Biochemical and Biophysical Research Communications **277,** 72–78 (2000)
doi:10.1006/bbrc.2000.3634, available online at http://www.idealibrary.com on IDEAL®

Lysophosphatidic Acid Enhances Collagen Gel Contraction by Hepatic Stellate Cells: Association with Rho-Kinase

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Received August 22, 2000

We studied the effect of lysophosphatidic acid (LPA) on collagen gel contraction by cultured rat hepatic stellate cells (HSCs) in association with the function of Rho-kinase, one of the target molecules of small GTPase Rho. Binding studies showed a single class-binding site of LPA on HSCs. LPA enhanced the contraction of a collagen lattice seeded with HSCs. LPA increased the number of HSCs with polygonal morphology that contained actin stress fibers, and enhanced the phosphorylation of myosin light chain and the assembly of focal adhesion kinase and RhoA around fibronectin-coated beads seeded on HSCs. The electric cell-substrate impedance sensor system showed that LPA enhanced adhesion of HSC to extracellular substrate. All the effects of LPA were suppressed by Y-27632, Rho-kinase inhibitor. These data support the notion that LPA is involved in modulating HSC morphology, its attachment to surrounding extracellular matrix and its contraction by a mechanism involving Rho-kinase.

Key Words: **hepatic stellate cells; collagen gel contraction; lysoposphatidic acid; Rho-kinase; focal adhesion; actomyosin-interaction; electric cell-substrate imedance sensor.**

Hepatic stellate cells (HSCs; previously called Ito cells, lipocytes, or fat-storing cells) are located in the perisinusoidal space of Disse, surrounding the sinusoids with numerous dendrite-like processes (1). They have several physiological functions, other than storage and metabolism of retinoid and production of extracellular matrix proteins (2). They express several cytoskeletal proteins and corresponding motor proteins, and contain membrane receptors for several kinds of vasoactive agents. HSCs have been shown to be contractile in response to these agents, suggesting that they play a role in modulating portal blood flow and in contraction of fibrous septa (3). HSCs are the main producers of type I collagen and participate in remodeling of the extracellular matrix.

Lysophosphatidic acid (LPA) is a product of phospholipid metabolism. It is produced and released into blood and/or other biological fluids mainly by activated platelets during blood-clotting. LPA is produced by various cell types and exerts diverse biological effects on a variety of cells, including induction of proliferation, contraction, and/or migration (4, 5). Production of LPA is increased in many pathological conditions (6–9). Exogenous LPA binds to surface G protein-coupled receptors and its biological activities are mediated in part by the cytosolic small GTPase Rho. This modulates cell morphology by organizing the actin cytoskeleton and regulates several actomyosin-dependent cellular processes (10, 11). Among the target molecules of Rho, Rho-kinase (12) (termed also ROCK (isoforms I (13) and II (14)), or ROKα (15)) has been recently shown to participate in the induction of focal adhesions and stress fibers in Swiss 3T3 cells (16), contraction of smooth muscle by mediating calcium sensitization (17, 18), and tumor invasion (19).

Abbreviations used: LPA, lysophosphatidic acid; HSC, hepatic stellate cell; Y-27632, (R)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide; FAK, focal adhesion kinase; MLC, myosin light chain; ECIS, electric cell-substrate impedance sensor system.

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0006-291X/00 $35.00

The vasoactive effect of LPA on HSC as well as its effect on remodeling of the extracellular matrix by these cells remain to be fully elucidated. Thus, in this paper, we studied the role of Rho-kinase on LPA-mediated contraction of a collagen gel and on the role of the actomyosin system in cell-matrix interaction and gel contraction.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle medium (DMEM) and Dulbecco's phosphate buffered saline (−) [PBS(−)] were provided by Nissui Pharmaceutical Co. (Tokyo, Japan). Twenty-four-well uncoated plastic culture plates were purchased from Falcon (Lincoln Park, NJ); culture chamber slides, from Nunc Inc. (Naperville, IL); fetal calf serum (FCS), from GIBCO (Gaithersberg, MD); and LPA (L-α-lysophosphatidic acid, oleoyl), bovine serum albumin (BSA) and fibronectin, from Sigma Chemical Co. (St. Louis, MO). [^{3}H]LPA (specific activity, 1850 GBq/mmol) was a product from NEN Life Science Products, Inc. (Boston, MA). Y-27632 [(R)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide] (17) was kindly provided by Yoshitomi Pharmaceutical Industries Ltd. (Osaka, Japan), and dissolved with DMEM. The following antibodies were shown to be monospecific by Western blot analysis using goat anti-rabbit immunoglobulin (Ig) conjugated with peroxidase or rabbit anti-mouse Ig conjugated with peroxidase (Amersham International plc; Buckinghamshire, England): anti-focal adhesion kinase (anti-FAK) and anti-RhoA antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal antibody against phosphorylated serine 19 of myosin regulatory light chain (anti-MLC-pS19) was previously described (20). Tetramethylrhodamine isothiocyanate (TRITC)-labeled phalloidin, TRITC-conjugated goat anti-mouse IgG antibody and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody were obtained from Sigma.

Animals. Male Sprague-Dawley rats (300 to 400 g; Shizuoka Laboratory Animal Center, Shizuoka, Japan) were fed a commercial pelleted diet and water *ad libitum* as previously described (21), and used in all the experiments. All animal study protocols conformed to National Research Council criteria for humane care.

Cell isolation and culture. HSCs were isolated from the rats as previously described (22). The cells were seeded on uncoated plastic culture dishes and cultured in DMEM containing 10% FCS. The medium was changed 24 h later and then every other day. The confluent cells were subcultured 10 days after seeding and used in the experiments.

LPA binding assay. Binding of LPA to HSCs was examined as previously described (23), with some modifications. The cells were seeded on 24-well uncoated plastic culture plates at a starting density of 2.5×10^4 cells/cm^2, and cultured in DMEM containing 10% FCS for 24 h, in DMEM containing 0.5% FCS for 18 h, and then in serum-free DMEM for 6 h. The cells were rinsed twice with 500 μl of ice-cold binding buffer (120 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $CuSO_4$, 10 mM glucose, 25 mM Hepes [pH 7.4], 0.1% BSA). The cells were incubated for 120 min at 4°C in 200 μl of binding buffer containing various concentrations of [^{3}H]LPA, ranging from 10^{-8} to 10^{-6} M. Reactions were terminated by aspirating the buffer, and the cells were rapidly rinsed with 500 μl of cold binding buffer four times to remove unbound [^{3}H]LPA. The cells were solubilized in 1 N NaOH, and the radioactivity was measured as total binding in 2 ml of scintillation fluid in a liquid scintillation counter. Nonspecific binding was determined by counting the radioactivity bound to the cells incubated similarly with [^{3}H]LPA in the presence of 100-fold excess amount of cold LPA. Specific binding was calculated by subtracting the nonspecific binding from the total binding. Affinity (K_d) and capacity (B_{max}) of the binding sites of LPA were calculated by a nonlinear least-square curve fitting.

Collagen gel-contraction assay. Contractility of HSCs was evaluated using collagen gel lattices on 24-well culture plates as described previously (24, 25), with some modifications. Wells were filled with Dulbecco's PBS(−) with 0.02% glucose (PBS) containing 1% BSA for at least 1 h at 37°C, then washed twice with PBS and air dried. Four parts type I collagen (Nippon Meat Packers, Osaka, Japan), 5 parts 2× DMEM and 1 part 0.2 M Hepes were combined at 4°C, resulting in a final collagen concentration of 1.2 mg/ml. The collagen solution was poured into the wells, and incubated for 1 h at 37°C to allow gelation.

HSCs were layered on top of the formed lattices at a starting density of 1.5×10^5 cells/cm^2, and cultured in DMEM with 10% FCS for 72 h, in serum-free DMEM for 5 h, and then in DMEM with or without 10^{-7} to 10^{-4} M of Y-27632 for an hour. LPA was added to the culture media at a concentration of 10^{-7} to 10^{-5} M, just before the lattices were detached by gentle circumferential dislodgement of the lattice using a 200 μl micro-pipette tip. Contraction of the lattices was serially monitored as a change in lattice diameter, and the lattice area was calculated.

F-actin staining. HSCs were plated on culture chamber slides at a starting density of 2.5×10^4 cells/cm^2. After a 72-h culture in DMEM with 10% FCS and a 5-h culture in serum-free DMEM followed by a 1-h culture in DMEM with or without 10^{-5} M of Y-27632, 10^{-5} M of LPA was added to the medium. After a 30-min incubation with LPA at 37°C, the cells were washed, fixed in PBS containing 4% paraformaldehyde, then treated with Triton X-100. For visualization of F-actin, the cells were stained overnight with TRITC-labeled phalloidin. The samples were observed by fluorescence microscopy with photographic recording of the data.

Immunoblotting for phosphorylated myosin light chain. HSCs were plated at a starting density of 1.5×10^5 cells/cm^2. After a 72-h culture in DMEM containing 10% FCS and a 5-h culture in serum-free DMEM followed by a 1-h culture in DMEM with or without 10^{-5} M of Y-27632, 10^{-5} M of LPA was added to the medium. Ten minutes later, the cells were harvested. Protein content was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA). After boiling in sample buffer (62.5 mM Tris–HCl [pH 6.8] containing 1% sodium dodecyl sulfate (SDS), 10% glycerol, 2% 2-mercaptoethanol and 1 mM orthovanadate) for 5 min, identical amounts of protein were separated by SDS–polyacrylamide gel electrophoresis (PAGE) (15% acrylamide), and transferred to a sheet of polyvinylidene difluoride (PVDF) membrane (Amersham). To block nonspecific binding, the membrane was soaked in blocking agent derived from skim milk (Block Ace; Yukijirushi Co., Ltd., Tokyo, Japan) for 1 h at room temperature. Then, it was incubated with anti-MLC-pS19 overnight at 4°C. Incubation with secondary antibody was performed for 2 h at room temperature. Immunoreactive proteins were visualized using a chemiluminescence kit (Amersham), and recorded in a chemiluminescence recording system (LAS 1000; Fuji-film, Tokyo, Japan).

Seeding of fibronectin-coated beads and immunocytochemistry. Latex beads (mean diameter, 10 μm; Polysciences, Inc., Warrington, PA) were coated with ligands as previously described (26, 27). Briefly, fibronectin or BSA was dissolved at a concentration of 0.1 mg/ml in 10 mM Tris–HCl [pH 8.0] containing 2 M LiBr and 1 mM dithiothreitol. Latex beads were incubated in the mixture at a concentration of 10^7 beads/ml for 1 h at 37°C, and then dialyzed against 100 volumes of 10 mM acetate [pH 5.5] containing 0.4 mM dithiothreitol.

HSCs were plated on culture chamber slides at a starting density of 2.5×10^4 cells/cm^2. After 72 h of culture in DMEM with 10% FCS and a 5-h culture in serum-free DMEM followed by a 1-h culture in DMEM with or without 10^{-5} M of Y-27632, LPA at a final concentration of 10^{-5} M or vehicle was added to the culture medium. Soon after, the beads were seeded on the cells at a cell-to-bead ratio of 1:2. After a 30-min incubation with the beads at 37°C, the cells were

washed, fixed in PBS with 4% paraformaldehyde, and treated with Triton X-100. Then, they were incubated with anti-FAK or anti-RhoA overnight, followed by incubation with TRITC or FITC-conjugated secondary antibodies overnight, respectively. The samples were observed by fluorescence microscopy with photographic recording of the data.

Evaluation of cell adhesion to extracellular substrate. Cell adhesion to extracellular substrate was evaluated by the electric cell-substrate impedance sensing system (ECIS; Applied BioPhysics, Inc., Troy, NY) as previously described by Giaever and Keese (28). Briefly, cells were cultured on a small gold electrode (area, 10^{-4} cm^2) deposited on a tissue culture vessel. A small alternating current signal (1 μA) at a frequency of 4000 Hz was passed between the small electrode and a larger counter electrode (area, 10^{-1} cm^2) placed at a distance. The voltage between the two electrodes was recorded.

HSCs were plated at a starting density of 2.5×10^4 cells/cm^2. After a 24-h culture in DMEM containing 10% FCS and a 6-h culture in serum-free DMEM followed by a 40-min culture in DMEM with or without 10^{-5} M of Y-27632, 10^{-5} M of LPA was added to the medium. Resistance, which reflects the extent of cell-substrate adhesion, was monitored as described previously (29). The resistance was normalized as a ratio to the initial resistance measured just before the addition of Y-27632.

Data analysis. Data were given as the mean and the standard error of the mean. The statistical analysis of the results was performed using the paired Student's *t* test.

RESULTS AND DISCUSSION

We first examined the binding of LPA to HSCs. In preliminary experiments, [^{3}H]LPA bound to HSCs in a time and temperature-related manner, and maximal specific binding reached a plateau at 120 min after the addition of LPA. Accordingly, the cells were incubated for 120 min in all experiments. Because it has been reported that LPA is degraded to free fatty acids when incubated with membrane preparations, in our experiments to measure binding of LPA to HSCs, we added $CuSO_4$ to the reaction mixture to inhibit the degradation of LPA (22). As shown in Fig. 1, HSCs exhibited saturable binding of LPA. By Scatchard analysis, HSCs showed a single class-binding site for LPA with a K_d of 7.8×10^{-7} M and a B_{max} of 5.2×10^7 sites/cell. The half-maximal binding of [^{3}H]LPA to HSCs was calculated to be 2×10^{-7} M. This value is one-rank higher in order compared with that in the study using neuronal cells (30). LPA receptors have been cloned as vzg-1 (31)/Edg-2 (32), Edg-4 (33), and Edg-7 (34) and reported to couple to at least three distinct G proteins: Gq, which links the receptor to phospholipase C; Gi, which mediates activation of Ras-MAPK pathway; and $G_{12/13}$, which mediates Rho activation (4). However, it remains to be elucidated which type(s) of receptors is expressed in HSCs and is responsible for LPA-mediated biological activities.

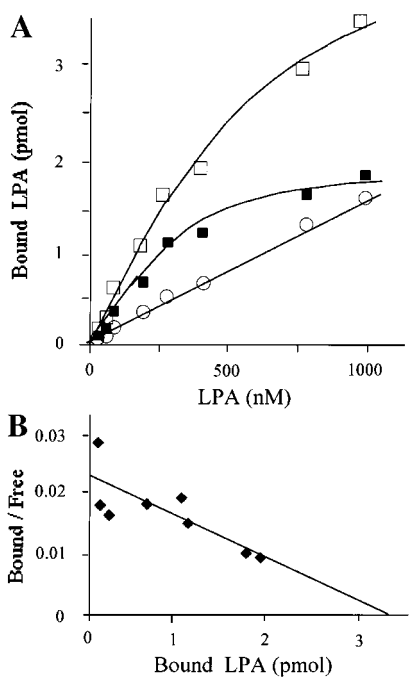

FIG. 1. Equilibrium binding of [^{3}H]LPA to cultured rat HSCs. (A) Saturation experiments were carried out for 120 min with three independent cultures; a typical result is shown. Each point is the mean value of triplicate determinations. Open square, total binding; open circle, nonspecific binding; closed square, specific binding. (B) Scatchard plots of the data presented in A.

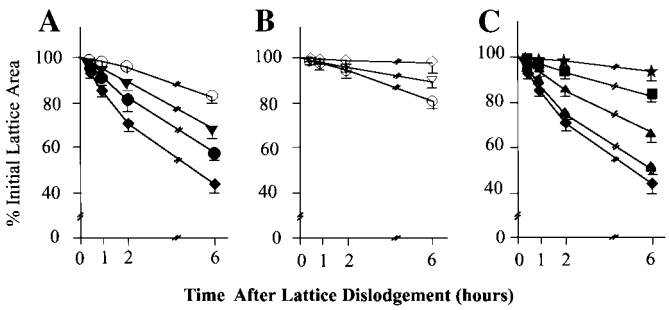

FIG. 2. Changes in area of collagen lattices seeded with rat HSCs. (A) Various concentrations of LPA were added to the culture media at the time of lattice dislodgement: ○, control; ▼, 10^{-7} M of LPA; ●, 10^{-6} M of LPA; and ◆, 10^{-5} M of LPA. (B) Various concentrations of Y-27632 were added to the culture media an hour before lattice dislodgement: ○, control; △, 10^{-7} M of Y-27632; and ◇, 10^{-6} M of Y-27632. (C) Various concentrations of Y-27632 were added to the culture media an hour before addition of 10^{-5} M of LPA and lattice dislodgement: ◆, LPA; ♠, LPA $+10^{-7}$ M of Y-27632; ▲, LPA + 10^{-6} M of Y-27632; ■, LPA + 10^{-5} M of Y-27632; and ★, LPA + 10^{-4} M of Y-27632. Data are presented as mean values with standard errors of three determinations.

We further examined the effects of LPA on the capacity of HSCs to contract collagen gels. This procedure is considered to be an *in vitro* equivalent of tissue contraction occurring during wound healing (24). As shown in Fig. 2A, the lattices seeded with HSCs were contracted over time after dislodgment from the wall even in the nontreated control. When LPA was added to the culture media, the contraction of the lattices was enhanced in a dose-related manner. When the cells were incubated with Y-27632, a specific inhibitor of Rho-kinase, contraction of the lattice was inhibited in a

dose-dependent manner, irrespectively of the amount of LPA added (Figs. 2B and 2C). HSCs cultured in serum-free DMEM containing 10^{-4} M of Y-27632 for 7 h were 100% viable as determined by the trypan blue-exclusion test. When the lattices seeded with the cells were incubated in serum-free DMEM with or without 10^{-4} M of Y-27632 for 7 h and then in DMEM with 10% FCS for 24 h, the contraction of the lattices after the addition of LPA did not differ between the groups treated or untreated with 10^{-4} M of Y-27632 (data not shown). Therefore, Y-27632 at the concentrations used in this study is not cytotoxic to the cells and its inhibitory effect is fully reversible. LPA is a stimulator of Rho (4, 5) and Rho-kinase acts downstream of Rho (11), Thus, based on our results, we postulate Rho-kinase is involved in LPA-mediated enhancement of gel-contraction by HSCs. It is speculated that cumulative traction exerted by the cells on collagen fibrils within the collagen gel would lead to rearrangement of the matrix (24). However, molecular mechanisms involved in rearrangement of the extracellular matrix, as well as intracellular signaling events occurring during contraction remain to be established.

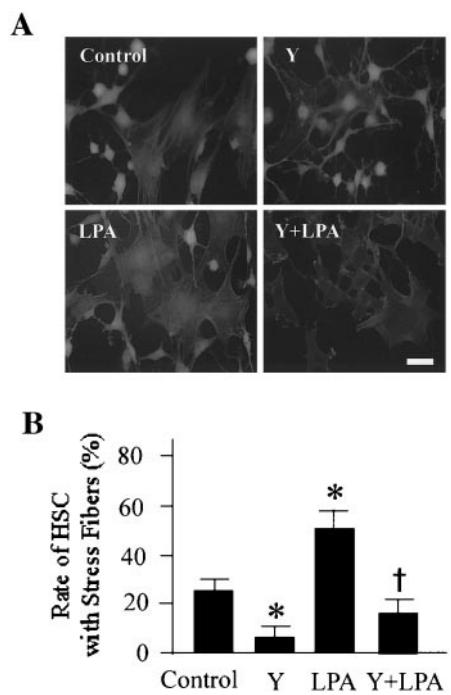

FIG. 3. Fluorescent staining of rat HSCs with rhodamine-conjugated phalloidin for actin stress fibers. (A) The cells were cultured in DMEM containing 10% FCS, and then in serum-free DMEM for 5 h followed by an hour culture in DMEM with or without 10^{-5} M Y-27632. Then, they were cultured for further 30 min after the addition of 10^{-5} M of LPA to the media. Control, nontreated cells; Y, cells in media with Y-27632 alone; LPA, cells in media with LPA alone; Y+LPA, cells in media with Y-27632 and LPA. White bar: 50 μm. (B) Percent of cells showing evident stress fibers for each group presented in A. Data are presented as mean values with standard errors of three experiments. *$P < 0.05$ compared with control; †$P < 0.05$ compared with Y.

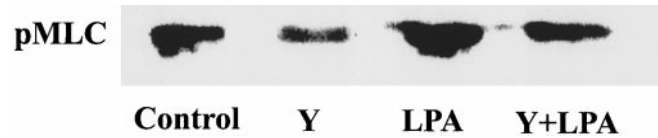

FIG. 4. Western blot analysis of phosphorylated MLC in rat HSCs. The cells were cultured in DMEM containing 10% FCS for 72 h and in serum-free DMEM for 5 h followed by an hour culture in DMEM with or without 10^{-5} M Y-27632. Then, 10^{-5} M of LPA or vehicle was added to the medium, and the cells were harvested 10 min later. A representative immunoblot from three independent experiments is presented. Control, nontreated; Y, treated with Y-27632 alone; LPA, treated with LPA alone; Y+LPA, treated with Y-27632 and LPA.

Some serum-deprived control HSCs were polygonal in shape and contained actin stress fibers (Fig. 3A). In HSCs treated with 10^{-5} M of LPA for 30 min, the assembly of stress fibers was more prominent than in the control cells. When HSCs were incubated with 10^{-5} M of Y-27632 for an hour, they shrank, became round and had fewer stress fibers. However, when 10^{-5} M of LPA was added to the culture medium after preincubation with Y-27632, some of the cells regained their polygonal morphology and showed actin fibers. The percent of cells with evident stress fibers is shown in Fig. 3B for the groups presented in Fig. 3A, and was shown to be significantly increased in cells treated with LPA as compared with untreated control and decreased in those treated with Y-27632. When LPA was added to the culture medium of HSCs preincubated with Y-27632, the number of cells with stress fibers was significantly increased as compared with cells incubated with Y-27632 only. These data suggest that LPA and Y-27632 alter HSC morphology and cytoskeletal organization by inducing a dynamic reorganization of actin filaments.

To study whether contractility of HSCs was associated with increased α-smooth muscle actin level, we performed Western blot analysis on cell lysates. Treatment with 10^{-5} M of LPA and/or 10^{-5} M of Y-27632 for 24 h did not affect concentration of α-smooth muscle actin in HSCs (data not shown). Thus, the transformation from G-actin to F-actin may be induced without change in the total actin protein expression in HSCs stimulated by LPA. Moreover, LPA and Y-27632 showed stimulatory and inhibitory effects, respectively, on phosphorylation of MLC, a key pathway for actomyosin interaction (Fig. 4). The signal inducing actomyosin interaction is transduced through Ca^{2+}/calmodulin-dependent myosin light chain kinase (35), or Rho-kinase (36); the former directly phosphorylates MLC and the latter enhances phosphorylation of MLC by regulating myosin phosphatase. Our data support an involvement of the Rho-kinase pathway.

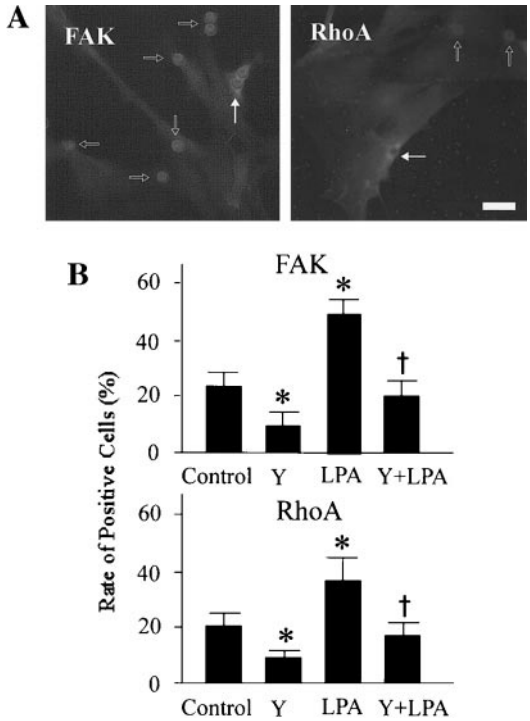

FIG. 5. Immunocytochemistry for FAK and RhoA of rat HSCs seeded with fibronectin-coated beads. The cells were cultured in DMEM containing 10% FCS, and in serum-free DMEM for 5 h followed by an hour culture in DMEM with or without 10^{-5} M Y-27632. Then, the beads were seeded on the cells followed by an additional 30-min culture. In some groups, 10^{-5} M of LPA was added to the media at the time of bead seeding. (A) Closed arrows indicate the beads with the accumulation of FAK or RhoA to its circumference in nontreated control cells; and open arrows, the beads without the accumulation of FAK or RhoA. White bar represents 30 μm. (B) Percent of cells with clustering of FAK or RhoA to the circumference of the beads. Data are presented as mean values with standard errors of three experiments. Control, nontreated; Y, treated with Y-27632 alone; LPA, treated with LPA alone; Y+LPA, treated with Y-27632 and LPA. *$P < 0.05$ compared with control; †$P<0.05$ compared with Y.

To elucidate the effect of LPA on the HSC attachment to extracellular matrix, we studied the intracellular movement of signal peptides related to focal adhesions, i.e., FAK and RhoA, and the cellular adhesion to the extracellular substrate using ECIS. Focal adhesions, to which actin stress fibers attach, transmit cellular tension to the extracellular matrix. When fibronectin-coated beads were seeded on HSCs, some HSCs showed clustering of FAK and/or RhoA around the beads (Fig. 5A). When albumin-coated beads were seeded on the cells as a negative control, such clustering was not elicited (data not shown). Figure 5B shows the percent of cells with clustering of FAK or RhoA to the circumference of the fibronectin-coated beads. In the cells treated with 10^{-5} M of LPA for 30 min, the percent of cells with clustering of FAK or RhoA was higher than that in the untreated control, whereas it was decreased in the cells pretreated with 10^{-5} M of Y-27632. The decrease in the percent caused by Y-27632 pretreatment was attenuated by the further addition of LPA. LPA elicits the recruitment of FAK to focal adhesions (37) and RhoA to subplasmalemma (38). It was also reported that Rho-kinase contributes to formation of focal adhesion (16) and further activation of Rho (39). Based on these observations and findings described in this communication, we suggest that Rho-kinase is involved in LPA-induced formation of focal adhesions and recruitment of FAK and RhoA.

In the electric cell-substrate impedance sensing system (ECIS), the voltage between a small gold electrode seeded with cells and a larger counter electrode placed at a distance, is measured on charging a constant small alternating current. Because cell membrane has a very high impedance, the attachment of cells to the small electrode causes the blockage of electric flow, resulting in an increase of the impedance. Thus, the obtained resistance reflects the cell-attached area of the electrode. Besides, ECIS can monitor the cellular attachment over time. In the present experiment, we applied ECIS for the examination of the adhesion of HSCs for the first time. HSCs were seeded loosely on the electrode. The initial resistance levels measured just before the addition of reagents were similar for all the measurements, suggesting that the cells were seeded on the electrode at a similar density in each determination. Figure 6 shows the change of resistance measured by ECIS in HSCs treated with LPA and/or Y-27632. The normalized resistance was significantly increased in the cells treated with LPA compared with

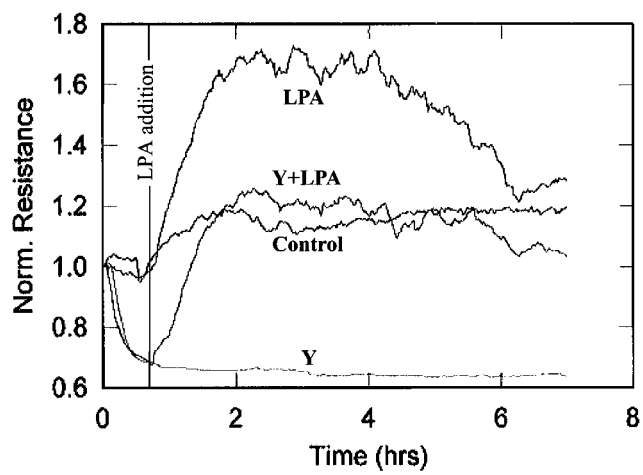

FIG. 6. Changes of resistance determined by ECIS in rat HSCs treated with LPA and/or Y-27632. When HSCs were cultured in DMEM with or without 10^{-5} M of Y-27632 for 40 min, 10^{-5} M of LPA was added to the media. A typical representative tracing from four replicate experiments is presented. In all the measurements, the initial resistance measured just before the addition of Y-27632 was ranging from 3000 to 3500 Ω. Ordinate represents the resistance normalized as a ratio to the initial resistance measured just before the addition of Y-27632. Control, nontreated; Y, treated with Y-27632 alone; LPA, treated with LPA alone; Y+LPA, treated with Y-27632 and LPA.

the untreated control, whereas it was decreased in the cells treated with Y-27632. When LPA was added to HSCs preincubated with Y-27632, the resistance was increased compared with the cells incubated with Y-27632 only. These results suggest that adhesion of HSCs to extracellular substrate is augmented with the LPA treatment, while it is suppressed in the presence of Y-27632.

Recently, attention has been paid to the role of platelets in the pathogenesis of liver injury. The activation of platelets was noted in patients with acute or chronic liver disease (40, 41). In the rat hepatic allograft preservation injury, prolongation of cold preservation or rewarming before reperfusion accelerated liver injury in concordance with the increase of activated and degranulated platelets which adhered to endothelial cells (42). It has been reported that the serum level of LPA reached a range of 2 to 20 μM in the process of blood-clotting (5, 43). In patients with several kinds of cancers, plasma levels of LPA were elevated to levels comparable to that of serum (44, 45).Thus, the concentrations of LPA used here are considered to occur *in vivo*, although the physiological and/or pathological roles of LPA *in vivo* should be further investigated.

From our observations, it is possible to consider that LPA enhances the tension of cultured HSCs, resulting in the enhancement of the contraction of collagen gel lattices. In conclusion, LPA can enhance the remodeling of collagen matrix by HSCs, in a manner supporting the involvement of Rho-kinase through modulation of cellular morphology and attachment to extracellular matrices.

ACKNOWLEDGMENTS

The authors thank Dr. Masaaki Higashihara (Kitasato University, Kanagawa, Japan) for experimental advice. This work was supported in part by NIH Grant RO1 AA09231 (M.R.) and was started at the Marion Bessin Liver Research Center of the Albert Einstein College of Medicine (Bronx, NY). This work was also supported by grants-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan (09670512).

REFERENCES

1. Wake, K. (1999) Hepatic stellate cells. *In* Liver Diseases and Hepatic Sinusoidal Cells (Tanikawa, K., and Ueno, T., Eds.), pp. 56–65, Springer-Verlag, Tokyo.
2. Friedman, S. L. (1993) Seminars in medicine of the Beth Israel Hospital, Boston. The cellular basis of hepatic fibrosis. Mechanisms and treatment strategies. *N. Engl. J. Med.* **328,** 1828–1835.
3. Bissell, D. M. (1998) Hepatic fibrosis as wound repair: A progress report. *J. Gastroenterol.* **33,** 295–302.
4. Moolenaar, W. H., Kranenburg, O., Postoma, F. R., and Zondag, G. C. M. (1997) Lysophosphatidic acid: G-protein signalling and cellular responses. *Curr. Opin. Cell Biol.* **9,** 168–173.
5. Goetzl, E. J., and An, S. (1998) Diversity of cellular receptors and functions for the lysophospholipid growth factors lysophosphatidic acid and sphingosine 1-phosphate. *FASEB J.* **12,** 1589–1598.
6. Fukami, K., and Takenawa, T. (1992) Phosphatidic acid that accumulates in platelet-derived growth factor-stimulated Balb/c 3T3 cells is a potential mitogenic signal. *J. Biol. Chem.* **267,** 10988–10993.
7. Valet, P., Pages, C., Jeanneton, O., Daviaud, D., Barbe, P., Record, M., Saulnier-Blache, J. S., and Lafontan, M. (1998) Alpha2-adrenergic receptor-mediated release of lysophosphatidic acid by adipocytes. A paracrine signal for preadipocyte growth. *J. Clin. Invest.* **101,** 1431–1438.
8. Fourcade, O., Simon, M. F., Viode, C., Rugani, N., Leballe, F., Ragab, A., Fournie, B., Sarda, L., and Chap, H. (1995) Secretory phospholipase A2 generates the novel lipid mediator lysophosphatidic acid in membrane microvesicles shed from activated cells. *Cell* **80,** 919–927.
9. Siess, W., Zangl, K. J., Essler, M., Bauer, M., Brandl, R., Corrinth, C., Bittman, R., Tigyi, G., and Aepfelbacher, M. (1999) Lysophosphatidic acid mediates the rapid activation of platelets and endothelial cells by mildly oxidized low density lipoprotein and accumulates in human atherosclerotic lesions. *Proc. Natl. Acad. Sci. USA* **96,** 6931–6936.
10. Ridley, A. J., and Hall, A. (1992) The small GTP-binding protein Rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70,** 389–399.
11. Narumiya, S., Ishizaki, T., and Watanabe, N. (1997) Rho effectors and reorganization of actin cytoskeleton. *FEBS Lett.* **410,** 68–72.
12. Matsui, T., Amano, M., Yamamoto, T., Chihara, K., Nakafuku, M., Ito, M., Nakano, T., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996) Rho-associated kinase, a novel serine/threonine kinase, as a putative target for the small GTP binding protein Rho. *EMBO J.* **15,** 2208–2216.
13. Ishizaki, T., Maekawa, M., Fujisawa, K., Okawa, K., Iwamatsu, A., Fujita, A., Watanabe, N., Saito, Y., Kakizuka, A., Morii, N., and Narumiya, S. (1996) The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. *EMBO J.* **15,** 1885–1893.
14. Nakagawa, O., Fujisawa, K., Ishizaki, T., Saitoh, Y., Nakao, K., and Narumiya, S. (1996) ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming serine/threonine protein kinase in mice. *FEBS Lett.* **392,** 189–193.
15. Leung, T., Manser, E., Tan, L., and Lim, L. (1995) A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. *J. Biol. Chem.* **270,** 29051–29054.
16. Amano, M., Chihara, K., Kimura, K., Fukata, Y., Nakamura, N., Matsuura, Y., and Kaibuchi, K. (1997) Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. *Science* **275,** 1308–1311.
17. Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yagagami, K., Inui, J., Maekawa, M., and Narumiya, S. (1997) Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* **389,** 990–994.
18. Yoshii, A., Iizuka, K., Dobashi, K., Horie, T., Harada, T., Nakazawa, T., and Mori, M. (1999) Relaxation of contracted rabbit tracheal and human bronchial smooth muscle by Y-27632 through inhibition of Ca^{2+} sensitization. *Am. J. Respir. Cell Mol. Biol.* **20,** 1190–1200.
19. Itoh, K., Yoshioka, K., Akedo, H., Uehata, M., Ishizaki, T., and Narumiya, S. (1999) An essential part for Rho-associated kinase in the transcellular invasion of tumor cells. *Nature Med.* **5,** 221–225.
20. Chihara, K., Amano, M., Nakamura, N., Yano, T., Shibata, M.,

Tokui, T., Ichikawa, H., Ikebe, R., Ikebe, M., and Kaibuchi, K. (1997) Cytoskeletal rearrangements and transcriptional activation of c-fos serum response element by Rho-kinase. *J. Biol. Chem*. **272,** 25121–25127.

21. Ikeda, H., Yatomi, Y., Yanase, M., Satoh, H., Nishihara, A., Kawabata, M., and Fujiwara, K. (1998) Effects of lysophosphatidic acid on proliferation of stellate cells and hepatocytes in culture. *Biochem. Biophys. Res. Commun*. **248,** 436–440.
22. Friedman, S. L., and Roll, F. J. (1987) Isolation and culture of hepatic lipocytes, Kupffer cells, and sinusoidal endothelial cells by density gradient centrifugation with Stractan. *Anal. Biochem*. **161,** 1233–1247.
23. Thomson, F. J., Perkins, L., Ahern, D., and Clark, M. (1994) Identification and characterization of a lysophosphatidic acid receptor. *Mol. Pharmacol*. **45,** 718–723.
24. Montesano, R., and Orci, L. (1988) Transforming growth factor β stimulates collagen-matrix contraction by fibroblasts: Implications for wound healing. *Proc. Natl. Acad. Sci. USA* **85,** 4894–4897.
25. Rockey, D. C., Housset, C. N., and Friedman, S. L. (1993) Activation-dependent contractility of rat hepatic lipocytes in culture and in vivo. *J. Clin. Invest*. **92,** 1795–1804.
26. Curtis, A. S., McGrath, M., and Gasmi, L. (1992) Localised application of an activating signal to a cell: Experimental use of fibronectin bound to beads and the implications for mechanisms of adhesion. *J. Cell Sci*. **101,** 427–436.
27. Clancy, R. M., Rediske, J., Tang, X., Nijher, N., Frenkel, S., Philips, M., and Abramson, S. B. (1997) Outside-in signaling in the chondrocyte: Nitric oxide disrupts fibronectin-induced assembly of a subplasmalemmal actin/RhoA/focal adhesion kinase signaling complex. *J. Clin. Invest*. **100,** 1789–1796.
28. Giaever, I., and Keese, C. R. (1984) Monitoring fibroblast behavior in tissue culture with an applied electric field. *Proc. Natl. Acad. Sci. USA* **81,** 3761–3764.
29. Noiri, E., Hu, Y., Bahou, W. F., Keese, C. R., Giaever, I., and Goliogorsky, M. S. (1997) Permissive role of nitric oxide in endothelin-induced migration of endothelial cells. *J. Biol. Chem*. **272,** 1747–1752.
30. van der Bend, R. L., Brunner, J., Jalink, K., van Corven, E. J., Moolenaar, W. H., and van Blitterswijk, W. J. (1992) Identification of a putative membrane receptor for the bioactive phospholipid, lysophosphatidic acid. *EMBO J*. **11,** 2495–2501.
31. Hecht, J. H., Weiner, J. A., Post, S. R., and Chun, J. (1996) Ventricular zone gene-1 (vzg-1) encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex. *J. Cell Biol*. **135,** 1071–1083.
32. An, S., Dickens, A., Bleu, T., Hallmark, O. G., and Goetzl, E. J. (1997) Molecular cloning of the human Edg2 protein and its identification as a functional cellular receptor for lysophosphatidic acid. *Biochem. Biophys. Res. Commun*. **231,** 619–622.
33. An, S., Bleu, T., Hallmark, O. G., and Goetzl, E. J. (1998) Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. *J. Biol. Chem*. **273,** 7906–7910.
34. Bandoh, K., Aoki, J., Hosono, H., Kobayashi, S., Kobayashi, T., Murakami-Murofushi, K., Tsujimoto, M., Arai, H., and Inoue, K. (1999) Molecular cloning and characterization of a novel human G- protein- coupled receptor, Edg 7, for lysophosphatidic acid. *J. Biol. Chem*. **274,** 27776–27785.
35. Ikebe, M., and Hartshorne, D. J. (1985) Phosphorylation of smooth muscle myosin at two distinct sites by myosin light chain kinase. *J. Biol. Chem*. **260,** 10027–10031.
36. Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996) Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* **273,** 245–248.
37. Barry, S. T., and Critchley, D. R. (1994) The RhoA-dependent assembly of focal adhesions in Swiss 3T3 cells is associated with increased tyrosine phosphorylation and the recruitment of both pp125FAK and protein kinase C-δ to focal adhesions. *J. Cell Sci*. **107,** 2033–2045.
38. Kranenburg, O., Poland, M., Gebbink, M., Oomen, L., and Moolenaar, W. H. (1997) Dissociation of LPA-induced cytoskeletal contraction from stress fiber formation by differential localization of RhoA. *J. Cell Sci*. **110,** 2417–2427.
39. Hirose, M., Ishizaki, T., Watanabe, N., Uehata, M., Kranenburg, O., Moolenaar, W.,H., Matsumura, F., Maekawa, M., Bito, H., and Narumiya, S. (1998) Molecular dissection of the Rho-associated protein kinase (p160ROCK)-regulated neurite remodeling in neuroblastoma N1E-115 cells. *J. Cell Biol*. **141,** 1625–1636.
40. Hughes, R. D., Lane, D. A., Ireland, H., Langley, P. G., Gimson, A. E., and Williams, R. (1985) Fibrinogen derivatives and platelet activation products in acute and chronic liver disease. *Clin. Sci. Colch*. **68,** 701–707.
41. Luzzatto, G., Fabris, F., Gerunda, G. E., Zangrandi, F., and Girolami, A. (1987) Failure of two anti-platelet drugs (indobufen and dipyridamole) to improve thrombocytopenia in liver cirrhosis. *Acta Hematol*. **77,** 101–106.
42. Cywes, R., Packham, M. A., Tietze, L., Sanabria, J. R., Harvey, P. R., Phillips, M. J., and Strasberg, S. M. (1993) Role of platelets in hepatic allograft preservation injury in the rat. *Hepatology* **18,** 635–647.
43. Tokumura, A., Iimori, M., Nishioka, Y., Kitahara, M., and Sakashita, M. (1994) Lysophosphatidic acids induce proliferation of cultured vascular smooth muscle cells from rat aorta. *Am. J. Physiol*. **267,** C204–C210.
44. Xu, Y., Shen, Z., Wiper, D. W., Wu, M., Morton, R. E., Elson, P., Kennedy, A. W., Belinson, J., Markman, M., and Casey, G. (1998) Lysophosphatidic acid as a potential biomarker for ovarian and other gynecologic cancers. *JAMA* **280,** 719–723.
45. Sasagawa, T., Okita, M., Murakami, J., Kato, T., and Watanabe, A. (1999) Abnormal serum lysophospholipids in multiple myeloma patients. *Lipids* **34,** 17–21.