Injury-elicited differential transcriptional regulation of phospholipid growth factor receptors in the cornea

DE-AN WANG,¹ HAIMING DU,¹ JONATHAN H. JAGGAR,¹ DAVID N. BRINDLEY,² GABOR J. TIGYI,¹ AND MITCHELL A. WATSKY¹

¹Department of Physiology, University of Tennessee Health Sciences Center, Memphis, Tennessee 38163; and ²Signal Transduction Research Group, Heritage Medical Research Centre, University of Alberta, Edmonton, Alberta, Canada T6G 2S2

Received 11 July 2002; accepted in final form 13 August 2002

Wang, De-An, Haiming Du, Jonathan H. Jaggar, David N. Brindley, Gabor J. Tigyi, and Mitchell A. Watsky. Injury-elicited differential transcriptional regulation of phospholipid growth factor receptor transcripts in the cornea. Am J Physiol Cell Physiol 283: C1646–C1654, 2002. First published August 14, 2002; 10.1152/ajpcell.00323.2002.—The phospholipid growth factors (PLGFs), including lysophosphatidic acid (LPA), have been implicated in corneal wound healing. PLGF concentrations and activities are elevated after corneal injury. Using real-time PCR, we quantified receptor mRNA levels in the healing rabbit cornea. In intact corneas, transcripts for S1P1, LPA1, and LPA3 receptor subtypes were detected, as was lipid phosphate phosphatase 1 (LPP1). After wounding, the trend for endothelium and keratocytes was for significant decreases in transcript numbers for the three receptor subtypes, whereas epithelial cells showed increased transcript numbers, except for an S1P1 decrease in healing cells. LPP1 transcript numbers were decreased in keratocytes and endothelium, although LPP-specific activity was unchanged. LPA-elicited Ca²⁺ transients were significantly reduced in the healing endothelium. Consistent with reduced LPA3 receptor numbers, diocetylglycerol pyrophosphate, a selective antagonist, reduced LPA-induced Ca²⁺ transients 2.7-fold in nonwounded epithelium but only 1.5-fold in wound-healing endothelium. These data for the first time establish physiologically relevant differential changes in the expression of PLGF receptor subtypes and provide evidence for the changing role of LPA3 receptors in endothelial cells.

LYSOPHOSPHATIDIC ACID (LPA) and sphingosine 1-phosphate (S1P) are lysophospholipid mediators with growth factor-like effects (12, 32) that have been implicated in wound healing in selected tissues including the cornea (2, 23, 31, 36). The extracellular actions of LPA and S1P are mediated by the endothelial differentiation gene family of G protein-coupled receptors. One cluster within this family, comprising the LPA1, LPA2, and LPA3 receptors, specifically recognizes LPA (3, 11), whereas the other cluster, consisting of S1P1, S1P2, S1P3, S1P4, and S1P5, is specifically activated by S1P (18). Each receptor has a distinct coupling pattern to various G proteins (15, 18, 28). Transient heterologous expression of LPA1/2/3 receptors results in the LPA-induced activation of intracellular Ca²⁺ transients (8, 9).

LPA and S1P facilitate wound closure in monolayers of endothelial cells isolated from adult bovine aorta and human umbilical vein (23). In vivo, LPA treatment stimulates wound closing and increases neoeptithelial thickness in the skin (2). LPA accelerates intestinal epithelial wound healing by promoting intestinal epithelial cell migration and proliferation in vitro and in vivo (2, 31). In corneas, LPA stimulates proliferation in the three major cell types of the cornea (epithelial cells, keratocytes, and endothelial cells) in a dose-dependent manner (24, 36). This LPA-induced proliferative response is pertussis toxin sensitive in the corneal epithelial cells and keratocytes (36). Keratocytes from wounded corneas express an LPA-activated Cl⁻ current that is also activated by an increase in cell volume and serum (35). LPA activation of the Cl⁻ current in these cells is receptor mediated, and keratocytes isolated from nonwounded corneas do not express this current (24, 35). Biochemical examination of phospholipids present in the aqueous humor and lacrimal gland fluid from the rabbit eye has detected several phospholipid growth factors (PLGFs) including LPA, phosphatidic acid, alkenyl glycerophosphate, and lysophosphatidylserine (24). Injury to the cornea has resulted in an increased production of these PLGFs (24). All these data suggest that LPA, S1P, and their natural analogs might be involved in maintaining the integrity of the normal cornea and in promoting wound healing and/or cellular recovery after injury. This hypothesis presumes that PLGF responsiveness, determined by the expression of PLGF receptors, is maintained in the different cell types of the healing cornea.

The purpose of the present study was to investigate the expression of PLGF receptors in the three major cell types of the cornea and to assess wound healing-induced changes in their mRNA expression pattern. In

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
addition, we determined the expression of mRNA encoding lipid phosphate phosphatase 1 (LPP1) as well as lipid phosphatase activity. LPP1 has been shown to degrade LPA at the cell surface (4, 22). Transcripts for S1P1, LPA1, and LPA3 receptors were detected in all three cell types, as was LPP1 mRNA. In endothelial cells and keratocytes, LPA and S1P receptor transcripts decreased during wound healing. In contrast, epithelial cell LPA1 and LPA3 expression increased during wound healing. In agreement with the decreased abundance of mRNA for the LPA3 receptor subtype in endothelial cells isolated from wound-healing corneas, LPA-elicited Ca^{2+} -transients were significantly diminished and lost their sensitivity to dioctylglycerol pyrophosphate (DGPP), an LPA3-selective antagonist. LPP1 message was decreased during wound healing in keratocytes and endothelial cells, although LPP1 activity was unchanged. These results support the hypothesis that PLGF receptors mediate the effects of these lipid mediators in corneal cells and that cell type-specific and differential transcriptional regulation of PLGF receptor expression might play a significant physiological role during corneal wound healing.

**MATERIALS AND METHODS**

*Rabbit corneal wounding and cell isolation.* Corneas from anesthetized rabbits were freeze-wounded with the use of a liquid nitrogen-cooled brass probe by using a protocol described previously (35). At 44–72 h postwound, rabbits were killed, their eyes were enucleated, and the corneas were dissected from the globes. This was the time frame during which significant changes in LPA-activated Cl^{-} current activity were found (35, 36). Buttons from injured areas were trephined to isolate the wound-healing zone. Cells from the trephined button, surrounding rim (the peri-wound area), and control corneas (no wounding) were isolated. Epithelial cells were scraped from the corneas, endothelium was peeled off with Descemet’s membrane, and keratocytes were isolated by collagenase digestion of the stroma as described previously (35). All work was carried out in accordance with the Guiding Principles for Research Involving Animals and Human Beings.

**RNA extraction and reverse transcriptase reaction.** RT-PCR experiments utilized a total of 18 wounded corneas and 6 control corneas. Cells from the corneas were pooled, and total RNA was extracted from harvested cells using TRIzol (Life Technologies, Gaithersburg, MD). RNA was digested by using RNase-Free DNase I (2 U/μl; Stratagene, La Jolla, CA) in the presence of RNaseOUT (6 U/μl; Life Technologies) for 20 min at 37°C to eliminate genomic DNA contamination.

Total RNA (2 μg) was used as a template for cDNA synthesis by random primers using the ThermoScript RT-PCR system (Life Technologies) (33). The resulting cDNA was diluted 10-fold with diethyl pyrocarbonate-treated water (Sigma-Aldrich, St. Louis, MO) and was used as a template for standard and real-time PCR.

Primers were designed to highly conserved mammalian PLGF receptor cDNA sequences or to the rabbit sequences, where available (Table 1). Sequences of the rabbit LPA1, and S1P3 cDNA were kindly provided by Dr. James Rae (Mayo Clinic, Rochester, MN). Thirty-five PCR cycles, each consisting of 10 s at 94°C, 30 s at 55°C, and 1 min at 72°C, were performed. Amplification products were cloned into the pCRII-TOPO vector by using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) for sequencing. Primer compatibility with rabbit tissue was tested by using rabbit brain stem as a positive control.

To monitor for contamination with genomic DNA, we also subjected all RNA samples to PCR (without reverse transcriptase treatment), and the resulting PCR products were analyzed on ethidium bromide-stained agarose gels. All samples subjected to DNase I digestion were found to be free of DNA contamination. To quantify receptor gene copy numbers in the samples, we developed standards using fragments of each of the receptors cloned into the pCRII-TOPO vector. Corresponding cRNAs were synthesized by using SP6 RNA polymerase with the mCAP mRNA capping kit (Stratagene). The cRNA concentration was measured with the GeneQuant RNA/DNA calculator (Pharmacia Biotech, Cambridge, UK). The yield of total RNA was measured by absorbance at 260 nm. The 260- to 280-nm (260/280) ratios of RNA samples were always >2.1 (7). The cRNA copy number was calculated according to the following equation: copy number = [(concentration × volume/molecular weight]) × 6.28 × 10^{23}. The yield and quality of RNA were also checked by agarose gel electrophoresis.

**Receptor cloning.** Rabbit orthologs of several PLGF receptors, LPP1, and β-actin were cloned to ensure appropriate design of real-time PCR primers. Mouse primers (Table 1) were used to amplify transcripts for LPA1, S1P1, LPP1, and β-actin. LPA1, transcripts were amplified by using the sequence data provided by Dr. Rae. Products were subcloned into TOPO TA vector for sequencing.

**Real-time PCR.** Real-time PCR experiments utilized a total of 44 wounded corneas (72 h after wounding) and 10 control corneas. As with the RT-PCR experiments, cells were pooled before RNA extraction. PCR primers were designed

### Table 1. Primers for RT-PCR and cloning

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1P1</td>
<td>5’-CAGGATCAGCTGAGCTGAAAT-3’</td>
<td>GTGCTAGTCTGAGGCGATCAT-5’</td>
</tr>
<tr>
<td>LPA1</td>
<td>5’-CAGCAGCTGAGCTGAAAT-3’</td>
<td>ACTAGTCTGAGGCGATCAT-5’</td>
</tr>
<tr>
<td>S1P3</td>
<td>5’-CAGCAGCTGAGCTGAAAT-3’</td>
<td>ACTAGTCTGAGGCGATCAT-5’</td>
</tr>
<tr>
<td>LPA2</td>
<td>5’-CAGCAGCTGAGCTGAAAT-3’</td>
<td>ACTAGTCTGAGGCGATCAT-5’</td>
</tr>
<tr>
<td>S1P2</td>
<td>5’-CAGCAGCTGAGCTGAAAT-3’</td>
<td>ACTAGTCTGAGGCGATCAT-5’</td>
</tr>
<tr>
<td>LPA3</td>
<td>5’-CAGCAGCTGAGCTGAAAT-3’</td>
<td>ACTAGTCTGAGGCGATCAT-5’</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5’-CAGCAGCTGAGCTGAAAT-3’</td>
<td>ACTAGTCTGAGGCGATCAT-5’</td>
</tr>
<tr>
<td>LPP1</td>
<td>5’-CAGCAGCTGAGCTGAAAT-3’</td>
<td>ACTAGTCTGAGGCGATCAT-5’</td>
</tr>
</tbody>
</table>

S1P, sphingosine 1-phosphate; LPA, lysophosphatidic acid; LPP1, lipid phosphate phosphatase 1.
using the Primer Express software (Table 2; Applied Biosystems, Foster City, CA). PCR reactions were performed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). The SYBR Green PCR core reagents kit (Applied Biosystems) was used for the PCR reaction. The reaction contained 25 μl of SYBR reagent, 2 μl of diluted cDNA, and 30 nM primers in a 50-μl volume. The thermal cycling conditions involved an initial denaturation step at 95°C for 10 s and an extension step at 65°C for 1 min. Experiments were performed in triplicate for each data point. Quantitative values were obtained from the threshold cycle value (Ct), which is the point where a significant increase of fluorescence is first detected. The transcript number of rabbit β-actin was quantified as an internal RNA control, and each sample was normalized on the basis of its β-actin content. The relative PLGF receptor gene expression level of each sample was also normalized to the nonwounded control group (calibrator). Calculation of mRNA copy number was done by using the formulas available at http://dorakmt.tripod.com/genetics/realt ime.html (17). Final results, expressed as N-fold difference in PLGF receptor expression relative to β-actin and the nonwounded control, termed N, were calculated as $N = \frac{2^{ΔCt} \text{sample} - \Delta Ct \text{calibrator}}$, where ΔCt values of the sample and calibrator were determined by subtracting the average Ct value of a PLGF receptor gene from the average Ct value of the β-actin gene.

**Determination of lipid phosphate phosphatase activity.** Cells from a total of 16 wounded (72 h after wounding) and 8 control rabbit corneas were examined. Cells were pooled from several corneas (separated as control, wound-healing buttons, and peri-wound rims) into individual tubes such that there were three individual groups for each category examined (n = 3 for each assay). Tissue samples were homogenized in a buffer solution containing (in mM, unless otherwise noted) 1% Nonidet, 10% glycerol, 50 HEPES, 137 NaCl, 1 MgCl₂, 1 CaCl₂, 10 NaHPO₄, 5 μg/ml aprotinin, 1 phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μM okadaic acid. Okadaic acid was employed to inhibit protein phosphatase activities because LPP activity is inhibited by vanadate and fluoride. Total LPP activity was measured by using [³H]palmitate-labeled phosphatidate (final concentration 0.6 mM) in the presence of 5 mM N-ethylmaleimide and 8 mM Triton X-100. The formation of [³H]diacylglycerol was determined after its purification with basic alumina (25).

**Ratiometric intracellular Ca²⁺ measurements.** Isolated corneal cells were incubated at room temperature for 20 min in NaCl Ringer’s solution (in mM: 145 NaCl, 5 KCl, 2.5 CaCl₂, 5 glucose, and 5 HEPES) containing the ratiometric fluorescent Ca²⁺ indicator fura 2-AM (2 μM) and 0.001% (wt/vol) Pluronic acid, during which time the cells adhered to a glass coverslip in a recording chamber. Cells were then washed for 10 min in bath solution to allow deesterification of the indicator dye. Cells were then alternately excited at 340 or 380 nm using a PC-driven Hyperswitch (Ionoptix, Milton, MA). Background-corrected fura 2 ratios were collected at 510 nm every 0.2 s with the use of a photomultiplier tube and PC-based acquisition software (Ionoptix). Ca²⁺ fluorescence data were collected from single cells or groups of less than three attached cells. Intracellular Ca²⁺ concentrations ([Ca²⁺]) were calculated by using the Grynkiewicz equation (16)

$$[\text{Ca}^{2+}] = K_d \left( \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \right)$$

where R is the 340/380 ratio; Rₘᵢₙ and Rₘₐₓ are the minimum and maximum ratios determined in Ca²⁺-free and saturating Ca²⁺ solutions, respectively; S₁₋₂ is the Ca²⁺-free-to-Ca²⁺ replete ratio of emissions at 380-nm excitation; and Kₐ is the dissociation constant for fura 2. Rₘᵢₙ, Rₘₐₓ, S₁, and S₂ were determined at the end of each experiment and in separate experiments by increasing the Ca²⁺ permeability of cells with ionomycin (10 μM) and perfusing cells with a high-Ca²⁺ (in mM: 140 KCl, 2.5 NaCl, 10 CaCl₂, and 5 glucose) or Ca²⁺-free solution (in mM: 140 KCl, 2.5 NaCl, 5 glucose, and 1 EGTA). The in situ apparent dissociation constant (Kₐ) for fura 2 used in this study was 224 nM (16).

Data are expressed as resting [Ca²⁺], or as the peak LPA-induced elevation in [Ca²⁺], i.e., LPA peak [Ca²⁺]—baseline [Ca²⁺], for control and wound-healing cells. Inhibition of the LPA-induced intracellular Ca²⁺ transients by DGPP, the LPA₂ receptor subtype-selective antagonist (8), was determined by the following experimental paradigm. Cells were first exposed to LPA (500 nM), followed by a 15-min washout in bath solution. DGPP (2 μM) was then applied for 15 min before a second application of LPA in the continued presence of DGPP. After a 15-min washout with bath solution, LPA (500 nM) was applied a second time to assess desensitization and/or rundown. The LPA-induced intracellular Ca²⁺ transient was calculated as the mean of the first and second application.

**Statistical methods.** Student’s t-test was used for testing the null hypothesis that wounding elicited no statistically significant differences in mRNA abundance or baseline Ca²⁺ concentration, or that DGPP had no significant effect on the size of Ca²⁺ transients elicited by LPA. ANOVA was used to compare phosphatase activity levels between groups. P values <0.05 were considered significant.

**RESULTS**

**S1P receptor transcripts in the corneal tissue.** RT-PCR with the use of gene-specific primers based on the conserved transmembrane segments of the mouse S1P₁, S1P₂, S1P₄, and S1P₅ receptors (Table 1) detected only S1P₁ transcripts in corneal endothelium, keratocytes, and epithelium (Fig. 1). The mouse S1P₅, S1P₄, and S1P₅ receptor primers did amplify rabbit mRNAs encoding their respective receptor transcripts present in rabbit brain stem, indicating that the lack of product in these reactions was not due to inadequacy of the primers (data not shown). The rabbit-specific primer to the S1P₃ receptor did not detect S1P₃ receptor message in the corneal cell types (Fig. 1), although

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1P₁ (rabbit)</td>
<td>5′-agtgtggaatgtcagtcagatg</td>
<td>AGGGCCAGGGACCTTCTGCA174-3′</td>
</tr>
<tr>
<td>LPA₁ (rabbit)</td>
<td>5′-tttggcagagggactagttgaatgg</td>
<td>CTTGAATGTTGTCGCTAGTTGCA174-3′</td>
</tr>
<tr>
<td>LPA₃ (rabbit)</td>
<td>5′-gccctctgccttcctctgatgt</td>
<td>GGAAAACACGGGTTGACA175-3′</td>
</tr>
<tr>
<td>β-Actin (rabbit)</td>
<td>5′-tacctggcaggggggtgat</td>
<td>CTCTTGCTCTGGGCTGTCGCTG173-3′</td>
</tr>
<tr>
<td>LPP1 (rabbit)</td>
<td>5′-ttgcattgcagtcagttgcaagt</td>
<td>GGTCACACAAACATCCAAAGATG338-3′</td>
</tr>
</tbody>
</table>
this primer readily amplified S1P3 transcripts present in the rabbit brain stem (data not shown). These results indicate that among the four S1P receptor subtypes, transcripts for the S1P1 receptor were the only ones ubiquitously expressed in all three corneal cell types.

**LPA receptor transcripts in the corneal tissue.** Gene- and species-specific primers based on LPA1 receptors were used to assess the presence of this receptor subtype. LPA1 receptors were readily detectable in the three cell types of the cornea (Fig. 1). When primers based on the conserved putative transmembrane segments of the mouse orthologs of LPA2 and LPA3 receptors were used, amplification product was only detected for LPA3. When the same set of primers was used with mRNA isolated from rabbit brain stem, products were obtained that agreed in size with the expected size of the LPA receptor fragments (data not shown). The present results suggest that LPA1 and LPA3 receptors, but not LPA2 transcripts, are expressed in the cornea.

**Receptor cloning.** Rabbit β-actin transcript was amplified, cloned, sequenced, and submitted to GenBank under the accession number AF404278. With the use of primers specific to rabbit β-actin, transcripts were detected in every mRNA specimen, indicating that the quality of the RNA was adequate. Sequence alignment of the mouse and rabbit β-actin orthologs showed a high degree of conservation (92%; data not shown).

The S1P1 receptor primer yielded a product in corneal endothelium, keratocytes, and epithelium that was cloned and sequenced. Sequences obtained from multiple clonal isolates yielded a single sequence corresponding to that of the rabbit ortholog of S1P1, which was submitted to GenBank under the accession number AF404275. Sequence alignment of the mouse and rabbit S1P1 receptor orthologs showed a high degree of conservation (89%; data not shown).

The primer set used to detect LPA1 and LPA3 receptors in corneal endothelium, keratocytes, and epithelium yielded two products that were cloned and sequenced. Sequence analysis showed two sequences obtained from multiple clonal isolates that represented the rabbit orthologs of the LPA1 and LPA3 receptors. The LPA3 sequence was deposited in GenBank under accession number AF404276. Sequence alignment of the mouse and rabbit LPA1 and LPA3 receptor orthologs also showed high degrees of conservation (92 and 83%, respectively; data not shown).

The LPP1 primers yielded a single product in all three corneal cell types that was also cloned and sequenced. This sequence represented the rabbit ortholog of LPP1 that was deposited in GenBank under accession number AF404277. Sequence alignment of the mouse and rabbit LPP1 receptor orthologs showed a high degree of conservation (86%; data not shown).

**Real-time PCR.** Quantitative real-time RT-PCR was utilized for the determination of mRNA copy numbers of the S1P1, LPA1 and LPA3 receptors and LPP1 enzyme, which were present in all three corneal cell types. Because β-actin had unchanged mRNA levels in control, wounded, and peri-wound rim cells, it was chosen as an endogenous RNA control (Fig. 2).

**Epithelium.** In the epithelium, S1P1 receptor transcripts were relatively low in abundance, constituting ~4,000 copies per 2 μg of RNA (Fig. 2). S1P1 receptors showed a decrease in the wound-healing area, whereas they were increased in the peri-wound rim. Both of these changes were statistically significant compared with the nonwounded epithelium. LPA1 receptors were the least abundant transcripts in the epithelium and showed a statistically significant approximately two-fold increase in the wound-healing button and a modest but significant increase in abundance in the peri-wound rim. LPA3 receptor transcripts were the most abundant in the epithelium, with copy numbers in the...
most abundant PLGF receptor in keratocytes. The copy number of LPA\(_3\) decreased significantly in the wound-healing button and peri-wound rim. The abundance of LPA\(_3\) in the rim, although reduced relative to nonwounded control, remained significantly higher compared with the wound-healing button.

**Endothelium.** Endothelial cells contained the highest copy number of S1P\(_1\) receptor transcripts among the cells of the cornea, with copy numbers in the range of 10\(^4\) copies per 2 \(\mu\)g of RNA (Fig. 2). The abundance of S1P\(_1\) transcripts decreased modestly but significantly during wound healing. Interestingly, S1P\(_1\) mRNA decreased substantially (~3.5-fold) in the endothelium of the peri-wound. LPA\(_3\) copy numbers detected in the endothelium were comparable to those seen in keratocytes. This receptor showed a modest decrease after wounding from 1.1 \(\times\) 10\(^4\) in nonwounded controls to 9.2 \(\times\) 10\(^2\) copies per 2 \(\mu\)g of RNA. As seen for S1P\(_1\), the expression of LPA\(_1\) transcripts was markedly reduced in the rim. LPA\(_3\) was the most abundant transcript detected in the endothelium, with copy numbers as high as ~3.5 \(\times\) 10\(^5\) per 2 \(\mu\)g of RNA. Expression of LPA\(_3\) transcript showed a >10-fold decrease in the wound-button and almost a 10-fold decrease in the endothelium of the peri-wound rim.

In summary, quantitative RT-PCR analysis revealed cell type- and receptor type-specific changes in the abundance of PLGF receptors mRNA transcripts for S1P\(_1\), LPA\(_1\) and LPA\(_3\) during wound healing in the cornea. LPA\(_3\) transcripts were found to be the most abundant in every corneal cell type studied, suggesting that this receptor could play a role in the maintenance of corneal integrity. In addition, the LPA\(_3\) receptor mRNA was downregulated in the inner two cell types of the cornea, whereas it was upregulated in the outer, epithelial cell layer. To seek a functional correlation between the downregulation of LPA receptor transcripts, we compared LPA-elicited Ca\(^{2+}\) responses in endothelial cells (see below).

**LPP1 transcripts and lipid phosphate phosphatase activity.** LPP1 transcripts were detected in all cell types of the cornea (Fig. 3A). Epithelial cells had the lowest LPP1 transcript numbers, which were not affected by wounding (Fig. 3B). Keratocytes and endothelial cells had higher LPP1 transcript copy numbers than epithelial cells, with the wounded region and rim having lower copy numbers than their control counterparts (although not significantly lower in keratocyte rim cells).

Keratocytes had the highest lipid phosphate phosphatase activity among the three cell types, which was 5.5- and 1.4-fold higher than that found in epithelial and endothelial cells, respectively (Fig. 3C). No significant changes were found in total lipid phosphate phosphatase activity during wound healing compared with nonwounded controls (Fig. 3C).

Changes in LPA-elicited Ca\(^{2+}\) responses in the healing endothelium. LPA evokes a transient elevation in [Ca\(^{2+}\)]\(_c\) in a variety of cell types. However, LPA regulation of intracellular Ca\(^{2+}\) has not been investigated in corneal cells. In addition, we sought to investigate...
the physiological consequences of changes in LPA receptor subtypes during wound healing on Ca\(^{2+}\) signaling in corneal cells. Endothelial cells were the only corneal cell type we found to respond to LPA with a change in [Ca\(^{2+}\)]\(_i\) (Fig. 4; epithelial cell and keratocyte data not shown).

[Ca\(^{2+}\)]\(_i\)- and LPA-induced intracellular Ca\(^{2+}\) transients were measured in control endothelial cells and endothelial cells isolated from wounded corneas (7 h after wounding). Resting [Ca\(^{2+}\)]\(_i\) in wound-healing endothelial cells (49 ± 24 nM; n = 5) was significantly lower than in nonwounded cells (113 ± 20 nM; n = 4; Fig. 4A). Consistent with the effects of LPA in other cell types, LPA at a concentration of 500 nM, which is

![Graphs and images](image_url)
a concentration ~10-fold higher than the $K_d$ to the receptors, elicited a transient increase in $[Ca^{2+}]_i$ with a mean peak size of 278 ± 65 nM ($n = 4$) in nonwounded cells (Fig. 4B). In contrast, the same concentration of LPA only elevated $[Ca^{2+}]_i$ by 48 ± 13 nM ($n = 5$) in wound-healing cells. These results agree with the hypothesis that downregulation of LPA receptors could be responsible for the reduced LPA responsiveness of the endothelium.

To further substantiate the hypothesis that LPA receptor downregulation is responsible for the reduced $Ca^{2+}$ response, we used DGPP, a selective antagonist of the LPA3 receptor with a $K_i$ value of 104 nM. This is substantially lower than its $K_i$ to LPA2, which is ~7 μM (8). LPA2 transcript numbers decreased by 80% in the wounded endothelium, whereas LPA2 transcript numbers decreased by only 10%; thus, we hypothesized that the relative contribution of the LPA3 receptor to $Ca^{2+}$ signaling should be the most significantly affected (reduced) in endothelial cells from healing corneas. In support of this hypothesis, 2 μM DGPP reduced the LPA-induced $Ca^{2+}$ transient 2.7-fold in control cells but only 1.5-fold in the endothelium from the wound-healing button (Fig. 4). These data also suggest that the LPA2 receptor is responsible for most of the LPA-induced $Ca^{2+}$ elevation in control cells.

**DISCUSSION**

Several different growth factor receptors are involved in corneal wound healing, including receptors for platelet-derived growth factor, epidermal growth factor, transforming growth factor-$eta$, fibroblast growth factor, and hepatocyte growth factor (10, 21, 26, 27, 29, 37). For this reason, we have included anti-CD3 plus anti-CD28-induced apoptosis of T cells in our experiment (14). Anti-Fas-, anti-CD2-, and anti-CD3 antibodies were used to determine whether the activation of PLGF receptors could be explained by LPA receptor mRNA induction during corneal wound healing. Our results are not consistent with this hypothesis and indicate that in keratocytes and endothelial cells the opposite occurs: the LPA receptor transcript number is decreased following wounding, at least at the time point we examined. These results lead us to conclude that the Cl$^-$ channel activation must be the result of changes not directly related to the transcriptional regulation of LPA and S1P receptor mRNAs. Additional possibilities include posttranscriptional regulation of receptor activity and/or activation or amplification of signaling pathways leading from the remaining receptors to the Cl$^-$ channel.

We have used PCR primers based on mouse and rabbit sequences to detect the different PLGF receptor subtypes present in the three cell types of the rabbit cornea and have found a restricted expression pattern of only LPA1, LPA3, and S1P1 receptors. As part of this study, we cloned the rabbit orthologs of the LPA1, LPA3, S1P1 PLGF receptors as well as LPP1 and β-actin, which was necessary for adequate real-time PCR primer design. These rabbit orthologs all showed an identity >80% with mouse sequences.

LPP1 decreases LPA responsiveness in different cell types (1, 20, 22, 39). For this reason, we have included LPP1 mRNA determination in our PCR experiments and have measured lipid phosphate phosphatase activity in the three different corneal cell types during wound healing. The present results show that the abundance of LPP1 transcript decreases after wounding in keratocytes and endothelial cells but not in epithelial cells. However, total lipid phosphate phosphatase activities were unchanged in all cell types during wound healing. This observation suggests that either the decrease in keratocyte and endothelial cell LPP1 mRNA levels is not associated with changes in activity or that other types of lipid phosphatases are also involved in the degradation of LPA in the tissues.

Little is known about the transcriptional regulation of PLGF receptors. Interestingly, S1P1 was originally isolated as a phorbol ester-inducible early response gene from vascular smooth muscle cells (19). Incubation of human blood T lymphocytes with mitogenic stimuli in vitro significantly alters the expression level of mRNA encoding PLGF receptors. These activation-mediated changes in T cell LPA1/2 receptor mRNA abundance correlate with major changes in LPA-mediated IL-2 production (14). Anti-Fas-, anti-CD2-, and anti-CD3 plus anti-CD28-induced apoptosis of T cells had no effect on the LPA1/2 or S1P2 mRNA abundance, whereas C6-ceramide substantially decreased LPA1/2 receptor mRNA abundance without affecting the S1P2 mRNA abundance (13). The present study provides the first comprehensive quantitative analysis of transcriptional regulation of PLGF receptors transcripts in tis-
Ca²⁺ LPA receptors to the mobilization of intracellular 


coupling efficiency of the signaling pathway linking 

more experiments are necessary to exclude changes in 

affected by the wound healing process, not just the area 

changes in PLGF receptor transcript numbers in the 

and endothelial cells. In addition, we found signifi- 

receptor subtypes also show a transcriptional regula- 

during wound healing. LPA1 and LPA3 expression in-

levels in 

This work was supported by National Institutes of Health grants 

$^{14}$OH10013 (to D. N. Brindley), and Canadian Institutes of Health Research Grant MT10504 (to D. N. Brindley).

REFERENCES

1. Alderton F, Darroch P, Sambi B, McKie A, Ahmed IS, Pyne N, and Pyne S. G-protein-coupled receptor stimulation of the p42/p44 mitogen-activated protein kinase pathway is attenuated by lipid phosphate phosphatases 1, 1a, and 2 in human embry- 

2. Balazs L, Okolincany J, Ferrebee M, Tolley B, and Tigyi G. Topical application of the phospholipid growth factor lysophospho-


5. Crawford KM, MacCallum DK, and Ernst SA. Agonist-in-


7. Dracheva S, Marras EA, Elhakem SL, Kramer FR, Davis KL, and Haroutunian V. N-methyl-D-aspartic acid receptor expression in the dorsolateral prefrontal cortex of elderly pa-


12. Goetzl EJ and An S. Diversity of cellular receptors and func-


320 PAGES

Homeostasis in the endothelium 

The control cornea, the epithelium is exposed to tears and the external environment, while the barrier created by the tight junctions between these cells limits keratocytes and endothelial cells to exposure to factors only within the anterior segment of the eye. After wounding, how-

however, the epithelial barrier is disrupted and all cell types are exposed to tears and external factors, al-

though this exposure ends as soon as the epithelial barrier is restored. We speculate that factors regulat-

ing the expression of the LPA receptor genes are predom-

inant in the tear and aqueous humor, because these are the biological fluids that bathe the epithelial and endothelial layers. Our earlier report provided evidence for the increased generation of LPA and its analogs in postinjury lacrimal gland fluid and aqueous humor. The transcriptional upregulation of LPA recep-

tors together with the increased production of the li-

gand following injury further supports our hypothesis of a role for these factors during corneal epithelial wound healing.

LPA-induced changes in [Ca²⁺]ᵢ were only observed in the corneal endothelial cells. Previous work has 

found that corneal endothelium intracellular Ca²⁺ can be increased through receptor-mediated pathways by histamine (5, 6), purinergic agonists (5, 30), bradykinin (5, 40), and endothelin-1 (5, 38). Adrenergic agonists decreased [Ca²⁺]ᵢ (34). None of these studies were performed in rabbit cells. The current study clearly shows that LPA can be added to this list and is the first agonist shown to elevate intracellular Ca²⁺ levels in rabbit corneal endothelial cells. Moreover, we detected a correlation between the decrease in LP₃ receptor mRNA and the changes in the DGPP-sensitive com-

ponent of the Ca²⁺ response in endothelial cells. Clearly, 

more experiments are necessary to exclude changes in 

the coupling efficiency of the signaling pathway linking 

LPA receptors to the mobilization of intracellular Ca²⁺. Nevertheless, our data establish a distinct 

change in the pharmacological properties of LPA recep-

tors regulating Ca²⁺ homeostasis in the endothelium during wound healing.

Altogether, the present study for the first time provides evidence that physiological conditions occurring during wound healing affect the expression of PLGF receptors mRNA and that these changes are accompa-

nied by differences in the pharmacology of LPA-in-

duced Ca²⁺ responses. These results open many new questions regarding the mechanisms underlying these changes, which must be addressed in future studies.

We thank J. Dewald for help in performing the LPP assays. This work was supported by National Institutes of Health grants EY-12821 (to M. A. Watsky) and HL-61469 (to G. J. Tigyi), National Science Foundation Grant IBN-9728147 (to G. J. Tigyi), and Cana-

C1653
C1654  PLGF RECEPTORS AND LPP1 IN THE HEALING CORNEA


