Vascular Remodeling Induced by Naturally Occurring Unsaturated Lysophosphatidic Acid In Vivo

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Background—We previously identified unsaturated (16:1, 18:1, and 18:2) but not saturated (12:0, 14:0, 16:0, and 18:0) lysophosphatidic acids (LPAs) as potent factors for vascular smooth muscle cell (VSMC) dedifferentiation. Unsaturated LPAs strongly induce VSMC dedifferentiation via the coordinated activation of the extracellular signal–regulated kinase (ERK) and p38 mitogen–activated protein kinase (p38MAPK), resulting in the proliferation and migration of dedifferentiated VSMCs. Here, we investigated the effects of 18:1 and 18:0 LPAs (as representative unsaturated and saturated LPAs, respectively) on the vasculature in vivo.

Methods and Results—Rat common carotid arteries (CCAs) were treated transiently with 18:1 or 18:0 LPA and then examined by histological and biochemical analyses. The 18:1 but not 18:0 LPA potently induced vascular remodeling that was composed primarily of neointima. The incorporation of [3 H]18:1 LPA into the CCAs revealed that a sufficient amount of unmetabolized [3 H]18:1 LPA to induce VSMC dedifferentiation was present in the vascular wall. The 18:1 LPA–induced neointimal formation in vivo was also dependent on the coordinated activation of ERK and p38MAPK. Unlike balloon-injured CCAs, the 18:1 LPA–treated CCAs showed a histological similarity to human atherosclerotic arteries.

Conclusions—This is the first report demonstrating a role for a naturally occurring unsaturated LPA in inducing vascular remodeling in vivo and provides a novel animal model for neointimal formation. (Circulation. 2003;108:1746-1752.)

Key Words: kinases ■ cells ■ muscle, smooth ■ phenotype ■ atherosclerosis

A n early change in atherosclerosis is characterized by neointimal formation resulting from the proliferation and migration of dedifferentiated vascular smooth muscle cells (VSMCs). The inflammatory response and oxidization stress in the vasculature are proposed to be important for the development and progression of atherosclerosis. Recent studies have shown that growth factors, cytokines, and oxidative products of LDL are candidate molecules for atherogenic factors.1,2 The identities of the critical factors for atherogenesis, however, remained unclear, because the cultured VSMCs used in these studies were passaged cells that display a dedifferentiated phenotype. To study the molecular mechanism underlying the phenotypic modulation of VSMCs from the differentiated phenotype to the dedifferentiated one, we established a culture system of VSMCs and visceral SMCs that maintains the differentiated phenotype as defined by cell morphology, ligand-induced contractility, and the expression of SMC molecular markers.3–5 Using this culture system, we previously demonstrated that the insulin-like growth factor–I–triggered phosphatidylinositol 3-kinase (PI3K)/protein kinase B [PKB(Akt)] pathway is critical for maintaining the differentiated VSMC phenotype, whereas the coordinated activation of the extracellular signal–regulated kinase (ERK) and p38 mitogen–activated protein kinase (p38MAPK) pathways triggered by other growth factors induces VSMC dedifferentiation. Thus, we proposed that changes in the balance between the strengths of the PI3K/PKB(Akt) pathway and the ERK and p38MAPK pathways determine the VSMC phenotype.5–3 Using this differentiated VSMC culture system, we identified unsaturated but not saturated lysophosphatidic acids (LPAs) as potent and major VSMC dedifferentiation factors in human serum.5 We also found that unsaturated (16:1, 18:1, and 18:2) LPA–induced VSMC dedifferentiation is mediated through the activation of both the MAPK pathways.5 LPAs are bioactive phospholipids affecting various cell types.6 However, there have been no reports regarding the effect of LPAs on vascular remodeling in vivo except for their effect on blood pressure.7

Here, we compared the effects of 18:1 and 18:0 LPAs, as representative unsaturated and saturated LPAs,5,8 respectively, on the vasculature in vivo. Treatment of common carotid arteries (CCAs) with 18:1 but not 18:0 LPA potently
induced neointimal formation. A sufficient amount of 18:1 LPA to induce VSMC dedifferentiation was incorporated into the vascular wall. Like the VSMCs in culture, the coordinated activation of the ERK and p38MAPK pathways in the CCAs was critical for the 18:1 LPA–induced vascular remodeling. Histological analyses revealed a strong similarity between the 18:1 LPA–treated CCAs and human atherosclerotic arteries. This is the first report demonstrating the role of a naturally occurring unsaturated LPA in inducing vascular remodeling and provides a novel animal model for neointimal formation.

**Methods**

**Materials**

Monoclonal antibodies against α-smooth muscle actin (α-SMA), calponin, and 18:1 LPA were purchased from Sigma. Polyclonal antibodies against ERK, p38MAPK, monocyte chemoattractant protein-1 (MCP-1), p-65 nuclear factor-κB (NF-κB), and IκB-α were purchased from Santa Cruz Biotechnology. Monoclonal antibodies against CD68 and caldesmon (CaD) were purchased from Roche Diagnostics and Ylem, respectively. Polyclonal antibody against von Willebrand factor (vWF) was purchased from Dako. PD98059 and SB203580 were purchased from Calbiochem. 18:0 LPA was prepared as described previously.

**Histological Examination**

Animal experiments were conducted according to the ethical guidelines of Osaka University Medical School. Male Sprague-Dawley rats (7 weeks old; Japan Animal Company, Osaka, Japan) were anesthetized with sodium pentobarbital (50 mg/kg). The left internal carotid artery (CA) and the CCA proximal to the neck were transiently clipped, and a PE10 tube (Becton Dickinson) was carefully inserted into the left external CA without mechanical injury to the endothelial cells of the CCA. The CCA was washed 5 times with 100 mL of the base medium (0.2% BSA and 0.1% DMSO in PBS), and then the CCA was filled with 100 μL of the base medium containing the indicated reagents. After 30 minutes, the left external CA was ligated, and the CCA was reperfused with circulating blood. Balloon injury of the CCA was performed as described elsewhere. To retrieve the CCAs, rats were deeply anesthetized and perfused transcardially with 4% paraformaldehyde in 0.1 mol/L PBS. The CCAs were excised and embedded in paraffin, and the sections were stained with hematoxylin and eosin (HE). Human atherosclerotic arteries were obtained by surgery according to the guidelines of the Ethical Committee of Iwate Medical University, fixed in 4% paraformaldehyde, and analyzed as described above. For fresh-
frozen sections, the CCAs were embedded in OCT compound (Sakura Finetek) and frozen in liquid nitrogen.

Sections 6 μm thick were prepared from the paraffin-embedded or fresh-frozen CCAs and human arteries and incubated with the indicated antibodies. The LSAB2 avidin-biotin complex and DAB (Dako) or secondary antibodies conjugated with Alexa 488 or 546 (Molecular Probes) were used to detect the marker proteins. The nuclei were stained with Hoechst 33258. Fluorescence images were collected by use of a microscope with appropriate filters and MetaMorph software. In situ hybridization was performed with a rat high molecular weight CAD (h-CAD)—specific probe amplified by reverse transcriptase–polymerase chain reaction of rat aortic mRNAs with primers 5′-GTAGAGGAGATGGTAGAAGA-3′ and 5′-CTCTTCCCTTCTGA-AGCCGG-3′ (AB049626). For fluorescence in situ hybridization, a digoxigenin-labeled antisense RNA probe was hybridized to the CCA sections at 45°C. The signals were amplified with the TSA-AP system (New England Nuclear) and detected with Fast Red TR (Roche Diagnostics).

The incorporation and localization of LPA in the CCAs were determined by use of [3H]18:1 LPA (New England Nuclear). The CCAs treated with the indicated amounts of [3H]18:1 LPA (1.8 TBq/mmol) as described above were retrieved at 10 minutes and 2 and 7 days after reperfusion. After washing with PBS, a piece of each CCA was lysed in NCS-II (Amersham Bioscience), and the radioactivity was counted. The lipid fraction from the [3H]18:1 LPA- treated CCAs was separated by 2D thin-layer chromatography (TLC),5 and autoradiography was performed using Kodak BioMax MS film with EN 3 HANCE Spray (Perkin Elmer) at 80°C to detect the radioactive signals retained in the CCAs. Unmetabolized [3H]18:1 LPA was identified by authentic 18:1 LPA. The rest of the [3H]18:1 LPA–treated CCAs were frozen, and frozen sections 10 μm thick were coated with Hypercoat EM-1 photographic emulsion (Amersham Biosciences).

Statistical Analysis

The statistical significance of differences in the neointimal areas of CCAs was determined by the Kruskal-Wallis test and the Mann-Whitney U test. A value of P<0.05 was considered significant.

Results

Unsaturated LPA–Induced Neointimal Formation

We compared the effects of 18:1 and 18:0 LPA on the vasculature in vivo. The 18:1 LPA (1 μmol/L)–treated CCAs (n=6) on day 7 showed typical neointimal formation, and their neointimal areas were expanded further on day 21. In contrast, vehicle (n=6) or 18:0 LPA (1 μmol/L) (n=6) had no effect on the CCAs on days 7 and 21 (Figure 1, A and B). The neointima was detectable when the CCAs were treated with 0.3 μmol/L 18:1 LPA (n=6) (data not shown) but was much more intensive when 1 μmol/L 18:1 LPA was used. Quantification of the neointimal areas and neointimal/medial area ratios of the CCAs treated with 18:1 LPA (n=32), 18:0 LPA (n=13), and vehicle (n=12) on day 21 indicated that 18:1 LPA specifically induced neointimal formation (Figure 1C).

To determine the [3H]18:1 LPA incorporation into the CCAs, we measured the radioactivity in the CCAs. The radioactivity 10 minutes after treatment was 8.17±0.86×10^5 cpm/mg tissue (n=3) and was reduced thereafter (1.80±0.89×10^5 cpm/mg tissue on day 2 [n=3], 0.57±0.10×10^5 cpm/mg tissue on day 7 [n=3]). Autoradiography of the [3H]18:1 LPA–treated CCAs 10 minutes after treatment revealed that the radiolabeling was localized primarily to the subendothelial and inner medial VSMC layers (n=3) (Figure 2A). 2D TLC showed that a large proportion of the [3H]18:1 LPA incorporated into the CCAs was metabolized but that a significant amount (<10%) of the total radioactivity) of unmetabolized [3H]18:1 LPA was also present; the content of unmetabolized [3H]18:1 LPA in the CCAs was estimated to be 33.7±6.1 nmol/kg tissue (n=3) (Figure 2B).

Involvement of ERK and p38MAPK in 18:1 LPA–Induced Neointimal Formation

To investigate the signaling pathways involved in 18:1 LPA–induced neointimal formation, we assayed the MAPK activities in the 18:1 LPA– or 18:0 LPA–treated CCAs (each time point, n=3). The 18:1 but not 18:0 LPA markedly activated both ERK and p38MAPK in the CCAs 30 minutes after treatment, and these activations were maintained for >3 hours (Figure 3A). PKB(Akt) was activated only by 18:1 LPA (data not shown). Pretreatment with either PD98059 or SB203580 (n=3) inhibited the 18:1 LPA–induced ERK or p38MAPK activation, respectively. Simultaneous pretreat-
ment with PD98059 and SB203580 (n=3) blocked the 18:1 LPA–induced ERK and p38MAPK activation (Figure 3B). Consistent with the 18:1 LPA–induced MAPK activation in vivo, pretreatment with either PD98059 or SB203580 significantly but only partially suppressed 18:1 LPA–induced neointimal formation (n=4), and simultaneous pretreatment with both inhibitors completely suppressed it (n=4) (Figure 3C). Taken together, these data indicate that 18:1 LPA–induced neointimal formation in vivo depends on the coordinated activation of the ERK and p38MAPK pathways.

Different Effects of 18:1 and 18:0 LPAs on Vascular Cells

There are 2 alternative scenarios for neointimal formation induced by 18:1 LPA: the neointima is induced indirectly by factors released from 18:1 LPA–primed endothelial cells or by the direct action of 18:1 LPA on medial VSMCs. To address both scenarios, we analyzed the LPA-induced responses of endothelial cells and VSMCs in culture and in vivo as monitored by the NF-κB signaling. The degradation of IκB-α in endothelial cells was induced by both 18:1 and 18:0 LPAs within 30 minutes, but that in differentiated VSMCs was induced only by 18:1 LPA (n=3) (Figure 4A). Consistent with these culture experiments, 18:1 LPA induced the nuclear localization of NF-κB in both endothelial cells and medial VSMCs of the CCAs for 30 minutes (Figure 4B) and at least 3 hours (data not shown), whereas 18:0 LPA did so only in endothelial cells but not in medial VSMCs (Figure 4B). Thus, endothelial cells and VSMCs in culture and in vivo exhibited different responses to unsaturated and saturated LPAs.

Characterization of LPA-Induced Neointimal Formation

We performed the histological characterization of 18:1 LPA–treated CCAs (each time point, n=6) (Figure 5). As revealed by vWF staining, there were no histological changes or denudation of endothelial cells in the CCAs treated with vehicle or 18:1 LPA on day 2 (data not shown), suggesting that the LPA treatment did not damage the endothelial layer.
Immunohistochemical staining showed no changes in the expression of the α-SMA, calponin, and CaD on day 2. However, in situ hybridization revealed the marked reduction of h-CaD transcripts in the inner medial VSMC layer on day 2, indicating the phenotypic modulation of VSMCs. These results are in line with our previous studies, in which unsaturated LPAs induced the rapid and irreversible dedifferentiation of VSMCs in culture. On days 7 and 21, the expression of α-SMA, calponin, and CaD proteins in most neointimal cells was reduced, and this reduction was more prominent in the inner medial VSMC layer than in the outer medial VSMC layer, suggesting that neointimal cells were derived largely from medial VSMCs. The expression of MCP-1 in endothelial cells of 18:1 LPA–treated CCAs was weak on day 2 but was marked in the neointima on days 7 and 21 (data not shown). Consistent with this, deposits of macrophages that stained positive with the anti-CD68 antibody were less significant on day 2, whereas they were detected in the neointima on day 7 and were prominent on day 21.

**Histological Similarity Between 18:1 LPA–Induced CCAs and Human Atherosclerotic Arteries**

The balloon-injured model has been used to analyze vascular remodeling. We compared histological features between 18:1 LPA–treated CCAs (21 days after treatment, n=32), balloon-injured CCAs (21 days after injury, n=6), and human atherosclerotic arteries (n=18). In the neointima of human atherosclerotic arteries (Figure 6, B and F) and of 18:1 LPA–treated CCAs (Figure 6, C and G), the expression of calponin was dramatically downregulated to undetectable levels. In contrast, the calponin expression in the neointima of balloon-injured arteries was at the same level as in the medial VSMC layer (Figure 6, D and H). The expression of vWF in the neointima of human atherosclerotic arteries (Figure 6, B and F) and of 18:1 LPA–treated CCAs (Figure 6, C and G) was restricted to the endothelium, whereas that of the balloon-injured arteries was detected in the neointima in addition to the endothelium (Figure 6, D and H). Thus, histological features of 18:1 LPA–treated CCAs showed a similarity to those of human atherosclerotic arteries, whereas those of balloon-injured arteries did not.

**Discussion**

This is the first report demonstrating that 18:1 but not 18:0 LPA is a potent factor for inducing vascular remodeling, composed primarily of neointima (Figure 1). The 18:1 LPA–induced vascular remodeling was mediated through the coordinated activation of ERK and p38MAPK, given that 18:1 LPA activated both MAPKs and simultaneous pretreatment with PD98059 and SB203580 completely suppressed 18:1 LPA–induced neointimal formation (Figure 3). Consistent with this, these MAPK pathways are critical for 18:1 LPA–induced VSMC dedifferentiation in culture, indicating that the same signaling pathways regulate the VSMC phenotype.
in vivo and in culture. In addition, there was a histological similarity between the 18:1 LPA-treated CCAs and human atherosclerotic arteries (Figure 6).

We demonstrated previously that the apparent IC_{50} values of unsaturated LPAs to induce VSMC dedifferentiation in culture ranged from 20 to 30 nmol/L.\(^5\) As shown in Figure 2, the amount of unmetabolized \[^{3}H\]18:1 LPA retained in the whole CCAs was 33.7 ± 6.1 nmol/kg tissue, and the radioactive signals were restricted to the subendothelial and inner medial VSMC layers in the CCA. The concentration of unmetabolized \[^{3}H\]18:1 LPA in these layers was therefore calculated to be 200 to 300 nmol/L, suggesting that a sufficient amount of unmetabolized 18:1 LPA to induce VSMC dedifferentiation was present in the subendothelial and inner medial VSMC layers. It has been reported that the 18:1 LPA–induced endothelial activation involves a morphological change in the cell from a cobblestone-like shape to a round one, resulting in a sparse cell-to-cell contact.\(^{12,13}\) Such 18:1 LPA–induced endothelial cell activation might enable the incorporation of 18:1 LPA into the vascular wall in vivo. 18:1 LPA also activates the expression of proinflammatory genes in cultured endothelial cells.\(^{14}\) Our present study demonstrated that by monitoring the NF-κB signaling, 18:1 LPA activated only endothelial cells (Figure 4). These results suggest that vascular remodeling induced by 18:1 LPA treatment might be primarily a result of the direct effect of 18:1 LPA on medial VSMCs in the CCAs, rather than the endothelial cell–mediated indirect effect. Furthermore, the phenotypic modulation of medial VSMCs in the CCAs was detected within 2 days after 18:1 LPA treatment, whereas the MCP-1 expression followed by macrophage infiltration into the vascular wall was observed on days 7 and 21 after treatment (Figure 5). Thus, these results indicated that the phenotypic modulation of medial VSMCs precedes macrophage infiltration during vascular remodeling.

It has been reported that LPAs are generated by at least 3 different routes: degradation of phosphatidic acid by phospholipase A\(_2\) and/or A\(_1\) released from activated platelets, hydrolysis of lysophospholipids by lysophospholipase D, and mild oxidation of LDL.\(^{15}\) It remains unclear which of these is the major route for generating unsaturated LPAs that act as atherogenic factors. It has been reported that the amount of unsaturated LPAs released from activated platelets is very low.\(^{8}\) In the case of atherogenesis, however, platelet-derived unsaturated LPAs could play a role in atherogenesis, because the concentration of LPAs released in the area close to activated platelets is thought to be very high. The second and third possible sources of LPAs are LDL oxidation and atherosclerotic lesions. Siess et al\(^{13}\) demonstrated that mild oxidation of LDL forms LPAs and that human atherosclerotic lesions contain high levels of LPAs. Three types of LPA receptor (LPA1/Edg2, LPA2/Edg4, and LPA3/Edg7) have been identified.\(^{15}\) We previously demonstrated, using reverse transcriptase–polymerase chain reaction, that LPA1 and LPA3 but not LPA2 receptor messages are expressed in VSMCs.\(^{5}\) These 3 LPA receptors, however, show affinities for saturated and unsaturated LPAs to different degrees.\(^{16,17}\) Taken together, our previous\(^{5}\) and present studies suggest the possibility that a novel unsaturated LPA-specific receptor rather than any of these identified LPA receptors may be involved in the unsaturated LPA–induced vascular remodeling.
formed atherosclerotic lesions are likely to be involved in the development and progression of atherosclerosis. Our vascular remodeling system should provide a useful animal model for neointimal formation.

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References

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