neointima of balloon-injured carotid arteries, particularly in inflammatory cells (such as leukocytes) and neointimal VSMCs. The present results suggest that the 12-LO pathway may play a role in the development of restenosis.

LO products can play a role in VSMC growth and migration, including that induced by angiotensin II and PDGF. In the present study, we observed that treatment of RVSMCs with 12-LO Rz could partially inhibit fibronectin production and PDGF-induced migration. These results suggest that 12-LO Rz-mediated reduction in neointimal thickening is mediated, at least in part, through the inhibition of VSMC migration and matrix deposition. However, other mechanisms may also be operative. Because increased 12-LO expression was evident in neointimal VSMCs as well as inflammatory cells, the Rz effects may be a consequence of blocking 12-LO mRNA in monocytes/macrophages, neointimal VSMCs, and endothelial cells. This is supported by recent observations that 12-LO products such as 12-HETE can directly induce the adhesion of monocytes to endothelial cells.

Figure 7. Effect of the 12-LO Rz on neointimal thickening in balloon-injured rat carotid arteries. Rz (10 μg) mixed with lipofectin was instilled into injured arteries for 30 minutes immediately after injury. Twelve days after injury, carotid arteries were fixed and processed as described in Methods. A, 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) arteries from 1 rat. B, Immunostaining with 12-LO antibody.
There has been increasing interest in the development of exogenous Rz-based therapies. However, very few studies have evaluated Rz-based approaches to prevent restenosis. A recent study showed that a chimeric Rz directed to PCNA was highly effective in reducing neointima formation induced by stents in a porcine model.29 Another study demonstrated the effectiveness of an adenovirus encoding an Rz to c-myb mRNA both in vitro (in reducing VSMC proliferation) and in vivo (in attenuating neointimal thickening).30 Novel therapies to prevent restenosis are important, particularly for diabetic patients, who have significantly greater rates of restenosis. The 12-LO pathway is upregulated in VSMCs and endothelial cells under high-glucose conditions.9,28 Hence, a 12-LO Rz could serve as a novel therapeutic tool for restenosis, especially for diabetic patients.

The present study does not fully address the mechanism by which the LO pathway mediates restenosis. LO products can induce VSMC growth,7,16 monocyte adhesion to endothelial cells,28 and possibly endothelial cell apoptosis by various mechanisms. These lipids can mediate the expression of oncogenes, activate key growth and stress-related mitogen-activated protein kinases, induce oxidant stress, and activate transcription factors.6,8 Thus, LO products could activate signaling cascades, culminating in the expression of genes mediating key events in the pathogenesis of atherosclerosis and restenosis.

12-LO Rz could block neointimal thickening by 70% to 80%. However, it blocked RVSMC fibronectin and PDGF-induced migration by only 30% to 50%. This is not surprising because, as discussed earlier, the in vivo Rz effects may represent its net action on RVSMCs, leukocytes, and endothelial cells. Similarly an Rz to c-myb was more effective in vivo than in cell culture.31 This was attributed to the cleavage activity not being rate-limiting in vivo. However, our data also suggest that PDGF has 12-LO–independent effects also.

The direct in vivo significance of 12-LO activation in atherosclerosis has recently been demonstrated by studies showing that crossbreeding of a leukocyte-type 12-LO
knockout mouse with the atherosclerosis-susceptible apolipoprotein E–deficient mouse dramatically decreases lesion development in the latter.31 Our studies support the feasibility of Rz-based technology and therapies to evaluate specific gene pathways involved in vascular disease.

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Figure 10. Histomorphometric analyses of in vivo effects of 12-LO Rz, MRz, and SRz (10 μg each) on neointimal thickening. Results shown are mean±SEM of intima-to-media ratios obtained from 10 rats with Rz, 8 rats with MRz, and 5 rats with SRz. *P<0.001 vs injured by paired analysis.
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