Lysophosphatidic Acid and Platelet-Derived Growth Factor Synergistically Stimulate Growth of Cultured Rat Mesangial Cells (44184)

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Abstract. **Lysophosphatidic acid (LPA) is a structurally simple, platelet-derived phospholipid, capable of eliciting a variety of physiological responses. We have demonstrated previously that LPA elicited a marked contractile response in rat mesangial cells (Inoue CN, Forster HG, Epstein M. Circ Res 77:888–896, 1995). In the present study, we examined the potential of this vasoactive substance to induce mesangial cell proliferation. Serum-starved quiescent rat mesangial cells were incubated with either LPA or in combination with platelet-derived growth factor (PDGF). DNA synthesis was assessed by [^{3}H]thymidine incorporation after 24 hr, and cell numbers were determined at 0, 4, and 7 days. LPA- (1 n*M*–30 μ*M*) stimulated mesangial cell DNA synthesis in a dose-dependent manner. The DNA synthesis stimulated by PDGF (1–100 ng/ml) was characterized by a bell-shaped response curve with a maximum at 40 ng/ml PDGF. The ability of LPA (30 μ*M*) to synergize PDGF was observed over the entire range of PDGF concentrations (1–100 ng/ml). Under optimal concentrations of LPA/PDGF (30 μ*M*40 ng/ml, respectively), mesangial cells displayed a 67-fold increase in [^{3}H]thymidine incorporation, and a 1.9-fold (Day 4) and 2.5-fold (Day 7) increase in cell number as compared with that of quiescent mesangial cells. With an *in vitro* assay with myelin basic protein as the substrate, both LPA and PDGF induced stimulation of mitogen-activated protein (MAP) kinase activity. In addition, LPA augmented PDGF-induced increase in MAP kinase activity. In summary, these results demonstrate that LPA is mitogenic alone and also acts synergistically in combination with PDGF to promote mesangial cell proliferation. We postulate that these actions of LPA have the potential to play a crucial role in the mitogenic response of mesangial cells seen in a wide array of inflammatory and thrombotic glomerular disorders.**

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Proliferation of glomerular mesangial cells is a characteristic feature of several types of experimental and human glomerulonephritis, including IgA nephropathy and lupus nephritis (1). The mechanisms responsible for stimulating the proliferation of mesangial cells in glomerulonephritis, however, have not been fully delineated. Recent reports have demonstrated that the platelet may play an important role in mediating mesangial cell growth (2, 3). Many inflammatory substances that are released from platelets in response to glomerular injury, such as growth factors, vasoactive amines, thromboxane, and proteases, have been postulated to participate in mesangial cell proliferation (4). Among these, platelet-derived growth factor (PDGF) is thought to constitute one of the most potent mitogens for glomerular mesangial cells (5). In addition, increasing evidence has demonstrated that PDGF is produced by other cell types, including endothelial cells and activated macrophages/monocytes, and by mesangial cells themselves (6, 7). Thus, in the case of glomerular injury PDGF released

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from coagulating platelets as well as that produced by endothelial, mesangial, and inflammatory cells could conceivably act in an autocrine, paracrine manner to stimulate mesangial cell proliferation (4).

Recently, lysophosphatidic acid (LPA), the structurally simplest and naturally occurring phospholipid, has attracted investigative interest as a platelet metabolite capable of stimulating significant growth activity in cultured fibroblasts, epithelial cells, and smooth muscle cells (8, 9). As a normal constituent of serum in an albumin-bound form (10), LPA has also been reported to be produced and released in large quantities by activated platelets during blood clotting (11, 12).

Recent studies from our laboratory have demonstrated that LPA evokes a marked contraction of cultured rat mesangial cells that involves activation of the phosphoinositide cascade and mobilization of Ca^{2+} from intracellular Ca^{2+} stores (13). Because several reports have demonstrated that vasoactive substances that elicit mesangial cell contraction such as arginine vasopressin (AVP) and endothelin 1 (ET-1) can also act as mitogens for mesangial cell growth (14), we undertook the present studies to determine whether LPA could also exhibit mitogenic properties in our model of cultured rat mesangial cells.

In the present study, we present evidence that LPA stimulates DNA synthesis *in vitro*. Furthermore, LPA acts with PDGF to increase synergistically DNA synthesis and cell number in cultured rat mesangial cells. The co-mitogenic action of these two platelet-derived substances was further analyzed by measuring mitogen-activated protein (MAP) kinase activity *in vitro*. Our data may provide a theoretical framework for postulating a role for LPA and platelets for the initiation and exacerbation of glomerular inflammatory and thrombotic diseases.

Materials and Methods

Materials. L-α-Lysophosphatidic acid(1-oleoyl) (LPA), recombinant platelet-derived growth factor ($PDGF_{BB}$ homodimer), serotonin (5-hydroxytryptamine hydrochloride), platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), angiotensin II (Ang II), arginine vasopressin (AVP), pepstatin A, and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co. (St. Louis, MO). ET-1 was purchased from Peninsula (Belmont, CA). Pertussis toxin (PTX) was obtained from Calbiochem (La Jolla, CA). RPMI-1640, fetal calf serum (FCS), trypsin, and free fatty acid–poor fraction V bovine serum albumin (BSA) were obtained from GIBCO (Grand Island, NY). Aprotinin and leupeptin were obtained from Boehringer-Mannheim (Indianapolis, IN). Anti-rat MAP kinase R2 (*erk* 1-CT) antibody-coupled Protein A agarose and MAP kinase assay kit were from UPstate Biotechnology Incorporated (Lake Placid, NY). [Methyl-^{3}H]thymidine (67 Ci/mmol) was obtained from Amersham Corporation (Arlington Heights, IL). [γ-^{32}P]ATP (6000 Ci/mmol) was from Dupont, NEN Research Products (Boston, MA).

Culture of Rat Mesangial Cells. Isolated glomeruli were prepared from the kidney cortices of male Sprague-Dawley rats weighing 75–100 g by consecutive sieving with three different stainless steel meshes (106, 150, and 75 μm), as reported previously by Kreisberg *et al.* (15). The glomeruli were then digested with collagenase and seeded onto plastic culture flasks. Cells were maintained in culture in RPMI-1640 medium supplemented with 20% FCS, ITS premix, penicillin (50 U/ml), and streptomycin (50 μg/ml), at 37°C in 5% CO_2 atmosphere. Mesangial cell growth predominated by Day 14 of the primary culture and the first subculture was performed on Day 21. Cells were utilized in this study in the 3rd to the 10th passage because they satisfied the previously reported criteria for mesangial cells (15).

[^{3}H]Thymidine Incorporation in Mesangial Cells. DNA synthesis was measured as the amount of [^{3}H]thymidine incorporated into trichloroacetic acid (TCA)–precipitable material. Mesangial cells were plated in 24-well culture dishes and incubated with RPMI-1640 supplemented with 20% FCS until they became subconfluent. Cells were then brought to quiescence by incubation for 2 days in serum-free RPMI-1640 containing 0.1% BSA. The quiescent cells were exposed to the growth factors for 24 hr, and [^{3}H]thymidine (1 μCi/ml) was added 6 hr before harvest. At the end of the 6-hr pulsing period, medium was removed by aspiration, followed by duplicate washes with phosphate-buffered saline (PBS). Ice-cold 10% TCA was then added to the wells and the dishes were kept on ice for 1 hr. Cells were then washed twice with 5% TCA, and TCA-precipitated material was then solubilized with 0.1 *N* NaOH. Radioactivity was determined using liquid scintillation counting.

Determination of Mesangial Cell Proliferation. To determine the mesangial cell replication, we subcultured mesangial cells from the 8th to the 10th passage in 24-well culture dishes and incubated with RPMI-1640 supplemented with 20% FCS until they reached approximately 40% confluence. Because preliminary studies from this laboratory demonstrated that the exposure of the mesangial cells to serum-free media for more than 4 days resulted in loss of cell viability and detachment of the cells from culture dishes, we modified culture conditions for the cell growth studies. After cells were made quiescent by incubation for 2 days in serum-free RPMI-1640 containing 0.1% BSA, the stimulation of mesangial cell proliferation by growth factors was carried out in RPMI-1640/0.1% BSA medium containing 0.5% FCS. By adding FCS, we could maintain cell viability and attachment to culture dishes for 7 days. Cell counts were performed on triplicate wells at Day 0, 4, and 7. Mesangial cells were washed with PBS twice and then incubated in 0.05% trypsin with 0.53 m*M* EDTA for 15 min at 37°C. Detached mesangial cells were then resuspended in a RPMI medium containing 0.2% trypan

blue and the number of viable cells were determined in a counting chamber.

Measurement of MAP Kinase Activity. Quiescent mesangial cells in 60-mm diameter dishes were stimulated with LPA (30 μ*M*), PDGF (40 ng/ml), or combination of LPA (30 μ*M*) and PDGF (40 ng/ml) in RPMI containing 0.1% BSA for 5, 10, 25, and 50 min. To stop the reaction, the cells were washed twice with ice-cold buffer containing 50 m*M* Tris/HCl (pH 7.5) and 150 m*M* NaCl. Cells were then scraped into 350 μl of ice-cold lysis buffer (50 m*M* Tris/HCl, pH 7.5, 150 m*M* NaCl, 1 m*M* EGTA, 1% NP-40, 0.25% sodium deoxycholate, 1 m*M* sodium orthovanadate, 1 m*M* NaF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 m*M* PMSF). Cells were homogenized by 10 passes through a 26-gauge needle fitted to a 1-ml syringe, and the homogenate was centrifuged for 10 min at 14000*g*. Lysate containing 120 μg of protein was diluted to the concentration of 1 μg/ml with PBS, and was immunoprecipitated using anti-MAP kinase antibody coupled to Protein A agarose at 4°C for 12 hr with agitation. Immune complexes were then washed four times with PBS and once with MAP kinase assay buffer (20 m*M* MOPS, pH 7.2, 25 m*M* β-glycerol phosphate, 5 m*M* EGTA, 1 m*M* sodium orthovanadate, 1 m*M* dithiothreitol). MAP kinase activity was measured by *in vitro* kinase assay using myelin basic protein (MBP) as a substrate. The assay was performed according to the manufacturer's instruction at 30°C for 15 min in a final volume of 40 μl containing 18.75 m*M* $MgCl_2$, 125 μ*M* ATP, 5 μ*M* protein kinase C inhibitor peptide, 0.5 μ*M* protein kinase A inhibitor peptide, 5 μ*M* compound R24571, 0.5 mg/ml MBP, 1 μCi of [γ-^{32}P]ATP and 10 μl of sample. The kinase reaction was terminated by spotting the aliquot (25 μl) of reaction mixture on P81 phosphocellulose paper squares. Free [γ-^{32}P]ATP was eluted by four washes in 0.75% of phosphoric acid and radioactivity on the filter was measured by liquid scintillation counting.

Statistical Analysis. Data were presented as mean ± SEM. Data were analyzed by one-way analysis of variance followed by the unpaired *t* test. A value of $P < 0.05$ was considered statistically significant.

Results

Effects of LPA and PDGF on Mesangial Cell DNA Synthesis. Quiescent subconfluent mesangial cells were exposed to either LPA (1 n*M*–30 μM) or PDGF (1 ng/ml to 100 ng/ml) in serum-free RPMI containing 0.1% BSA for 24 hr. DNA synthesis was assessed by incorporation of [^{3}H]thymidine into DNA during the last 6 hr of the incubation period. As shown in Figure 1, LPA induced a dose-dependent increase in [^{3}H]thymidine incorporation into DNA to reach plateau levels at concentrations of 10 μ*M* and higher. PDGF also stimulated progressive increases in [^{3}H]thymidine incorporation in the range of 1 ng/ml to 40 ng/ml. PDGF concentrations higher than 40 ng/ml resulted in statistically significant decreases in [^{3}H]thymidine incorporation into mesangial cells, showing a bell-shaped dose-response curve (Fig. 2).

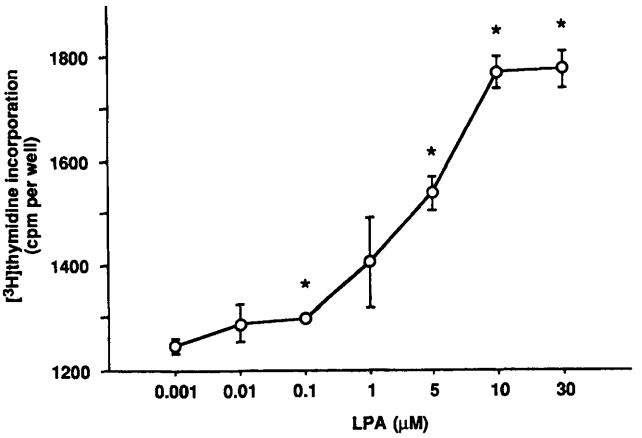

Figure 1. Dose-response curve of LPA-induced DNA synthesis in rat mesangial cells. Quiescent cells were incubated in serum-free RPMI-1640 containing 0.1% BSA with the indicated concentrations of LPA for 24 hr. [^{3}H]Thymidine was present during the last 6 hr of stimulation, and incorporation of [^{3}H]thymidine into TCA-precipitable material was counted. LPA induced a dose-dependent increase in [^{3}H]thymidine incorporation to reach plateau levels at concentrations of 10 μ*M* and higher. Values represent mean ± SEM of three independent experiments, each performed in triplicate. *$P < 0.05$ versus control.

Synergistic Effects of LPA on PDGF-Induced DNA Synthesis. We next examined the effect of LPA to modify DNA synthesis of mesangial cells stimulated by PDGF. For these experiments, quiescent mesangial cells were stimulated with increasing concentrations of PDGF (1–100 ng/ml) and LPA (30 μ*M*). As shown in Figure 3, LPA progressively enhanced PDGF-induced DNA synthesis. At 30 μ*M* LPA together with 40 ng/ml PDGF, DNA

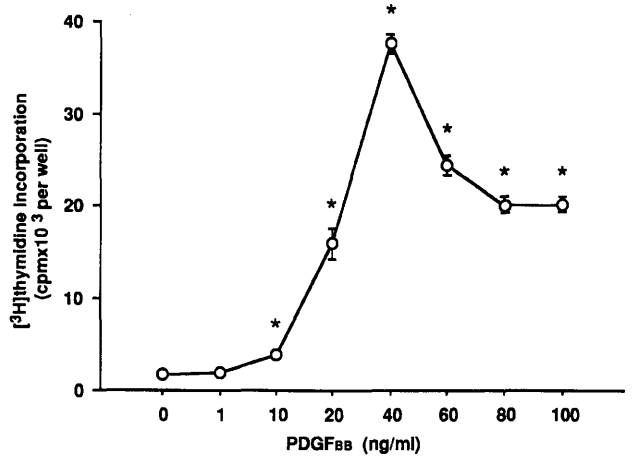

Figure 2. Quiescent rat mesangial cells were incubated with the indicated concentrations of $PDGF_{BB}$ and assayed for [^{3}H]thymidine incorporation as in Figure 1. PDGF stimulated progressive increases in [^{3}H]thymidine incorporation in the range of 1–40 ng/ml. PDGF concentrations higher than 40 ng/ml resulted in statistically significant decreases in [^{3}H]thymidine incorporation into mesangial cells, demonstrating a bell-shaped dose-response curve. Values represent mean ± SEM of two independent experiments, each performed in quadricate. *$P < 0.05$ versus control.

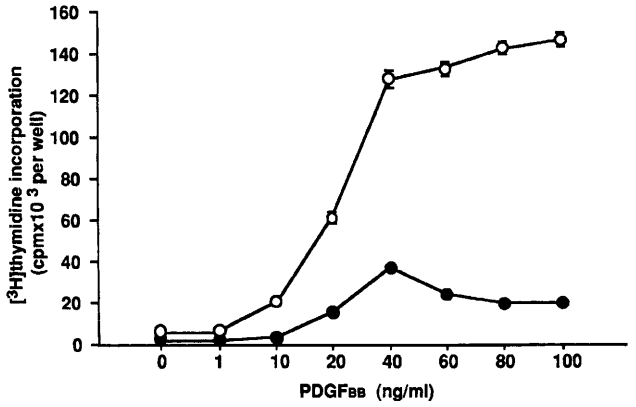

Figure 3. Synergistic effect of LPA on $PDGF_{BB}$-induced DNA synthesis. Quiescent rat mesangial cells were stimulated with the indicated concentrations of $PDGF_{BB}$ in the absence (●) or the presence (○) of LPA (30 μ*M*) for 24 hr. [^{3}H]Thymidine was present during the last 6 hr of stimulation. LPA/$PDGF_{BB}$ combinations were effective over a wide rage of concentrations. Values represent mean ± SEM of two independent experiments, each performed in quadricate.

synthesis was approximately 3.5 times higher when compared with that of PDGF alone (128,235 ± 3,658 cpm/well vs 37,726 ± 953 cpm/well, $n = 4$, $P < 0.05$). It should be noted that at doses of PDGF of 40 ng/ml and higher, the combined effects of LPA (30 μ*M*) and PDGF to stimulate DNA synthesis of mesangial cells reached plateau levels.

Effects of LPA and PDGF on Mesangial Cell Proliferation. To confirm the formulation that the enhanced [^{3}H]thymidine incorporation was associated with mesangial cell proliferation, we performed cell counts on cultures on Days 0, 4, and 7 (Fig. 4). Thirty micromolar LPA slightly increased cell number by Day 4 (29,253 ± 314 cells/well, $n = 3$) and Day 7 (32,837 ± 1,773 cells/well, $n = 3$), values not different from control quiescent dishes

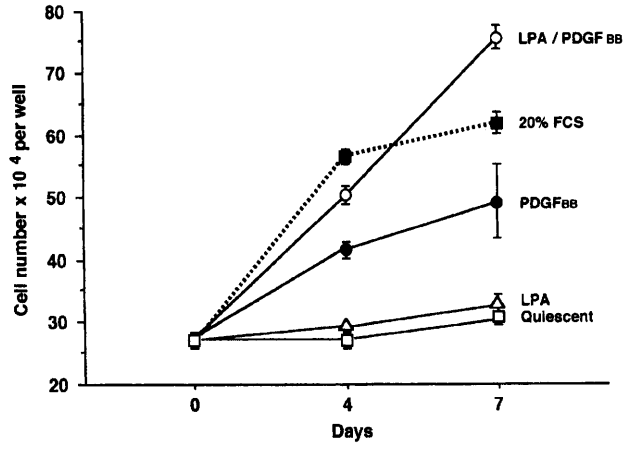

Figure 4. Effect of LPA on proliferation of rat mesangial cells. Mesangial cells plated in 24-well plates were serum-starved for 2 days in RPMI containing 0.1% BSA, and subsequently the medium was changed to RPMI containing 0.1% BSA and 0.5% FCS with the addition of LPA (30 μ*M*; △), $PDGF_{BB}$ (40 ng/ml; ●), or LPA/$PDGF_{BB}$ (30 μ*M*/40 ng/ml; ○). FCS (20%) was used as a positive control (■). Total cell number per well were counted at the day indicated. Values represent mean ± SEM of two independent experiments, each performed in triplicate.

(Day 4, 27,200 ± 1,353 cells/well; Day 7, 30,333 ± 821 cells/well, $n = 3$; NS). PDGF (40 ng/ml) promoted a significant increase in cell number by Day 4 (41,670 ± 1,243 cells/well, $n = 3$, $P < 0.05$ versus control quiescent cells) and Day 7 (49,371 ± 6,041 cells/well, $n = 3$, $P < 0.05$). Exposure of mesangial cells to both LPA (30 μ*M*) and PDGF (40 ng/ml) disclosed a potent mitogenic action with cell number increasing linearly to 50,417 ± 1,530 cells/well by Day 4 and 75,750 ± 1,893 cells/well by Day 7 ($n = 3$, $P < 0.05$ vs control quiescent cells). In concert with the [^{3}H]thymidine incorporation studies, these data indicate that LPA is weakly mitogenic by itself but acts as a potent comitogen together with PDGF in rat mesangial cells.

The mitogenic response of the mesangial cells treated with LPA/PDGF was compared to that of 20% FCS-treated dishes. In the presence of 20% FCS, mesangial cell number increased to 56,930 ± 342 cells/well by Day 4 ($n = 3$), a significantly higher value compared with that of LPA/PDGF-treated dishes (50,417 ± 1,530 cells/well, $n = 3$, $P < 0.05$). However, by Day 7 the LPA/PDGF-treated dishes showed a significantly higher cell number than that of 20% FCS–treated dishes (75,750 ± 1,893 cells/well vs 61,959 ± 1,710 cells/well, $n = 3$, $P < 0.05$). These results indicate that by Day 4, 20% FCS-treated dishes showed a higher cell growth rate compared with the LPA/PDGF-treated dishes, but between Days 4 and 7 the growth of 20% FCS–treated dishes was attenuated, whereas PDGF/LPA-treated dishes continued to grow, outstripping the cell number of 20% FCS–treated dishes.

In order to further characterize the difference in cell growth curves between 20% FCS-stimulated cells and LPA/PDGF-stimulated cells, the shapes of the mesangial cells grown under these two culture conditions were morphologically observed using phase-contrast microscopy after 7 days of culture. Mesangial cells grown in 20% FCS manifested the characteristically elongated, spindle cell shape in parallel arrays. After reaching confluence, cells stopped proliferating and remained in monolayers (Fig. 5A). Conversely, mesangial cells stimulated with LPA/PDGF were smaller, assumed a polygonal morphology, and continued to grow in multilayers despite increased cell density (Fig. 5B). From these results, we concluded that the difference in cell number between 20% FCS–treated and LPA/PDGF-treated mesangial cells derive from their different growth properties and cell shapes.

MAP Kinase Activity Assay. To assess the mechanism of LPA's growth stimulatory activities in mesangial cells, we measured MAP kinase activity after stimulation of quiescent mesangial cells by LPA (30 μ*M*), PDGF (40 ng/ml), or LPA/PDGF (30 μ*M*/40 ng/ml) by an immune complex kinase assay using an anti-rat MAP kinase antibody (see Materials and Methods). This method allows a specific and reproducible measurement of the phosphotransferase activity of MAP kinase in immunoprecipitates to MBP. Figure 6 shows the time course of MAP kinase activity. LPA induced a transient increase in MAP kinase activity with

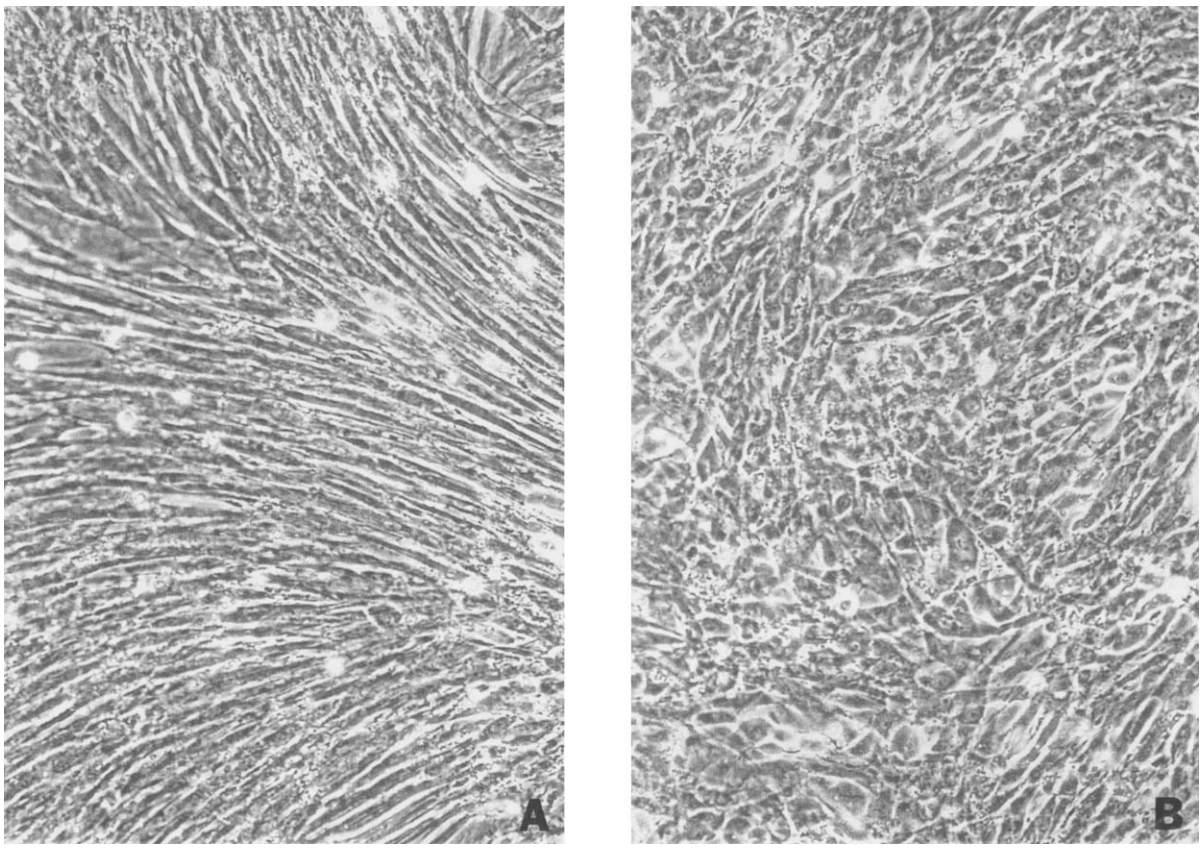

Figure 5. Morphological changes in rat mesangial cells stimulated with 20% FCS (A) or LPA (30 μ*M*)/$PDGF_{BB}$ (40 ng/ml)(B). Serum-starved mesangial cells were stimulated to proliferate by changing medium to RPMI 1640/20% FCS or RPMI 1640/0.1% BSA/0.5% FCS containing LPA/$PDGF_{BB}$. Phase-contrast micrographs were photographed 7 days after the addition of growth factors. The 20% FCS-treated cells appeared elongated and spindle-shaped and cell proliferation was attenuated at high cell density. On the other hand, LPA/$PDGF_{BB}$-treated cells were bipolar or polygonal in shape and continued to grow in multilayers despite high cell density. (Magnification: ×400.)

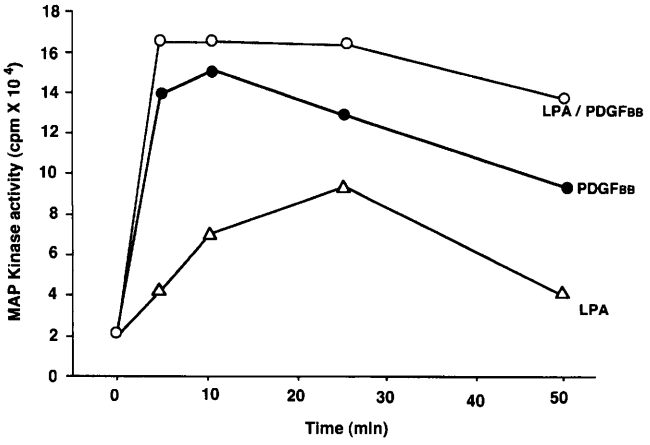

Figure 6. Time course of the activation of MAP kinase activity by LPA, $PDGF_{BB}$, or LPA/$PDGF_{BB}$. Quiescent rat mesangial cells were stimulated with LPA (30 μ*M*; △), $PDGF_{BB}$ (40 ng/ml; ●), or LPA (30 μ*M*)/$PDGF_{BB}$ (40 ng/ml; ○) for indicated times and cell lysates were immunoprecipitated with anti-rat MAP-kinase antibody. Immunoprecipitates were subjected to the *in vitro* MAP kinase assay using MBP as a substrate. Similar results were obtained in two other experiments.

maximal effect at 25 min (4.5-fold increase versus basal value). Thereafter, MAP kinase activity was declined. PDGF induced a peak MAP kinase activity 10 min after stimulation (7.2-fold) followed by a gradual decrease in activity till 50 min. When cells were stimulated with LPA together with PDGF, peak MAP kinase activity was observed at 10 min (7.9-fold) and higher levels of MAP kinase activity were sustained throughout the experiment (50 min).

Sensitivity to Pertussis toxin on DNA Synthesis and MAP Kinase Activity. In order to further define the molecular basis for LPA-induced proliferation, we tested whether LPA-induced DNA synthesis and LPA's synergistic action with PDGF were affected by pertussis toxin (PTX), which inactivates G_i or G_0 proteins (16). As shown in Figure 7, pretreatment of rat mesangial cells with 100 ng/ml PTX for 4 hr inhibited [^{3}H]thymidine incorporation in response to LPA by 31% (4,238 ± 385 cpm/well versus 2,914 ± 328 cpm/well, $n = 4$, $P < 0.05$). On the other hand, pretreatment with PTX did not inhibit the mitogenic response to PDGF (36,340 ± 1,677 cpm/well versus 42,235 ± 1,325 cpm/well, $n = 4$, $P > 0.05$). When tested against the mitogenic actions of the combination of LPA and PDGF, pretreatment with PTX reduced the [^{3}H]thymidine incorporation by 21% (102,037 ± 3,483 cpm/well versus 80,342 ± 3,594 cpm/well, $n = 4$, $P < 0.05$).

The sensitivity of PTX for LPA-induced MAP kinase activity was further tested using *in vitro* MAP kinase assay

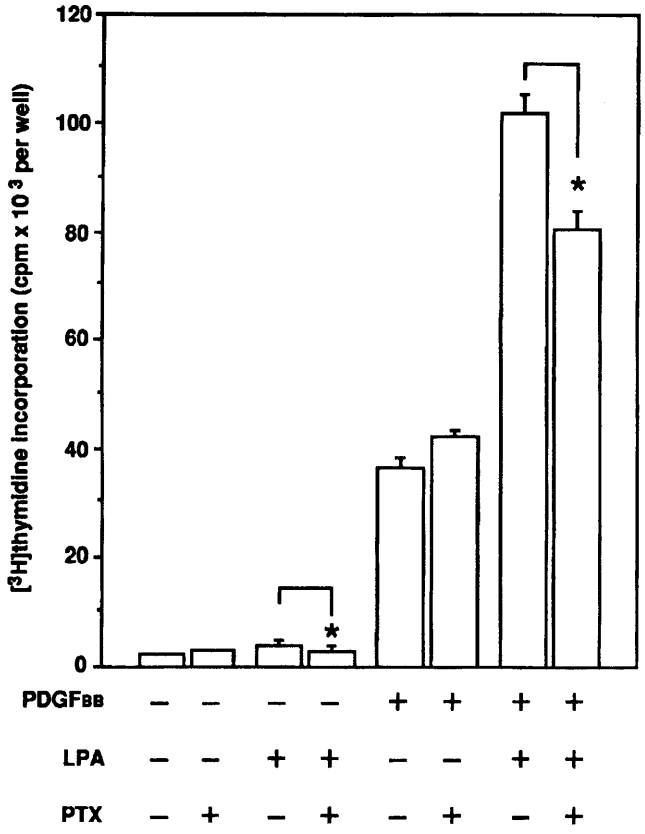

Figure 7. Inhibition of LPA-induced and LPA/$PDGF_{BB}$-induced DNA synthesis by pertussis toxin (PTX) in mesangial cells. Quiescent rat mesangial cells were pretreated with 100 ng/ml PTX for 4 hr in RPMI containing 0.1% BSA, followed by the stimulation of either LPA (30 μ*M*), $PDGF_{BB}$ (40 ng/ml), or LPA/$PDGF_{BB}$ (30 μ*M*/40 ng/ml) for 24 hr. [^{3}H]Thymidine was present during the last 6 hr of the stimulation. Control cultures received the same stimulations but were not exposed to PTX. Data are mean ± SEM (n = 4) from four independent experiments. *P < 0.05 compared with each control dish.

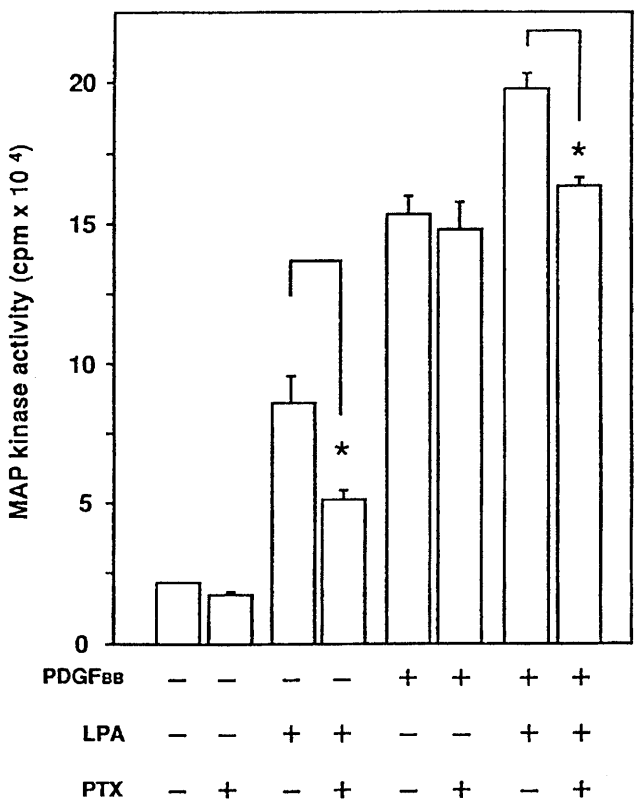

Figure 8. Inhibition of LPA-induced and LPA/$PDGF_{BB}$-induced MAP kinase activation by pertussis toxin (PTX) in mesangial cells. Quiescent rat mesangial cells were pretreated with 100 ng/ml PTX for 4 hr in RPMI containing 0.1% BSA, followed by the stimulation of either LPA (30 μ*M*), $PDGF_{BB}$ (40 ng/ml), or LPA/$PDGF_{BB}$ (30 μ*M*/40 ng/ml) for 25 min. Cell lysates were immunoprecipitated with anti-rat MAP kinase antibody, and the immunoprecipitates were subjected to *in vitro* MAP kinase assay using MBP as a substrate. Data are mean ± SEM (n = 3). *P < 0.05 compared with each control dish.

system as MBP as a substrate. For this study, MAP kinase activity was examined 25 min after the stimulation by the agonists (Fig. 8). Pretreatment of the cells with PTX (100 ng/ml, 4 hr) significantly inhibited MAP kinase activity stimulated by LPA (85,413 ± 9,899 cpm versus 50,781 ± 3,880 cpm, n = 3, P < 0.05). On the other hand, PTX pretreatment did not inhibit MAP kinase activity stimulated by PDGF (153,397 ± 6,113 cpm versus 148,078 ± 9,505 cpm, n = 3, P > 0.05). Pretreatment with PTX significantly inhibited the increase in MAP kinase activity induced by the combined stimulation of LPA with PDGF (197,602 ± 5,314 cpm versus 162,887 ± 3,495 cpm, n = 3, P < 0.05).

These results suggest that a PTX-sensitive G protein is involved in LPA-induced mesangial cell MAP kinase signaling pathways as well as in DNA synthesis, and that this signaling pathway is distinct from the PDGF-mediated signaling pathway, which is insensitive to PTX.

Comparison of the Mitogenic Action of LPA with Other Vasoactive Agents. The ability of LPA to stimulate mesangial DNA synthesis was then compared with that of other well-known vasoactive agents. Serotonin (10 μ*M*), ET-1 (0.1 μ*M*), PAF (10 μ*M*), Ang II (1 μ*M*), and AVP (1 μ*M*) induced modest but statistically significant (1.5- to 2.3-fold) increases in [^{3}H]thymidine incorporation compared with control quiescent dishes (Fig. 9A). The combination of LPA (30 μ*M*) with each of these vasoactive agents demonstrated an additive effect to enhance the DNA synthesis, displaying a 2.2- to 3.3-fold increase in DNA synthesis compared with control quiescent mesangial cells (Fig. 9B). On the other hand, the addition of PDGF (40 ng/ml) displayed remarkable synergistic enhancement of DNA synthesis in cultures stimulated by each of these vasoactive agents. Among these dishes, the LPA/PDGF combination was more potent (60-fold) than the combination of PDGF with other vasoactive agents (17- to 43-fold) (Fig. 9C). Moreover, as shown in Figure 9D, when LPA was added to each of the cultures of serotonin/PDGF-, ET-1/PDGF-, PAF/PDGF-, Ang II/PDGF-, or AVP/PDGF-treated dishes, all of these dishes demonstrated an extremely high DNA synthetic capability, displaying up to 67- to 100-fold increases in [^{3}H]thymidine incorporation over control dishes. These results indicate that although LPA confers only a mild additive effect to diverse vasoactive agents including serotonin, ET-1, PAF, Ang II, and AVP, it acts synergistically with PDGF to enhance DNA synthesis in rat

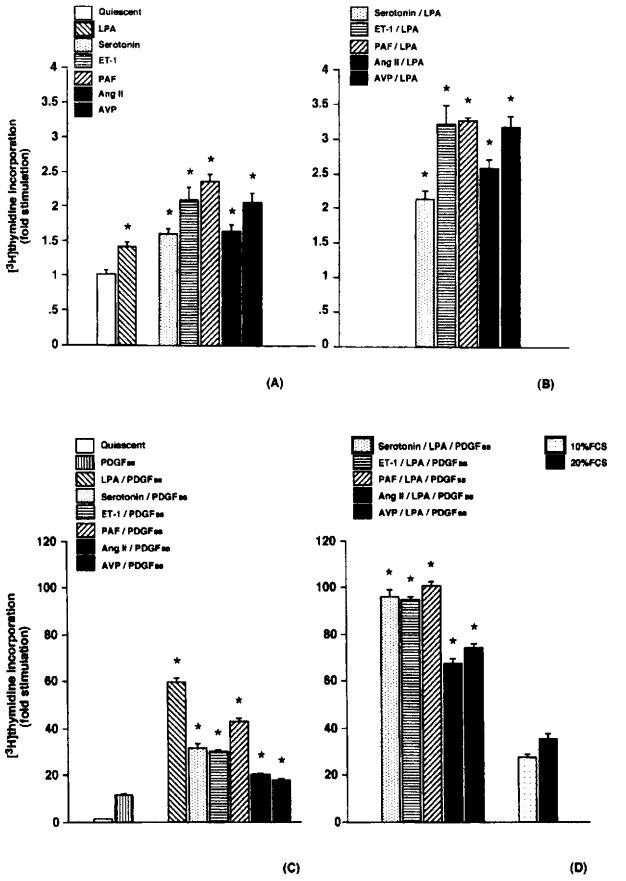

Figure 9. (A) Effects of LPA, serotonin, ET-1, PAF, Ang II, and AVP on mesangial cell DNA synthesis in serum-free RPMI-1640 containing 0.1% BSA. Quiescent rat mesangial cells were treated for 24 hr with each of the following agents including LPA (30 μ*M*), serotonin (10 μ*M*), ET-1 (0.1 μ*M*), PAF (10 μ*M*), Ang II (1 μ*M*), or AVP (1 μ*M*), and the [^{3}H]thymidine incorporation was measured during the last 6 hr. Data are mean ± SEM ($n = 6$) and are presented as fold increase in [^{3}H]thymidine incorporation over the control values. Control [^{3}H]thymidine incorporation was 1,242 ± 113 cpm/well. $^*P < 0.05$ versus control quiescent dishes. (B) Effects of LPA on serotonin–, ET-1–, PAF–, Ang II–, and AVP–induced DNA synthesis in rat mesangial cells in serum-free RPMI-1640 containing 0.1% BSA. LPA (30 μ*M*) additively augments the DNA synthesis induced by serotonin (10 μ*M*), ET-1 (0.1 μ*M*), PAF (10 μ*M*), Ang II (1 μ*M*), or AVP (1 μ*M*). Data are mean ± SEM ($n = 6$). $^*P < 0.05$ versus LPA values. (C) Effects of $PDGF_{BB}$ on serotonin–, ET-1–, PAF–, Ang II–, and AVP–induced DNA synthesis in rat mesangial cells in serum-free RPMI-1640 containing 0.1% BSA. $PDGF_{BB}$ (40 ng/ml) synergistically increases the DNA synthesis induced by serotonin (10 μ*M*), ET-1 (0.1 μ*M*), PAF (10 μ*M*), Ang II (1 μ*M*), or AVP (1 μ*M*). Data are mean ± SEM ($n = 6$). $^*P < 0.05$ versus $PDGF_{BB}$ values. (D) Effects of LPA in combination with $PDGF_{BB}$ on serotonin–, ET-1–, PAF–, Ang II–, and AVP–induced DNA synthesis in serum-free RPMI-1640 containing 0.1% BSA. The simultaneous addition with LPA (30 μ*M*) and $PDGF_{BB}$ (40 ng/ml) synergistically augments the DNA synthesis induced by serotonin (10 μ*M*), ET-1 (0.1 μ*M*), PAF (10 μ*M*), Ang II (1 μ*M*), or AVP (1 μ*M*). Data are mean ± SEM ($n = 6$). $^*P < 0.05$ versus LPA/$PDGF_{BB}$ values.

mesangial cells and that the synergistic effect of LPA in combination with PDGF was further observed under the presence of other vasoactive agents described above. It should be noted, however, that the increase in DNA synthesis obtained by the combination of LPA/PDGF with the vasoactive agents did not necessarily lead to the acceleration of mesangial cell growth; the growth rate was essentially the same as that by the combination of LPA and PDGF.

Discussion

Several lines of evidence have clearly delineated the effects of vasoactive agents on glomerular ultrafiltration (17). Recently, utilizing cell culture techniques investigators have also demonstrated the potential of many of the vasoactive agents to stimulate glomerular mesangial cell growth. Vasoconstrictors such as AVP, epinephrine, ET-1, and serotonin have all demonstrated mitogenic properties on mesangial cells (18, 19). On the other hand, the data are conflicting regarding the *in vitro* mitogenic potential of Ang II on mesangial cell proliferation (19, 20). The production of diacylglycerol and inositol triphosphate by the hydrolysis of phosphoinositide with the subsequent increase in cytosolic free calcium and protein kinase C are now thought to play a role in modifying cellular processes such as metabolism and the progression to mitosis (21).

However, several exceptions to this correlation have been reported, and the effector systems that participate in the mitogenic action of these vasoactive agents have not been fully determined. For example, $PDGF_{BB}$, a potent mitogen for mesangial cells, has not manifested vasoconstrictive properties but has been reported to stimulate IP_3 production and increases in $[Ca^{2+}]_i$ (22). In addition, a larger number of nonvasoactive agents such as interleukin-1, phorbol ester, and epidermal growth factor (EGF) are mitogenic but do not produce a rapid $[Ca^{2+}]_i$ transient in cultured mesangial cells (19). In summary, it is readily apparent that the mechanisms mediating mitogenesis are complex and diverse, and it is impossible to generalize on the role of calcium in mediating these events.

Recent studies from this laboratory have demonstrated that LPA elicited a marked contractile response in rat mesangial cells that was accompanied by an increase of IP_3 and a transient rise in $[Ca^{2+}]_i$ in rat mesangial cells (13). These observations prompted us to investigate whether this platelet-derived phospholipid also had proliferative properties for mesangial cells. In the present study, we present evidence that LPA is weakly mitogenic as assessed by [^{3}H]thymidine incorporation in serum-free media, but acts synergistically with PDGF, extending and amplifying the mitogenic action of this well-established growth factor. Furthermore, as demonstrated in our cell replication studies, LPA/PDGF-stimulated mesangial cell growth levels were nearly equivalent to those attained under "maximal culture conditions"—that is, 20% FCS–supplemented media (a population doubling time of 4.6 days in LPA/PDGF-treated dishes versus 3.6 days in 20% FCS–treated dishes).

We next conducted investigations to elucidate the molecular basis of the mitogenic action of LPA and its synergistic action with PDGF. The mitogens capable of triggering the G_0 to S phase transition in the cell cycle can be classified into two groups: one is the PTX-sensitive group

consisting of factors that bind to a G protein–coupled receptor, whereas the PTX-insensitive group is composed of growth factors known to activate receptor tyrosine kinases (23). In the present study, the mitogenic action of LPA in rat mesangial cells was partially (31%) inhibited by pretreatment with PTX, whereas PDGF-induced DNA synthesis was unaffected by PTX. These results support the evidence that PDGF exerts its mitogenic effects through the activation of tyrosine kinases without involving PTX-sensitive G protein–mediated signaling pathways (24), whereas LPA acts at least in part through PTX-sensitive G protein–mediated signaling pathways to stimulate DNA synthesis. Furthermore, as shown in Figure 3, the combination of LPA and PDGF synergistically increased mesangial DNA synthesis and this potent mitogenic action was also partially (21%) inhibited by PTX in analogy with LPA alone (Fig. 7). In concert, these data indicate that PDGF-induced pathways and LPA-induced signaling pathways are distinct, but suggest that they may also share some common signaling pathways leading to an amplification of mitogenic signals.

Recently, MAP kinase pathway has been demonstrated to behave at certain points as a switch kinase, capable of integrating signals from tyrosine kinase receptors as well as G protein–coupled receptors (25). Therefore, we decided to investigate the effects of LPA and PDGF on MAP kinase activation in mesangial cells. As shown in Figure 6, both PDGF and LPA stimulated the MAP kinase activity in mesangial cells. These results indicate that MAP kinase cascade was shared by LPA- and PDGF-induced signaling pathways in rat mesangial cells. Furthermore, stimulation with the combination of LPA/PDGF demonstrated a sustained elevation in MAP kinase activity as compared with those stimulated by either of LPA or PDGF alone. In the system of fibroblasts, sustained activation of MAP kinase was demonstrated to be associated with cell proliferation, whereas transient increases in MAP kinase activity did not lead to mitogenic action (26, 27). In analogy with this description, it is tempting to suggest that the highly sustained MAP kinase activation by the combination of LPA and PDGF may be the cause of the synergistic stimulation of mesangial cell proliferation. In this relevance, the modest mitogenic action of LPA may be explained by its transient activation of MAP kinases.

Recently, Hill *et al.* suggested that PDGF-induced activation of the serum response element (SRE) could be potentiated by Rho-mediated signaling pathways are activated by LPA (28). Thus, alternatively it may be hypothesized that distinct signaling pathways are activated by PDGF and LPA at the SRE, a regulatory element that activates the oncogene c-*fos* promoter (29). In such a case, the partial inhibition of LPA- or LPA/PDGF-induced DNA synthesis by PTX might be explained by the existence of Rho-mediated signaling pathway which is insensitive to PTX (30). Further studies are required to verify whether the Rho-mediated signaling pathway is involved in the synergistic mitogenic response by PDGF and LPA in mesangial cells.

In our experiments, the synergistic effects of LPA with PDGF were similar to that observed when glomerular mesangial cells were stimulated with the combination of PDGF and one of the following vasoactive agents: serotonin, PAF, ET-1, Ang II, or AVP. Over the past several years, all of the cellular receptors for these vasoactive agents have been cloned and demonstrated to belong to the G protein–coupled superfamily of receptors (31–34). LPA has been also demonstrated to interact with a G protein–coupled receptor (35). Therefore, it is reasonable to postulate that PDGF exerts its synergistic mitogenic effects with those diverse vasoactive agents by involving the G protein–mediated signaling pathways.

The possibility of synergistic enhancement at the level of the PDGF receptor cannot be overlooked. Bobik *et al.* suggested that an increase in the number of $PDGF_{BB}$ receptors by stimulation with Ang II may mediate the synergistic enhancement of DNA synthesis by Ang II/$PDGF_{BB}$ in rat vascular smooth muscle cells (36). On the other hand, Naftilan *et al.* (37) suggested that Ang II may act synergistically with $PDGF_{BB}$ through the endogenous production of the PDGF-A chain in rat smooth muscle cells. In a series of preliminary studies in this laboratory, exogeneously added $PDGF_{AA}$ did not enhance the LPA- or $PDGF_{BB}$-induced DNA synthesis in rat mesangial cells (data not shown). Therefore, it seems unlikely that the synergistic enhancement of mitogenic action by LPA and PDGF could be generated at the level of $PDGF_{AA}$ receptor or *via* the endogenous production of $PDGF_{AA}$. In the present study, however, $PDGF_{BB}$ induced a bell-shaped dose-response relationship until LPA was added, then the dose-response curve took on sigmoidal characteristics (Fig. 3). Therefore, the possibility cannot be ruled out that LPA may increase the expression of $PDGF_{BB}$ receptors and vice versa. Binding assay studies could help verify these possibilities.

It is becoming increasingly clear that at least two distinct signaling pathways are involved in the activation of MAP kinase in mesangial cells: one pathway through sequential activation of Ras and Raf, and the other pathway through protein kinase C (PKC) and as yet unidentified intermediate kinases. The Ras-dependent pathways have been demonstrated to be inhibited by the elevation of intracellular cAMP levels, whereas the PKC-dependent pathway is insensitive to cAMP elevation (38). A number of vasoactive agents that stimulate receptors coupled to G proteins activate MAP kinase in a PKC-dependent manner (39). Although we did not identify the precise mechanisms whereby LPA exerts MAP kinase activation, the characteristic features of LPA as a vasoactive agent suggests that LPA activates MAP kinase in a PKC-dependent manner.

The presence of LPA has been demonstrated in aged plasma (12) and sera of several animals including humans (10, 40). Tokumura *et al.* demonstrated that the major plasma phospholipid, lysophosphatidylcholine, can yield LPA at concentrations greater than 70 μM when cleaved by phospholipase D (40). As we have demonstrated in this

study, this concentration of LPA is sufficient to induce the DNA synthesis of mesangial cells when added exogenously. Alternatively, LPA is produced and released by activated cells as well as mesangial cells by stimulation of Group II phospholipase A_2 (PLA_2) (41, 42). Because Group II PLA_2 is considered to play a role in the initiation and propagation of inflammatory responses in mesangial cells (43), it is reasonable to suggest that LPA, produced in the circulation as well as by the mesangial cell itself, could act in an autocrine and paracrine manner to promote mesangial cell proliferation under similar inflammatory conditions of the glomeruli.

Finally, our present data allow us to speculate on a role for LPA in regard to platelet-mediated mesangial cell proliferation. Mounting evidence has implicated infiltrating platelets in mediating glomerular mesangial cell proliferation in response to glomerular injury (4, 44, 45). PDGF is regarded as the most crucial mitogen for mesangial cell proliferation among the many kinds of growth factors produced by platelets. In addition to its ability to stimulate mesangial DNA synthesis exogenously, PDGF can be produced endogenously in mesangial cells in response to many stimuli including EGF, TNF-α, bFGF, ET-1, and thrombin (7, 46, 47), suggesting that PDGF participates as an intermediary for other growth factors. Therefore, locally produced PDGF in clotting platelets as well as in the glomerular microcirculatory bed could act in an autocrine and paracrine manner to stimulate mesangial cell replication. In this context, our data demonstrating that LPA has a potential to enhance the mitogenic activity of PDGF, in concert with the ability of LPA to stimulate platelet aggregation (12), suggest that LPA may enhance positive feedback in the initial aggregation response and possibly in platelet-mediated mesangial cell proliferation.

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