A Major Human Oral Lysophosphatidic Acid Species, LPA 18:1, Regulates Novel Genes in Human Gingival Fibroblasts

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Background: The small bioactive lipid lysophosphatidic acid (LPA) plays critical roles in both normal physiology and inflammation in many systems. However, its actions are just beginning to be defined in oral biology and pathophysiology.

Methods: Microarray analysis was used to test the hypothesis that human gingival fibroblasts (GFs) would show significant changes in wound-healing and inflammation-related gene transcripts in response to a major human salivary and gingival crevicular fluid LPA species, 18:1, and that they would express transcript for the major LPA-producing enzyme autotaxin. The microarray results were validated for three highly relevant upregulated inflammatory transcripts using quantitative reverse transcription-polymerase chain reaction (QRT-PCR). Liquid chromatography-tandem mass spectrometry was used to assay time-dependent LPA species production by GFs.

Results: LPA 18:1 significantly regulated 20 GF novel and 27 known genes linked to the control of inflammation ($P \leq 0.01$). QRT-PCR validation of interleukin (*IL*)*-8, IL-11,* and suppressor of cytokine signaling 2 (*SOCS2*) messenger RNAs confirmed statistically significant differences from control ($P \leq 0.05$). Autotaxin transcript was present, and GFs were found to produce multiple LPA species in a time-dependent manner.

Conclusions: The upregulation of transcripts for known GF proinflammatory (*IL-6, IL-8*) and anti-inflammatory (*IL-11*) ILs, along with *SOCS2*, shows that LPA transiently regulates a complex set of GF genes critical to periodontal wound healing and inflammation. These results implicate LPA exerting actions on GFs that are compatible with functioning as a mediator in oral fibroblast biology and inflammatory responses. Therefore, LPA may potentially modulate/regulate periodontal inflammation. *J Periodontol 2015;86:713-725.*

KEY WORDS

Cytokines; fibroblasts; gene expression; humans; inflammation; lysophospholipids.

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Chronic inflammatory periodontal disease appears to result from overactive proinflammatory pathways and failure to activate inflammation-resolving pathways.[1] Identifying new mediators that contribute to regulating wound healing and inflammatory processes in oral cells is essential to continue unraveling the understanding of their biology and for developing new therapeutic approaches to control and/or heal chronic periodontitis (CP).

Lysophosphatidic acid (LPA; 1-acyl-*sn*-glycerol-3-phosphate) controls multiple aspects of cell survival, growth, migration, activation, and inflammation in most cell types. In vivo, LPA exists as multiple molecular species that have a fatty acid of varying chain length and degree of unsaturation covalently attached via an acyl, alkyl, or alkenyl linkage. These species differ in affinities for the individual LPA receptors (LPARs) and can elicit varying cellular effects, because LPARs differ in their G-protein–coupling properties and activation of intracellular signaling pathways.[2] However, LPA 18:1 has been, and remains, the most commonly used species for the vast majority of reported studies.

The laboratory of the present authors has shown that gingival fibroblasts (GFs) and periodontal ligament fibroblasts (PDLFs) from multiple young, healthy

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male and female patients showed no detectable differences in a variety of physical responses to LPA 18:1 (including mitogenesis, growth, chemotaxis, and in vitro wound healing).[3-5] However, LPA 16:0 and 18:0 preferentially elicit their $[Ca^{2+}]_i$ responses.[6]

LPAR expression on GFs and PDLFs from multiple donors was profiled by flow cytometry, and it was shown that they express the first five (LPA1 through LPA5)[7] of the six LPARs cloned to date. The expression of so many LPAR subtypes by GFs supports the proposition that LPA plays an essential role in GF biology.

LPA is present in all normal bodily fluids but contributes to pathology in different systems when levels are elevated.[8] The present authors have shown that LPAs 18:1, 18:0, and 16:0 are 10-fold increased over normal (to pharmacologic levels) in the saliva and gingival crevicular fluid (GCF) of patients with moderate to severe periodontitis.[9]

In 2006, a microarray pilot study with LPA 18:1–stimulated GFs from a single patient was performed to determine immediate-early and delayed-early gene regulation (unpublished data; DR C, TP McV, and AO). The dose of LPA used was 10 μM, the optimal concentration from the in vitro wound-healing assays; cell-cycle progression requires micromolar LPA doses.[10] Based on these data, it was hypothesized that exposure to LPA would modulate GF expression of various categories of critical genes that orchestrate wound-healing and inflammatory responses.

Microarray approaches have been reviewed previously,[11,12] including three relevant studies using LPA 18:1–stimulated immortalized mouse embryo fibroblasts (MEFs), mouse preosteoblasts, and human dermal fibroblasts,[13-15] which showed that LPA controls inflammation-related transcripts. However, despite its critical and extensive role in regulating physiologic and pathophysiologic processes in other systems,[11,12] the potential of LPA to regulate transcriptional activity has not been examined in the oral system. Whereas other groups have used microarrays to investigate gene transcriptional differences between degrees of periodontal inflammation from healthy versus gingivitis and periodontally diseased sites, based on the published body of work of the present authors, the aim is first to begin to understand the effects of a single major human salivary LPA species, LPA 18:1,[16] on healthy human GF gene transcription.

Using primary GFs in culture, microarray analysis was used to uncover gene expression changes correlated with LPA treatment. The significantly changed genes and the derived biologic networks reported suggest an immediate (2-hour) and later (8-hour) regulation of genes involved in orchestrating inflammatory-type responses of GFs to LPA 18:1.

MATERIALS AND METHODS

Oral Fibroblast Isolation and Culture

The authors used attached gingival tissue from healthy, non-smoking dental patients of White descent, under no medication, and without evidence of periodontal disease (five males and four females, aged 25 to 35 years; mean age: 27 years). The study was approved by the Institutional Review Board of Creighton University, Omaha, Nebraska. Informed written consent was obtained from all donors. GFs were isolated as described previously.[3]

LPA Treatment

GFs (pass 2) were seeded at 1×10^4 cells/2 mL per 35-mm dish in cell culture media‖ with 10% serum¶ and antibiotics.# They were incubated in a 5% CO_2, 37°C humidified atmosphere for 2 days until they were ≈90% confluent. The cells were then washed four times with a balanced salt solution** and serum starved for 24 hours in serum-free media.

Oleoyl-LPA (18:1)†† stock (1×10^{-2} M) was prepared in 0.25% albumin‡‡ in media. Each donor's cells were treated individually for 2 or 8 hours with 2 mL 10-μM LPA in serum-free media. Untreated controls received an equal volume of 0.25% albumin in serum-free media.

RNA Isolation

Each donor's cells were then trypsinized, washed once with ice-cold phosphate-buffered saline, held on ice, and counted. The nine donors' cells were grouped into three pools of 3,000 cells, each containing 1,000 cells per donor: A = donors one to three (two males and one female); B = donors four to six (one male and two females); and C = donors seven to nine (two males and one female). The experimental design is shown in supplementary Figure 1A in online *Journal of Periodontology*. The pools were lysed directly in a commercial proprietary buffer§§ and quick-frozen on dry ice for shipment to the company.

Microarray Analysis

LPA-induced gene transcription for each treatment pool was assessed commercially‖‖ using commercial arrays.¶¶ The raw data were deposited at the National Center for Biotechnology Information Gene Expression Omnibus database as record GSE57496.

‖ Dulbecco modified Eagle medium, Life Technologies, Thermo Fisher Scientific, Waltham, MA.
¶ Defined fetal bovine serum, Hyclone, Logan, UT.
# Penicillin/streptomycin, Life Technologies, Thermo Fisher Scientific.
** Hank's Balanced Salt Solution, Life Technologies, Thermo Fisher Scientific.
†† Avanti Polar Lipids, Alabaster, AL.
‡‡ Delipidated (fraction V) bovine serum albumin, Sigma, St. Louis, MO.
§§ Miltenyi Biotec, Auburn, CA.
‖‖ Miltenyi Biotec.
¶¶ Whole Human Genome Oligo Microarray Analysis, Array G4112F, Agilent Technologies, Santa Clara, CA.

Data Analyses

The company designated genes that showed less than or equal to, or greater than or equal to two-fold differences between the signal from the LPA-treated samples and their untreated controls as significantly changed; their gene analysis program[##] level of significance is $P \leq 0.01$. The average fold change for each gene was determined by averaging the three replicated microarray experiments at each LPA treatment time (2 and 8 hours). Patient pools (A, B, C) were analyzed for intragroup variation between 2- and 8-hour gene regulation using the Wilcoxon signed-rank test.

Data were analyzed to explore molecular interaction networks by uploading the gene list to a program.[***] Functional gene ontology (GO) categories were determined with a program[17] and used other programs[18-20] to redundantly explore gene associations and relations with the inflammatory process. Novel LPA-regulated genes were verified (to the best of the authors' knowledge) using two databases.[†††‡‡‡]

Quantitative Reverse Transcription-Polymerase Chain Reaction

Quantitative reverse transcription-polymerase chain reaction (QRT-PCR) was performed to validate the microarray results using three biologic replicates for four of the upregulated genes at the 8-hour time point. Total 8-hour RNA (LPA-positive [LPA^+] of LPA-negative [LPA^-]) was isolated.[§§§] Using random hexamers with RT,[‖‖‖] 1 μg total RNA was converted to complementary DNA. Primers for human 18S ribosomal RNA (rRNA),[21] β-actin,[22] and suppressor of cytokine signaling 2 (SOCS2)[23] were synthesized.[¶¶¶] Using the interleukin (IL)-8 and IL-11 labeled primer sets[###] and labeled 18S, β-actin, and SOCS2 primers, PCR assays were performed in triplicate (see supplementary Fig. 1B in online *Journal of Periodontology*). Relative quantitation of mRNA abundance was normalized to 18S rRNA. A paired *t* test (one-tailed) was performed[****] on the average normalized gene expression (cycle threshold difference [ΔCT]) of LPA^+ versus LPA^- treatment from the three biologic replicates to validate the observed increase in gene expression seen by microarray analysis.

GF Production of LPA

GF cells (5×10^4) were seeded into T-75 flasks (one per time point) and grown in media with 10% serum until just confluent. The cells were serum starved in media overnight and then washed 10 times with 10 mL balanced salt solution to further remove any residual LPA present from the serum used to grow the cells. The cells were re-fed with 5 mL serum-free media and then incubated in a 5% CO_2, 37°C humidified atmosphere. The conditioned media from the cells were collected at 2, 4, 6, 8, or 24 hours for LPA species analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

LC-MS/MS Analyses

The instrument specifications, software and setting conditions, mobile phase, and method validation have been reported previously; LPA calibration curves were prepared in serum-free media as described.[9]

RESULTS

Some donor-dependent variation in responses to LPA have been reported previously for human retinal pigmented epithelium,[24] but intragroup analyses of the early (2 hours) versus later (8 hours) gene responses showed no statistical differences ($P = 0.88$ to 0.09) for relative fold regulation of LPA-induced genes, as determined using the Wilcoxon signed-rank test.

Analysis of microarray data of triplicate patient/treatment pools (mock, 2-hour LPA, 8-hour LPA) showed that LPA treatment regulated 382 GF genes known to be involved in controlling the inflammatory response. For inflammation-related genes more than two-fold regulated, either negatively or positively, there are a subset of overrepresented GO categories ($P<0.01$). The overrepresented biologic themes were not statistically different between the 2- and 8-hour time points for the three groups. These overrepresented GO categories, listed in supplementary Table 1 in online *Journal of Periodontology*, are known to be processes critical in GF biology and in the establishment and clinical course of periodontal disease.

The fold change range for all significantly regulated genes was 2.1 to 114.7 versus control at either 2 or 8 hours. All transcripts (with the exception of CC chemokine ligand 20 [1.6- to 3.2-fold] and some of the metallothionein isoforms) were at least three-fold regulated in all (or in at least two of the three) patient pools tested. The inflammation-related genes regulated by LPA at 2 and 8 hours are shown in Tables 1 through 3: 1) the top transcripts; 2) the novel transcripts; and 3) those already known to be expressed by GFs.

Three highly relevant, upregulated inflammation-related genes seen in the microarray study were validated using QRT-PCR. The average fold increases

Rosetta Resolver Gene Expression Data Analysis System, Rosetta Biosoftware, Microsoft, Redmond, WA.
*** Ingenuity Pathway Analysis software, Ingenuity Systems, Redwood City, CA.
††† PubMed, National Center for Biotechnology Information, Bethesda, MD.
‡‡‡ Scirus, Elsevier, Amsterdam, The Netherlands.
§§§ MMLV Reverse Transcriptase, RNeasy Mini Kit, Qiagen, Valencia, CA.
‖‖‖ MMLV Reverse Transcriptase, RNeasy Mini Kit, Qiagen.
¶¶¶ Integrated DNA Technologies, Coralville, IA.
FAM-, Taqman-, and SYBR Green-labeled primer sets, Applied Biosystems, Life Technologies, Thermo Fisher Scientific.
**** StepOne Software v.2.1, Applied Biosystems, Thermo Fisher Scientific.

Table 1.

Top Inflammation- and Wound Healing–Related Genes Regulated by LPA

RefSeq. No.	Gene ID	Gene Name	Fold Change (2 hours)	RefSeq. No.	Gene ID	Gene Name	Fold Change (8 hours)
Upregulated transcripts				Upregulated transcripts (cont.)			
NM_025195	*TRIB1*	Tribbles 1 (only in top 10 at 2 hours)	34.8 ± 6.1	NM_000584	*IL8*	Interleukin 8	114.7 ± 64
NM_003877	*SOCS2*	Suppressor of cytokine signaling 2	31.7 ± 27	NM_003877	*SOCS2*	Suppressor of cytokine signaling 2	46.0 ± 37
NM_000584	*IL8*	Interleukin 8	30.6 ± 13	NM_182790	*NAMPT*	Nicotinamide phosphoribosyltransferase/ visfatin/PBEF	31.5 ± 24.9
NM_182790	*NAMPT*	Nicotinamide phosphoribosyltransferase/ visfatin/PBEF	30.3 ± 20	NM_003485	*GPR68/OGR1*	G-protein-coupled receptor 68/ovarian cancer G-protein-coupled receptor 1	18.8 ± 12.8
NM_002309	*LIF*	Leukemia inhibitory factor	28.9 ± 7.0	NM_00514	*GDNF*	Glial-derived neurotrophic factor	14.2 ± 8.0
NM_000963	*PTGS2*	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	27.7 ± 6.1	NM_000882	*IL12A*	Interleukin 12A	12.8 ± 6.0
NM_000710	*BDKRB1*	Bradykinin B1 receptor	16.3 ± 2.7	NM_005953	*MT2A*	Metallothionein 2A isoform	10.6 ± 4.1
NM_000641	*IL11*	Interleukin 11	15.7 ± 1.7	NM_176891	*IFNE*	Interferon ε E1	10.3 ± 6.0
NM_00514	*GDNF*	Glial-derived neurotrophic factor	15.1 ± 9.4	NM_000963	*PTGS2*	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	8.0 ± 2.8
NM_002090	*CXCL3*	Chemokine (C-X-C motif) ligand 3	12.7 ± 0.6	NM_002090	*CXCL3*	Chemokine (C-X-C motif) ligand 3	6.9 ± 0.1
Downregulated transcripts				Downregulated transcripts (cont.)			
NM_016123	*IRAK4*	Interleukin-1 receptor-associated kinase 4	3.7 ± 0.7	NM_001548	*IFIT1*	Interferon-induced protein with tetratricopeptide repeats 1	15.6 ± 8.0
NC_000008.10	*NADPH*	Nicotinamide adenine dinucleotide phosphate	3.6 ± 0.7	NM_172174	*IL15*	Interleukin 15	10.8 ± 6.9
NM_001548	*IFIT1*	Interferon-induced protein with tetratricopeptide repeats 1	3.3 ± 0.4	NM_000880	*IL7*	Interleukin 7	9.2 ± 3.1
NM_000586	*IL2*	Interleukin 2	3.2 ± 0.9	NM_000959	*PTGFR*	Prostaglandin F receptor (FP)	8.6 ± 3.5
NM_144717	*IL20Rβ*	Interleukin 20 receptor β chain	2.9 ± 0.2	NC_000008.10	*NADPH*	Nicotinamide adenine dinucleotide phosphate	5.3 ± 0.8
NM_172174	*IL15*	Interleukin 15	2.3 ± 0.1	NM_144717	*IL20Rβ*	Interleukin 20 receptor β chain	4.5 ± 0.6
				NM_000586	*IL2*	Interleukin 2	3.5 ± 1.2

RefSeq. = reference sequence database.

Table 2.

Novel Inflammation- and Wound Healing–Related Genes Regulated by LPA

RefSeq. No.	Gene ID: Description	Fold Change							
		Donor Pool A		Donor Pool B		Donor Pool C		Mean ± SE	
		2 hours	8 hours	2 hours	8 hours	2 hours	8 hours	2 hours	8 hours
Upregulated transcripts									
NM_001627	*ALCAM*: activated leukocyte cell adhesion molecule	3.4	2.4	3.6	6.6	−2.0	3.8	1.7 ± 1.8	4.3 ± 1.2
NM_139314	*ANGPTL4*: angiopoietin-like 4 (*ANGPTL4*), transcript variant 1	4.4	4.5	6.6	4.0	6.7	2.7	5.9 ± 0.8	3.8 ± 0.5
NM_002089	*CXCL2*: chemokine (C-X-C motif) ligand 2	8.5	3.6	10.8	3.3	4.3	3.3	7.8 ± 1.9	3.4 ± 0.1
NM_005232	*EPHA1*: Ephrin type-A receptor 1	3.9	2.4	4.4	4.5	20.9	5.7	9.7 ± 5.6	4.2 ± 1
NM_000882	*IL12A*: interleukin 12A (natural killer cell stimulatory factor 1)	5.5	15.0	1.0	21.0	−1.1	2.4	1.8 ± 1.9	12.8 ± 6
NM_001014336	*IL31*: interleukin 31	9.7	2.9	11.2	4.2	8.1	5.1	9.6 ± 0.9	4.0 ± 0.6
NM_001570	*IRAK2*: interleukin 1 receptor-associated kinase 2	3.5	1.5	11.3	6.5	12.3	9.0	9.0 ± 2.8	5.6 ± 2.2
NM_176891	*IFNE1*: interferon ε E1	5.3	5.6	4.7	3.5	9.2	22.0	6.4 ± 1.4	10.3 ± 6
NM_005384	*NFIL3*: nuclear factor, interleukin 3-regulated	4.1	1.1	5.2	−1.5	6.1	−1.2	5.1 ± 0.6	−0.5 ± 0.8
NM_000595	*LTA*: lymphotoxin-α	3.0	−1.1	2.5	1.1	2.9	2.9	2.8 ± 0.2	1.0 ± 1.2
NM_00514	*GDNF*: glial-derived neurotrophic factor	8.7	9.4	33.6	29.0	2.8	4.2	15.1 ± 9.4	14.2 ± 8
NM_182790	*PBEF1*: visfatin, nicotinamide phosphoribosyltransferase (*NAMPT*)	9.4	4.9	12.3	8.4	69.3	81.3	30.3 ± 20	31.5 ± 24.9
NM_003877	*SOCS2*: suppressor of cytokine signaling 2	3.5	6.4	6.9	11.8	84.7	119.9	31.7 ± 27	46.0 ± 37
NM_003485	*GPR68/OGR1*: G-protein-coupled receptor 68	4.6	10.1	2.1	2.4	15.3	44.0	7.3 ± 4.0	18.8 ± 12.8
NM_006794	*GPR75*: G-protein-coupled receptor 75 (binds [C-C motif chemokine 5]/regulated on activation normal T-cell expressed and secreted [RANTES])	4.0	1.6	8.6	3.2	14.2	2.1	8.9 ± 3	2.3 ± 0.5
NM_021972	*SPHK1*: sphingosine kinase, transcript variant 1	3.8	5.5	4.8	5.2	3.2	3.3	3.9 ± 0.5	4.7 ± 0.7
Downregulated transcripts									
NM_023039	*ANKRA2*: ankyrin repeat, family A (*RFXANK*-like)	3.5	2.5	3.2	3.5	3.4	3.4	3.4 ± 0.1	3.1 ± 0.3
NM_144717	*IL20Rβ*: interleukin 20 receptor β	2.6	4.5	2.9	3.4	3.2	5.6	2.9 ± 0.2	4.5 ± 0.6
NM_001548	*IFIT1*: interferon-induced protein with tetratricopeptide repeats 1	2.7	9.7	4.0	5.6	3.2	31.4	3.0 ± 0.4	15.6 ± 8.0
NM_016123	*IRAK4*: interleukin 1 receptor-associated kinase 4	2.4	2.7	4.5	1.9	4.1	+1.6	3.7 ± 0.7	1.0 ± 1.3

RefSeq. = reference sequence database.

Table 3.

Other Known Inflammation- and Wound Healing–Related Genes Regulated by LPA

RefSeq. No.	Gene ID: Description	Fold Change							
		Donor Pool A		Donor Pool B		Donor Pool C		Mean ± SE	
		2 hours	8 hours	2 hours	8 hours	2 hours	8 hours	2 hours	8 hours
Previously reported upregulated transcripts									
NM_002090	*CXCL3*: chemokine (C-X-C motif) ligand 3	12.7	6.3	17.3	8.8	8.1	5.6	12.7 ± 0.6	6.9 ± 0.1
NM_004591	*CCL20*: chemokine (C-C motif) ligand 20	1.6	2.5	3.2	2.2	1.2	2.4	2.0 ± 0.6	2.4 ± 0.1
NM_000600	*IL6*: interleukin 6	5.4	3.1	2.7	1.7	3.3	2.6	3.8 ± 0.8	2.5 ± 0.4
NM_000584	*IL8*: interleukin 8	55.5	235.7	25.1	89.2	11.3	19.3	30.6 ± 13	114.7 ± 64
NM_000641	*IL11*: interleukin 11	19.0	6.0	13.2	4.7	15.0	4.8	15.7 ± 1.7	5.2 ± 0.4
NM_172200	*IL15Rα*: interleukin 15 receptor α	1.3	3.2	1.7	4.2	7.6	6.5	5.3 ± 1	3.6 ± 2.0
U58146	*IL6ST*: alternatively spliced interleukin 6 receptor β chain (Partial cds)	3.8	1.8	4.8	2.6	5.7	−1.1	4.8 ± 0.5	1.1 ± 1.1
NM_002309	*LIF*: leukemia inhibitory factor	21.7	4.0	22.1	10.5	43.0	2.7	28.9 ± 7.0	5.7 ± 2.4
NM_000710	*BDKRB1*: B1 bradykinin receptor	20.0	9.4	11.0	4.0	18.0	7.2	16.3 ± 2.7	6.9 ± 1.6
NM_003999	*OSMR*: oncostatin M receptor	2.2	2.2	3.0	4.8	3.0	12.9	2.7 ± 0.3	6.6 ± 3.2
NM_000963	*PTGS2*: prostaglandin-endoperoxide synthase 2 (COX-2)	31.9	11.3	15.6	2.4	35.5	10.2	27.7 ± 6.1	8.0 ± 2.8
NM_014452	*TNFRSF21*: tumor necrosis factor receptor superfamily, member 21/DR6	4.1	7.5	−1.1	3.7	15.7	3.3	6.2 ± 5.0	4.8 ± 1.4
NM_025195	*TRIB1*: *Homo sapiens* tribbles homolog 1 (*Drosophila*)	25.8	4.2	32.2	4.1	46.4	6.9	34.8 ± 6.1	5.1 ± 0.9
MT isoforms (all only significantly induced at 8 hours)									
NM_005946	MT-1A	1.1	3.1	1.0	2.5	−1.0	2.1	0.4 ± 0.7	2.6 ± 0.3
NM_005947	MT-1B	1.4	4.3	1.2	3.0	−1.0	2.3	0.5 ± 0.8	3.2 ± 0.6
NM_005950	MT-1G	1.5	2.6	1.1	2.3	1.0	2.4	1.2 ± 0.1	2.4 ± 0.1
NM_005951	MT-1H	1.0	4.1	−1.5	3.0	−1.1	2.3	−0.5 ± 0.8	3.1 ± 0.5
NR_001447	MT-1L	−1.3	4.6	−1.0	3.0	−1.1	2.5	−1.1 ± 0.1	3.4 ± 0.6
NM_005952	MT-1X	1.1	4.8	−1.1	3.7	−1.0	2.5	−0.3 ± 0.7	3.7 ± 0.7
NM_005953	MT-2A	3.4	18.3	1.8	9.0	1.3	4.4	2.2 ± 0.6	10.6 ± 4.1
NM_203330	*CD59*: *CD59* molecule, complement regulatory protein, transcript variant 1	−1.1	5.6	−1.3	2.1	−1.1	2.2	−1.1 ± 0.1	3.3 ± 1.2

Table 3. (continued)
Other Known Inflammation- and Wound Healing–Related Genes Regulated by LPA

RefSeq. No.	Gene ID: Description	Fold Change							
		Donor Pool A		Donor Pool B		Donor Pool C		Mean ± SE	
		2 hours	8 hours	2 hours	8 hours	2 hours	8 hours	2 hours	8 hours
Previously reported downregulated transcripts									
NM_001735	*C5*: complement component 5	3.9	3.0	4.2	5.8	6.5	18.5	4.9 ± 0.8	9.1 ± 4.7
NM_000586	*IL2*: interleukin 2	2.2	2.0	2.4	5.8	4.9	2.8	3.2 ± 0.9	3.5 ± 1.2
NM_000880	*IL7*: interleukin 7	1.9	7.6	2.1	15.4	1.8	4.5	1.9 ± 0.1	9.2 ± 3.1
NM_172174	*IL15*: interleukin 15	2.4	4.9	2.1	2.9	2.3	24.6	2.3 ± 0.1	10.8 ± 6.9
NM_000895	*LTA4H*: leukotriene A-4 hydrolase	2.0	2.0	2.2	4.2	1.8	3.8	2.0 ± 0.1	3.3 ± 0.7
NM_000959	*PTGFR*: prostaglandin F receptor, transcript variant 1	1.6	5.2	1.3	4.9	3.7	15.7	2.2 ± 0.8	8.6 ± 3.5

RefSeq. = reference sequence database.

were 118.7-fold (*IL8*), 2.1-fold (*IL11*), and 4.1-fold (*SOCS2*). Actin alpha-1 (*ACTA1*), encoding α-actin, a well-known LPA target gene,[12] was increased 3.6-fold in LPA^+-treated GFs. With the exception of *IL11*, the LPA^+-associated increase in normalized ΔCT values for these genes (*IL8*, *SOCS2*, and *ACTA1*) were statistically significant ($P < 0.05$, paired one-tailed t test).

Pathway analysis†††† identified five major transcription factors through the integration of the LPA-induced genes. The top five LPA-regulated transcription factors were nuclear factor-κB (NF-κB) complex, forkhead box L2 (FOXL2), signal transducer and activator of transcription 3 (STAT3), NF-κB1, and v-rel avian reticuloendotheliosis viral oncogene homolog A (RELA) (highly induced; $P < 0.001$) at both 2 and 8 hours. Published evidence relates these factors to the infectious process, inflammation, and/or chronic periodontal disease.[25-29]

Adding to this laboratory's findings that LPA species differentially affect GF Ca^{2+} signaling responses,[6] LPA significantly induced phosphoinositide 3-kinase δ (PIK3CD) and protein kinase Cγ message at 2 hours ($P < 0.001$).

Diacylglycerol kinase ε (DGK) messenger RNA (mRNA) was significantly induced ($P < 0.001$). DGK makes alkyl-LPAs, which show distinct pharmacologies at LPARs and potently drive platelet aggregation.[30] The microarray analysis also revealed that GFs express ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin [ATX]; encoded by ENPP2) mRNA. ATX is a secreted lysophospholipase D that converts lysophosphatidylcholine and related substrates into LPA, thus confirming the results seen in Figure 1; analysis of serum-free conditioned media from human GFs (as well as PDLFs [data not shown]) showed that they produce nanomolar LPA in a time-dependent manner, demonstrating the capacity for feed-forward autocrine signaling in culture and likely in vivo.

Figure 2 shows the software-generated†††† model of gene relations contained within the top upregulated and downregulated genes identified (Table 1). Prostaglandin G/H synthase-2 (PTGS2; cytochrome *c* oxidase-2 [COX-2]) transcript was in the top upregulated transcripts (2.4- to 35.5-fold), confirming the ability of LPA to regulate GF prostaglandin (PG) production.

An exclusive gene significantly regulated only at 2 hours in all three pools (A, B, C) was identified by the software:§§§§ the basic leucine zipper transcriptional repressor nuclear factor, IL3-regulated (*NFIL3/E4BP4*) (Fig. 2). It is an inducible activator/repressor of inflammatory and apoptotic genes expressed at low levels by many cell types;[31] the very early regulation

†††† Ingenuity Pathway Analysis software, Ingenuity Systems.
‡‡‡‡ Grow tool in Ingenuity Pathway Analysis software, Ingenuity Systems.
§§§§ Ingenuity Pathway Analysis software, Ingenuity Systems.

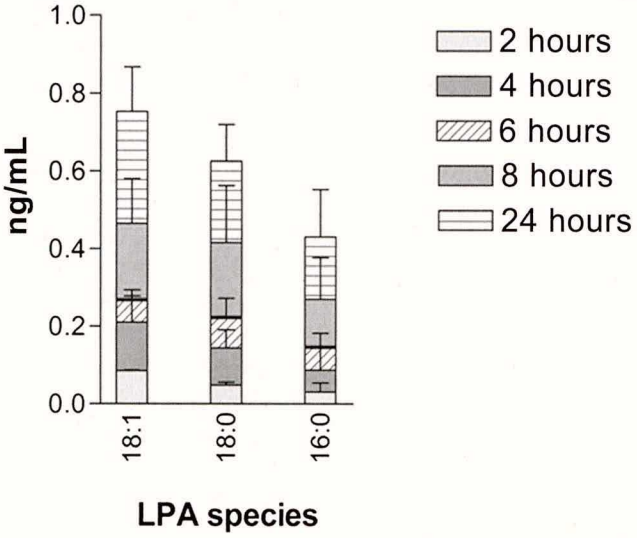

Figure 1.
GFs produce LPA. Serum-free conditioned media from GFs were assayed for the presence of the 18:1, 18:0, and 16:0 acyl-LPA species by LC-MS/MS analyses at the time points shown (n = 2; ± SEM).

of this transcription factor (shown interacting with PTGS2 [COX-2] in Fig. 2) shows LPA rapidly activating GF genes involved in the control of immunity and inflammation.

DISCUSSION

To the best of the authors' knowledge, this study provides the first evidence that LPA 18:1 exerts complex regulation on genes involved in orchestrating the inflammatory responses of GFs. Although protein data are not available, the stimulation of three genetically different pools of GFs with LPA reproducibly and specifically stimulated/inhibited transcription of the same genes in each pool, suggesting that LPA has the potential to regulate GF behavior during periodontal inflammation. The full biologic ramifications of this pattern of gene regulation remain to be investigated in additional studies.

For the main novel LPA-regulated software‖ analysis–identified genes that have the capacity to play a role in inflammation and periodontal breakdown, their context and "big picture" integration were discussed (Fig. 3). These genes regulate the factors affecting GFs: 1) neutrophils; 2) cellular responses to bacterial lipopolysaccharide (LPS); 3) inflammatory mediator production/regulation; 4) the bone breakdown that takes place during periodontal inflammation; and 5) their interaction with LPA and its receptor subtypes in this orchestrated response. Discussion of the other identified genes shown that play vital supporting roles can be found in the supplementary Appendix A in online *Journal of Periodontology*.

The just-published ENCODE Project[32] uncovered many DNA and gene expression pattern variations between mice and humans, which share only ≈70% of the same protein-encoding gene sequences. Although many inflammation-related genes were regulated by LPA 18:1 in retrovirally immortalized MEFs,[13] all major regulated primary GF genes were different except for *IL6*, *IL11*, *PTGS2* (*COX-2*), and Tribbles 1 (*TRIB1*), highlighting the novelty of the study findings. MEFs also only express LPA1, LPA2, and LPA4,[13] whereas the authors' previous work shows that GFs express LPA1 through LPA5 by flow cytometry.[7]

Critically, unlike MEFs,[13] GFs do express ATX. Therefore, human GFs are capable of both producing LPA and responding to exogenous LPA; for the present study, autocrine actions, if any, would be negligible, because GFs produce nanomolar LPA (Fig. 1), and micromolar LPA was used for treatment. This autocrine capacity, together with the large number of LPARs they express,[7] underscores the potential regulatory importance of LPA to GF biology in health and disease.

Neutrophils

LPA and its generating enzyme ENPP2/ATX are essential for lymphocyte transmigration.[11] Neutrophils initiate the inflammatory response in the periodontium and are statistically elevated in human periodontally diseased sites. They express LPA1 through LPA3 and respond to nanomolar LPA levels; LPA dose-dependently stimulates their respiratory burst.[33] ATX increases inflammation by upregulating neutrophil integrins.[11] LPA concentrations can reach very high local levels, such as the 10-fold pharmacologic (micromolar) increases the authors have found in saliva and GCF from patients with moderate to severe periodontitis.[9] High local LPA production has been proposed to result in LPAR supersaturation[34] because ATX appears not to be inhibited by its product.

Unless neutrophils "class switch" and make lipoxins (small anti-inflammatory lipid mediators) and anti-inflammatory PGs,[35] their delayed apoptosis means continued or worsening tissue breakdown from their attempts to kill pathogens. The authors suggest that the pharmacologically elevated salivary and GCF LPA concentrations reported previously[9] would likely enhance this damage because high local LPA concentrations in the inflamed periodontium would stimulate the respiratory burst of neutrophils.

Leukotriene A-4 hydrolase (*LTA4H*) makes LTB4, a potent leukocyte and neutrophil chemoattractant. LTs and PGs produce edema, inflammatory cell recruitment, collagen breakdown, and the bone resorption characteristic of chronic periodontal disease. Of singular importance, LTA4H has an aminopeptidase

‖ Ingenuity Pathway Analysis software, Ingenuity Systems.

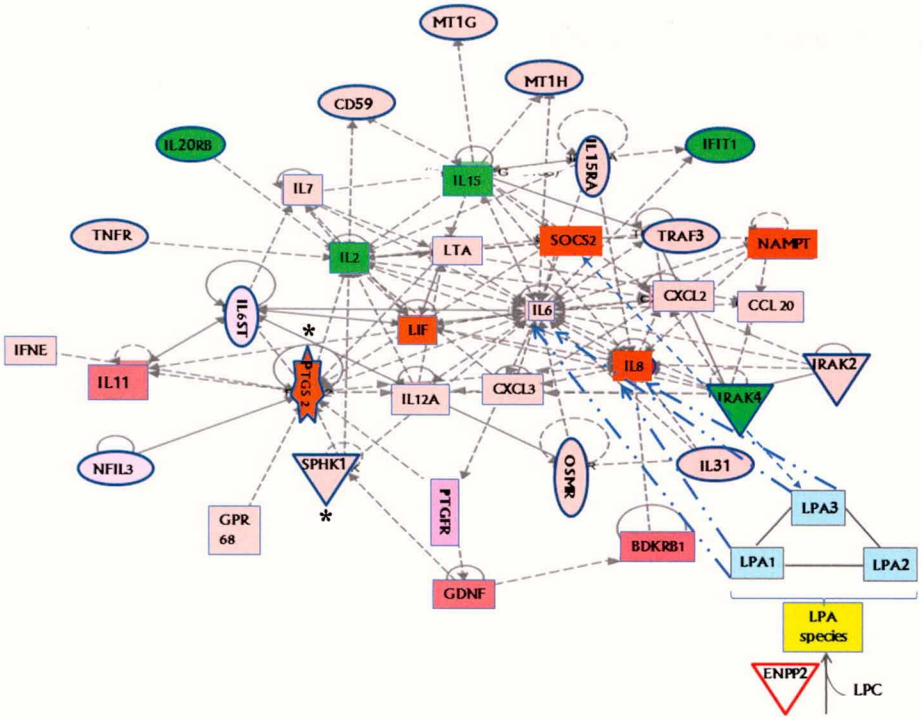

Figure 2.

*Graphic molecular representation showing putative gene interaction networks. Top LPA-regulated genes. Analysis was performed using analysis software.¶¶¶¶ Calculated networks of interacting gene products are represented by nodes. The molecule class is represented by the software legend shapes. Shaded nodes are genes represented in the input list. Reddish shades indicate upregulation, and green ones indicate repression. The biologic relation between two nodes is represented as an edge (line). All edges are supported by canonical information in the software knowledge base and/or published peer-reviewed reference(s). The nodal relations are shown as solid lines (direct interaction) or dashed lines (indirect interactions), indicating functional interaction or physical association. Arrowhead direction shows direction of the effect. These gene relations are primarily contained within the top upregulated and downregulated genes identified (Tables 1 through 3): seven of seven novel top upregulated genes: glial-derived neurotrophic factor (GDNF), G-protein-coupled receptor 68 (GPR68), IL12A, interferon ε E1 (IFNE), nicotinamide phosphoribosyltransferase (NAMPT), SOCS2, and sphingosine kinase 1 (SPHK1); and three of three top novel downregulated genes: interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), IL1 receptor-associated kinase 4 (IRAK4), and IL20 receptor β chain (IL20RB). Previously known genes expressed by GFs include five top upregulated genes: bradykinin B1 receptor (BDKRB1), IL8, IL11, leukemia inhibitory factor (LIF), and PTGS2; and three top downregulated genes: IL2, IL7, and IL15. *PTGS2 and SPHK1 are starred to emphasize their contributory roles in inflammation. Added to the diagram is ENPP2 (ATX), shown making LPA species from substrates, such as lysophosphatidylcholine (LPC). These LPA species would then bind to some of the main LPARs characterized on GFs: LPA1, LPA2, and LPA3. LPA1 and LPA3—identified as participating in inflammation in other human systems—are shown interacting with IL6 and IL8, implicating these LPAR subtypes in the regulation of periodontal inflammation/periodontal disease.*

activity and degrades the neutrophil chemoattractant Pro-Gly-Pro (PGP). PGP is a biomarker for neutrophil persistence in the lung. LTA4H degrades PGP, thus helping to resolve lung inflammation.[36] Notably, LPA downregulated *LTA4H* (Table 3).

Periodontal disease and rheumatoid arthritis (RA) share many commonalities because the inflammatory breakdown of the alveolar bone is similar to the inflammatory breakdown of joints in RA; neutrophils accumulate in the joints of patients with RA. LPA plays a critical role in inducing synovial fibroblast (RA synovial fibroblast [RASF]) COX-2 in collaboration with inflammatory cytokines.[37] That ATX-made LPA is the pathogenic driver of RA supports the contention of the relevance of LPA to the oral system.

PIK3CD was significantly (4.7-fold) induced by LPA. It controls migration and invasion of synoviocytes in RA and regulates their actin cytoskeleton and lamellipodium formation during platelet-derived growth factor stimulation.[38]

Nicotinamide phosphoribosyltransferase (*NAMPT*)/pre-B-cell colony-enhancing factor (*PBEF*)/visfatin is another key mediator in RA. It was one of the most highly upregulated GF transcripts. Like LPA,[9] it is found in GCF; concentrations in patients with periodontitis were significantly higher and fell after treatment.[39] *PBEF* knockdown in RASFs inhibited both basal and Toll-like receptor (TLR) ligand-induced production of IL6, IL8, matrix metalloproteinases 1 and 3. The probable molecular interactions for *NAMPT* are shown in Figure 2.

In comparing the present results with those found in a previous study of LPA 18:1–stimulated human diploid fibroblasts,[15] only two LPA-regulated genes were shared: sphingosine kinase 1 (*SPHK1*) and *IL8*. *SPHK1* induction was higher in the present study (3.9- and 4.7-fold) versus the earlier study (1.3- and 1.1-fold). For *IL8*, the previous study's findings of fold changes of 1.5- and 1.1-fold at 1 and 8 hours, respectively, was much lower than the 30.6- and 114-fold induction seen in the present study at 2 and 8 hours. This was not surprising, because the inflammatory responses of GFs also differ from those of other fibroblasts by virtue of their constant exposure to complex oral flora.[40]

SPHK makes sphingosine 1-phosphate (S1P), a master regulator of immune cell activation, trafficking, and the cytokine network.[41] *SPHK1* suppression of

¶¶¶¶ Ingenuity Pathway Analysis software, Ingenuity Systems.

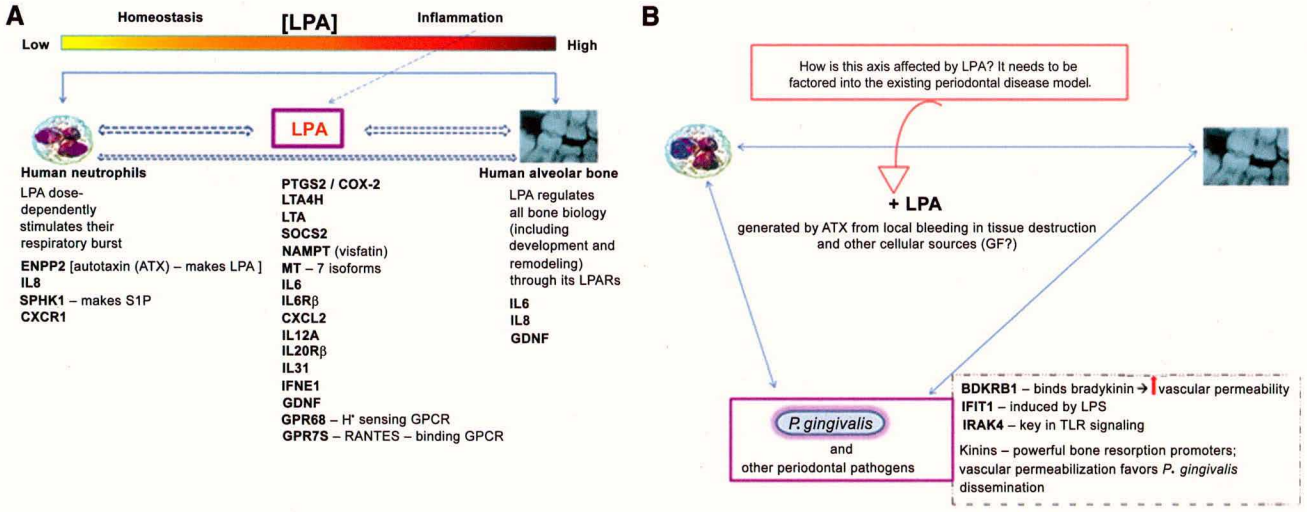

Figure 3.

Graphic representation of LPA-regulated GF genes: a theoretical emphasis on those affecting/interacting with neutrophils and bone in inflammation. ***A)*** *Transcripts for cytokines, immune mediators in several families, and their receptors were regulated by LPA. The CXC chemokines are known neutrophil attractants. Regulated genes highly significant to periodontal homeostasis and inflammation are shown under each category of emphasis/action.* ***B)*** *The P. gingivalis/periodontal pathogen–neutrophil–periodontium/alveolar bone axis in periodontal disease. P. gingivalis and its gingipains interact with the kinin system, fueling vasodilation and inflammation. Currently, LPA is not factored into these interactions; given the results of this study, it is proposed that its roles in this system need to be determined.*

c-Jun N-terminal protein kinase (JNK) activity in the lung prevents inflammation and neutrophil-driven injury in the mouse LPS model of infection,[42] showing the critical role of *SPHK1* in the immune response to LPS. In LPS-stimulated human monocytic cells, ATX and LPA3 were upregulated coordinately through a mechanism requiring *SPHK1*.[43] Of great interest, in bacterial pneumonia, patients' neutrophils expressed more LPA1 and showed significantly increased chemotaxis to LPA and S1P; additionally, LPA1 heterodimerized with an IL8 receptor, chemokine (C-X-C motif) receptor 1 (CXCR1), thus affecting neutrophil function.[44] Furthermore, because LPA1 through LPA3 also heterodimerize with S1P receptors,[45] the authors of this study proposed that the LPA upregulation of *SPHK1* in GFs may be an attractive, unexplored pathway in regulating GF inflammatory responses. The IPA-generated molecular interaction pathways (Fig. 2) suggest this, notably linking *SPHK1* to *PTGS2* (COX-2), IL2, IL6, and glial-derived neurotrophic factor.

Angiogenesis and Bone Breakdown in Periodontal Inflammation

LPA1 nulls show osteopenia and decreased osteogenesis. Significantly, LPA-induced IL6 and IL8 control receptor activator of NF-κB ligand expression in osteoblasts; the role of LPA in directly controlling bone mass and composition via LPA1 and LPA4 has been reviewed.[46] Given these findings and the present results, the authors believed that local LPA may participate in controlling bone balance in periodontitis.

GF Responses to Bacterial LPS and Porphyromonas gingivalis

SOCS2 was the second most highly induced transcript (Table 1). In vivo, it regulates pathogen/LPS-induced chemokine production, and *SOCS2* nulls showed uncontrolled proinflammatory cytokine production and abnormal leukocyte infiltration.[47]

Periodontal tissue from patients with CP has significantly higher levels of *SOCS1* through *SOCS3* than healthy controls.[48] Because *SOCS2* was the second most highly induced transcript at both time points, LPA may have the potential to modulate critical anti-inflammatory circuits in GFs through *SOCS2*, which regulates other SOCS proteins. LPA3 is the likely receptor involved with *SOCS2;* an indirect molecular pathway is suggested in Figure 2.

The bradykinin (BK) B1 receptor (*BDKRB1*) upregulates in inflammation.[49] *P. gingivalis* proteases (gingipains) degrade cytokines, complement system components, and enhance vascular permeability through direct BK release.[50] BK stimulates IL6 and IL8 production by human GFs challenged with IL1β or tumor necrosis factor-α and rapidly induces COX-2 expression in IL1-primed human GFs.[51] Most notably, however, *P. gingivalis* LPS also upregulates BK receptors through mechanisms involving neutrophil influx.[52]

Inflammatory Mediator Production and Regulation

IL8 was the third most upregulated transcript at 2 hours and the most highly induced at 8 hours.

Although the LPA induction of *IL6* and *IL8* message in healthy GFs was much higher than the reported numbers (no more than two-fold) for pass 2 GFs explanted from inflamed human periodontium,[53] a valid comparison cannot be made, because the authors of the previous study grew their cells in 10% serum, which contains micromolar LPA in addition to many other growth factors.[11] LPA was absent from the oral research literature at the time, so they unavoidably reported LPA-interactive effects.

TRIB1 (Table 1) was the most highly upregulated transcript at 2 hours. Inflammatory stimuli induce *TRIB1*, which regulates TLR-mediated signaling[54] and is therefore central in periodontal disease. Human GFs constitutively express TLR1 through TLR9, and LPS triggers production of inflammatory cytokines important in periodontal disease.[55,56] IL1 receptor-associated kinase 4 (*IRAK4*) (Table 1) was the top downregulated molecule at 2 hours. *IRAK1* activation is key to TLR signaling, and *IRAK4* can activate or induce the degradation of *IRAK1*.[56]

IL11 levels are significantly lower in GCF from periodontitis sites.[57] IL11 enhances Wnt (wingless-type MMTV integration site family) signaling and bone formation.[58] IL11 is also indispensable for osteoclast activity.[59] The combined strong upregulation of *COX-2, IL6, IL8,* and *IL11* further suggests that LPA present in the periodontal pocket is likely to contribute in multiple complex ways to the balance of soft and hard tissue breakdown and repair in periodontal disease.

CONCLUSIONS

Sugiura et al.[16] advanced the hypothesis that LPA regulates normal oral wound healing. However, it has received relatively little attention aimed at deciphering its role in oral homeostasis and periodontal disease. One of the two major sources of LPA is activated platelets, which are always present in the inflamed periodontium because of the tissue breakdown and bleeding characteristic of moderate to severe periodontal disease. The results of this study now suggest that LPA is a likely modulator of GF inflammatory responses and their interactions with neutrophil/lymphocyte chemotactic and immune events in the periodontium. Ultimately, LPA may participate in regulating inflammation in periodontal disease, in which the balance of breakdown of gingival tissue, PDL, and alveolar bone in response to periodontal pathogens determines disease status and progression. The authors believe that LPA merits additional investigation of its regulatory role in the oral system and that its contributions remain to be determined using animal models of periodontal disease.

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