GPR92/LPA5 lysophosphatidate receptor mediates megakaryocytic cell shape change induced by human atherosclerotic plaques

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Aims
Oxidative processes and vascular inflammation underlying atherosclerosis lead to an accumulation of lysophosphatidic acid (LPA) molecules in the atheromatous intima. LPA, a platelet-activating component of human atherosclerotic plaques, possibly contributes to atherothrombus formation after plaque rupture. Human platelets express mRNA for the G protein-coupled receptors LPA1–7 that derive from megakaryocytes. The aim of our study was to identify the functional LPA receptor(s) in human platelets by silencing individual LPA receptors in megakaryocytic (MK) cells.

Methods and results
We studied shape change of two human MK cell lines (Meg-01, Dami) by turbidometry, phase-contrast and scanning electron microscopy. They showed upon LPA stimulation a rapid, Rho-kinase-mediated shape change similar to that of human platelets. By qRT–PCR analysis we found expression of LPA1–7 in both cell lines; LPA4 and LPA5 were the most abundant receptor transcripts. In both Meg-01 and Dami cells, the rank order of activation by LPA species was similar to that found in platelets: alkyl-LPA 18:1 > alkyl-LPA 16:0 > acyl-LPA 18:1 > alkyl-LPA 18:0. Knock-down of individual LPA receptors by siRNA showed that LPA-mediated activation of MK cells was mediated by LPA5, but not by LPA1 – 4,6,7. Importantly, we found that human atherosclerotic plaque and lipid-rich core induced shape change of Dami cells, and that this effect was inhibited after LPA5 silencing.

Conclusions
Our findings indicate that LPA5 mediates LPA-induced shape change of MK cells and support its involvement in atherosclerotic plaque and lipid-rich core-mediated platelet activation. This receptor could be an attractive novel target for antithrombotic therapy.

Keywords
Atherosclerosis • Thrombosis • Plaque rupture • Platelets • Receptors

1. Introduction
During the progression of atherosclerosis, oxidative processes and vascular inflammation lead to an accumulation of lysophosphatidic acid (LPA) in the arterial intima.1–3 LPA consists of various molecular species with different platelet-activating potency.4,5 In atherosclerotic plaques, LPA molecules of high platelet-activating potency have been identified that mediate the initial platelet response—shape change—by the plaque lipid-rich core, and induce synergistically with other platelet stimuli (ADP, epinephrine) aggregation of isolated platelets.6 Moreover, LPA at concentrations approaching those found in vivo induces platelet activation in whole blood.5 Thus, after rupture of lipid-rich atherosclerotic plaques, LPA exposed to circulating platelets might contribute to the formation of intravascular thrombi responsible for acute coronary syndrome, myocardial infarction, and ischaemic stroke.1,2
LPA binds to G-protein-coupled receptors (GPCRs) on the platelet surface. The signal emitted by the activated receptor(s) is transduced by G_{12/13} proteins to activation of Rho, Rho-kinase, LIM-kinase 1, and subsequent phosphorylation of cytoskeletal proteins such as myosin light chain, moesin, and cofilin. These biochemical events cause specific actin remodelling leading to platelet shape change. Cytosolic Ca^{2+} increase and Rac activation are not involved in LPA-induced shape change.

LPA GPCRs are divided into two subfamilies: one is composed of three members, LPA_1, LPA_2, and LPA_3 belonging to the Endothelial Differentiation Gene (EDG-) subfamily and the second is the purinoreceptor (P2Y) cluster of GPCRs. This subfamily has grown considerably in recent years, and it now consists of five LPA receptors: LPA_4 (GPR23), LPA_5 (GPR92), LPA_6 (GPR87), LPA_7 (P2Y5), and LPA_8 (P2Y10). However, there remain some doubts whether LPA_4, LPA_6, and LPA_8 are functional LPA receptors or not.

Human platelets express mRNA for LPA_{1-7}, and the most abundant are LPA_4 and LPA_5 transcripts. The expression of LPA receptors at the protein level is unknown due to the lack of specific antibodies. A previous study has suggested a role of LPA_4 and LPA_3 in LPA-induced platelet activation, whereas two recent studies favour the involvement of other receptors such as LPA_4 and LPA_5 in LPA-induced platelet shape change. However, firm evidence that these receptors mediate platelet stimulation by LPA and plaque lipid-rich core is lacking: The LPA response of platelets did not match with the pharmacological properties of the heterologously expressed LPA_4 and LPA_5 receptors, and the pharmacological receptor agonists and antagonists used were not selective for LPA_5. The role of the newly discovered LPA receptors LPA_6 (GPR87) and LPA_7 (P2Y5) in LPA-mediated platelet activation has not been studied so far. Therefore, the functional platelet LPA receptor(s) remains elusive.

In the present study, we set out to identify the functional platelet LPA receptor by applying siRNA interference technology to selectively knock-down LPA_1–7 receptors in megakaryocytes. Platelets derive from megakaryocytes, and, based on studies showing the presence of various platelet receptors on the surface of megakaryocytes, we reasoned that megakaryocytes might also express functional platelet LPA receptors. In line with this assumption, a previous study used human megakaryocytic (MK) cell lines to discover the P2Y_1 receptor in platelets.

**2. Methods**

**2.1 Materials**

LPA species were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and Echelon Biosciences (Salt Lake City, UT, USA). Human thrombin (T-7009) was from Sigma-Aldrich. Fatty acid-free bovine serum albumin was obtained from Fluka (Taufkirchen, Germany). The RNasey mini kit, Omniscript reverse transcriptase kit, and QuantFast SYBR Green RT–PCR kit were from Qiagen (Hilden, Germany). Alexa Fluor-546 phalloidin was from Molecular Probes (Eugene, OR). Y-27632 [(+)-(R)-trans-4-(1-aminomethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride] was obtained from Merck Biosciences GmbH (Schwalbach, Germany), and Collagen (Horm) was obtained from Nycomed Pharma (Munich, Germany).

**2.2 Isolation of human carotid atherosclerotic plaques and lipid-rich core**

The investigation conforms with the principles outlined in the Declaration of Helsinki for use of human tissue. Atherosclerotic tissue specimens were obtained from patients who had undergone operations for high-grade carotid stenosis as described previously. Written consent of the patients was obtained, and the study was approved by the Ethics Committee of the Faculty of Medicine of the University of Munich. Atherosomatous plaques were carefully dissected from other regions of the atherosclerotic tissue specimens. The plaque samples were weighed, homogenized in an ice-cold N_2 saturated buffer (150 mM NaCl, 1 mM EDTA, pH 7.4), and stored at −80°C. In some atheromatous plaque specimens, it was possible to excise the lipid-rich core from the surrounding fibrous capsule. The concentrations of atheromatous plaque and lipid-rich core were adjusted to 50 and 100 mg wet weight/mL, respectively.

**2.3 Cultures of MK cell lines and measurement of shape change**

Dami and Meg-01 (American Type Culture Collection, ATCC) cells were grown in plastic culture flasks in RPMI 1640 medium supplemented with 10% FCS, 100 U/mL of penicillin, and 100 U/mL of streptomycin at 37°C in a humidified atmosphere with 5% CO_2. They were subcultured twice a week to maintain a concentration of 0.5 × 10^6 cells/mL.

For measurement of shape change, MK cells were washed twice in PBS and resuspended in HEPES–Tyrode’s buffer (15 mM HEPES, 140 mM NaCl, 2.7 mM KCl, 1 mM MgCl_2, 0.1% glucose, 3.75 mM NaH_2PO_4, 24 mM NaHCO_3, pH 7.5) to obtain a cell density of 4 × 10^6 cells/mL. Shape change of the stirred (1100 rpm) MK suspension was measured by the decrease of light transmission at 37°C in a LABOR aggregometer (Fresenius, Germany).

**2.4 Microscopy of fixed and living cells**

Shape change of Dami and Meg-01 cells was morphologically examined after phalloidin staining of F-actin as described previously. Phase contrast microscopy revealed the shape change of platelets in the MK cell lines. Platelet shape change increased in the presence of LPA species and was inhibited by Y-27632.

**Figure 1** Expression of LPA_{1-7} transcripts in Dami and Meg-01 cells. (A) LPA_{1-7} receptor mRNA expression as shown by PCR. (B) Relative abundance of LPA receptor transcripts in Dami and Meg-01 cell lines measured by quantitative RT–PCR. Values represent the mean ± SD from three different experiments.
and fluorescence microscopy was performed using a Nikon TE2000 microscope. The imaging of living cells was carried at 37°C in an incubation chamber. The images were taken with a cooled CCD camera and analysed using the manufacturer’s software NIS elements 3.0.

2.5 Scanning electron microscopy

Dami and Meg-01 cells were prepared as described above and samples were exposed to LPA or solvent for 2 min whilst stirring in the aggregometer and then fixed with 3% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.3). Samples were then filtered through a 0.2-µm Nucleopore filter followed by 10 mL of prefiltered distilled water. Filters were dehydrated with graded ethanol (from 35 to 100%), soaked for 10 min in hexamethyldisilazane, dried, and coated with a thin layer of gold (Bal-Tec Sputter Coater SCD 050, Balzers Union AG, Balzers, Liechtenstein). Imaging was performed using a Zeiss Supra 55 VP scanning electron microscope.

2.6 Quantitative PCR of LPA receptor mRNA in MK cells

Total cellular RNA was extracted from Dami or Meg-01 cells using the RNeasy mini kit (Qiagen), treated with RNase-free DNase. For the reverse-transcription synthesis of cDNA, the Omniscript reverse transcriptase kit with random hexamer primers was used. Quantitative RT–PCR was then performed in a Real-Time PCR iCycler using the QuantFast SYBR Green RT–PCR Kit according to the manufacturer’s protocols. The primer sets were as follows: LPA1 (forward) CACAGTCAGCAAAGTGGTGTGATG and (reverse) TCTCCGAGTATTGGGTGTCTTG; LPA2 (forward) GGCACAGCCTCCGCTTAATC and (reverse) CAATGAGCATGACCAGCCGGCTGA; LPA3 (forward) GGCACATGCTCAATGGG and (reverse) ATGATGAGGAAGGCCATGAG; LPA4 (forward) ACCCACCCTGACTTGAAGG and (reverse) AGAGTTGCAAGGCCAACAGGT; LPA5 (forward) CTCCGTGGTGAAGGGTGTTA and (reverse) ATGATGAGGAAGGCCATGAG; LPA6 (forward) GGCAGTGCCAAGACAAAA and (reverse) GGAGAAAACTG; LPA7 (forward) GGAACTAAACAAAACTAA and (reverse) GTCTCTAGTGTGGCAGGTAACAA; β-actin (forward) CACCCACCTCTACAATGTGAG and (reverse) CAGAGCGGTACCAGGATAGG.

The RT–PCRs were performed in triplicate on each cDNA template along with triplicate reactions of the housekeeping gene, β-actin. Negative control was obtained by performing real-time RT–PCR without cDNA. All RT–PCR products were verified by melting-curve analysis and agarose gel electrophoresis. The LPA receptor mRNA expression levels

![Figure 2](https://academic.oup.com/cardiovascres/article-abstract/90/1/157/285072)
were determined by the comparative Ct method, normalizing expression to β-actin.

2.7 siRNA transfection

The human LPA receptors siGENOME SMARTpool siRNAs and control non-targeting siRNA were obtained from Dharmacon (Lafayette, CO, USA). Sequences of siRNA against LPA_1–7 are listed in Supplementary material online. Table. siRNA transfection was performed as described previously. Briefly, prior to this procedure, cells were pelleted by centrifugation at 200 g for 5 min then washed twice with PBS. After a final centrifugation, cells were resuspended at 5 x 10^5 cells/mL in serum/antibiotic-free RPMI 1640 medium with oligofectamine-complexed siRNA duplexes. Transfections were carried out twice with a 24-h interval and fetal calf serum replenished to 10% 4 h post-transfection. The cells were used for experiments at 72 h after transfection. The percentage of relative gene expression was calculated as the relative amount of LPA receptor mRNA in cells transfected with LPA receptor targeting siRNA compared with that of cells transfected with the non-targeting control siRNA which was set to 100%.

2.8 Statistical analysis

Values given are mean ± SD. Significant difference was determined by the paired Student’s t-test or other tests as appropriated. A P-value of <0.05 was considered statistically significant. Logarithmic median effective concentrations (LogEC_{50}) were calculated from respective binding curves using the web-based BioDataFit software.

3. Results

3.1 Expression of LPA receptor mRNAs in human MK cell lines

Both Dami and Meg-01 cells expressed LPA_1–7 transcripts. Agarose gel electrophoresis of the PCR products revealed fragments of the expected sizes (Figure 1A). DNA sequencing of the PCR products confirmed the correct sequences of the LPA receptors. Similar to platelets, quantitative RT–PCR analysis of LPA receptors showed that LPA_4 and LPA_5 receptors were the most abundant in Dami and Meg-01 cell lines (Figure 1B). Expression of LPA_4 transcripts was similar in both cell lines, whereas LPA_5 was more prominent in Dami cells. Interestingly, in Dami cells, LPA_3 receptor expression was also pronounced, and similar to that of LPA_4.

3.2 The effect of LPA on MK cell lines

In order to analyse whether LPA can also activate MK cell lines, stirred Dami and Meg-01 cell suspensions were exposed to LPA, and shape change was measured by the decrease of light transmission in an aggregometer. Figure 2A shows that LPA (50 nM) rapidly induced shape change of Dami cells which was maximum within 60 s. Moreover, similar to platelets, LPA-mediated shape change in MK cells was reversible (see Supplementary material online. Figure S1).

Previously it has been shown that LPA stimulates platelet shape change through activation of the Rho/Rho-kinase pathway. Therefore, we examined whether Dami cell activation by LPA was also Rho-kinase dependent. Pre-incubation of Dami cells with the Rho-kinase inhibitor Y-27632 (20 μM) completely inhibited LPA-induced shape change (Figure 2A). Moreover, similar to platelets, the pre-incubation of MK cells with albumin dose-dependently inhibited the LPA-elicted shape change response with an IC_{50} of 2.84 ± 1.06 and 4.14 ± 1.02 μM, in Dami and Meg-01 cells, respectively (see Supplementary material online, Figure S2).

To observe the shape change morphologically, MK cells were fixed and visualized by phase-contrast and fluorescence microscopy after F-actin staining with Alexa Fluor 546 phalloidin (Figure 2B). Unstimulated Dami and Meg-01 cells (control) were spherical and only a few cells had pseudopods (Figure 2B). Upon LPA stimulation, the cell surface became irregular; Meg-01 cells formed mainly multiple pseudopods and extensive membrane blebbing at several sites on cell surface.

![Figure 2](https://academic.oup.com/cardiovascres/article-abstract/90/1/157/285072/fig2)

**Figure 2** Concentration–response curves of Dami and Meg-01 cells stimulated by various LPA species. Suspensions of Dami (A) and Meg-01 (B) cells were stirred at 37°C for 2 min and then exposed to increasing concentrations of LPA species. The decrease in light transmission was measured. Values represent the mean ± SD from three different experiments.

![Figure 3](https://academic.oup.com/cardiovascres/article-abstract/90/1/157/285072/fig3)

**Figure 3** Comparison of activities of different molecular species of LPA on MK cells and platelets

### Table 1 Comparison of activities of different molecular species of LPA on MK cells and platelets

<table>
<thead>
<tr>
<th>LPA species</th>
<th>Dami (μM)</th>
<th>Meg-01 (μM)</th>
<th>Platelets (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkyl-LPA 16:0</td>
<td>2.80 ± 0.13</td>
<td>1.76 ± 0.08</td>
<td>1.66 ± 0.28</td>
</tr>
<tr>
<td>Alkyl-LPA 18:1</td>
<td>2.46 ± 0.29</td>
<td>2.02 ± 0.14</td>
<td>2.80 ± 0.13</td>
</tr>
<tr>
<td>Acyl-LPA 18:1</td>
<td>1.84 ± 0.04</td>
<td>1.48 ± 0.09</td>
<td>2.18 ± 0.48</td>
</tr>
<tr>
<td>Alkyl-LPA 18:0</td>
<td>1.52 ± 0.08</td>
<td>1.16 ± 0.07</td>
<td>1.66 ± 0.28</td>
</tr>
</tbody>
</table>

Dami and Meg-01 LogEC_{50} values were calculated from the concentration–response curves using the web-based BioDataFit software (n = 3, mean ± SD). Platelet EC_{50} values are from Ref. 20.

These results suggest that LPA activates MK cells through Rho-kinase-dependent pathways, and the receptor expression patterns vary between Dami and Meg-01 cells.
the cell, whereas Dami cells formed pseudopods and often long pro-
trusions (Figure 2B). Scanning electron microscopy of MK cells showed
that untreated cells had numerous microvillous projections; after LPA
activation, Dami and Meg-01 cells formed multiple pseudopods and
membrane blebs, and Dami cells produced long protrusions
(Figure 2D).

To observe the shape change in more detail, live microscopy of
Dami and Meg-01 cells was performed (see Supplementary material
online, Videos). Also under non-stirring conditions, LPA rapidly
induced shape change of MK cells within 2 min. Dami cells in contrast
to Meg-01 dramatically formed long protrusions (1–3 per cells).
Meg-01 cells produced mostly short pseudopods and extensive mem-
brane blebbing at several sites of the cell. By analysis of Dami cells
before and 2 min after LPA stimulation, we found that the cell diam-
eter was reduced from $12.64 \pm 1.32$ to $11.4 \pm 1.93$ μm ($P = 0.019$),
and the length of protrusions was $10 \pm 3$ μm.

3.3 MK cell lines and platelets show similar structure–activity relationships
The LPA response curves of platelets show a preference of alkyl-LPA
over acyl-LPA.2 We studied LPA molecular species on MK cells which
had shown different platelet activating potency.7,20 The concentra-
tion–response curves of four LPA species tested were similar in the
two MK cell lines. The rank order of potency was: alkyl-LPA
18:1 > alkyl-LPA 16:0 > acyl-LPA 18:1 > alkyl-LPA 18:0 (Figure 3,
Table 1). The EC50 values of the different LPA species were in a
similar range in Dami, Meg-01, and platelets (Table 1).

3.4 LPA induces Dami and Meg-01 cell activation through the LPA5 receptor
Since Dami and Meg-01 cells express all known LPA receptors, we
applied the siRNA interference technology to identify the functionally
relevant LPA receptor subtypes in MK cell lines. LPA receptors were
down-regulated by specific siRNAs targeting LPA1–7. Real-time PCR
analysis showed a 60–80% reduction of LPA1–7 in Dami cells
(Figure 4A). The effect of LPA receptor down-regulation was analysed
by shape change measurement in the aggregometer as well as by
phase-contrast microscopy (Figure 4B–D). Silencing of the LPA5
receptor reduced the LPA-induced activation of Dami cells to $34 \pm $10% of control ($P < 0.001; n = 6) In addition, down-regulation of
LPA5 in Meg-01 cells also inhibited the LPA-induced activation
(Figure 4D). Silencing of LPA5 receptor had no effect on

![Figure 4](https://academic.oup.com/cardiovascres/article-abstract/90/1/157/285072)

**Figure 4** LPA5 knock-down inhibits shape change of Dami and Meg-01 cells. (A) LPA1–7 mRNA levels after selective receptor knock-down. Dami
cells were treated for 72 h with specific LPA receptor siRNA or non-target siRNA. The expression level was analysed by RT–PCR. (B) Effect of LPA
receptor silencing on LPA-induced Dami cell shape change as measured by the decrease of light transmission. Dami cells treated for 72 h with specific
LPA receptor siRNA or non-target siRNA (control) were exposed to acyl-LPA 16:0. Values are mean $\pm$ SD ($n = 3$; significance was tested by
ANOVA, Bonferroni was used as post-test $^*P < 0.001$). (C) Quantification of activated cells after contrast microscopy. Dami cells treated for 72 h with
specific LPA5 receptor siRNA or non-target siRNA (control) were exposed to acyl-LPA 16:0. Cells were fixed and counted at $\times 40$ magnification, and
the total numbers of cells in 10 randomly selected fields were determined. Values are mean $\pm$ SD ($n = 3$). (D) Representative tracings of LPA-induced
shape change of Dami and Meg-01 cells after treatment with specific LPA receptor siRNA or non-target siRNA.
thrombin-induced shape change in both MK cell lines (see Supplementary material online, Figure S3).

3.5 The involvement of LPAS receptor in lipid-rich core- and plaque-induced MK cell activation

Human plaque homogenates contain platelet-activating collagenous structures and various LPA molecules which activate platelets, whereas the plaque lipid-rich core contains LPA species but lacks collagenous structures.

In order to identify the possible role of LPAS in plaque-mediated platelet activation, we first analysed whether lipid-rich core, plaque, and collagen can induce shape change of Dami cells. We used concentrations of these stimuli that induced maximal platelet activation as shown in previous studies.

Plaque and lipid-rich core induced shape change as observed by the decrease in light transmission, phase-contrast microscopy, and scanning electron microscopy (Figure 5A and C; see Supplementary material online, Figure S4). In contrast, collagen did not activate these cells (Figure 5A). These results indicate that lipids such as LPA rather than collagen may mediate Dami cell shape change by plaque. Indeed, shape change of Dami cells induced by the lipid-rich core and plaque was inhibited after down-regulation of LPAS (Figure 5B and C). Thus, the LPAS receptor appears to mediate plaque-induced activation of Dami cells.

4. Discussion

The present study was undertaken to identify the functional LPA receptor(s) in human MK cells. We used these cells as a model system for platelets to allow a conclusion about the functional platelet LPA receptor. Previously, human MK cell lines have been successfully used to identify the P2Y1 receptor in platelets. In the present study, we found that two human MK cell lines (Dami and Meg-01) behaved similarly in many ways to platelets in their response to LPA: (a) the two MK cell lines showed a rapid shape change after exposure to low LPA concentrations; (b) the shape change was Rho-kinase mediated; (c) low concentrations of albumin inhibited the LPA-elicited shape change of these cells with a low IC50 comparable to the IC50 in platelets; and (d) we also studied the activity of four different LPA species in these MK cells and found that the structure–activity relationships of Dami and Meg-01 cells were not only similar to each other, but they were also comparable with that of platelets.

These similarities argue for the existence of the same functional LPA receptor(s) on the surface of the two MK cell lines and platelets.
Moreover, in both MK cell lines, we found transcripts for LPA1–7, and—similar to platelets—the LPA3 and LPA4 transcripts were the most abundant in both cell lines. The higher expression of LPA4 in Dami cells compared with that in Meg-01 cells may be due to different stages of maturation of the cell lines, i.e., Dami cells are more mature.29,30

In our previous studies, the identification of the functional LPA receptor(s) in platelets was carried out by using a pharmacological approach, and firm evidence that the LPA4 receptor mediates platelet stimulation by LPA and plaque lipid-rich core was lacking.25-27 In the present study, we applied siRNA interference technology which allowed the effective and specific silencing of individual LPA1–7 receptors in the MK cells. We found that LPA-induced activation was significantly inhibited after down-regulation of LPA3 in both cell lines but not after silencing of LPA1–4,6,7, indicating that LPA5 is the functional receptor mediating LPA-induced shape change. In contrast to Meg-01 cells, the effect of LPA in LPA4-down-regulated Dami cells was not completely abolished. This might be because Dami cells have a higher expression of LPA4 when compared with Meg-01 cells and that they still had some functional LPA5 receptors on their surface after LPA4 silencing.

It has been shown that LPA mediates plaque lipid-rich core- and plaque-induced platelet shape change.1,7 LPA contained in the lipid-rich core consists of several molecular species containing different fatty acids in acyl- and alkyl-linkage.7 The various LPA species activate platelets with different potencies.6,7,20 We have demonstrated in this study that atherosclerotic plaques and the plaque lipid-rich core are capable of inducing shape change of Dami and Meg-01 cells. This effect was inhibited after LPA3 depletion. We conclude that our findings provide strong evidence that LPA3 is the functional platelet LPA receptor stimulated by the lipid-rich core of atherosclerotic plaques. This receptor might be a suitable target for antithrombotic therapy.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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Conflict of interest: none declared

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