AD_____

Award Number: DAMD17-99-1-9108

TITLE: Lysophospholipid Receptors and Effects in Breast Cancer

PRINCIPAL INVESTIGATOR: Edward J. Goetzl, M.D.

CONTRACTING ORGANIZATION: University of California, San Francisco
San Francisco, California 94143-0962

REPORT DATE: July 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2002	Final (1 Jul 99 - 30 Sep 02)

4. TITLE AND SUBTITLE
Lysophospholipid Receptors and Effects in Breast Cancer

5. FUNDING NUMBERS
DAMD17-99-1-9108

6. AUTHOR(S):
Edward J. Goetzl, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California, San Francisco
San Francisco, California 94143-0962

E*Mail: egoetzl@itsa.ucsf.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

ABSTRACT

Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are widely-distributed stimuli of cellular growth and functions, which are generated enzymatically from precursors in membranes of activated normal cells and at much higher levels from many types of cancers. A subfamily of G protein-coupled receptors encoded by endothelial differentiation genes (Edg Rs) bind and transduce cellular signals from LPA (Edg-2, -4, -7) and S1P (Edg-1, -3, -5, -6, and –8). Human breast cancer cells (BCCs) express principally Edg-3, less Edg-4, very low levels of Edg-2 and Edg-5, and no Edg-1, -6, -7 or –8. BCCs have much higher levels of Edg-3 S1P Rs than normal human breast epithelial cells. These Edg Rs mediate proliferation of BCCs directly by signaling growth-related immediate-early genes, through a distinctive set of transcription factors, and indirectly by increasing BCC secretion of several autocrine protein growth factors. Delivery of LPA to BCCs by specific plasma protein carriers, such as gelsolin, and novel mechanisms for regulation of expression of Edg-3 Rs also may distinguish mechanisms of action of S1P and LPA in BCCs from those in normal breast epithelial cells. Detection of elevated levels of Edg-3 Rs and splice variants of Edg-3 Rs may become a diagnostic index of breast cancer and Edg R-directed antagonists may suppress growth and dissemination of breast cancers.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer	80
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION: (All references cited are from the publication list.)

A subfamily of eight structurally homologous G protein-coupled receptors (GPCRs) for the growth factors lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are encoded by endothelial differentiation genes (Edg Rs) and several more distantly homologous GPCRs are specific for related phospholipids. A nomenclature subcommittee of IUPHAR has this year renamed Edg Rs according to the preferred ligand and order of discovery (h). $S1P_1$ (Edg-1), $S1P_2$ (Edg-5), $S1P_3$ (Edg-3), $S1P_4$ (Edg- 6), and $S1P_5$ (Edg–8) Rs are specific for S1P, whereas LPA_1 (Edg-2), LPA_2 (Edg-4), and LPA_3 (Edg–7) are specific for LPA. Edg Rs transduce two distinct types of cellular responses to LPA and S1P (b,d,f,i). The first are growth-related and include direct nuclear signaling of immediate-early genes by induction of a distinctive set of transcription factors and indirect signaling through increases in both generation of autocrine protein growth factors and expression of their receptors. Suppression of apoptosis is in this cluster of responses to LPA and S1P. These responses would contribute to the unregulated cellular proliferation typical of breast cancer. The second are growth-independent and encompass a cluster of cytoskeleton-dependent functions, including adhesion, migration, contraction and secretion. The former responses in this cluster may mediate the tissue invasion and metastasis by breast cancers.

Results of numerous studies conducted during the term of this project have shown that many gender-specific cancers overexpress one or more LPA and/or S1P Rs (a,d,k). Our investigations in the first years of this project established that human breast cancer cells (BCCs) of several estrogen receptor-positive and –negative cultured lines express high levels of $S1P_3$ (Edg-3) Rs and LPA_2 (Edg-4) Rs, which respectively transduce S1P and LPA stimulation of proliferation by both direct and indirect mechanisms (a). Concentrations of S1P and LPA in plasma and other extracellular fluids are normally in the near micromolar range and often are higher in cancers, where their respective GPCRs are fully saturated (e). It became clear in the last year of the project that the most relevant goals were to elucidate mechanisms by which BCCs upregulate distinct S1P and/or LPA GPCRs and to determine which cancer-active agents might alter BCC expression of S1P or LPA Rs. The focus of all completed studies thus has been on Tasks 1 and 2, and we have addressed only a few aspects of Tasks 3 and 4 (j,k).

BODY:

Hypothesis and Tasks- The central hypothesis is that the upregulation of $S1P_3$ and LPA_2 GPCRs in BCCs contributes to the abnormal proliferation and spread of breast cancer, represents a distinctive functional marker of this malignancy, and may provide specific targets for novel therapeutic approaches.

1. Delineate determinants of expression of Edg Rs (S1P Rs and LPA Rs) by human cultured breast cancer cells (BCCs)

2. Characterize mechanisms of Edg R (S1P R and LPA R) enhancement of SRE-regulated transcription in human cultured breast cancer cells (BCCs)

3. Elucidate the pathways by which Edg Rs (S1P Rs and LPA Rs) transduce LPL enhancement of both secretion of IGF-II and expression of IGF-I-Rs

4. Investigate the mechanisms of Edg R (S1P R and LPA R) transduction of LPL enhancement of expression and functions of heparin-binding epidermal growth factor-like growth factor (HB-EGF)

Task 1-
Initial semi-quantitative RT-PCR studies of the rank-order of frequency of expression of mRNA encoding S1P Rs and LPA Rs by BCCs revealed Edg-3> Edg-4> Edg-5 ≥ Edg-2, without detectable Edg-1 or -7, in both the estrogen receptor-positive MCF-7 line and the estrogen receptor-negative MDA-MB-453 line (a). Subsequent semi-quantitative RT-PCR analyses of mRNA from five other lines of BCCs confirmed the more general relevance of this profile of Edg Rs. Edg R expression was re-quantified subsequently by TaqMan real-time PCR and Western blots with mouse monoclonal anti-Edg R peptide antibodies (k). Quantitative PCR has substantially improved our ability to determine precisely the levels of mRNA encoding each Edg R. For example, in the MDA-MB-453 line of human BCCs, Edg-3 seemed dominant quantitatively by conventional RT-PCR, but nearly equal levels of Edg-2, -4, and –5 also were detected. With real-time quantitative PCR, the respective mean representation of Edg-1, -2, -3, -4 and –5 was: 0, 1.4, 60, 32, and 2.2. The results with mRNAs isolated from normal breast epithelial cells showed quantitative differences in comparison with those from BCCs. All Edg R mRNA levels were lower in the normal breast epithelial cells and the rank-order of frequency of expression was different with Edg-3= Edg-4 >Edg-5= Edg-2, and no detectable Edg-1 or –7. Thus Edg R levels show quantitative differences between BCCs and normal breast epithelial cells, but not the major qualitative distinctions of ovarian cancer where Edg-4 R is expressed by all ovarian cancer cells and not by normal or immortalized ovarian surface epithelial cells.

Estrogen, the LPA and S1P ligands, and 1, 25-dihydroxy-vitamin D3 (VD3) all were examined initially for their effects on expression of Edg receptors (Edg Rs) by BCCs. The first results indicated that LPA, S1P and VD3 all were capable of regulating expression of the principal Edg Rs of BCCs except for estrogen, for which no effects were observed at physiological concentrations. At 1 nM to 1 uM, LPA downregulated Edg-2 Rs and less effectively Edg-4 Rs by respective mean maxima of up to 83% and 36% after 30 min at 37°C. Similarly, 1 nM to 1 uM S1P reduced expression of Edg-3 and Edg-5 Rs, respectively, by mean maxima of 59% and 90% after 30 min at 37°C. LPA had no effect on expression of Edg-3 and Edg-5 Rs, and S1P had no effect on expression of Edg-2 and Edg-4 Rs by MDA-MB-453 BCCs. The characteristics of ligand-independent alterations in expression of Edg Rs by VD3 were elucidated in a detailed series of experiments (k). A 24 h exposure to VD3 suppressed Edg R-encoding mRNA with VD3 concentration-dependence, similar effects on Edg-2, -3 and –5, but no suppression of Edg-4 R mRNA. Thus VD3 pretreatment effectively shifts the predominant sensitivity of BCCs from S1P to LPA. The increases in $[Ca^{2+}]i$ induced in MDA-MB-453 BCCs by S1P and LPA also were suppressed by pretreatment with VD3, with ligand concentration-

dependence and increased suppression in relation to the time of pretreatment. The magnitude of suppression of $[Ca^{2+}]i$ responses to S1P by VD3 were far greater than that of equivalent responses to LPA, as expected by the greater reduction in Edg-3 and Edg-5 Rs than Edg-2 Rs, and the lack of suppression of Edg-4 Rs. In later studies, incubation of MDA-MB-453 BCCs with 10 uM all-*trans* retinoic acid (RA) suppressed expression of Edg-2, -3 and –4 by more than 50%, but increased expression of Edg-5 significantly (k). After
24 h and 48 h with RA, mRNA encoding Edg-2, -3 and –4 were suppressed significantly, whereas that for Edg-5 was augmented by more than three-fold. Western blots of proteins extracted from untreated BCCs and those exposed to VD3 and RA confirmed findings at the level of mRNA (k).

Three distinct sets of MDA-MB-453 (453) BCCs thus were generated by their optimal exposure to VD3 and RA: native untreated BCCs have predominantly Edg-3 and –4 Rs, VD3-treated BCCs have decreased levels of Edg-3 and other Edg Rs but normal expression of Edg-4 Rs, and RA-treated BCCs have increased levels of Edg-5 Rs and decreased expression of all other Edg Rs. Biochemical studies of signal transduction and analyses of functional responses to LPLs were carried out in these three sets of BCCs. S1P and LPA both evoke concentration-dependent increases in $[Ca^{2+}]i$ in native 453 BCCs in which responses to LPA are greater and a higher multiple of the background than those to S1P, despite greater expression of Edg-3 than Edg-4 Rs (k). In VD3-treated 453 BCCs, $[Ca^{2+}]i$ responses to multiple concentrations of S1P but not to LPA were suppressed as is consistent with VD3-induced reduction in Edg-3, but not Edg-4 Rs (r5). In RA-treated 453 BCCs, in contrast, $[Ca^{2+}]i$ responses to LPA were prominently suppressed, in parallel with reductions in Edg-2 and Edg-4 Rs (k), whereas responses to S1P were only slightly suppressed, consistent with modest decreases in expression of Edg-3 concurrently with increases in Edg-5 Rs. Migration of native 453 BCCs was stimulated chemokinetically by 10^{-8} M to 10^{-6} M S1P and much less prominently by 10^{-6} M LPA (j,k). VD3 treatment suppressed significantly the chemokinetic responses to S1P, but not to LPA, as expected from results of receptor studies (k). Although not clearly predictable from results of analyses of changes in receptor expression, RA also decreased chemokinetic responses to S1P. Edg-3 Rs thus appear to mediate S1P-evoked chemokinesis. RA-induced increases in Edg-5 Rs do not compensate for the loss of Edg-3 Rs but instead raise Edg-5 to levels which suppress migration to non-lipid factors.

c) Task 2-

These studies proved more challenging than expected and required development of two additional capabilities, the first of which was activating monoclonal anti-Edg-3 and -4 R antibodies and the second was luciferase reporter constructs of two sub-components of the SRE element. The basic observation is highly reproducible, with striking enhancement of the SRE report by LPA and S1P (a). S1P exhibited 100-fold higher potency than LPA. VD3 suppressed SRE reports evoked by each stimulus, with far less difference than that observed for $[Ca^{2+}]i$ responses. These results raised questions about the Edg R-transduction of SRE responses to LPA and S1P. Newly-developed anti-Edg-3 and anti-Edg-4 R antibodies with agonist activity gave the same results as the primary LPA and S1P stimuli. The use of a new reporter construct composed of the Elk-1

substituent of SRE in a luciferase reporter plasmid demonstrated that LPA and S1P signals were transduced by their respective Edg Rs, as equivalent reports were evoked by S1P and anti-Edg-3 antibody, and by LPA and anti-Edg-4 antibody. In this system, VD3 suppressed luciferase responses to S1P, but not to LPA, as expected from the differential inhibition of expression of Edg-3 but not Edg-4 Rs.

Task 3-
The basal level of secretion of IGF-II was sufficiently high for analysis by immunoassays only in ER-positive BCCs, where it was increased significantly and progressively by 10^{-9} M to 10^{-6} M S1P and LPA up to mean levels four- to five-times the baseline. This response was dependent on Gi alpha and Ras/MEK more than Rho. The suppression of proliferative responses of BCCs to S1P and LPA by different neutralizing antibodies to IGF-II and to IGF-I-Rs reached a mean maximum of 41% and 51%, respectively, and indicated a substantial role for this amplification mechanism in the total stimulatory effect of LPA and S1P (a). That the level of IGF-II attained by LPA or S1P induction was capable of augmenting proliferation was proven directly by assessing effects of adding synthetic IGF-II alone to cultures of BCCs. Optimal levels of IGF-II increased SRE-luciferase reports by a mean of three-fold, as contrasted with 12-fold by 10^{-7} M LPA, supporting the possibility that S1P/LPA-evoked IGF-II mediates a substantial part of the proliferative response.

e) Task 4-
No work has been done on this project task since the original description (r4).

Key Research Accomplishments:
Development of anti-Edg-3 R and anti-Edg-4 R monoclonal antibodies with Edg receptor-dependent functional effects on BCCs.
Characterization and application of biochemical assays of Edg-3 R and Edg-4 R signaling in BCCs.
Finding that BCC migration through Matrigel on a micropore filter, as a model of trans-basement membrane passage into tissues, is altered by LPA and S1P in relation to Edg R expression.
Verification that 1, 25-dihydroxy-vitamin D3 selectively suppresses expression of Edg-3 Rs and to a lesser extent Edg-2 and Edg-5 Rs, but not Edg-4 Rs, whereas all-*trans* retinoic acid suppresses Edg-2, -4 and –3, while increasing Edg-5 Rs in BCCs by transcriptional mechanisms and to an extent that modifies functional responses.

Reportable Outcomes:

Publications-

Goetzl, E.J., Dolezalova, H., Kong, Y., and Zeng, L. Dual mechanisms for lysophospholipid induction of proliferation of human breast carcinoma cells. Cancer Res. 59: 4732-4737, 1999.

Goetzl, E.J., and Lynch, K.R. The omnific lysophospholipid growth factors. Ann. N.Y. Acad. Sci. 905: xi-viv, 2000.

Goetzl, E.J., Lee, H., Dolezalova, H., Kalli, K.R., Conover, C.A., Hu, Y.-L., Azuma, T., Stossel, T.P., Karliner, J.S., and Jaffe, R.B. Mechanisms of lysolipid phosphate effects on cellular survival and proliferation. Ann. N.Y. Acad. Sci. 905: 177-187, 2000.

Goetzl, E.J., An, S., and Lee, H. Lysophospholipid growth factor receptors. Cytokine Reference Database. Academic Press, pp. 2255-2260, 2000.

Goetzl, E.J., Lee, H., and Tigyi, G.J. Lysophospholipid growth factors. Cytokine Reference Database. Academic Press, pp. 1407-1418, 2000.

Goetzl, E.J. Pleiotypic mechanisms of cellular responses to biologically active lysophospholipids. Prostaglandins and other Lipid Mediators 64: 11-20, 2001.

Goetzl, E.J., Graeler, M., Huang, M.-C., and Shankar, G. Lysophospholipid growth factors and their G protein-coupled receptors in immunity, coronary artery disease, and cancer. The Scientific World Journal 2: 324-338, 2002.

Chun, J., Goetzl, E.J., Hla, T., Igarashi, Y., Lynch, K.R., Moolenaar, W., Pyne, S., and Tigyi, G. International Union of Pharmacology. XXXIV. Lysophospholipid Receptor Nomenclature. Pharmacol. Rev.54: 265-269.

Huang, M.-C., Graeler, M., Shankar, G., Spencer, J., and Goetzl, E.J. Lysophospholipid mediators of immunity and neoplasia. Biochimica Biophysica Acta 1582: 161-167, 2002.

Dolezalova, H., and Goetzl, E.J. Bidirectional regulation of expression of the G protein-coupled Edg-5 receptor for sphingosine 1-phosphate (S1P) in human MDA-MB-453 breast cancer cells (BCCs). FASEB J. 16: A926, 2002.

Dolezalova, H., Shankar, G., Huang, M.-C., Bikle, D.D., and Goetzl, E.J. Regulation of human breast cancer cell expression of $S1P_2$ (Edg-5) and $S1P_3$ (Edg-3) G protein-coupled receptors for sphingosine 1-phosphate by 1, 25-dihydroxy-vitamin D3 and all-trans retinoic acid. J. Cellular Biochem., in press.

Conclusions:

The results of over 3 years of research in this field clearly show that Edg-3 Rs are upregulated and predominate on BCCs, compared to normal human breast epithelial cells, and therefore that S1P is the major LPL growth factor signal for BCCs. Some other Edg Rs may be mainly inhibitory, as is the case for S1P-mediated suppression of BCC migration by unopposed signals from Edg-5 Rs. The involvement of both direct and indirect signaling from Edg-3 Rs in the biochemical and biological responses of BCCs to S1P also has been confirmed. Thus Edg-3 may be a marker for breast cancer and pharmacological antagonists specific for Edg-3 Rs may have therapeutic benefits in some breast cancer patients. These results also clearly distinguish the abnormalities in the Edg Rs of BCCs from those of ovarian cancer cells, where the predominant axis is LPA and Edg-4 and –7 Rs.

References

These are cited from the list of publications included under Reportable Outcomes, page 8.

[CANCER RESEARCH 59, 4732–4737, September 15, 1999]

Dual Mechanisms for Lysophospholipid Induction of Proliferation of Human Breast Carcinoma Cells[1]

Edward J. Goetzl,[2] Hana Dolezalova, Yvonne Kong, and Li Zeng

Departments of Medicine and Microbiology-Immunology, University of California Medical Center, San Francisco, California 94143-0711

ABSTRACT

Endothelial differentiation gene-encoded G protein-coupled receptors (Edg Rs) Edg-1, Edg-3, and Edg-5 bind sphingosine 1-phosphate (S1P), and Edg-2 and Edg-4 Rs bind lysophosphatidic acid (LPA). LPA and S1P initiate ras- and rho-dependent signaling of cellular growth. Cultured lines of human breast cancer cells (BCCs) express Edg-3 > Edg-4 > Edg-5 > or = Edg-2, without detectable Edg-1, by both assessment of mRNA and Western blots with rabbit and monoclonal mouse anti-Edg R antibodies. BCC proliferation was stimulated significantly by 10^{-9} M to 10^{-6} M LPA and S1P. Luciferase constructs containing the serum response element (SRE) of growth-related gene promoters reported mean activation of BCCs by LPA and S1P of up to 85-fold. LPA and S1P stimulated BCC secretion of type II insulin-like growth factor (IGF-II) by 2–7-fold, to levels at which exogenous IGF-II stimulated increased proliferation and SRE activation of BCCs. All BCC responses to LPA and S1P were suppressed similarly by pertussis toxin, mitogen-activated protein kinase kinase inhibitors, and C3 exoenzyme inactivation of rho, suggesting mediation by Edg Rs. Monoclonal anti-IGF-II and anti-IGFR1 antibodies suppressed proliferation and SRE reports of BCCs to LPA and S1P by means of up to 65%. Edg Rs thus transduce LPA and S1P enhancement of BCC growth, both directly through SRE and indirectly by enhancing the contribution of IGF-II.

INTRODUCTION

The lysolipid phosphate mediators LPA[3] and S1P are generated enzymatically from membrane lipid precursors of many different types of normal and malignant cells (1, 2). Extracellular LPA and S1P both stimulate cellular proliferation, differentiation, survival, adhesion, aggregation, and other specific functions (3–5). A recently characterized subfamily of at least five G protein-coupled receptors, which are encoded by *edg*s, bind and transduce signals from LPA or S1P (6–10). Two homology clusters with greater structural similarity and shared ligand specificity are composed of the *edg*-encoded G protein-coupled receptors (Edg Rs) Edg-1, Edg-3, and Edg-5 set of S1P Rs and Edg-2 and Edg-4 LPA Rs. The capacity of LPA and S1P to improve cellular survival is in part a result of suppression of apoptosis by several distinct mechanisms (11, 12). LPA and S1P stimulate cellular proliferation directly by eliciting the serum response factor and ternary complex factor transcription factors, which together bind to and activate the SRE in promoters of many immediate-early genes (13). The involvement of SRE-dependent mechanisms in mediating LPA and S1P enhancement of proliferation has not been examined carefully in malignant cells, nor has the possibility of effects of LPA and/or S1P on polypeptide growth factors necessary for optimal tumor growth.

Functional Edg receptors and proliferative responses to LPA and S1P thus were characterized in the ER-positive MCF-7 cultured line of human BCCs and the MDA-MB-453 ER-negative line of BCCs. The relative contributions of direct SRE-dependent induction of transcription and of enhancement of production of IGF-II in proliferative responses to LPA and S1P also were determined in these BCCs.

MATERIALS AND METHODS

Chemical Reagents and Antibodies. The sources of chemicals were: S1P and sphingosine (Biomol, Plymouth Meeting, PA); LPA, phosphatidic acid, 1-β-D-galactosyl-sphingosine (psychosine), and fatty acid-free BSA (Sigma Chemical Co., St. Louis, MO); and human IGF-II (Peprotech, Inc., Rocky Hill, NJ). Cells were treated with PTX (Calbiochem, Inc., La Jolla, CA), recombinant *Clostridium botulinum* C3 ADP-ribotransferase (C3 exoenzyme; List Biological Laboratories, Inc., Campbell, CA), which ADP-ribosylates rho specifically, and the MEK inhibitor 2′-amino-3′-methoxyflavone (PD98059; Calbiochem) as described (10, 14). Mouse monoclonal antibodies specific for substituent peptides of human Edg-3 (amino acids 1–21), Edg-4 (amino acids 9–27), and Edg-5 (amino acids 303–322) have been described (12, 15), the immunogens for which were selected from sequences of high homology among humans and rodents. The expected cross-reaction with corresponding rodent Edg Rs has been confirmed by the identical recognition of human and rat Edg-5 Rs. The cross-reactivity of each antibody with heterologous Edg proteins was <1%, as determined by Western blots of 0.1–100 μg of membrane proteins isolated from HTC4 rat hepatoma cells stably transfected with human Edg-2, Edg-3, Edg-4, or Edg-5 (12, 15). Each monoclonal IgG was purified by protein A affinity-chromatography (Pierce Chemical Co.) and used to develop Western blots at 0.1–0.3 μg/ml (15). A mouse monoclonal IgG1 that specifically neutralizes activity of human/rat IGF-II, but not IGF-I (Upstate Biotechnology, Inc., Lake Placid, NY), and a mouse monoclonal antibody, termed α-IR3, which blocks binding of IGF-II to IGFR1 (Oncogene Science, Cambridge, MA), were purchased. A rabbit polyclonal antiserum to rodent and human Edg-2 was kindly provided by Dr. Jerold Chun (University of California–San Diego, San Diego, CA).

Cell Culture and Quantification of Cellular Proliferation. Layers of ER-positive MCF-7 (ATCC # HTB-22) and ER-negative MDA-MB-453 (ATCC# HTB-131) human BCCs were cultured in DMEM with 4.5 g/100 ml of glucose, 10% FBS, 100 units/ml of penicillin G, and 100 μg/ml of streptomycin (complete DMEM) to 100% confluence and relayered every 3–4 days to 25–30% confluence. To assess proliferation, replicate layers of 1×10^4 BCCs were cultured in 48-well plates in complete DMEM for 4 h, washed once, and cultured for 20 h in serum-free DMEM. Some wells were pretreated with PTX for 6 h, C3 exoenzyme for 30 h, or MEK inhibitor for 2 h. Antisera were added, followed in 1 h by lipid stimuli and incubation for 72 h. Then wells were washed two times with Ca^{2-}- and Mg^{2+}-free Hanks' solution, and the cells were harvested in 0.2 ml of EDTA-trypsin solution for staining with trypan blue and eosin and quantification by microscopic counting of 10 1-mm^3 fields in a hemocytometer.

Reverse Transcription-PCR Analysis of Edg Rs. Total cellular RNA was extracted by the TRIzol method (Life Technologies, Inc., Grand Island, NY), from suspensions of BCCs and lines of stably transfected rat HTC4 hepatoma cells, that all had low background expression of native Edg Rs, and each overexpressed one recombinant human Edg R. A Superscript kit (Life Technologies, Inc.) was used for reverse transcription synthesis of cDNAs. PCR began with a "hot start" at 94°C for 3 min; Taq DNA polymerase was added, and amplification was carried out with 35 cycles of 30 s at 94°C, 2 min at 55°C, and 1 min at 72°C. Two μCi of [α-^{32}P]dCTP were added to some sets

Received 5/6/99; accepted 7/20/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] These studies were supported by Grant HL-31809 from the NIH and a grant (to E. J. G.) and fellowship (to H. D.) from the Department of the Army.

[2] To whom requests for reprints should be addressed, at Immunology and Allergy, UB8B, Box 0711, University of California Medical Center, 533 Parnassus, San Francisco, CA 94143-0711. Phone: (415) 476-5339; Fax: (415) 476-6915; E-mail: egoetzl@itsa.ucsf.edu.

[3] The abbreviations used are: LPA, lysophosphatidic acid; S1P, sphingosine 1-phosphate; *Edg*, endothelial differentiation gene; SRE, serum response element; ER, estrogen receptor; BCC, breast cancer cell; MEK, mitogen-activated protein kinase kinase; PTX, pertussis toxin; IGF-II, type II insulin-like growth factor; IGFR, IGF receptor; FBS, fetal bovine serum; RT-PCR, reverse transcription-PCR; G3PDH, glyceraldehyde 3-phosphate dehydrogenase.

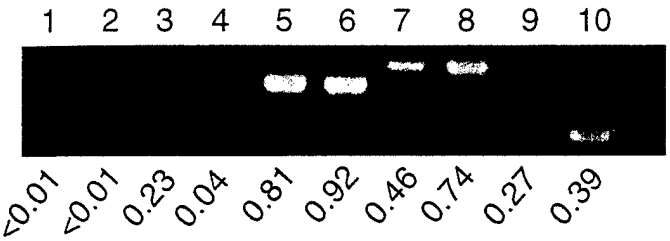

Fig. 1. RT-PCR semiquantification of mRNA encoding Edg Rs in MCF-7 and MDA-MB-453 cells. The volume of cDNA mixture from each type of BCC was selected to equalize the level of amplified G3PDH cDNA product. *Lanes 1, 3, 5, 7,* and *9* are from MDA-MB-453 cells, and *Lanes 2, 4, 6, 8,* and *10* are from MCF-7 cells. *Lanes 1* and *2,* Edg-1; *Lanes 3* and *4,* Edg-2; *Lanes 5* and *6,* Edg-3; *Lanes 7* and *8,* Edg-4; *Lanes 9* and *10,* Edg-5. The number *below* each lane represents the ratio of ^{32}P in cDNA for an Edg R to that for G3PDH.

of reaction mixtures to allow quantification of mRNA encoding each Edg R relative to that of the standard G3PDH (16). Oligonucleotide primer pairs were: G3PDH, 5′-dCCTGGCCAAGGTCATCCATGACAAC and 5′-dTGTCATACCAGGAAATGAGCTTGAC; Edg-1, 5′-CTACACAAAAAGCTTGGATCACTCA and 5′-CGACCAAGTCTAGAGCGCTTCCGGT (1100 bp): Edg-2, 5′-dGCTCCACACACGGATGAGCAACC and 5′-GTGGTCATTGCTGTGAACTCCAGC (621 bp); Edg-3, 5′-dCAAAATGAGGCCTTACGACGCCA and 5′-dTCCCATTCTGAAGTGCTGCGTTC (701 bp); Edg-4. 5′-dAGCTGCACAGCCGCCTGCCCCGT and 5′-dTGCTGTGCCATGCCAGACCTTGTC (775 bp); and Edg-5, 5′-CTCTCTACGCCAAGCATTATGTGCT and 5′-ATCTAGACCCTCAGACCACCGTGTTGCCCTC (500 bp). PCR products were resolved by electrophoresis in a 2 g/100 ml agarose gel with ethidium bromide staining. G3PDH and Edg R cDNA bands were cut from gels and solubilized for β-scintillation counting in 0.5 ml of sodium perchlorate solution at 55°C for 1 h (Elu-Quick; Schleicher and Schuell, Keene, NH). Initially, the G3PDH cDNA templates in several different-sized portions of each sample were amplified to determine volumes that would result in G3PDH bands of equal intensity for each sample. Relative quantities of cDNA encoding each Edg R also were calculated by the ratio of radioactivity to that in the corresponding G3PDH band (16).

Western Blots. Replicate suspensions of 1×10^7 BCCs, which had been incubated without or with LPA or S1P for 16 h, were washed three times with 10 ml of cold Ca^{2+}- and Mg^{2+}-free PBS, resuspended in 0.3 ml of cold 10 mM Tris-HCl (pH 7.4) containing a protease inhibitor mixture (Sigma Chemical Co., St. Louis, MO), 0.12 M sucrose, and 5% glycerol (v/v). After homogenization with a Teflon pestle on ice for 2 min at 250 rpm, each sample was centrifuged at $400 \times g$ for 5 min at 4°C, and the supernatant was centrifuged at $300,000 \times g$ for 30 min at 4°C. Each $300,000 \times g$ pellet was resuspended in 0.2 ml of 10 mM Tris-HCl (pH 7.4) with 1% (v/v) NP40, 5% glycerol, and protease inhibitor mixture and rehomogenized and incubated at 4°C for 2 h prior to centrifugation again at $300,000 \times g$. Aliquots of supernatant containing 1–100 μg of protein were mixed with 4× Laemmli's solution, heated to 100°C for 3 min, and electrophoresed in an SDS-12% polyacrylamide gel for 20 min at 100 V and 1.5 h at 140 V, along with a rainbow prestained set of molecular weight markers (DuPont NEN, Boston, MA or Amersham, Inc., Arlington Heights, IL). Proteins in each gel were transferred electrophoretically to a nitrocellulose membrane (Hybond; Amersham) for sequential incubation with 5 g% reconstituted nonfat milk powder to block unspecific sites, dilutions of mouse monoclonal anti-Edg R antibody, and then horseradish peroxidase-labeled goat anti-mouse IgG, prior to development with a standard ECL kit (Amersham).

RIA and Dot-Blot Quantification of IGF-II. RIAs were conducted according to the instructions of Research and Diagnostic Antibodies, Inc. (Berkeley, CA), after removal of some IGF binding proteins by Sep-Pak chromatography (Millipore Corp., Milford, MA), as directed (17). Dot-blot quantification of IGF-II was performed using a method in which binding proteins do not alter immunoreactivity of IGF-II in unprocessed cellular secretions (18).

Transfections and Reporter Assay. Replicate suspensions of $0.3–1 \times 10^5$ MCF-7 and MDA-MB-453 BCCs in 1 ml of complete DMEM were cultured in 12-well plates for 24 h to establish monolayers of 40–50% confluency. The monolayers were washed twice and covered with 1 ml of serum-free DMEM and lipotransfected with 100 ng/well of a SRE firefly luciferase reporter plasmid (8) and 5 ng/well of pRL-CMV Renilla luciferase vector (Promega Corp., Madison, WI) using FuGENE 6 (Boehringer Mannheim Corp., Indianapolis, IN). After 30 h of incubation, medium was replaced with fresh serum-free DMEM and anti-IGFR1 or anti-IGF-II mouse monoclonal antibodies or IgG1 isotype control was added, followed in 2 h by 10^{-10} M to 10^{-6} M LPA, S1P, or other lipids in serum-free DMEM with 0.1 mg/ml of fatty acid-free BSA. Some wells were pretreated with PTX for 6 h, C3 exoenzyme for 30 h, or MEK inhibitor for 2 h. After 4 h of incubation at 37°C, the luciferases were extracted in Reporter lysis buffer (Promega), and their activities were quantified sequentially by luminometry using Luciferase Assay and Stop & Glo reagents (Promega), with integration of light emitted during the 15 s after addition of each reagent (EG & G Berthold microplate luminometer, model LB96V). Firefly luciferase values were corrected for differences in apparent transfection efficiency by expression as a ratio with Renilla luciferase signals in the corresponding samples.

RESULTS

BCC Expression of Edg Receptors. mRNA encoding individual Edg Rs had been detected by Northern blotting in some human tumor cells (7–9). The relative levels of mRNA encoding each of the Edg Rs in BCCs now have been semiquantified by RT-PCR (Fig. 1). Several

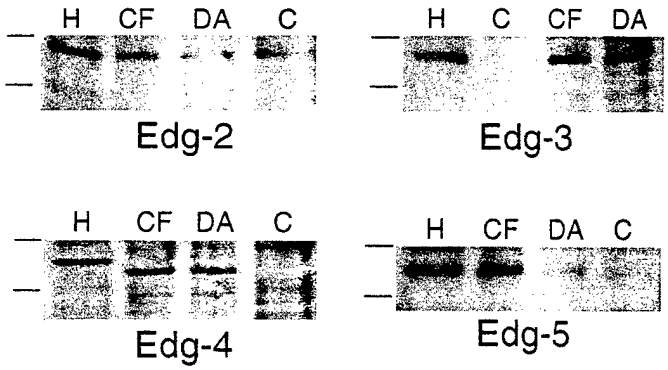

Fig. 2. Western blot analysis of the expression of Edg-2, Edg-3, Edg-4, and Edg-5 Rs by MCF-7 and MDA-MB-453 BCCs. The four samples analyzed for content of each Edg R are: *H,* 3 μg of protein extracted from HTC4 rat liver cells that were stably transfected with the respective Edg Rs; *C,* 10 μg of protein from control untransfected HTC4 cells; *CF,* 10 μg of protein from MCF-7 BCCs; and *DA,* 10 μg of protein from MDA-MB-453 BCCs. Blots were developed with rabbit anti-Edg-2 antiserum and anti-Edg-3, anti-Edg-4, and anti-Edg-5 mouse monoclonal antibodies. The marginal lines show the positions of M_r 45,000 and M_r 66,000 protein molecular weight markers.

Table 1 *Relative levels of mRNA encoding Edg receptors in BCCs*

All numbers are the mean ± SD of the results of three RT-radioPCR determinations of mRNA, where each value presented is the ratio of ^{32}P in the respective Edg R cDNA band to that in the G3PDH cDNA.

Type of BCC	Edg-1	Edg-2	Edg-3	Edg-4	Edg-5
MCF-7	0.00	0.04 ± 0.02	1.00 ± 0.08	0.70 ± 0.04	0.49 ± 0.09
MDA-MB-453	0.00	0.17 ± 0.05	0.81 ± 0.08	0.43 ± 0.03	0.19 ± 0.07
Edg-2 Tr[a]	<0.02	1.44 ± 0.17	0.18 ± 0.06	0.08 ± 0.06	0.06 ± 0.03
Edg-3 Tr	0.02	0.27 ± 0.09	1.29 ± 0.19	0.07 ± 0.05	0.03 ± 0.03
Edg-4 Tr	0.02	0.28 ± 0.03	0.16 ± 0.04	1.43 ± 0.28	0.07 ± 0.05
Edg-5 Tr	0.03	0.25 ± 0.07	0.17 ± 0.05	0.05 ± 0.04	1.36 ± 0.25

[a] Tr, HTC4 rat transfected hepatoma cells expressing a human recombinant Edg R.

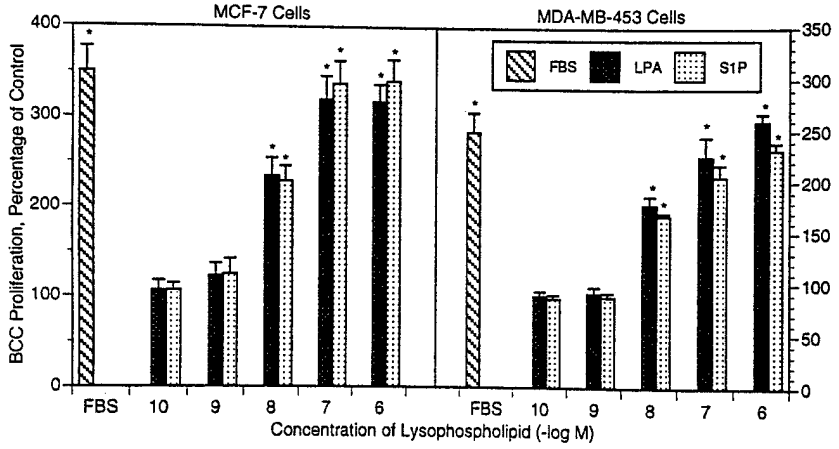

Fig. 3. Stimulation of proliferation of MCF-7 and MDA-MB-453 BCCs by LPA and S1P. *Columns*, means of the results of three studies performed in duplicate; *bars*, SD. FBS is the 2% FBS-positive control. The serum-free medium alone controls (100%) were 1.5, 1.4, and 1.5 × 10^4/well in the three studies of MCF-7 BCC proliferation and 1.1, 1.2, and 1.1 × 10^4/well in the three studies of MDA-MB-453 BCC proliferation. The levels of significance of increases above medium control proliferation were determined by a paired Student *t* test; *, $P < 0.01$.

different amounts of first-strand cDNAs prepared from MCF-7 and MDA-MB-453 BCCs were amplified initially to allow selection of a volume of each that provided equally intense cDNA bands for the internal standard G3PDH. With this standard approach, the mRNA from both human BCC lines was found to encode similarly high levels of Edg-3 R but had no detectable Edg-1 R message (Fig. 1). The ER-negative MDA-MB-453 BCCs had higher levels of mRNA encoding the Edg-2 R, whereas the ER-positive MCF-7 BCCs had higher levels of mRNA for Edg-4 and Edg-5.

RadioPCR has been used to assess levels of mRNA specific for other G protein-coupled Rs, but not Edg Rs (16). Thus, an initial study examined mRNA from four lines of rat HTC4 hepatoma cells, which were stably transfected with individual human Edg Rs 2 to 5 (Table 1). The rank order of levels of mRNA for endogenous Edg Rs in HTC4 cells prior to transfection was Edg-2 ≫ Edg-3 > Edg-4 > Edg-5, without detectable Edg-1 mRNA. The level of mRNA for the transfected Edg R in each line was much higher than background (Table 1). In this frame of reference, the levels of BCC mRNA encoding Edg-3 were nearly as high as the index transfectant and > Edg-4 > Edg-5 ≥ Edg-2, without any Edg-1 mRNA. The differences in relative amounts of mRNA for each Edg R between the two lines of BCCs were the same as for standard PCR (Fig. 1).

Western blots developed with polyclonal anti-Edg-2 R and monoclonal anti-Edg-3, anti-Edg-4, and anti-Edg-5 antibodies showed one predominant protein of expected size in extracts of each of the four lines of HTC4 cell transfectants (Fig. 2). Electrophoresis of over three times more protein from untransfected control HTC4 cells than transfectants did not show Edg-3, Edg-4, or Edg-5 protein antigen, but a faint band of Edg-2 protein was detected that might reflect the higher endogenous levels of mRNA encoding this R (Table 1). The results of BCC Western blots confirmed expression of Edg proteins representing both LPA R and S1P R subtypes, with a predominance of Edg-3 R in both BCC lines (Fig. 2). In contrast to expectations from PCR results, however, MCF-7 BCCs had higher levels of Edg-2 as well as Edg-4 and Edg-5 proteins than MDA-MB-453 BCCs. The Edg-4 R protein of both BCC lines was consistently M_r 2000–3000 smaller than the recombinant human Edg-4 R protein, but the basis for the difference has not yet been elucidated.

Functional and Biochemical Responses of BCCs to LPA and S1P. The proliferation of both lines of BCCs was assessed by counting viable cells after 72 h (Fig. 3). Proliferation of MCF-7 BCCs was increased significantly by 10^{-8} M to 10^{-6} M LPA and S1P to maximum levels similar to those attained by 2% FBS. In parallel studies of MDA-MB-453 BCCs, proliferative responses to LPA and S1P were similar to those of MCF-7 BCCs, with significant increases evoked by 10^{-8} M to 10^{-6} M LPA and S1P (Fig. 3).

Activation of SRE in the promoters of diverse growth-related genes is a fundamental characteristic of the growth-promoting potential of LPA and S1P. BCCs thus were transfected with an SRE-firefly luciferase construct and 1/20 the amount of a Renilla luciferase-CMV construct as an internal standard for consistency of transfection. LPA and S1P increased the mean levels of standardized luciferase luminometric activity in ligand concentration-dependent relationships by maxima of up to 37-fold and 85-fold, respectively, in MCF-7 BCCs (Fig. 4). Similar responses to the same concentrations of LPA and S1P

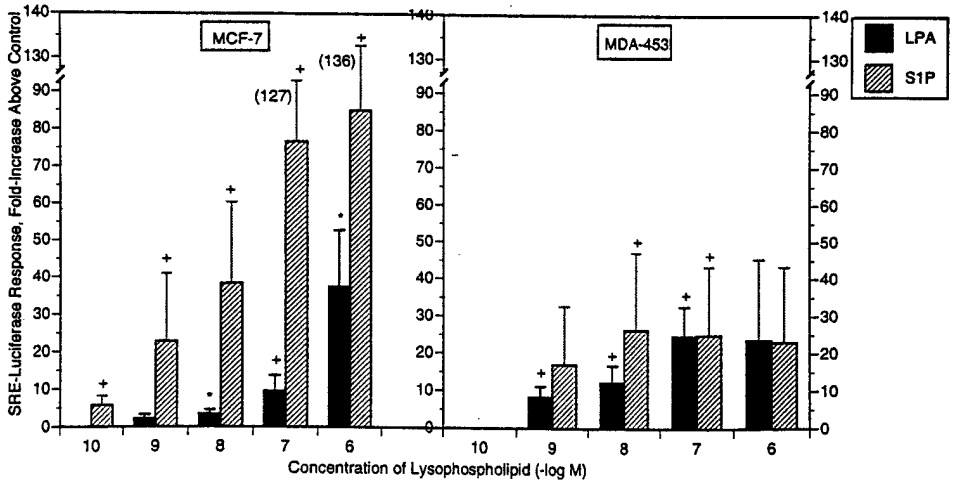

Fig. 4. SRE reporter assay of LPA and S1P stimulation of human BCCs. *Columns*, means of the results of three studies performed in duplicate; *bars*, SD. The medium alone control values were 1272, 957, and 352 luminometer units for MCF-7 BCCs and 269, 715, and 1401 for MDA-MB-453 BCCs. The statistical methods and symbols are the same as in Fig. 3, except that + = $P < 0.05$.

Table 2 *Pharmacological inhibition of LPA and S1P signaling to the SRE-Luciferase reporter in BCCs*

Each number is the mean of results of two studies performed in duplicate and presented as the percentage of inhibition of the control responses to 10^{-7} M LPA and 10^{-7} M S1P in serum-free DMEM without inhibitors (0% inhibition). Inhibitor conditions were 50 ng/ml of PTX for 6 h, 5 μM MEK inhibitor (MEK INH) for 1 h, and 10 μg/ml of C3 exoenzyme for 30 h.

	MCF-7 BCCs			MDA-MB-453 BCCs		
	PTX	MEK INH	C3 exoenzyme	PTX	MEK INH	C3 exoenzyme
LPA	74	41	41	80	69	75
S1P	60	37	44	78	61	79

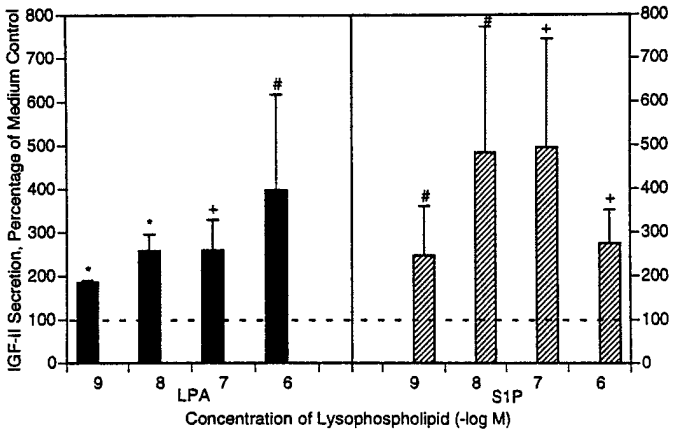

Fig. 5. Stimulation by LPA and S1P of MCF-7 BCC secretion of IGF-II. *Columns*, means of the results of three studies; *bars*, SD. Secretion of IGF-II in medium alone was 1.7, 3.0, and 4.1 ng/ml in the three studies. The statistical methods and symbols are the same as in Fig. 4, except that # = $P = 0.05$.

were detected in MDA-MB-453 BCCs, where the respective mean maxima were 24-fold and 26-fold.

Pharmacological inhibitors known to suppress one or more components of the pathways by which Edg Rs signal nuclear events were applied in BCCs transfected with the SRE-luciferase reporter. Suppression of Gi protein activity by PTX, the ras-mitogen-activated protein kinase pathway by a MEK inhibitor, and the rho pathway by C3 exoenzyme all substantially decreased nuclear signals from Edg receptors in both types of BCCs (Table 2).

Enhancement of BCC Secretion of IGF-II by LPA and S1P. Functional prominence of the IGF-II/IGFR1 system in many breast cancers suggested the possibility that part of the stimulation of proliferation of some lines of BCCs by LPA and/or S1P might be attributable to augmentation of secretion of IGF-II by one or both lysophospholipid mediators. Secretion of radioimmunoreactive IGF-II by MCF-7 BCCs was enhanced significantly by 10^{-9} M to 10^{-6} M LPA and S1P in concentration-dependent relationships where the maximal effects were attained by 10^{-6} M LPA and 10^{-8} M and 10^{-7} M S1P (Fig. 5). In two of the studies, neither 10^{-10} M LPA nor S1P affected release of IGF-II. At 10^{-6} M, but not 10^{-8} M, the phosphatidic acid and sphingosine biochemical precursors of LPA and S1P enhanced secretion of IGF-II with marginal statistical significance. A dot-blot immunoassay for IGF-II, which eliminates the blocking activity of IGF-binding proteins, gave similar results for MCF-7 BCCs. With 10^{-7} M LPA, 10^{-6} M LPA, 10^{-8} M S1P, and 10^{-7} M S1P, MCF-7 BCC-derived IGF-II was increased to respective means of 2.6-, 3.2-, 4.7-, and 5.5-fold above a mean unstimulated level of 2.2 ng/ml. Stimulation of MCF-7 BCC secretion of IGF-II by LPA and S1P was inhibited by PTX, MEK inhibition, and C3 exoenzyme sufficiently to implicate Gi and both the ras and rho pathways of signaling by the Edg receptors (Table 3). A greater involvement of signaling through the ras-raf-mitogen-activated protein kinase pathway than rho pathways may be predicted based on the higher effectiveness of the MEK inhibitor than C3 exoenzyme.

The level of secretion of IGF-II by LPA- and S1P-stimulated MDA-MB-453 BCCs was much lower than that by MCF-7 cells, and it was not possible to quantify accurately the very low IGF-II concentrations attained by unstimulated MDA-MB-453 cells. With 10^{-6} M LPA and 10^{-7} M S1P, the levels of IGF-II secreted by MDA-MB-453 BCCs attained means of 1.2 and 2.0 ng/ml, respectively. Because stimulated levels of IGF-II from MDA-MB-453 BCCs were only one-fifth of those from MCF-7 BCCs or lower and unstimulated levels were not reliably detectable, subsequent studies focused only on IGF-II mechanisms in MCF-7 BCCs. The capacity of human synthetic IGF-II to stimulate BCC proliferation, at concentrations in the range attained by incubation of MCF-7 BCCs with LPA and S1P, was examined to assess functional relevance of the observed endogenous increases. IGF-II increased MCF-7 BCC proliferation significantly, as determined by increases in cell counts after 72 h. MCF-7 BCC counts were increased by 1, 3, 10, and 30 ng/ml of IGF-II to respective means of 152, 234, 316, and 388% ($n = 2$) of serum-free medium control. The same range of concentrations of synthetic IGF-II also activated SRE in MCF-7 BCCs, as detected in the reporter assay (Table 4). The increases in SRE signal above control level were significant for all concentrations of IGF-II examined, and the increment in SRE signal attained by each higher concentration compared with the next lower concentration also was significant. The reduction in LPA-induced SRE signal by immunoneutralization of IGF-II was similar in magnitude to the maximum increase elicited by IGF-II alone (Table 4).

Suppression of MCF-7 BCC Responses to LPA and S1P by Anti-IGF-II and Anti-IGFR1 Antibodies. MCF-7 BCCs were preincubated with a range of concentrations of an IgG1 mouse neutralizing monoclonal anti-IGF II antibody, prior to introduction of 10^{-7} M LPA and S1P. The neutralizing antibody to IGF-II suppressed significantly both proliferative responses and SRE-luciferase reporter responses with antibody concentration dependence, whereas isotype-matched control IgG1 had no effect (Fig. 6). The effects of anti-IGFR1 antibody, which blocks binding of IGF-II to IGFR1, were examined in relation to the stimulatory effects of 10^{-7} M S1P on MCF-7 BCCs. At 1, 3, and 10 μg/ml, anti-IGFR1 antibody suppressed S1P-stimulated proliferation of MCF-7 BCCs, as assessed with cell counts, by means ± SD ($n = 3$) of 20 ± 4.6%, 32 ± 4.0%, and 41 ± 3.6% ($P < 0.01$ for all), respectively. At 3, 10, and 30 μg/ml, anti-IGFR1 antibody suppressed S1P-stimulated activation of the SRE-luciferase reporter in MCF-7 BCCs by means ± SD ($n = 3$) of 36 ± 7.8%, 47 ± 7.8%, and 51 ± 7.6% ($P < 0.01$ for all), respectively. In contrast, the IgG isotype control had no significant inhibitory effect, and anti-IGFR1 antibody did not suppress unstimulated proliferation of MCF-7 BCCs.

Table 3 *Pharmacological inhibition of LPA and S1P enhancement of MCF-7 BCC secretion of IGF-II*

Each value is the mean ± SD of the results of three studies. The significance of each level of inhibition was calculated by a paired Student *t* test. The levels of IGF-II in medium without an inhibitor were 7.4, 10, and 12 ng/ml for 10^{-7} M LPA and 6.1, 8.5, and 10 ng/ml for 10^{-7} M S1P.

	Lysophospholipid Signaling Inhibitor		
	PTX	MEK INH (mean inhibition ± SD)	C3 exoenzyme
LPA (10^{-7} M)	83 ± 14[a]	44 ± 8.3[a]	19 ± 15
S1P (10^{-7} M)	60 ± 19[b]	35 ± 3[a]	19 ± 2[b]

[a] $P < 0.01$.
[b] $P < 0.05$.

Table 4 *Activation of SRE-Luciferase reporter in MCF-7 BCCs by IGF-II*

Each value is the mean ± SD of the results of three studies. The significance of each level of stimulation relative to serum-free control without IGF-II or LPA (100%) was calculated by a paired Student *t* test. The levels of significance of differences between 1 and 3 ng/ml ($P < 0.01$), 3 and 10 ng/ml ($P < 0.05$), and 10 and 30 ng/ml ($P < 0.05$) of IGF-II and between LPA without and with anti-IGF-II neutralizing antibody ($P < 0.01$) were calculated by the same method.

IGF-II (ng/ml)					
1	3	10	30	LPA (10^{-7} M)	LPA (10^{-7} M) + anti-IGF-II (30 μg/ml)
178 ± 17[a]	209 ± 24[a]	260 ± 44[b]	316 ± 31[a]	1202 ± 152[a]	910 ± 165[a]

[a] $P < 0.01$.
[b] $P < 0.05$.

DISCUSSION

IGF-I and IGF-II potently stimulate proliferation of many types of normal and malignant cells (19, 20). The IGFR1 is a heterotetrameric complex with tyrosine kinase activity that binds and transduces signals from IGF-I and IGF-II similarly (21). IGFR2 differs structurally from IGFR1, lacks signal transduction functions, and does not mediate cellular proliferation (22). IGF-II is the predominant form in human cultured BCCs, stimulates BCC proliferation through IGFR1, and decreases the estrogen growth requirement of ER-positive BCCs (23). Estrogen is a potent stimulus of proliferation of ER-positive BCCs that concurrently enhances expression and secretion of IGF-II by such lines of BCCs (19). However, the possibility that the IGF system may not have a major role in estrogen enhancement of proliferation of some ER-positive BCCs was suggested by the lack of inhibition of estrogen stimulation when IGFR1 was blocked by a neutralizing monoclonal antibody (24). In contrast, stimulation of proliferation of BCCs by the lysolipid phosphate growth factors LPA and S1P appears to be mediated in part by IGF-II but is not dependent on the expression of ERs.

The ER-positive MCF-7 cells and ER-negative MDA-MB-453 cells both express Edg-2 and Edg-4 Rs for LPA and Edg-3 and Edg-5 Rs, but not Edg-1 Rs, for S1P, with quantitative differences in the respective levels (Figs. 1 and 2; Table 1). Significant ligand concentration-dependent stimulation of BCC proliferation by LPA and S1P was observed with both lines, irrespective of ER status (Fig. 3). Signaling of transcription of growth-related genes, as assessed by prominent enhancement of SRE-coupled luciferase activity, was increased significantly by proliferation-stimulating concentrations of LPA and S1P in both MCF-7 and MDA-MB-453 BCCs (Fig. 4). The suppression of SRE-coupled reporter responses to LPA and S1P by PTX and by inhibition of MEK and rho, in a pattern characteristic of signal transduction by Edg Rs, confirms the presence of functional Edg Rs in both BCC lines (Table 2).

LPA and S1P both significantly enhanced secretion of immunoreactive IGF-II by MCF-7 cells up to respective peaks 4- and 5-fold higher than control levels (Fig. 5). IGF-II secretion evoked by 10^{-7} M LPA or S1P was suppressed significantly by PTX and MEK inhibition and less significantly by C3 exoenzyme inactivation of rho, which also is consistent with Edg R mediation (Table 3). The role of IGF-II was explored first by investigating the stimulation of proliferation and SRE-luciferase activity in MCF-7 BCCs by a range of concentrations of purified synthetic IGF-II (Table 4). At concentrations elicited by LPA or S1P, the synthetic IGF-II evoked greater proliferation and SRE-luciferase activity than at concentrations attained by unstimulated MCF-7 BCCs. The role of native IGF-II was confirmed by defining the effects of neutralizing antibodies to IGF-II and IGFR1 on growth and SRE-reporter responses to 10^{-7} M LPA and S1P (Fig. 6). Both responses of MCF-7 cells were inhibited by means of up to 55 and 65%, respectively, without an effect of non-antibody isotype-identical IgG (Fig. 6). Thus, a substantial part of the stimulation of growth of some BCCs by LPA and S1P depends on increased release of IGF-II and its capacity to induce BCC proliferation.

A tentative integration of the present findings suggests distinctive functions for lysolipid phosphate mediators in BCC biology. At concentrations usually attained in serum and in some inflammatory and malignant exudates and plasma (1, 25, 26), LPA and S1P both exert dual effects on BCC proliferation. First, the SRE-luciferase responses not inhibited by anti-IGF-II or anti-IGFR1 neutralizing antibodies represent either direct nuclear signaling through Edg Rs or possibly the actions of other non-IGF protein growth factors elicited by the lysolipid phosphate mediators and capable of activating SRE. Second, LPA and S1P enhance generation and/or release of IGF-II by the

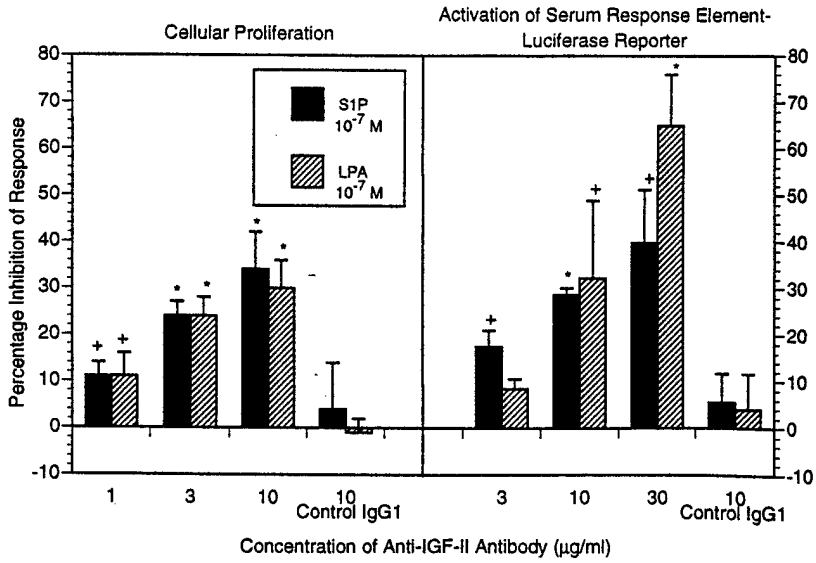

Fig. 6. Suppression of MCF-7 BCC responses to LPA and S1P by a neutralizing anti-IGF-II mouse monoclonal antibody. *Columns*, means of the results of three studies performed in duplicate; bars, SD. The control (0% inhibition) responses to 10^{-7} M LPA and S1P are shown in Figs. 3 and 4. The statistical methods and symbols are the same as in Fig. 4.

BCCs, irrespective of ER expression. The results of preliminary analyses of LPA and S1P production by BCCs showed very low endogenous levels, which would not have functional relevance. The sources of LPA and S1P, therefore, are likely to be cells other than the target BCCs, and these lysolipid phosphate growth factors thus would not appear to be autocrine stimuli in breast cancer. Rather, this class of mediators may function both as paracrine growth factors and by setting thresholds for secretory responses of one or more autocrine protein growth factors.

ACKNOWLEDGMENTS

We are grateful to Bethann Easterly for expert preparation of graphics.

REFERENCES

1. Fourcade, O., Simon, M. F., Viode, C., Rugani, N., Leballe, F., Ragab, A., Fournie, B., Sarda, L., and Chap, H. Secretory phospholipase A2 generates the novel lipid mediator lysophosphatidic acid in membrane microvesicles shed from activated cells. Cell, *80:* 919–927, 1995.
2. Spiegel, S., and Milstein, S. Sphingolipid metabolites: members of a new class of lipid second messengers. J. Membr. Biol., *146:* 225–233, 1995.
3. Durieux, M. E., and Lynch, K. R. Signaling properties of lysophosphatidic acid. Trends Pharmacol. Sci., *14:* 249–254, 1993.
4. Moolenaar, W. H., Kranenburg, O., Postma, F. R., and Zondag, G. Lysophosphatidic acid: G protein signaling and cellular responses. Curr. Opin. Cell Biol., *9:* 168–173, 1997.
5. Goetzl, E. J., and An, S. Diversity of cellular receptors and functions for the lysophospholipid growth factors lysophosphatidic acid and sphingosine 1-phosphate. FASEB J., *12:* 1589–1598, 1998.
6. Hecht, J. H., Weiner, J. A., Post, S. R., and Chun, J. *Ventricular zone gene-1 (vzg-1)* encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex. J. Cell Biol., *135:* 1071–1083, 1996.
7. An, S., Dickens, M. A., Bleu, T., Hallmark, O. G., and Goetzl, E. J. Molecular cloning of the human Edg-2 protein and its identification as a functional cellular receptor for lysophosphatidic acid. Biochem. Biophys. Res. Commun., *231:* 619–622, 1997.
8. An, S., Bleu, T., Huang, W., Hallmark, O. G., Coughlin, S. R., and Goetzl, E. J. Identification of cDNAs encoding two G protein-coupled receptors for lysosphingolipids. FEBS Lett., *417:* 279–282, 1997.
9. An, S., Bleu, T., Hallmark, O. G., and Goetzl, E. J. Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. J. Biol. Chem., *273:* 7906–7910, 1998.
10. Lee, M-J., Van Brocklyn, J. R., Thangada, S., Liu, C. H., Hand, A. R., Menzeleev, R., Spiegel, S., and Hla, T. Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG- 1. Science (Washington DC), *279:* 1552–1555, 1998.
11. Cuvillier, O., Rosenthal, D. S., Smulson, M. E., and Spiegel, S. Sphingosine 1-phosphate inhibits activation of caspases that cleave poly(ADP-ribose) polymerase and lamins during Fas- and ceramide-mediated apoptosis in Jurkat T lymphocytes. J. Biol. Chem., *273:* 2910–2917, 1998.
12. Goetzl, E. J., Kong, Y., and Mei, B. Lysophosphatidic acid and sphingosine 1-phosphate protection of T cells from apoptosis in association with suppression of Bax. J. Immunol., *162:* 2049–2056, 1999.
13. Hill, C. S., and Treisman, R. Differential activation of c-fos promoter elements by serum, lysophosphatidic acid, G proteins and polypeptide growth factors. EMBO J., *14:* 5037–5042, 1995.
14. An, S., Bleu, T., Zheng, Y., and Goetzl, E. J. Recombinant human Edg-2 and Edg-4 lysophosphatidic acid receptors mediate intracellular calcium mobilization. Mol. Pharmacol., *54:* 881–888, 1998.
15. Goetzl, E. J., Kong, Y., and Kenney, J. S. Lysophospholipid enhancement of human T cell sensitivity to diphtheria toxin by increased expression of heparin-binding epidermal growth factor. Proc. Assoc. Am. Physic., *111:* 1–11, 1999.
16. Kaltreider, H. B., Ichikawa, S., Byrd, P. K., Ingram, D. A., Kishiyama, J. L., Sreedharan, S. P., Warnock, M. L., Beck, J. M., and Goetzl, E. J. Upregulation of neuropeptides and neuropeptide receptors in a murine model of immune inflammation in lung parenchyma. Am. J. Resp. Cell. Mol. Biol., *16:* 133–145, 1996.
17. Crawford, B. A., Martin, J. L., Howe, C. J., Handelsman, D. J., and Baxter, R. C. Comparison of extraction methods for insulin-like growth factor-I in rat serum. J. Endocrinol., *134:* 169–176, 1992.
18. DeLeon, D. D., Terry, C., and Nissley, S. P. Direct detection of insulin like growth factor II (IGF-II) by chemiluminescence without interference by IGF binding proteins. Endocrinology, *134:* 1960–1963, 1994.
19. Stewart, A. J., Johnson, M. D., May, F. E. B., and Westley, B. R. Role of insulin-like growth factors and the type I insulin-like growth factor receptor in the estrogen-stimulated proliferation of human breast cancer cells. J. Biol. Chem., *265:* 21172–21178, 1994.
20. Yee, D. The insulin-like growth factor system as a target in breast cancer. Breast Cancer Res. Treat., *32:* 85–95, 1994.
21. Steele-Perkins, G., Turner, J., Edman, J. C., Hari, J., Pierce, S. B., Stover, C., Rutter, W. J., and Roth, R. A. Expression and characterization of a functional human IGF-I receptor. J. Biol. Chem., *263:* 11486–11492, 1988.
22. Roth, R. A. Structure of the receptor for IGF-II: the puzzle amplified. Science (Washington DC). *239:* 1269–1271, 1988.
23. Daly, R. J., Harris, W. H., Wang, D. Y., and Darbre, P. D. Autocrine production of insulin-like growth factor II using an inducible expression system results in reduced estrogen sensitivity of MCF-7 human breast cancer cells. Cell Growth Differ., *2:* 457–464, 1991.
24. Arteaga, C. L., and Osborne, C. K. Growth inhibition of human breast cancer cells *in vitro* with an antibody against the type I somatomedin receptor. Cancer Res., *49:* 6237–6241, 1989.
25. Xu, Y., Gaudette, D. C., Boynton, J., and Mills, G. B. Characterization of an ovarian cancer activating factor (OCAF) in ascites from ovarian cancer patients. Clin. Cancer Res., *1:* 1223–1232, 1995.
26. Xu, Y., Shen, Z., Wiper, D. W., Wu, M., Morton, R. E., Elson, P., Kennedy, A. W., Belinson, J., Markman, M., and Casey, G. Lysophosphatidic acid as a potential biomarker in ovarian and other gynecological cancers. J. Am. Med. Assoc., *280:* 719–723, 1998.

Preface: The Omnific Lysophospholipid Growth Factors

EDWARD J. GOETZL[a,c] AND KEVIN R. LYNCH[b]

[a]*Departments of Medicine and Microbiology-Immunology, University of California, San Francisco, California 94143-0711, USA*

[b]*Department of Pharmacology, University of Virginia Health, Sciences Center, Charlottesville, Virginia 22908, USA*

In the past three years, there have been striking advances in our understanding of the sources and biological roles of two major subfamilies of lysophospholipid (LPL) mediators. The designation "omnific" for these lipid factors describes their generation by many types of cells, albeit in different amounts, and their capacity to affect growth and functions of diverse cells in vertebrates and some lower-order organisms. The term also is a reminder of one of the major issues being addressed by investigators, which is the multiple mechanisms accounting for specificity of actions of each LPL. Lysophosphatidic acid (LPA) is the most prominent member of the lysoglycerol-containing phospholipid subfamily, which predominates quantitatively among lipid structural components of cellular membranes. Sphingosine-1-phosphate (S1P) is a highly active lysosphingophospholipid, which is structurally and functionally related to LPA. The subfamily of cellular lysosphingophospholipids are quantitatively diminutive in contrast to the subfamily of lysoglycerophospholipids, but exhibit great structural complexity and express protean biological effects similar to those of lysoglycerophospholipids. The LPLs of both subfamilies are related also by being products of metabolism of cellular membrane phospholipids, increasing in concentration transiently in relation to cellular responses, requiring carrier proteins for cellular presentation, moving and interacting with proteins in the planes of membranes, and potently influencing cellular proliferation and other functions through one or more subfamilies of G-protein-coupled receptors (GPCRs). Recent progress in our development of knowledge of every aspect of the cellular generation, recognition, and effects of LPLs has been promoted by discoveries of their distinctive biosynthetic and metabolic pathways, broad range of functional activities in addition to those related to growth, and use of the novel subfamily of endothelial differentiation gene (EDG)-encoded GPCRs (EDG Rs).

This volume reports the central points of new knowledge, current discussion, and occasional contention revealed in a conference held at the Rockefeller University on June 24–27, 1999. The principal areas of attention were novel pathways for the cellular generation of LPA and S1P, new subfamilies of GPCRs dedicated to LPLs, distinctive cellular activities and physiological functions of LPLs, the evolving definition of a pharmacophore for each subfamily of LPLs, and recently detected

[c]Address for correspondence: Edward J. Goetzl, M.D., University of California, UB8B, Box 0711, 533 Parnassus, San Francisco, CA 94143-0711. Voice: 415/476-5339; fax: 415/476-6915. egoetzl@itsa.ucsf.edu.

abnormalities in generation or effects of LPLs in human diseases. The volume serves also to highlight substantial gaps in our knowledge of LPL biology.

Three different stimulus-coupled cellular pathways for rapid synthesis and release of LPA and, by analogy, S1P were introduced at the conference and shown to link LPL biosynthesis to lipid degradation, lipid phosphorylation, and oxidation of complex membrane phospholipid precursors. The first pathway for LPA generation involves sphingomyelinase conditioning of cell-derived plasma membrane vesicles, phospholipase C (PLC)-, and/or PLD-dependent liberation of phosphatidic acid (PA) and its conversion to LPA by secretory PLA2 and possibly other PLs. In the second pathway, which is prominent in thrombin-activated platelets, diacylglycerol (DAG) kinase yields PA that is converted to LPA by the same phospholipase(s) as the first pathway. For the third pathway, production of LPA in minimally oxidized low-density lipoproteins results from specific oxidative degradation, analogous to that capable of generating derivatives of the phospholipid platelet-activating factor, designated PAF. Whatever the metabolic source, LPA is stored in some cells at concentrations of up to 30 to 60 μM, and is secreted and bound by serum albumin, gelsolin, and some other proteins for delivery to cellular targets. LPA may accumulate in extracellular fluids, including serum, malignant ascites, and inflammatory exudates, in concentrations as high as 10 μM. The limiting metabolic step in generation of S1P is the effective activity of sphingosine kinase, which has been cloned and overexpressed and inhibited in studies demonstrating its critical role in regulation of cellular functional responses.

Extracellular molecules that signal cells, such as LPA and S1P, are usually rapidly degraded, as was discussed by several speakers. The most plausible mechanism for biodegradation of LPLs is hydrolysis by the ectophosphohydrolases, lipid phosphate phosphohydrolases (LPPs). The three currently recognized LPPs show broad substrate specificity and relatively low apparent affinities for their substrates. However, their ubiquity, abundance, and localization at cell surfaces makes them attractive candidates for the primary step in destruction of extracellular LPLs.

The cellular recognition and effects of LPA, S1P, and perhaps some other LPLs were discussed and shown to be mediated by GPCRs, including those of the EDG subfamily, which are expressed in many different types of tissues. Tissue distribution and functional roles of two distantly related GPCRs termed psp24 and ovarian GPCR type 1 (OGR1) are less well documented than those of EDG Rs, but psp24 is selectively dedicated to LPA binding and signaling, and OGR1 to sphingosylphosphorylcholine and possibly another as yet unidentified LPL-like factor. Two structural and functional clusters of EDG Rs have been distinguished based on the degree of amino-acid sequence identity and preferred LPL ligand. The first includes EDG-1, -3, -5, and –8, which are 45% to 60% identical in amino-acid sequences and bind S1P with high affinity. The second cluster encompasses EDG-2, -4, and –7, which are 48% to 54% identical in amino-acid sequences and bind LPA with high specificity and affinity. It was reported at the conference that EDG-6, which is structurally between the two clusters but closer to the first, binds S1P with moderate affinity and high specificity. The S1P- and LPA-selective clusters of EDG Rs are approximately 35% identical overall. The EDG Rs also differ with respect to patterns of G-protein association and tissue distribution.

It was demonstrated at the conference that LPL growth factors influence a wide range of cellular functions, in addition to stimulation and regulation of cellular proliferation, which are both linked and not directly linked to cell growth. Enhancement of survival and suppression of apoptosis are growth-related effects of LPA and/or S1P, attributable to both unique and overlapping combinations of effects of LPLs. These include alterations in cellular production and secretion of protein growth factors and other cytokine survival factors, and in the cellular concentration and/or activity of proapoptotic and antiapoptotic proteins, caspases, and other effector molecules. Actions of LPLs on growth-unrelated cellular functions all involve changes in the cytoskeleton, and are manifested by a wide range of responses encompassing cell migration, interactions with other cells and/or the extracellular matrix, and activity of one or more ion channels. Some such cellular responses are evoked by only one class of LPLs, whereas others are induced by all LPLs when receptor expression permits cellular recognition. Far less was discussed at the conference and is known of integrated organ and systemic effects of LPLs.

The findings of most studies of the pathophysiological roles of LPLs reported at the conference have revealed definite abnormalities in their production and effects in relation to cardiovascular and neoplastic diseases. The most striking findings are in ovarian cancer, where tumor cells produce LPA in quantities sufficient to elevate local tissue, ascites, and plasma concentrations. Ovarian cancer cells also express increased levels of the EDG-4 LPA receptor, whereas little or none is seen in normal ovarian surface epithelial cells without or with cellular stimulation. Ovarian cancer cells secrete high levels of autocrine protein growth factors and express receptors for protein growth factors, including the angiogenic and vascular permeability-promoting protein termed vascular endothelial growth factor (VEGF), many of which are under partial control of LPA. Several other types of cancers appear to express higher levels of EDG-4 R than equivalent nonneoplastic cells from the same tissue. Some ovarian cancer cells also express elevated levels of EDG-2 and EDG-7 receptors. Differences in expression of EDG Rs in metastases compared to the primary tumor also exist, but have not been examined systematically. That such differences may be pathogenetically important is suggested by observations of LPLs altering tumor-cell adhesion, migration, homing, proliferation, and cytokine secretion.

Two basic approaches were described at the conference for designing LPA receptor agonists and antagonists to be more stable, soluble, and active than the primary mediators. Native variants of LPA, such as alkenyl-glycerophosphate and cyclic-phosphatidic acid and derivative analogs, interact with and desensitize the spectrum of LPA receptors with different specificities and potencies. These results illustrate the difficulties of drug development when each physiological and pathological setting is characterized by a different mixture of bioactive LPLs and LPL receptors. The second approach was to synthesize a broad array of substituted LPAs, which generated several analogs more potent than LPA but no apparent antagonists. These results have begun to define an LPA pharmacophore with the expected dependence on the phosphate group and fatty acid chain length, but also a surprising requirement for natural stereochemistry.

Although reports at the conference indicated initial definition of pharmacophores for each LPL mediator, these and several other potential synthetic agonists and an-

tagonists lack sufficient bioavailability, specificity, and potency for meaningful *in vivo* or even *in vitro* studies. Further development of LPL medicinal chemistry will provide the necessary tools for assigning LPL signaling events to specific receptors. It is hoped also that emerging sets of EDG R-neutralizing antibodies will both represent early tools for initial cellular and animal studies, and facilitate standardization of assays needed for drug discovery. Some of the remaining questions regarding LPL effects in complex tissues and organ systems may only be addressed definitively by genetic approaches, which modify levels of production of LPLs, LPL cellular transport systems, and extracellular carrier proteins, and receptor expression and signaling.

Many important milestones remain for future research designed to elucidate the activities of LPLs in development as well as in adult physiology and diseases. At a minimum, it will be critical to delineate the distinctive and compensatory functions of each set of LPL Rs in comprehensive studies of cellular signaling and responses, analyze the properties and effects of functionally active anti-EDG R antibodies and EDG R-specific drugs, establish and investigate multiple transgenes and knockouts of LPL Rs, and identify and characterize natural genetic anomalies of LPL generation and recognition. Complete elucidation of *in vivo* activities of LPLs will require discovery of potent and specific pharmacologic agents for animal and human uses. An appreciation of integrated systemic roles of these mediators also will necessitate investigations of interactions of LPLs with other mediator and receptor systems. A FASEB Summer Conference in this subject area will be held in Tucson, Arizona, in June 2001.

Development of Our Current Understanding of Bioactive Lysophospholipids

WOUTER H. MOOLENAAR[a]

Division of Cellular Biochemistry, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

ABSTRACT: **Lysophosphatidic acid (LPA) serves as the prototypic lysophospholipid mediator that acts through G-protein-coupled receptors to evoke a host of responses in numerous target cells. The hormone- and growth-factor-like activities of LPA, mediated by distinct G proteins, were discovered about 10 years ago. Since then, considerable progress has been made in our understanding of LPA receptor signaling, culminating in the recent identification of a growing family of heptahelical receptors specific for LPA and the structurally related lysolipid, sphingosine-1-phosphate (S1P). In addition to stimulating G_i-Ras-mediated cell proliferation, LPA and S1P induce rapid $G\alpha_{12/13}$-RhoA-mediated cytoskeletal changes underlying such diverse responses as neurite retraction, cell rounding, and enhanced tumor cell invasiveness. LPA also triggers inhibition of gap-junctional communication. This overview focuses on how our understanding of LPA as an intercellular lipid mediator has developed during the last decade.**

INTRODUCTION

Lysophosphatidic acid (LPA; 1-acyl-glycerol-3-phosphate) is one of the simplest natural phospholipids, and certainly also one of the most interesting. For several decades, LPA was just known as a key precursor in the biosynthesis of more complex lipids in both eukaryotic and prokaryotic cells. It was only during the early 1990s that LPA's first messenger role became evident, when it was established that LPA is a platelet-derived mediator that induces hormone- and growth-factor-like responses in its target cells through activation of specific G-protein-coupled receptors. LPA's function as a signaling molecule was discovered in our laboratory more or less by serendipity, as is outlined below. This chapter presents a brief history of how LPA's agonist role was unraveled, and it summarizes recent advances in our understanding of lysophospholipid signaling and cellular responses. More comprehensive background on lysophospholipid receptor signaling and new developments can be found in recent reviews.[1–5]

LPA AS A RECEPTOR LIGAND: A BRIEF HISTORY

First, here is a brief description of how we discovered LPA as a bioactive mediator. The story actually starts in the mid-1980s. At that time, there was enormous in-

[a]Corresponding author. Voice: +31-20-512 1971; fax: +31-20-512 1989.
wmoolen@nki.nl

73. HARPER, M.E., E. GLYNNEE-JONES, J. GODDARD, V.J. THURSTON & K. GRIFFITHS. 1996. Vascular endothelial growth factor (VEGF) expression in prostatic tumors and its relationship to neuroendocrine cells. Br. J. Cancer **74:** 910–916.
74. FERRER, F.A., L.J. MILLER, R.I. ANDRAWIS, S.H. KURTZMAN, P.C. ALBERTSEN, V.P. LAUDONE & D.L. KREUTZER. 1997. Vascular endothelial growth factor (VEGF) expression in human prostate cancer: *in situ* and *in vitro* expression of VEGF by human prostate cancer cells. J. Urol. **157:** 2329–2333.
75. DANIELPOUR, D. 1996. Induction of transforming growth factor-beta autocrine activity by all-*trans*-retinoic acid and 1-alpha, 25-dihydroxyvitamin D3 in NRP-152 rat prostatic epithelial cells. J. Cell. Physiol. **166:** 231–239.
76. GATELY, S., P. TWARDOWSKI, M.S. STACK, M. PATRICK, L. BOGGIO, D.L. CUNDIFF, H.W. SCHNAPER, L. MADISON, O. VOLPERT, N. BOUCK, J. ENGHILD, H.C. KWAAN & G.A. SOFF. 1996. Human prostate carcinoma cells express enzymatic activity that converts human plasminogen to the angiogenesis inhibitor, angiostatin. Cancer Res. **56:** 4887–4890.
77. GATELY, S., P. TWARDOWSKI, M.S. STACK, D.L. CUNDIFF, D. GRELLA, F.J. CASTELLINO, J. ENGHILD, H.C. KWAAN, F. LEE, R.A. KRAMER, O. VOLPERT, N. BOUCK & G.A. SOFF. 1997. The mechanism of cancer-mediated conversion of plasminogen to the angiogenesis inhibitor angiostatin. Proc. Natl. Acad. Sci. U.S.A. **94:** 10868–10872.
78. NIE, D., G.G. HILLMAN, T. GEDDES, K. TANG, C. PIERSON, D.J. GRIGNON & K.V. HONN. 1998. Platelet-type 12-lipoxygenase in a human prostate carcinoma stimulates angiogenesis and tumor growth. Cancer Res. **58:** 4047–4051.

Mechanisms of Lysolipid Phosphate Effects on Cellular Survival and Proliferation

EDWARD J. GOETZL,[a,b,c] HSINYU LEE,[b] HANA DOLEZALOVA,[b] KIMBERLY R. KALLI,[d] CHERYL A. CONOVER,[d] YU-LONG HU,[e] TOSHI AZUMA,[f] THOMAS P. STOSSEL,[f] JOEL S. KARLINER,[b,g] AND ROBERT B. JAFFE[e]

Departments of [b]Medicine and [c]Microbiology-Immunology, University of California, San Francisco, California 94143, USA

[d]Department of Internal Medicine, Mayo Clinic and Foundation, Rochester, Minnesota 55905, USA

[e]Reproductive Endocrinology Center, University of California, San Francisco, California 94143, USA

[f]Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115, USA

[g]Veterans Affairs Medical Center, San Francisco, California 94121, USA

ABSTRACT: **The specificity of cellular effects of lysolipid phosphate (LLP) growth factors is determined by binding to endothelial differentiation gene-encoded G protein–coupled receptors (EDG Rs), which transduce diverse proliferative and effector signals. The primary determinants of cellular responses to LLPs are the generative and biodegradative events, which establish steady-state concentrations of each LLP at cell surfaces, and the relative frequency of expression of each EDG R. There are major differences among types of cells in the net effective generation of the LLPs, lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), and in their profile of expression of EDG Rs. The less well characterized secondary determinants of cellular specificity of LLPs are high-affinity binding proteins with carrier and cell-presentation functions, cell-selective regulators of expression of EDG Rs, and cellular factors that govern coupling of EDG Rs to G protein transductional pathways. The roles of components of the LLP-EDG R system in normal physiology and disease processes will be definitively elucidated only after development of animal models with biologically meaningful alterations in genes encoding EDG Rs and the discovery of potent and selective pharmacological probes.**

GENERATION, TRANSPORT, CELLULAR PRESENTATION, AND ACTIONS OF LYSOLIPID PHOSPHATE MEDIATORS

Primary and Secondary Mechanisms for Specificity

Lysophosphatidic acid (LPA), sphingosine 1-phosphate (S1P), and other structurally related lysolipid phosphates (LLPs) have major effects on diverse cellular func-

[a]Address for correspondence: Edward J. Goetzl, University of California, UB8B, Box 0711, 533 Parnassus, San Francisco, CA 94143-0711. Voice: 415/476-5339; fax: 415/476-6915. egoetzl@itsa.ucsf.edu

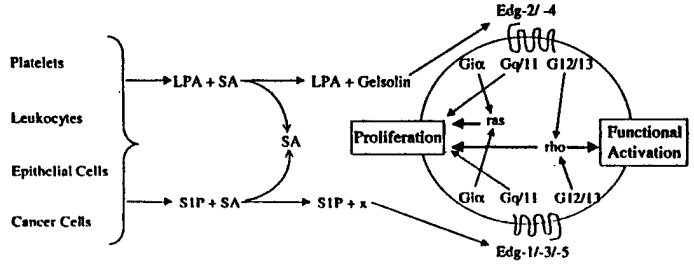

FIGURE 1. Generation, transport, and effects of lysolipid phosphate mediators. SA = serum albumin; x = postulated high-affinity S1P-binding protein.

tions, including initiation and regulation of proliferation, enhancement of survival, suppression of apoptosis, promotion of differentiation, and stimulation of cytoskeletal filament–based functions of many types of cells[1–4] (FIG. 1). LLPs are generated from precursors stored in membranes and secreted by platelets, macrophages, epithelial cells, and some cancer cells in amounts sufficient to establish micromolar concentrations in plasma normally and in other extracellular fluids during tissue reactions. LPA and S1P are both almost entirely bound by proteins in biological fluids. Serum albumin is a low-affinity and high-capacity carrier for LPA and S1P, whereas the actin-cleaving protein gelsolin is a high-affinity and low-capacity carrier for LPA, but not S1P.[5] It is presumed that S1P is bound by other, as yet unidentified, high-affinity proteins (FIG. 1). The present hypothesis is that LPA is bound by serum albumin and gelsolin in plasma and some other normal extracellular fluids, but almost exclusively by gelsolin at the surface of myocytes and other gelsolin-producing cells, where gelsolin exerts a predominant role in affinity-linked cellular presentation of LPA to EDG-2 and -4 Rs.[5] Normal plasma concentrations of gelsolin bind LPA with sufficient avidity to prevent optimal interactions with cellular receptors. In contrast, at the concentrations of approximately 5% to 15% of that of normal plasma found in many reactive extracellular fluids, gelsolin presents LPA to some types of cells with greater effectiveness than serum albumin.

LLPs resemble polypeptide growth factors (PGFs) in their capacity to evoke many cellular responses other than proliferation, act as autocrine and paracrine mediators, and signal cells through receptor-coupled transductional pathways, which alter transcriptional activities of growth-related genes directly and by amplification mechanisms.[3,4] Major differences between LLPs and PGFs are the cell membrane phospholipid precursor sources of LLPs, as contrasted with *de novo* synthesis of PGFs; multiple phospholipase- and phosphohydrolase-dependent enzymatic pathways for biodegradation of LLPs, as distinguished from proteolysis of PGFs; and their respective uses of G protein–coupled receptors (GPCRs) and protein tyrosine kinase receptors.

The central problem of biological specificity of the omnific LLPs, in contrast to PGFs, derives from the capacity of so many types of cells to produce and respond to LLPs. The primary determinants of specificity are the generative and biodegradative events, which establish steady-state concentrations of each LLP at cell surfaces, and

the structures, signaling pathways, and prevalence of each LLP receptor, which determine the net binding of LLPs and the characteristics of transduction. The secondary determinants of specificity are high-affinity carrier proteins and cell-selective presentation mechanisms, regulators of LLP receptor expression and signaling, and many other concurrently expressed mediator systems that modify cellular responses to LLPs. Thus, the next critical research goals for increasing our understanding of the distinctive roles of LLPs in normal physiology and disease processes are (1) to identify cell-selective factors that alter production, secretion, and biodegradation of each LLP, (2) to characterize the sources, nature, and cell-selective functions of LLP-binding proteins responsible for LLP transport in blood and other fluids and tissues, and for delivery to cellular receptors, (3) to delineate the distribution of LLP receptors on cells within each major organ system normally and in disease states, (4) to determine which factors regulate expression and signaling properties of LLP cellular receptors normally and in disease states, and (5) to define the major interactions between LLPs, PGFs, and other mediators of cellular functions.

In a few instances, it already has been demonstrated tentatively that an LLP is generated at greater than normal rates or that expression of one LLP receptor is expressed at higher than usual levels in relation to a developmental event, normal cellular response, or pathological process. Similarly, it has been shown experimentally in several model systems that LLPs may alter cellular production of PGFs or responsiveness to PGFs, and thereby increase the target cell selectivity of action of the LLPs. More conclusive correlation of these alterations in activity of the LLP mediator system with physiological and pathological events *in vivo* will require the availability of potent and selective pharmacological agents, functionally active antibodies to LLP receptors, and animal models with genetically overexpressed or deleted LLP receptors.

CELLULAR PATTERNS OF EXPRESSION OF EDG RECEPTORS FOR LLPs

Two subfamilies of G protein–coupled receptors (GPCRs), which are encoded by endothelial differentiation genes (*edgs*) and thus are designated EDG Rs, are dedicated to LPA, S1P, and related LLP mediators[6–11] (FIG. 1). The EDG Rs discovered so far may be considered in two homology and functional clusters based on both amino acid sequence identity and principal LLP ligand.[3,4] The first encompasses EDG-1, -3, -5, and -8, which are 45–60% amino acid sequence identical and bind S1P with high specificity.[6,9] The second includes EDG-2, -4, and -7, which exhibit 40–50% amino acid sequence identity and bind LPA with high affinity, but not S1P or other sphingolipids.[7,8,10] The EDG Rs all couple to three or more types of G proteins and transduce decreases in $[cAMP]_i$ through Gi, increases in $[Ca^{2+}]_i$ by augmenting phospholipase C activity through Gq/11 and beta/gamma dimers, and induction of PI_3 kinase, p125 focal adhesion kinase (FAK), and phospholipase D by activating rho through G12/13.[3,4] Induction of activity of serum response element (SRE) and subsequent transcriptional events by EDG Rs requires recruitment both of ternary complex factor (TCF) through Gi and ras, or through Gq/11 and the mitogen-activated protein (MAP) kinases ERK 1 and 2, and of serum response factor

(SRF) through G12/13 and rho.[12] All EDG Rs analyzed to date signal both nuclear transcriptional events and increases in $[Ca^{++}]_i$.[13–15] For each EDG R, however, a different G protein or combination of G proteins may serve as the predominant link to any one biochemical pathway.[16,17]

Assessment of mRNAs encoding EDG Rs by Northern blot and semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) techniques and of EDG R proteins by Western blots has provided preliminary, but often distinctive, profiles of their expression in several human and rodent organ systems. EDG-1 Rs dominate in endothelial cells and are present in lower amounts in some normal and anaplastic epithelial, neural, and myocytic cells, but are not detected in any cells of the T lymphocyte lineage from thymocytes to mature T helper and T suppressor cells. The EDG-2 R appears in dividing neurons of the periventricular zone in the developing murine brain and then disappears from postmitotic neurons of the adult brain.[7] In adult rodent and human brain cells, EDG-2 R is expressed only by oligodendrocytes.[18] EDG-4 R is absent at the level of protein and mRNA from freshly isolated normal human ovarian epithelial cells and SV40 virus–immortalized cultured lines of ovarian epithelial cells, but is expressed at high levels in all human ovarian cancer cell lines and tissues examined to date.[19,20] Rat cardiac myocytes express all EDG Rs except EDG-1, and the levels are increased by hypoxia and adrenergic agonists.[5] LPA acts through upregulated EDG-2 and EDG-4 Rs to protect cardiomyocytes from apoptosis induced by hypoxia and/or adrenergic stimulation. For the immune system, EDG-4 LPA R and EDG-6 R, for which the LLP ligand has not been identified as yet,[21] are the most densely represented in T cells, but also EDG-2, -3, and -4 are detected in some lines of human malignant T cells.[22,23] EDG-3 and -5 Rs are widely expressed in epithelial cells and fibroblasts. The results of studies to date indicate that levels of EDG Rs in thymocytes and T cells are altered substantially and differentially by cellular activation and some apoptosis-inducing agents. For example, ceramides, which enter T cells and evoke apoptosis, downregulate EDG-2 and EDG-4 LPA receptors, but not any of the S1P-specific EDG-Rs.[22] With the exception of the EDG-1 R,[6] however, very few examples of exclusive involvement of one type of EDG R and its signaling pathway have been delineated in relation to target cell specificity of LPA or S1P.

EFFECTS OF LLPs ON CELLULAR SURVIVAL AND PROLIFERATION

Regulation of T Lymphocyte Susceptibility to Apoptosis and Expression of Autocrine Polypeptide Growth Factors

LLPs affect cellular proliferation by four, often interactive, mechanisms. The first is enhancement of serum response element (SRE) activity in promoters of immediate-early growth–related genes.[12] The second is induction of cellular production and secretion of one or more polypeptide growth factors.[24,25] The third is sensitization of some types of cells to the effects of a polypeptide growth factor. This mechanism has been observed in cells for which LLPs alone have only weak activity, such as mesangial cells.[26] The fourth and rarest mechanism is inhibition of proliferation, as has been observed for some myelocytes in which LPA increases the intracellular

concentration of cyclic AMP ($[cAMP]_i$).[27] The results of recent studies of the roles of LLPs in cellular survival and proliferation often have revealed alterations in the cellular concentration, localization, or activity of one or two functionally relevant proteins of the target cells, which encompass diverse growth factors, receptors for growth factors, and other growth-related control proteins. Some of these mechanisms are well illustrated by the findings of investigations of T cell responses to LPA and S1P.

In the initial studies, LPA and S1P had striking effects on T cell susceptibility to apoptosis due to alterations in cellular levels of proteins of the Bcl-2 family and of the caspase cluster.[22,28] LPA and S1P also increased T cell sensitivity to diphtheria toxin (DT) as a result of enhanced T cell expression of the receptor for diphtheria toxin, which is heparin-binding epidermal growth factor–like growth factor (HB-EGF).[23] Cultured Tsup-1 cells of a human $CD4^+8^+3^{low}$ lymphoblastoma line express EDG-2, -3, -4, and -5 Rs, but not EDG-1 R, as determined by both RT-PCR analyses and Western blots.[22,23] Tsup-1 cell apoptosis was induced by antibodies to CD2, CD3 plus CD28 in combination, and Fas and by cell-permeant ceramide, and was assessed by morphological characteristics, increases in end-labeling of free 3′-OH groups of DNA, and release of radioactively labeled fragments of DNA. At 10^{-10} M to 10^{-7} M, both LPA and S1P protected Tsup-1 cells from apoptosis evoked by antibodies to surface proteins.[22] In contrast, S1P, but not LPA, suppressed apoptosis elicited by C6-ceramide. The failure of LPA to prevent ceramide-induced apoptosis of Tsup-1 cells was partially due to suppression by ceramide of the expression of EDG-2 and -4 Rs, but not EDG-3 and -5 Rs.[22] At 10^{-9} M to 10^{-7} M, both LPA and S1P suppressed Tsup-1 cell content of the apoptosis-promoting protein Bax without altering levels of Bcl-2 or Bcl-x_L.

The LPA and S1P suppression of Bax mediated by EDG Rs was shown by selectively reducing expression of EDG-2 and -4 together and of EDG-3 and -5 together through transfection of Tsup-1 cells with pools of the respective antisense cDNAs in plasmids expressing hygromycin resistance to allow enrichment of transfectants. Levels of suppression of EDG-2 and EDG-4 Rs that inhibited reductions in Bax by LPA prevented LPA protection from apoptosis.[22] Similarly, suppression of EDG-3 and -5 that inhibited reductions in Bax by the lower concentrations of S1P prevented S1P protection from apoptosis. At levels of S1P $\geq 10^{-7}$ M, prevention of Tsup-1 cell apoptosis correlated best with inhibition of activity of caspases 3, 6, and 7, but levels of LPA $> 10^{-7}$ M did not inhibit caspase activities in Tsup-1 cells or prevent apoptosis.

Other investigations of the effects of LPA and S1P on T cell survival revealed striking sensitization of Tsup-1 cells to the action of diphtheria toxin (DT). After 4 h of exposure of Tsup-1 cells to 1–10 ng/mL of DT, protein synthesis was suppressed by 11% to 72% and the levels of suppression were increased significantly by 10^{-9} M to 10^{-6} M LPA or S1P.[23] Heparin-binding epidermal growth factor–like growth factor (HB-EGF) is a plasma membrane protein of T cells, which binds to EGF Rs and matrix proteoglycans, and is the cellular receptor for DT. Under conditions that enhanced sensitivity to DT, LPA and S1P increased Tsup-1 cell expression of HB-EGF, as assessed by Western blots.[23] Direct evidence for the involvement of increased levels of HB-EGF in LLP enhancement of Tsup-1 cell sensitivity to DT was provided by HB-EGF neutralizing antibodies, which blocked the DT-sensitizing activity of

LPA and S1P. None of a range of analogues of LPA and S1P or other phospholipids mimicked the effects of the parent LLPs, and specific inhibitors of pathways of signaling characteristic of EDG Rs reduced LPA and S1P stimulation of both expression of HB-EGF and increased sensitivity to DT.

To confirm the roles of EDG Rs, Tsup-1 cells were transfected with EDG-2 plus EDG-4 antisense cDNA in mammalian expression plasmids encoding hygromycin resistance and incubated with hygromycin to augment the percentage of Tsup-1 cells with antisense suppression of EDG-2 and -4, as reflected in Western blots. Antisense reduction of EDG-2 and -4, but not EDG-3 and -5, prevented both increases in HB-EGF and enhanced sensitivity to DT induced by LPA, but not S1P.[23] Transfection of Tsup-1 cells with EDG-3 plus -5 antisense plasmids in the same protocol, to suppress immunodetectable EDG-3 and -5 proteins, prevented increases in both HB-EGF expression and sensitivity to DT elicited by S1P, but not LPA. In the absence of DT, such increased expression of HB-EGF may amplify LPA and S1P stimulation of T cell proliferation through greater juxtacrine activation of endogenous EGF Rs and heightened interactions of T cells with matrix proteoglycans. In preliminary studies of two lines of Jurkat human T cell transfectants stably overexpressing both EDG-3 Rs and EDG-4 Rs, 10^{-10} M to 10^{-7} M S1P and LPA respectively increased Jurkat T cell proliferation by up to 6-fold, as assessed by increased uptake of ^{3}H-thymidine. In wells precoated with heparan sulfate, the proliferation-enhancing effects of both LPA and S1P were increased further by a mean maximum of 3-fold. This effect is presumed to be attributable to increased expression of HB-EGF since neutralizing anti-HB-EGF antibody eliminated the stimulatory effect of heparan sulfate.

EFFECTS OF LLPs ON HUMAN BREAST CANCER CELLS

Cultured lines of estrogen receptor–positive (ER+) and ER– human breast cancer cells (BCCs) express EDG-2, -3, -4, and -5 Rs, without detectable EDG-1 R, as assessed by semiquantitative RT-PCR analyses and Western blots.[25] The rank order of prevalence in two lines of ER+ BCCs was EDG-3 ≥ -4 ≥ -5 ≫ -2 Rs and in two lines of ER– BCCs was EDG-3 > -4 ≫ -5 = -2 Rs (FIG. 2). Thus, both ER+ and ER– BCCs were predicted to respond to LPA and S1P. Detailed studies of the functional effects of LPA and S1P were conducted with the MCF-7 (ER+) and MDA-MB-453 (ER–) lines of human BCCs.[25] LPA and S1P at 10^{-8} M to 10^{-6} M enhanced proliferation of both BCC lines significantly after 72 h, as assessed by cell counts and ^{3}H-thymidine uptake, to maximal levels of 2.5- to 4-fold higher than that of control BCCs in serum-free medium alone. The level of SRE activity in BCCs transiently transfected with an SRE-luciferase reporter, which was used as an index of nuclear responses to proliferation-inducing LLP signals, was increased within 4 h by respective mean maxima of 37-fold and 85-fold by LPA and S1P in MCF-7 BCCs and by 24-fold and 26-fold in MDA-MB-453 BCCs.[25]

To examine the growth amplification mechanisms recruited by the LLPs (FIG. 2), their effects on secretion of the predominant type II insulin-like growth factor (IGF-II) were examined in MCF-7 BCCs that had readily quantifiable baseline levels not detected in culture media conditioned by the MDA-MB-453 BCCs. Significant in-

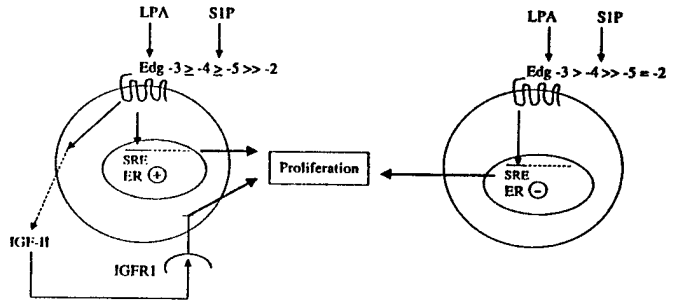

FIGURE 2. Lysolipid phosphate effects on human breast cancer cells. ER = estrogen receptor; SRE = serum response element; IGF-II = type II insulin-like growth factor; IGFR1 = type 1 insulin-like growth factor receptor.

creases in secretion of IGF-II by MCF-7 BCCs were evoked by 10^{-9} M to 10^{-6} M LPA and S1P, to respective mean maxima of 3.2-fold and 5.5-fold higher than the unstimulated mean of 2.2 ng/mL.[25] To affirm the functional significance of increases of this magnitude, the SRE-luciferase and proliferative responses of MCF-7 BCCs to synthetic IGF-II were examined in the range of increases elicited by LLPs. Concentration-dependent increases in proliferation were observed in response to 3 to 30 ng/mL of IGF-II, up to a mean maximal increase of 388% with 30 ng/ml of IGF-II. Similar increases in SRE-luciferase activity were evoked by the same concentrations of IGF-II, up to a mean maximal increase of 316% with 30 ng/mL of IGF-II. Known pharmacological inhibitors of EDG R signaling suppressed significantly and to the same extent LPA and S1P enhancement of BCC proliferation and IGF-II secretion.[25] The capacity of neutralizing monoclonal anti-IGF-II antibody to decrease BCC proliferative and SRE-luciferase responses to LLPs by up to 33% and 65%, respectively, confirmed the functional importance of the amplifying contribution of IGF-II recruited by LLPs. Thus, LLPs augment growth of BCCs through multiple EDG Rs by the dual mechanisms of direct nuclear signaling and stimulation of secretion of relevant quantities of IGF-II and perhaps other PGFs (FIG. 2).

EFFECTS OF LLPs ON HUMAN OVARIAN CANCER CELLS

As high levels of LPA in plasma and ascitic fluid of patients with ovarian cancer correlate with a poor prognosis, it was considered important to investigate the expression and functions of EDG Rs in human ovarian cancer cells (OCCs) as compared to nonmalignant ovarian surface epithelial cells (OSE). Analyses of mRNA encoding EDG Rs by semiquantitative RT-PCR showed that EDG-2 and -4 were the predominant Rs (FIG. 3). The most distinctive finding was of high levels of EDG-4 R mRNA in numerous established lines of OCCs, but not in SV40-immortalized nonmalignant OSE (IOSE) or normal human OSE.[19,20] In contrast, the level of EDG-2 R mRNA in IOSE and OSE cells was equal to or greater than that in OCCs, and both

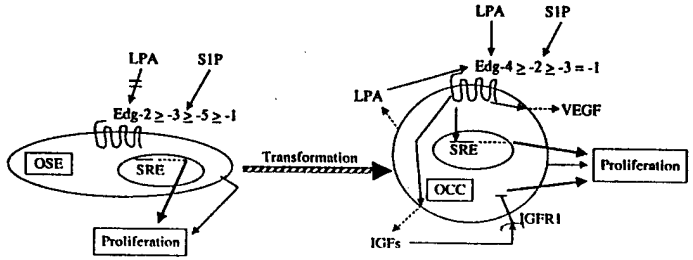

FIGURE 3. Lysolipid phosphate effects on human ovarian cancer cells. OCC = ovarian cancer cell; OSE = normal ovarian surface epithelial cell; SRE = serum response element; IGFs = a mixture of types I and II insulin-like growth factors; IGFR1 = type 1 insulin-like growth factor receptor.

EDG-3 and -5 R mRNA were consistently higher in IOSE and OSE cells than in OCCs.[19] EDG-1 R was expressed at similarly low levels in all lines of ovarian cells. Western blots supported the findings of higher levels of EDG-4 R in OCCs than in nonmalignant ovarian epithelial cells and higher levels of EDG-2, -3, and -5 Rs in IOSE and OSE cells than in OCCs[19] (FIG. 3). Thus, it was expected that OCCs would be more responsive functionally to LPA, and likewise nonmalignant ovarian cells to S1P.

LPA stimulated proliferation of the OV202 primary line of OCCs, but not IOSE 29 cells, as assessed by increases in uptake of ^{3}H-thymidine and cell counts[19] (FIG. 3). LPA evoked significant mean increases in uptake of ^{3}H-thymidine by OV202 cells of 1.7-fold at 10^{-6} M, 4.0-fold at 10^{-8} M, and 14-fold at 10^{-8} M, respectively, after 1, 3, and 5 days of stimulation. SRE-luciferase activity of OV202 OCC transfectants, which represents one index of immediate-early gene responses to EDG R signaling, was increased significantly by 10^{-9} M to 10^{-6} M LPA up to a mean maximum of 3-fold, whereas there was no response of IOSE 29 cell transfectants. In contrast, as predicted from the expression profile of EDG Rs, the SRE-luciferase responses to S1P were greater for IOSE 29 cells than OV202 cells.[19] OV202 OCC generation of IGF-II, which is a potent mitogen for OCCs, was increased significantly by 10^{-8} M and 10^{-7} M LPA and S1P to maximal levels of approximately 10-fold higher than medium alone. LPA also may promote ovarian tumor growth by increasing angiogenesis through stimulation of secretion of vascular endothelial growth factor (VEGF), which is the same protein as vascular permeability factor (VPF). LPA increased secretion of VEGF/VPF by the OVCAR-3 line of human OCCs up to a mean maximum of 4-fold, through a transcriptional activation mechanism, without influencing VEGF/VPF secretion by IOSE 29 cells[20] (FIG. 3). Pharmacological inhibitors of EDG R transduction suppressed similarly LLP stimulation of OCC proliferation, IGF-II generation, and VEGF production and secretion.[19,20] The capacity of some OCCs to secrete functionally relevant amounts of LPA suggests that the LLP-EDG R axis may be an autocrine growth and angiogenesis system in ovarian cancer (FIG. 3). The upregulation of VEGF/VPF also may contribute to the ascites, which is so characteristic of the local peritoneal invasion by ovarian cancer. EDG-4 R may be a marker for malignant transformation of ovarian epithelial cells, as well as a transducer of proliferation by direct nuclear signaling and enhancement of secretion of IGFs and other PGFs.

SUMMARY AND RESEARCH PLANS

Cells in many organ systems produce LLPs and express EDG Rs in often distinctive and defining patterns. The signals transduced by EDG Rs, which stimulate cellular survival and proliferation, and evoke cellular functional responses, include direct nuclear messages, increases in the levels of endogenous mediators, enhancement of sensitivity to endogenous and exogenous factors, and amplification or reorientation of one or more of the basic signaling pathways. Biological specificity of the LLP-EDG R systems is regulated at many levels, but presently the roles of high-affinity transport and presentation proteins, the relative levels of expression of each EDG R, and cell-selective amplification mechanisms appear to be more important determinants than production and degradation of the LLPs. One clear exception is some malignancies, such as ovarian cancer, where the combination of production of large amounts of LPA and expression of high levels of EDG-2 and -4 Rs can create an autocrine growth system. In addition to conducting further studies of the basic characteristics of the LLP-EDG R system, it is critical to develop mouse models with genetically altered EDG Rs and appropriately specific and potent pharmacological agonists and antagonists for *in vivo* investigations.

ACKNOWLEDGMENTS

We are grateful to Bethann Easterly for expert graphics. This research was supported by Grant No. HL31809 from the National Institutes of Health, Grant No. CRP 1 PF0265 from the California Department of Health Services, and a grant from the Department of the Army.

REFERENCES

1. SPIEGEL, S. & S. MILSTEIN. 1995. Sphingolipid metabolites: members of a new class of lipid second messengers. J. Membr. Biol. **146:** 225–233.
2. MOOLENAAR, W.H., O. KRANENBURG, F.R. POSTMA & G. ZONDAG. 1997. Lysophosphatidic acid: G protein signaling and cellular responses. Curr. Opin. Cell Biol. **9:** 168–173.
3. GOETZL, E.J. & S. AN. 1998. Diversity of cellular receptors and functions for the lysophospholipid growth factors lysophosphatidic acid and sphingosine 1-phosphate. FASEB J. **12:** 1589–1598.
4. AN, S., E.J. GOETZL & H. LEE. 1998. Signaling mechanisms and molecular characteristics of G protein–coupled receptors for lysophosphatidic acid and sphingosine 1-phosphate. J. Cell. Biochem. Suppl. **30/31:** 147–157.
5. GOETZL, E.J., H. LEE, T. AZUMA, T.P. STOSSEL & J.S. KARLINER. 1999. Gelsolin (GS) binding of lysophosphatidic acid (LPA) and enhancement of presentation of LPA to rat cardiac myocytes (RCMs). FASEB J. **13A:** 1423.
6. LEE, M-J., J.R. VAN BROCKLYN, S. THANGADA, C.H. LIU, A.R. HAND, R. MENZELEEV, S. SPIEGEL & T. HLA. 1998. Sphingosine-1-phosphate as a ligand for the G protein–coupled receptor EDG-1. Science **279:** 1552–1555.

7. HECHT, J.H., J.A. WEINER, S.R. POST & J. CHUN. 1996. Ventricular zone gene-1 (vzg-1) encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex. J. Cell Biol. **135:** 1071–1083.
8. AN, S., M.A. DICKENS, T. BLEU, O.G. HALLMARK & E.J. GOETZL. 1997. Molecular cloning of the human Edg2 protein and its identification as a functional cellular receptor for lysophosphatidic acid. Biochem. Biophys. Res. Commun. **231:** 619–622.
9. AN, S., T. BLEU, W. HUANG, O.G. HALLMARK, S.R. COUGHLIN & E.J. GOETZL. 1997. Identification of cDNAs encoding two G protein–coupled receptors for lysosphingolipids. FEBS Lett. **417:** 279–282.
10. AN, S., T. BLEU, O.G. HALLMARK & E.J. GOETZL. 1998. Characterization of a novel subtype of human G protein–coupled receptor for lysophosphatidic acid. J. Biol. Chem. **273:** 7906–7910.
11. GOETZL, E.J. & S. AN. 1999. A subfamily of G protein–coupled cellular receptors for lysophospholipids and lysosphingolipids. *In* Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation, and Related Diseases. Plenum. New York.
12. HILL, C.S. & R. TREISMAN. 1995. Differential activation of c-fos promoter elements by serum, lysophosphatidic acid, G proteins, and polypeptide growth factors. EMBO J. **14:** 5037–5042.
13. DURIEUX, M.E. & K.R. LYNCH. 1993. Signaling properties of lysophosphatidic acid. Trends Pharmacol. Sci. **14:** 249–254.
14. AN, S., T. BLEU, Y. ZHENG & E.J. GOETZL. 1998. Recombinant human G protein–coupled lysophosphatidic acid receptors mediate intracellular calcium mobilization. Mol. Pharmacol. **54:** 881–888.
15. AN, S., T. BLEU & Y. ZHENG. 1999. Transduction of intracellular calcium signals through G protein–mediated activation of phospholipase C by recombinant sphingosine 1-phosphate receptors. Mol. Pharmacol. **55:** 1–8.
16. MAO, J., H. YUAN, W. XIE, M.I. SIMON & D. WU. 1998. Specific involvement of G proteins in regulation of serum response factor–mediated gene transcription by different receptors. J. Biol. Chem. **273:** 27118–27123.
17. MONTANER, S., R. PERONA, L. SANIGER & J.C. LACAL. 1999. Activation of serum response factor by RhoA is mediated by the nuclear factor–kappaB and C/EBP transcription factors. J. Biol. Chem. **274:** 8506–8515.
18. WEINER, J.A., J.H. HECHT & J. CHUN. 1998. Lysophosphatidic acid receptor gene vzg-1/lpa1/edg-2 is expressed by mature oligodendrocytes during myelination in the postnatal murine brain. J. Comp. Neurol. **398:** 587–598.
19. GOETZL, E.J., H. DOLEZALOVA, Y. KONG, Y.-L. HU, R.B. JAFFE, K.R. KALLI & C.A. CONOVER. 1999. Distinctive expression and functions of the type 4 endothelial differentiation gene-encoded G protein-coupled receptor for lysophosphatidic acid in ovarian cancer. Cancer Res. **59:** 5370–5375.
20. HU, Y-L., E.J. GOETZL, G.B. MILLS, N. FERRARA & R.B. JAFFE. 1999. Induction of vascular endothelial growth factor expression by lysophospatidic acid in normal and neoplastic human ovarian epithelial cells. Proc. 81st Annu. Meet. of the Endocrine Society, P1-602 (Abstr.).
21. GRALER, M.H., G. BERNHARDT & M. LIPP. 1998. Edg6, a novel G protein–coupled receptor related to receptors for bioactive lysophospholipids, is specifically expressed in lymphoid tissue. Genomics **53:** 164–169.
22. GOETZL, E.J., Y. KONG & B. MEI. 1999. Lysophosphatidic acid and sphingosine 1-phosphate protection of T cells from apoptosis in association with suppression of Bax. J. Immunol. **162:** 2049–2056.
23. GOETZL, E.J., Y. KONG & J.S. KENNEY. 1999. Lysophospholipid enhancement of human T cell sensitivity to diphtheria toxin by increased expression of heparin-binding epidermal growth factor. Proc. Assoc. Am. Physicians **111:** 259–269.
24. PIAZZA, G.A., J.L. RITTER & C.A. BARACKA. 1995. Lysophosphatidic acid induction of transforming growth factors alpha and beta: modulation of proliferation and differentiation in cultured human keratinocytes and mouse skin. Exp. Cell Res. **216:** 51–64.
25. GOETZL, E.J., H. DOLEZALOVA, Y. KONG & L. ZENG. 1999. Dual mechanisms for lysophospholipid induction of proliferation of human breast carcinoma cells. Cancer Res. **59:** 4732–4737.

26. INOUE, C.N., Y.H. KO, W.B. GUGGINO, H.G. FORSTER & M. EPSTEIN. 1997. Lysophosphatidic acid and platelet-derived growth factor synergistically stimulate growth of cultured rat mesangial cells. Proc. Soc. Exp. Biol. Med. **216:** 370–378.
27. TIGYI, G., D.L. DYER & R. MILEDI. 1994. Lysophosphatidic acid possesses dual action in cell proliferation. Proc. Natl. Acad. Sci. U.S.A. **91:** 1908–1912.
28. CUVILLIER, O., D.S. ROSENTHAL, M.E. SMULSON & S. SPIEGEL. 1998. Sphingosine 1-phosphate inhibits activation of caspases that cleave poly (ADP-ribose) polymerase and lamins during Fas- and ceramide-mediated apoptosis in Jurkat T lymphocytes. J. Biol. Chem. **273:** 2910–2916.

Lysophospholipid Growth Factor Receptors

Edward J. Goetzl*, Songzhu An and Hsinyu Lee

University of California at San Francisco, Departments of Medicine and Microbiology, 533 Parnassus at 4th, UB8B, Box 0711, San Francisco, CA 94143-0711, USA

*corresponding author tel: 415 476 5339, fax: 415 476 6915, e-mail: egoetzl@itsa.ucsf.edu

DOI: 10.1006/rwcy.2000.23010.

SUMMARY

The endothelial differentiation gene-encoded G protein-coupled receptors (Edg Rs) Edg-1, -3, -5, and -8 bind sphingosine 1-phosphate (S1P) and Edg-2, -4, and -7 bind lysophosphatidic acid (LPA), resulting in transduction of cellular signals for proliferation, increased survival, reduced apoptosis, adherence, migration, secretion, and other specific functions. The diversity of signals emanating from each Edg R is in part attributable to coupling to multiple G proteins. One or more Edg Rs are expressed prominently in the cardiovascular, pulmonary, nervous, endocrine, genitourinary, hematological, and immune systems. Expression of Edg Rs is regulated by developmental and postdevelopmental physiological events. The levels of Edg Rs increase in distinctive patterns in many diseases, as exemplified by high levels of Edg-4 in *ovarian cancer*, whereas none is detected in normal ovarian surface epithelial cells. Although several analogs of LPA and S1P have altered agonist or antagonist activities, potent pharmacological agents have not been identified to date.

BACKGROUND

LPA, S1P, sphingosylphosphorylcholine and other structurally related lysophospholipid (LPL) growth factors have major effects on many different types of cells, which include initiation and regulation of cellular proliferation, enhancement of survival, suppression of apoptosis, promotion of differentiation, and stimulation of diverse cytoskeleton-based functions (Moolenaar, 1995; Tokomura, 1995; Spiegel and Merrill, 1996; Goetzl and An, 1998). LPLs are generated enzymatically from precursors stored in cell membranes and are secreted principally by platelets, macrophages, some other types of leukocytes, epithelial cells, and some cancer cells. The amounts secreted are sufficient to establish micromolar concentrations of LPA and S1P in plasma and S1P in plasma normally and in extracellular fluids during tissue reactions. Extracellular LPA and S1P are both almost entirely bound by serum proteins (Tigyi and Miledi, 1992; Thumser *et al.*, 1994). LPLs resemble protein growth factors in their ability to alter transcriptional activities of growth-related genes, amplify direct stimulation of cellular proliferation by recruiting other growth factor systems, and evoke many cellular responses other than proliferation and proliferation-related events (An *et al.*, 1998c; Goetzl and An, 1998).

Discovery

The LPLs and protein growth factors differ principally in their respective uses of G protein-coupled receptors (GPCRs) and protein tyrosine kinase receptors. LPA and S1P bind specifically to different members of a family of GPCRs, which are encoded by endothelial differentiation genes (Edgs) and thus are designated tentatively as Edg Rs (Hla and Maciag, 1990; MacLennan *et al.*, 1994; Yamaguchi *et al.*, 1996; Chun *et al.*, 1999). Edg-1 was discovered initially as a GPCR of unknown ligand specificity, which was expressed by endothelial cells only after

differentiation (Hla and Maciag, 1990). Edg-2 was first found on proliferating neurons in the periventricular zone of developing embryonic mice (Hecht *et al.*, 1996). LPA evoked shape changes and proliferation in these Edg-2 R-expressing neurons, and both their Edg-2 Rs and the responses to LPA disappeared after completion of CNS development. The primary determinants of cellular responses to LPLs are the generative and biodegradative events, which establish steady-state concentrations of each protein-bound LPL at cell surfaces, and the relative frequency of expression of each Edg R.

Alternative names

There are no alternative names for the Edg receptor family, but individual receptors have been called by other terms, such as ventricular zone gene 1 (vzg-1)-encoded receptor for Edg-2 (Hecht *et al.*, 1996).

Structure

Two subfamilies of the Edg Rs have been distinguished based on their degree of amino acid sequence identity and preferred LPL ligand (**Table 1**) (Goetzl and An, 1998; Chun *et al.*, 1999). The first encompasses Edg-1, -3, -5, and -8, which are 45–60% identical in amino acid sequences and bind S1P with high affinity (Hla and Maciag, 1990; MacLennan *et al.*, 1994; Yamaguchi *et al.*, 1996; An *et al.*, 1997b; Lee *et al.*, 1998a,b; Glickman *et al.*, 1999). The second includes Edg-2, -4, and -7, which are 48–54% identical in amino acid sequences and bind LPA with high specificity and affinity (Hecht *et al.*, 1996; An *et al.*, 1997a, 1998a; Bandoh *et al.*, 1999). Mechanisms of regulation of expression, G protein associations, predominant signaling pathways, pattern of tissue representation, and involvement in human diseases are now being defined for each Edg R.

Main activities and pathophysiological roles

The principal roles of Edg Rs are to bind LPA and/or S1P with high affinity and specificity, and to transduce G protein-dependent signals to cellular proliferation, survival, and functions.

GENE

The genomic structures of Edg-1, -3, and -5 differ fundamentally from those of Edg-2 and -4. The over-45 kb murine Edg-2 gene was successfully defined and shown to be composed of at least five separate exons on chromosome 4 (Contos and Chun, 1998). Divergent cDNA sequences are located upstream from a single common exon sequence, which encodes most of the open reading frame of Edg-2 and ends at an intron in the middle of transmembrane domain VI. In contrast, murine Edg-1 has only two exons, with a

Table 1 Encoding genes and structures of Edg LPA and S1P receptors

Human receptor	Ligand	Chromosomal location (human/mouse)	Protein size (aa no.)	G protein coupling	Signaling		
					A	C	E
Edg-1	S1P	1p21.1–3	381	i	I	S	S
Edg-3	S1P	9q22.1–2	378	i, q, 12/13	I	S	S
Edg-5	S1P	19p13.2/9	354	i, q, 12/13	I	S	S
Edg-8	S1P	ND	400				
Edg-6	ND	19p13.3	384	–	–	–	–
Edg-2	LPA	ND/4 (6)	364	i, 12/13	I	S	N/S
Edg-4	LPA	19p12	351 (382)[a]	i, q, 12/13	I	S	S
Edg-7	LPA	ND	353	q, s	S	S	N/S

A, adenylyl cyclase; C, increase in $[Ca^{2+}]_i$; E, ERK activity; ND, not determined; I, inhibit; S, stimulate; N, no effect; –, insufficient data; N/S, no effect in some cells, stimulation in other cells.

[a]The number of amino acids in the wild type is 351 and in the mutant is 382.

single coding exon, an intron separating the transcriptional start site from the open reading frame, and no intron in transmembrane domain VI (Liu and Hla, 1997). Genes encoding several other Edg Rs have very recently been defined structurally or sequenced by the Human Genome Project (Table 1). A recently recognized mutant of Edg-4 R results from a deletion of a single base near the 3′ end of the open reading frame, which leads to a frameshift, loss of the termination codon, and replacement of the four amino acid C-terminus of the wild-type Edg-4 with a completely different 35 amino acid peptide (Table 1). No promoter elements of any Edg R have been analyzed further as yet.

Accession numbers

Edg-1: AF022137, M312104
Edg-3: AF022139, X83864
Edg-5: AF034780
Edg-6: AJ000479
Edg-2: U80811
Edg-4: AF011466
Edg-7: AF127138

Chromosome location and linkages

See Table 1.

There is approximately a 26–28% homology in sequence between Edg Rs and some cannabinoid GPCRs.

PROTEIN

Description of protein

The Edg-1 to Edg-8 genes encode distinct but related seven transmembrane domain GPCRs (Table 1). Based on amino acid sequence identity, Edg-1, -3, -5, and -8 belong to one structural cluster and Edg-2, -4, and -7 are members of a second structural cluster. The amino acid sequence of Edg-6 lies between those of the two major clusters (Graler *et al.*, 1998). Edg Rs share other structural features which have not as yet been definitively linked to function. The *N*-linked glycosylation sites of the N-terminus and multiple potential sites of phosphorylation in intracellular regions are typical of all GPCRs and are preserved in the Edg Rs. In contrast, the disulfide bond most often formed between cysteines in the first and second extracellular loops in other GPCRs is most often formed between the second and third extracellular loops of Edg Rs. In Edg-4 R, an alanine replaces the proline typical of the seventh transmembrane domain sequence NPXXY of other GPCRs. An Src homology 2 (SH2) segment exists in the intracellular face of Edg-5, but has not been shown to express characteristic functions. The mRNAs encoding some of the Edg Rs have the AU-rich sequence AUUUA in their 3′ untranslated region, which is an mRNA-stabilizing structure typical of growth-related immediate-early genes. A GPCR originally reported as an orphan (An *et al.*, 1995), has been proven to bind the LPL sphingosylphosphorylcholine with high specificity and affinity, resulting in the expected intracellular signals (Xu *et al.*, 2000).

Distinctive patterns of tissue distribution of each Edg R have been mapped principally by semiquantitative PCR techniques, northern blots, *in situ* hybridization and western blots with monoclonal antibodies to multiple substituent peptides (An *et al.*, 1997a,b, 1998a; Hecht *et al.*, 1996; Liu and Hla, 1997; MacLennan *et al.*, 1997; Weiner *et al.*, 1998) (Table 2). Another type of GPCR has been identified on *Xenopus* oocytes and some rodent cells, which is specific for LPA but structurally unrelated to Edg Rs (Guo *et al.*, 1996).

Cell types and tissues expressing the receptor

These are listed in **Table 2.**

SIGNAL TRANSDUCTION

Each of the Edg-1 to -8 receptors usually couples to multiple G proteins, which results in concurrent initiation of diverse signaling pathways that may be amplified by crosstalk between these pathways (Goodemote *et al.*, 1995; Brindley *et al.*, 1996; Lee *et al.*, 1996; van Koppen *et al.*, 1996; An *et al.*, 1998b; Erickson *et al.*, 1998; Fukushima *et al.*, 1998; Okamota *et al.*, 1998; Zondag *et al.*, 1998; Windh *et al.*, 1999). The association of each Edg R with G_i leads to striking ligand-induced enhancement of the Ras/extracellular signal-regulated kinase (ERK) pathway central to LPA and S1P activation of cellular proliferation (Table 1). The $\beta\gamma$ subunits of G_i may couple ERKs to Edg Rs, whereas the α chain of G_i mediates the defining inhibition of adenylyl cyclase. The biochemical prerequisites for G_i recruitment of ERKs include an Src-related tyrosine kinase, Pyk 2 kinase, and probably PI-3 kinase, based on the

Table 2 Human Edg receptor tissue distribution and abnormalities in disease states

	Tissue distribution	Expression/mutations in diseases
Edg-1	Endothelial cells, many other types of cells	
Edg-3	Heart, many other types of cells	Increased in *breast cancer*
Edg-5	Heart, embryonic nervous system, many other types of cells	
Edg-8	Brain	
Edg-6	Lymphoid tissues, leukocytes, lung	
Edg-2	Embryonic nervous system, many other types of cells	
Edg-4	Leukocytes, testes, many other types of cells	Increased in breast cancer, increased in *ovarian cancer* (mutant form)
Edg-7	Pancreas, heart, prostate, testes, lung, ovary	

ability of selective inhibitors of PI-3 kinase to suppress LPA and S1P induction of ERK activity.

Edg-3, -4, -5, and -7 receptors couple with and activate G_q, resulting in sequential stimulation of phospholipase C, release of diacylglycerol leading to enhancement of protein kinase C activity and of inositol trisphosphate (IP_3), which consequently elicits pertussis toxin-insensitive increases in $[Ca^{2+}]_i$. In contrast, G_i mediates the pertussis toxin-sensitive part of LPA- and S1P-evoked increases in $[Ca^{2+}]_i$ transduced by Edg-3, -4, and -5, and the principal effect transduced by Edg-1 and -2 in some types of cells through recruitment of phospholipase C. The proposed capacity of S1P to act as intracellular messenger and thereby mobilize Ca^{2+} directly, without binding to a cell surface receptor, is independent of IP_3 and dependent on sphingosine kinase activity in some cells, but the cellular target protein for this action of S1P remains to be identified (Spiegel and Merrill, 1996; Kohama *et al.*, 1998; Melendez *et al.*, 1998).

LPA and S1P also evoke cellular proliferation and cytoskeleton-dependent functions through Rho-mediated pathways, which are initiated by engagement of any of the Edg Rs (Moolenaar, 1995; Spiegel and Milstien, 1995; Fromm *et al.*, 1997) (Table 1). In some cellular settings, Edg-1 may not couple to Rho. The elicitation of SRF transcriptional activity, regulation of cellular pH, and induction of formation of actin stress fibers and focal adhesion complexes by LPA and S1P are all Rho-mediated through $G\alpha_{12/13}$ or, in rare circumstances, through $G\alpha_q$ (Hill and Treisman, 1995; Hill *et al.*, 1995; Fromm *et al.*, 1997). The GTPase-activating factor Lsc/p115RhoGEF links $G\alpha_{13}$ with Rho and enables Rho, which in turn activates serine/threonine kinases, phospholipase D, SRF, PI-3 kinase, p125 focal adhesion kinase, and myosin light chain phosphatase (Hart *et al.*, 1998). Many effects of LPA and S1P on complex cellular responses involve integrated engagement of multiple signaling pathways.

DOWNSTREAM GENE ACTIVATION

Transcription factors activated

Transcriptional activation of SRE by LPA and S1P suggested the possibility that recruitment of growth-related immediate-early genes with SRE in their promoter, such as c-fos and others critical for proliferative responses, might be an important mechanism mediating growth effects of the LPLs. In some cancer cells, LPA and S1P also upregulate expression of autocrine protein growth factors and their receptors through transcriptional mechanisms, which remain to be defined (Goetzl *et al.*, 1999a,b).

BIOLOGICAL CONSEQUENCES OF ACTIVATING OR INHIBITING RECEPTOR AND PATHOPHYSIOLOGY

All of the effects of LPA and S1P described in the chapter on the lysophospholipid growth factors are presumed to be transduced by the Edg Rs and related GPCRs. The LPLs are often acting in tissues responding to other cytokines and mediators, and it is not known how these factors influence expression and functions of receptors for LPLs. The absence of

native inactivating genetic defects for any of the receptors and of potent pharmacological agents presently precludes specific assignment of any of the LPL effects to one type of receptor. Some native variants of LPA and synthetic modifications of LPA or S1P have interacted with Edg Rs as partial agonists or antagonists (Bittman *et al.*, 1996; Liliom *et al.*, 1996; Lynch *et al.*, 1997; Fischer *et al.*, 1998; Tigyi *et al.*, 1999). These have increased our understanding of cellular events, but are not considered to be useful for *in vivo* investigations.

Further investigations of the recently described Edg-2 knockout mice (Contos *et al.*, 2000) and development of agonists and antagonists may soon permit useful assessments of the roles of each receptor. Early reports of increases in expression or *de novo* appearance of one or more Edg Rs in *malignant neoplasms*, when contrasted with the patterns of expression in nonmalignant cells of the same lineage, are of interest, but difficult to interpret currently (Goetzl *et al.*, 1999a,b). The most striking finding is the appearance of Edg-4 in *ovarian cancer*, without expression in normal or virally transformed ovarian surface epithelial cells (Goetzl *et al.*, 1999b). At least one ovarian cancer expressed a mutant Edg-4 characterized by increased length of the cytoplasmic tail. Ongoing studies are directed to elucidation of this mechanism and identification of additional mutants in ovarian and other neoplasms.

References

An, S., Tsai, C., and Goetzl, E. J. (1995). Cloning, sequencing and tissue distribution of two related G protein-coupled receptor candidates expressed predominantly in human lung tissue. *FEBS Lett.* **375**, 121–124.

An, S., Dickens, M. A., Bleu, T., Hallmark, O. G., and Goetzl, E. J. (1997a). Molecular cloning of the human Edg2 protein and its identification as a functional cellular receptor for lysophosphatidic acid. *Biochem. Biophys. Res. Commun.* **321**, 619–622.

An, S., Bleu, T., Huang, W., Hallmark, O. G., Coughlin, S. R., and Goetzl, E. J. (1997b). Identification of cDNAs encoding two G protein-coupled receptors for lysosphingolipids. *FEBS Lett.* **417**, 279–282.

An, S., Bleu, T., Hallmark, O. G., and Goetzl, E. J. (1998a). Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. *J. Biol. Chem.* **273**, 7906–7910.

An, S., Bleu, T., Zheng, Y., and Goetzl, E. J. (1998b). Recombinant human G protein-coupled lysophosphatidic acid receptors mediate intracellular calcium mobilization. *Mol. Pharmacol.* **54**, 881–888.

An, S., Goetzl, E. J., and Lee, H. (1998c). Signaling mechanisms and molecular characteristics of G protein-coupled receptors for lysophosphatidic acid and sphingosine 1-phosphate. *J. Cell Biochem.* **30/31**, (Suppl.), 147–157.

Bandoh, K., Aoki, J., Hosono, H., Kobayashi, S., Kobayashi, T., Murakami-Murofushi, K., Tsujimoto, M., Arai, H., and Inoue, K. (1999). Molecular cloning and characterization of a novel human G-protein-coupled receptor, edg7, for lysophosphatidic acid. *J. Biol. Chem.* **274**, 27776–27785.

Bittman, R., Swords, B., Liliom, K., and Tigyi, G. (1996). Inhibitors of lipid phosphatidate receptors: *N*-palmitol-serine and *N*-palmitoyl-tyrosine phosphoric acids. *J. Lipid Res.* **37**, 391–398.

Brindley, D. N., Abousalham, A., Kikuchi, Y., Wang, C.-N., and Waggoner, D. W. (1996). "Cross talk" between the bioactive glycerolipids and sphingolipids in signal transduction. *Biochem. Cell Biol.* **74**, 469–476.

Chun, J., Contos, J. J. A., and Munroe, D. (1999). A growing family of receptor genes for lysophosphatidic acid (LPA) and other lysophospholipids (LPs). *Cell Biochem. Biophys.* **30**, 213–242.

Contos, J. J. A., and Chun, J. (1998). Complete cDNA sequence, genomic structure, and chromosomal localization of the LPA receptor gene, IpA1/vzg-1/Gpcr26. *Genomics* **51**, 364–378.

Contos, J. J. A., Munroe, D., and Chun, J. (2000). Developmental role of Edg-2/LPA 1a in the mouse central nervous system. (in press).

Erickson, J. R., Wu, J. J., Goddard, J. G., Tigyi, G., Kawanishi, K., Tomei, L. D., and Kiefer, M. C. (1998). Edg-2/vzg-1 couples to the yeast pheromone response pathway selectively in response to lysophosphatidic acid. *J. Biol. Chem.* **273**, 1506–1510.

Fischer, D. J., Liliom, K., Guo, Z., Nusser, N., Virag, T., Murakami-Murofushi, K., Kobayashi, S., Erickson, J. R., Sun, G., Miller, D. D., and Tigyi, G. (1998). Naturally occurring analogs of lysophosphatidic acid elicit different cellular responses through selective activation of multiple receptor subtypes. *Mol. Pharmacol.* **54**, 979–988.

Fromm, C., Coso, O. A., Montaner, S., Xu, N., and Gutkind, J. S. (1997). The small GTP-binding protein rho links G protein-coupled receptors and G alpha12 to the serum response element and to cellular transformation. *Proc. Natl Acad. Sci. USA* **94**, 10098–10103.

Fukushima, N., Kimura, Y., and Chun, J. (1998). A single receptor encoded by vzg-1/lp A1/edg-2 couples to G proteins and mediates multiple cellular responses to lysophosphatidic acid. *Proc. Natl Acad. Sci. USA* **95**, 6151–6156.

Glickman, M., Malek, R. L., Kwitek-Black, A. E., Jacob, H. J., and Lee, N. H. (1999). Molecular cloning, tissue-specific expression, and chromosomal localization of a novel nerve growth factor-regulated G protein-coupled receptor, nrg-1. *Mol. Cell. Neurosci.* **14**, 141–152.

Goetzl, E. J., and An, S. (1998). Diversity of cellular receptors and functions for the lysophospholipid growth factors lysophosphatidic acid and sphingosine 1-phosphate. *FASEB J.* **12**, 1589–1598.

Goetzl, E. J., Dolezalova, H., Kong, Y., and Zeng, L. (1999a). Dual mechanisms for lysophospholipid induction of proliferation of human breast carcinoma cells. *Cancer Res.* **59**, 4732–4737.

Goetzl, E. J., Dolezalova, H., Kong, Y., Hu, Y.-L., Jaffe, R. B., Kalli, K. R., and Conover, C. A. (1999b). Distinctive expression and functions of the type 4 endothelial differentiation gene-encoded G protein-coupled receptor for lysophosphatidic acid in ovarian cancer. *Cancer Res.* **59**, 5370–5375.

Goodemote, K. A., Mattie, M. E., Berger, A., and Spiegel, S. (1995). Involvement of a pertussis toxin-sensitive G protein in the mitogenic signaling pathways of sphingosine 1-phosphate. *J. Biol. Chem.* **270**, 10272–10277.

Graler, M. H., Bernhardt, G., and Lipp, M. (1998). EDG6, a novel G-protein-coupled receptor related to receptors for bioactive lysophospholipids, is specifically expressed in lymphoid tissue. *Genomics* **53**, 164–169.

Guo, Z., Liliom, K., Fischer, D. J., Bathurst, I. C., Tomei, L. D., Kiefer, M. C., and Tigyi, G. (1996). Molecular cloning of a high-affinity receptor for the growth factor-like lipid mediator lysophosphatidic acid from *Xenopus* oocytes. *Proc. Natl Acad. Sci. USA* **93**, 14367–14372.

Hart, M. J., Jiang, X., Kozasa, T., Roscoe, W., Singer, W. D., Gilman, A. G., Sternweis, P. C., and Bollag, G. (1998). Direct stimulation of the guanine nucleotide exchange activity of p115 Rho-GEF by Galpha13. *Science* **280**, 2112–2114.

Hecht, J. H., Weiner, J. A., Post, S. R., and Chun, J. (1996). Ventricular zone gene-1 (vzg-1) encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex. *J. Cell Biol.* **135**, 1071–1083.

Hill, C. S., and Treisman, R. (1995). Differential activation of c-fos promoter elements by serum, lysophosphatidic acid, G proteins and polypeptide growth factors. *EMBO J.* **14**, 5037–5047.

Hill, C. S., Wynne, J., and Treisman, R. (1995). The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. *Cell* **81**, 1159–1170.

Hla, T., and Maciag, T. (1990). An abundant transcript induced in differentiating human endothelial cells encodes a polypeptide with structural similarities to G-protein-coupled receptors. *J. Biol. Chem.* **265**, 9308–9313.

Kohama, T., Olivera, A., Edsall, L., Nagiec, M. M., Dickson, R., and Spiegel, S. (1998). Molecular cloning and functional characterization of murine sphingosine kinase. *J. Biol. Chem.* **273**, 23722–23728.

Lee, M.-J., Evans, M., and Hla, T. (1996). The inducible G protein-coupled receptor edg-1 signals via the Gi/mitogen-activated protein kinase pathway. *J. Biol. Chem.* **271**, 11272–11279.

Lee, M.-J., Brocklyn, J. R. V., Thangada, S., Liu, C. H., Hand, A. R., Menzeleev, R., Spiegel, S., and Hla, T. (1998a). Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor edg-1. *Science* **279**, 1552–1555.

Lee, M.-J., Thangada, S., Liu, C. H., Thompson, B. D., and Hla, T. (1998b). Lysophosphatidic acid stimulates the G-protein-coupled receptor Edg-1 as a low affinity agonist. *J. Biol. Chem.* **273**, 22105–22112.

Liliom, K., Bittman, R., Swords, B., and Tigyi, G. (1996). *N*-Palmitoyl-serine and *N*-palmitoyl-tyrosine phosphoric acids are selective competitive antagonists of the lysophosphatidic acid receptors. *Mol. Pharmacol.* **50**, 616–623.

Liu, C. H., and Hla, T. (1997). The mouse gene for the inducible G-protein-coupled receptor Edg-1. *Genomics* **43**, 15–24.

Lynch, K. R., Hopper, D. W., Carlisle, S. J., Catalano, J. G., Zhang, M., and Macdonald, T. L. (1997). Structure/activity relationships in lysophosphatidic acid: the 2-hydroxyl moiety. *Mol. Pharmacol.* **52**, 75–81.

MacLennan, A. J., Browe, C. S., Gaskin, A. A., Lado, D. C., and Shaw, G. (1994). Cloning and characterization of a putative G-protein coupled receptor potentially involved in development. *Mol. Cell Neurosci.* **5**, 201–209.

MacLennan, A. J., Marks, L., Gaskin, A. A., and Lee, N. (1997). Embryonic expression pattern of H218, a G-protein coupled receptor homolog, suggests roles in early mammalian nervous system development. *Neuroscience* **79**, 217–224.

Melendez, A., Floto, R. A., Gillooly, D. J., Harnett, M. M., and Allen, J. M. (1998). FcgRI coupling to phospholipase D initiates sphingosine kinase-mediated calcium mobilization and vesicular trafficking. *J. Biol. Chem.* **273**, 9393–9402.

Moolenaar, W. H. (1995). Lysophosphatidic acid signalling. *Cell Biol.* **7**, 203–210.

Moolenaar, W. H., Kranenburg, O., Postma, F. R., and Zondag, G. (1997). Lysophosphatidic acid: G protein signaling and cellular responses. *Curr. Opin. Cell Biol.* **9**, 168–173.

Okamota, H., Takuwa, N., Gonda, K., Okazaki, H., Chang, K., Yatomi, Y., Shigematsu, H., and Takuwa, Y. (1998). Edg1 is a functional sphingosine-1-phosphate receptor that is linked via a Gi/o to multiple signaling pathways, including phospholipase C activation, Ca^{2+} mobilization, ras-mitogen-activated protein kinase activation, and adenylate cyclase inhibition. *J. Biol. Chem.* **273**, 27104–27110.

Spiegel, S., and Merrill Jr, A. H. (1996). Sphingolipid metabolism and cell growth regulation. *FASEB J.* **10**, 1388–1398.

Spiegel, S., and Milstien, S. (1995). Sphingolipid metabolites: members of a new class of lipid second messengers. *J. Membr. Biol.* **146**, 225–237.

Thumser, A. E., Voysey, J. E., and Wilton, D. C. (1994). The binding of lysophospholipids to rat liver fatty acid-binding protein and albumin. *Biochem. J.* **301**, 801–806.

Tigyi, G., and Miledi, R. (1992). Lysophosphatidates bound to serum albumin activate membrane currents in *Xenopus* oocytes and neurite retraction in PC12 pheochromocytoma cells. *J. Biol. Chem.* **267**, 21360–21367.

Tigyi, G. J., Liliom, K., Fischer, D. J., and Guo, Z. (1999). "Phospholipid Growth Factors: Identification and Mechanism of Action". CRC Press, Washington DC.

Tokumura, A. (1995). A family of phospholipid autocoids: occurrence, metabolism and bioactions. *Progr. Lipid Res.* **34**, 151–184.

van Koppen, C. J., zu Heringdorf, D. M., Laser, K. T., Zhang, C., Jakobs, K. H., Buneman, M., and Pott, L. (1996). Activation of a high affinity Gi protein-coupled plasma membrane receptor by sphingosine-1-phosphate. *J. Biol. Chem.* **271**, 2082–2087.

Weiner, J. A., Hecht, J. H., and Chun, J. (1998). Lysophosphatidic acid receptor gene vzg-1/lpA1/edg-2 is expressed by mature oligodendrocytes during myelination in the postnatal murine brain. *J. Comp. Neurol.* **398**, 587–598.

Windh, R. T., Lee, M.-J., Hla, T., An, S., Barr, A. J., and Manning, D. R. (1999). Differential coupling of the sphingosine 1-phosphate receptors edg-1, edg-3, and H218/edg-5 to the Gi, Gq, and G12 families of heterotrimeric G proteins. *J. Biol. Chem.* **274**, 27351–27358.

Xu, Y., Zhu, K., Hong, G., Wu, W., Baudhuin, L. M., Xiao, Y., and Damron, D. S. (2000). Sphingosylphosphorylcholine (SPC) as a ligand for ovarian cancer G protein coupled receptor 1 (OGR1). *Nature Cell Biol.* (in press).

Yamaguchi, F., Tokuda, M., Hatase, O., and Brenner, S. (1996). Molecular cloning of the novel human G protein-coupled receptor (GPCR) gene mapped on chromosome 9. *Biochem. Biophys. Res. Commun.* **227**, 608–614.

Zondag, G. C. M., Postma, F. R., Etten, I. V., Verlaan, I., and Moolenaar, W. H. (1998). Sphingosine 1-phosphate signalling through the G-protein-coupled receptor edg-1. *Biochem. J.* **330**, 605–609.

Lysophospholipid Growth Factors

Edward J. Goetzl*, Hsinyu Lee and G.J. Tigyi

University of California at San Franscisco, Departments of Medicine and Microbiology, 533 Parnassus at 4th, UB8B, Box 0711, San Francisco, CA 94143-0711, USA

*corresponding author tel: 415-476-5339, fax: 415-476-6915, e-mail: egoetzl@itsa.ucsf.edu

DOI: 10.1006/rwcy.2000.13004.

SUMMARY

Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are the biologically predominant members of a family of mediators termed lysophospholipid growth factors (PLGFs) or lysophospholipids (LPLs). These amphipathic phospholipids are generated by platelets, macrophages, other leukocytes, epithelial cells, and some tumors in amounts that result in micromolar concentrations in serum and some tissue fluids. A family of G protein-coupled receptors bind LPA and S1P to transduce signals stimulating cellular proliferation, differentiation, survival, and cellular functions. LPA and S1P have roles in hemostasis, cardiovascular regulation, wound healing, cytoprotection, immunity, inflammation, and organ system development normally, and in the pathogenesis of some types of *cancer*.

BACKGROUND

Discovery

Lysophosphatidic acid (LPA), sphingosine 1-phosphate (S1P), and other LPLs/PLGFs are amphipathic metabolic products of membrane phospholipids in stimulated cells, which are present in many mammalian physiological fluids at up to micromolar concentrations and have diverse effects on cellular proliferation, differentiation, survival, and functions (Moolenaar, 1995; Spiegel and Milstien, 1995; Tokumura, 1995; Spiegel and Merrill, 1996; Heringdorf *et al.*, 1997; Moolenaar *et al.*, 1997; An *et al.*, 1998a; Goetzl and An, 1998; Lee *et al.*, 1999b; Tigyi *et al.*, 1999; Goetzl and Lynch, 2000). The PLGFs/LPLs were discovered first chemically in the mid- to late 1800s among other organic solvent-extractable components of cells and tissues. The discovery of LPA as a biologically active acidic phospholipid dates back to the late 1940s and 1950s (Vogt, 1949, 1957; Gabr, 1956) when it was isolated and identified as a stimulator of intestinal smooth muscle contraction. The only unifying structural motif of LPLs/PLGFs is the ubiquitous presence of a phosphate ester moiety and a hydrocarbon chain, which are linked to glycerol or serine backbone.

Glycero-PLGFs/LPLs, exemplified by LPA, are members of the phospholipid family which predominates quantitatively among lipid structural constituents of cellular membranes. Sphingolipids were named for their shared sphingoid backbone and represent a quantitatively diminutive family compared with phospholipids. However, as shown by the protean biological activities of S1P, sphingolipids are one of the structurally and functionally most complex classes of biological mediators. As for our evolving understanding of other bioactive lipids, the appreciation of crucial roles of PLGFs/LPLs in cellular biology derives from recent observations of their rapid generation from cell membrane precursors, ability to directly regulate lipid-metabolizing enzymes (Baker and Chang, 2000), often transient appearance in relation to cellular responses, capacity to move with proteins in the planes of membranes, and potent effects on critical activities of cells both as intracellular messengers and extracellular mediators.

Alternative names

Lysoglycerophospholipids, lysosphingophospholipids, phosphatidates.

Structure

The LPLs all are complex lipids with one phosphate, one free hydroxyl group, and a medium- or long-chain fatty acid on a glycerol or sphingoid backbone. The glycerolipid PLGFs/LPLs usually contain one phosphate ester moiety and an ester- or ether-linked hydrocarbon chain. Although many of them contain a hydroxyl group in the sn-1 or sn-2 position of the glycerol backbone, its presence is not essential for biological activity, as naturally occurring 1,2-cyclic-phosphatidic acid (Fischer *et al.*, 1998; Liliom *et al.*, 1998; Kobayashi *et al.*, 1999), as well as short-chain (Jalink *et al.*, 1994) and/or long-chain phosphatidic acids (Siddiqui and English, 1996, 1997) elicit similar cellular responses. The sphingolipid phosphates implicated as extracellular mediators include S1P (Postma *et al.*, 1996), sphingosylphosphorylcholine, psychosine, glucopsychosine (Himmel *et al.*, 1998), and ceramide-1-phosphate (Gomez-Munoz *et al.*, 1995).

Main activities and pathophysiological roles

The PLGFs/LPLs serve as both extracellular mediators and intracellular messengers for many types of cells (see the sections on *In vitro* activities and *In vivo* biological activities). In addition to their primary effects, PLGFs/LPLs have prominent secondary effects on cells through their abilities to alter production of protein growth factors, act synergistically with other growth factors, and modify expression of receptors for protein growth factors.

The current intense focus on PLGFs/LPLs was initiated in part by the discovery that many of their effects as extracellular mediators for diverse types of cells are transduced by a novel family of highly specific G protein-coupled receptors (GPCRs) (see chapter on Lysophospholipid growth factor receptors). These GPCRs were identified first as elements of expression of immediate-early responses of endothelial cells to differentiating stimuli, and thus have been designated tentatively the endothelial differentiation gene receptors (Edg Rs). The consideration of PLGFs/LPLs in this section and of Edg Rs in the companion section will be directed principally to descriptions of their distinctive cytokine-like effects in cellular differentiation, survival, proliferation, and cytoskeleton-based functions, and of new findings about their possible roles in human diseases.

GENE AND GENE REGULATION

As PLGFs/LPLs are generated and biodegraded by complex cellular pathways involving multiple enzymes (**Table 1**), expression of the genes encoding pathway-controlling enzymes and their regulatory factors determines the net levels of each LPL during any tissue reaction. The predominant pathways and enzymes also may differ in each type of cell, which results in a level of complexity precluding at present a meaningful tabulation of genes controlling the family of PLGFs/LPLs.

Table 1 Rate-controlling and regulatory enzymes of lysolipid phosphate biosynthesis

Activity	LPA	S1P
Release and metabolism of membrane precursor (product)	Phospholipase D and/or phospholipase C + DAGK with sphingomyelinase (phosphatidic acid)	Sphingomyelinases (ceramide) Ceramidase (sphingosine)
Conversion to LPL (product)	Secretory PLA_2 (LPA)	Sphingosine kinase (S1P)
Biodegradation of LPL	Phosphatidate PH	PP-lyase
	Lysophospholipases	
	LPA acyltransferase	

DAGK, diacylglycerol kinase; PH, phosphohydrolase; PP, pyridoxal phosphate-dependent.

Accession numbers

See Protein accession numbers.

Chromosome location

The location of the gene for each lipid-metabolizing enzyme is available from standard databases.

Cells and tissues that express the gene

These studies are not complete, but available data suggest expression of genes encoding the LPL synthetic systems principally in megakaryocytes (platelets), macrophages, some other leukocytes, epithelial cells, and some tumor cells.

PROTEIN

Accession numbers

Critical lipid-metabolizing enzymes of the systems for generating LPA and S1P are as follows:
Human:
Acid sphingomyelinase: M59916
Neutral sphingomyelinase: AJ222801
Ceramidase: U70063
Phospholipase C (PLC): α D16234, γ M34667
Phospholipase D (PLD): L11701
Diacylglycerol kinase: X62535
Secretory PLA_2: AF112982
Lysophosphatidic acid acyltransferase: AF000237
Mouse:
Sphingosine kinase: AF068749

Sequence

These are available through accession numbers.

Description of protein

The enzymes responsible for production of LPA and S1P are phospholipases (PLs), sphingolipases, and highly specialized lipid kinases. After sphingomyelinase conditioning of plasma membrane vesicles released from activated platelets, leukocytes, epithelial cells, and some tumors, PLC- and/or PLD-dependent mechanisms liberate phosphatidic acid, which is converted to LPA by secretory PLA_2 and possibly other PLs (Table 1) (Gaits *et al.*, 1997). Analogously, S1P is generated by the sequential actions of sphingomyelinases, ceramidase, and sphingosine kinase (Table 1) (Hannun, 1994; Spiegel and Merrill, 1996; Schissel *et al.*, 1998; Tomiuk *et al.*, 1998). PLA_2 and sphingosine kinase are the dominant rate-controlling enzymes in the respective synthetic pathways (Fourcade *et al.*, 1995; Higgs *et al.*, 1998; Kohama *et al.*, 1998). Concurrent degradative activities of a series of lysophospholipases, lysolipid phosphatases, acyltransferases, and an S1P-specific lyase contribute significantly to the courses of appearance and net maximal concentrations of LPA and S1P attained in any reaction (Waggoner *et al.*, 1996; Eberhardt *et al.*, 1997; Kai *et al.*, 1997; Wang *et al.*, 1997; Brindley and Waggoner, 1998; Mandala *et al.*, 1998; Roberts *et al.*, 1998). The dependence of tissue and fluid concentrations of LPA and S1P on multiple LPL-generating and -metabolizing enzymes suggests that a genetic defect in any one will alter the respective downstream pathways with functional significance.

CELLULAR SOURCES AND TISSUE EXPRESSION

Cellular sources that produce

LPA was definitively characterized structurally and as a serum and incubated-plasma vasoactive and platelet-active factor in the late 1970s (Tokumura *et al.*, 1978; Schumacher *et al.*, 1979). Three stimulus-coupled cellular synthetic schemes have been implicated in LPA generation and, by analogy S1P production. (1) Generation by degradation of complex membrane phospholipids. After sphingomyelinase conditioning of plasma membrane vesicles released from activated cells, PLC- and/or PLD-dependent mechanisms liberate phosphatidic acid, which is converted to LPA by secretory PLA_2 and possibly other PLs (Table 1). (2) Generation by lipid kinases. In thrombin-activated platelets diacylglycerol kinase is thought to generate PA, which in turn is cleaved to LPA by secretory PLA_2 (Lapetina *et al.*, 1981a,b). (3) Generation by oxidative degradation. Siess and colleagues have shown the production of LPA in minimally oxidized low-density lipoprotein, by mechanisms similar to those that generate PAF-derivatives (Siess *et al.*, 1999). The serum concentration of LPA is micromolar, in contrast with nanomolar levels in fresh plasma, suggesting that platelets are a major source of LPA (Gerrard and

Robinson, 1984; Eichholtz *et al.*, 1993). Macrophages, some other types of leukocytes, many epithelial cells, ovarian cancer cells, and some other tumor cells also produce and retain LPA at rates which result in intracellular concentrations ranging up to 30–60 μM (Tokumura *et al.*, 1999). The interactions of LPA with various intracellular lipid-binding proteins have not been studied to date.

Extracellular LPA is bound by serum albumin with an apparent K_d of 350 nM, which enhances cellular delivery and effective potency (Tigyi and Miledi, 1992; Thumser *et al.*, 1994; Goetzl *et al.*, 1999c). Recent analyses have shown high-affinity binding of LPA to plasma gelsolin, with an apparent K_d of 6–7 nM, which delivers LPA to some types of cells more efficiently and with more potent activity than serum albumin (Goetzl *et al.*, 1999c). LPA binds to the two phosphatidylinositol diphosphate (PIP_2) sites of gelsolin in competition with PIP_2. Cellular delivery of LPA by gelsolin is most effective at concentrations of 1% to up to 10% of those in normal plasma. At concentrations < 10% of those in normal plasma, gelsolin traps LPA and prevents access to cells with an efficiency which may explain the lack of effect of LPA in plasma on endothelial cells normally. The fact that lower ratios of gelsolin to LPA may lead to decreased binding affinity has suggested negative site cooperativity. Thus plasma gelsolin may carry LPA in an inactive state and then deliver it to cells in an active state when gelsolin levels drop in plasma and tissue fluids, as a result of dilution and binding to actin released from injured cells. This possibility is supported by findings of gelsolin concentrations optimal for cellular delivery of LPA in fluids of burned tissues and airway secretions of inflammatory lung diseases (Goetzl *et al.*, 1999c).

Sphingolysolipid phosphate mediators, such as S1P, are formed during turnover and degradation of membrane sphingolipids by diverse sphingomyelinases and downstream enzymes in numerous types of cells (Spiegel and Milstien, 1995; Spiegel and Merrill, 1996) (Table 1). Plasma and serum concentrations of S1P are in the high nanomolar to micromolar range with extensive binding normally to serum albumin and possibly other proteins, but not gelsolin.

Eliciting and inhibitory stimuli, including exogenous and endogenous modulators

Platelet adherence and aggregation, induced by a wide range of platelet-activating factors, and activation of macrophages and fibroblasts by relevant cytokines stimulate generation of LPA and S1P. PDGF stimulation of fibroblasts and α_1-adrenergic stimulation of adipocytes lead to the secretion of LPA into the extracellular medium. Thrombin stimulation of platelets causes the release of stored S1P into the bloodstream (Yatomi *et al.*, 1995).

RECEPTOR UTILIZATION

Two distinct types of GPCRs bind LPLs specifically and consequently transduce diverse cellular signals by associating with one or more G proteins. The Edg R family is composed of two subfamilies in which members are linked by higher levels of structural homology and specific recognition of the same LPL ligand (see chapter on Lysophospholipid growth factor receptors). Edg-1, -3 and -5 exhibit approximately 50% amino acid sequence identity and bind S1P, but not LPA, with high-affinity (An *et al.*, 1997b, 1998a; Zondag *et al.*, 1998). Edg-2, -4, and -7 represent a second cluster of structural similarity and bind LPA, but not S1P, with high affinity (Hecht *et al.*, 1996; An *et al.*, 1997a, 1998b; Bandoh *et al.*, 1999). Each Edg R also shows a distinctive profile of association with G proteins, which explains in part the differences in cellular activities between members of a subfamily. The LPL ligands for Edg-6 and Edg-8 have not been defined to date. *Xenopus* oocytes and some rodent cells express a second type of GPCR for LPA, termed PSP24, which is modestly homologous with the GPCR for phospholipid platelet-activating factor but not with Edg Rs (Guo *et al.*, 1996; Kawasawa, *et al.*, 1998). PSP24 transduces LPA-evoked oscillatory Cl^-currents through activation of the inositol trisphosphate-Ca^{2+} system and is most highly expressed in the central nervous system of mice, but its physiological roles are still under investigation.

IN VITRO ACTIVITIES

In vitro findings

The capacity of S1P to act as a potent intracellular messenger was suggested initially by its compartmentalized generation and concentration in cells responding to protein growth factors (Rani *et al.*, 1997; Melendez *et al.*, 1998). The facts that inhibitors of the sphingosine kinase, which controls synthesis of S1P, suppressed transduction of signals from receptors for some protein growth factors selectively and that exogenous S1P reversed this suppression support a role for S1P as an intracellular messenger (Spiegel and

Merrill, 1996). LPA has been implicated as an intracellular mediator of synaptic vesicle formation (Schmidt *et al.*, 1999).

The principal biological activities of LPA and S1P are as extracellular mediators, which have three basic types of effects (**Table 2**). The first are growth-related and include proliferation, differentiation, enhanced survival, and decreased sensitivity to apoptosis of diverse types of cells (Van Corven *et al.*, 1989, 1993; Tigyi *et al.*, 1994; Tokumura *et al.*, 1994; Piazza *et al.*, 1995; Wu *et al.*, 1995; Cuvillier *et al.*, 1996; Seufferlein *et al.*, 1996; Inoue *et al.*, 1997; Levine *et al.*, 1997; Herrlich *et al.*, 1998; Holtsberg *et al.*, 1998; Koh *et al.*, 1998; Dixon *et al.*, 1999; Goetzl *et al.*, 1999a; Hisano *et al.*, 1999; Pebay *et al.*, 1999; Pyne *et al.*, 1999; Sato *et al.*, 1999). The second are cytoskeleton-based functional effects, which include shape change, altered adherence, chemotaxis, contraction, and secretion (Tigyi and Miledi, 1992; Imamura *et al.*, 1993; Kolodney and Elson, 1993; Bornfeldt *et al.*,

Table 2 Biological activities of LPA and S1P

Activities	LPA	S1P
Growth-related effects		
Stimulate cellular proliferation	Fibroblasts	Fibroblasts
	Renal tubular cells	Monocytes
	Mesangial cells	T cells
	Smooth muscle cells (vascular)	Cancer cells
	Keratinocytes	
	T cells	
	Cancer cells	
Increase cellular survival	Macrophages	
	B lymphocytes	
Suppress apoptosis	Renal tubular cells	Fibroblasts
	Cardiac monocytes	Endothelial cells
	T cells	T cells
	Monocytes	Monocytes
		Oocytes
Cytoskeleton-based responses		
Cell morphology	Neurite retraction	Neurite retraction
Actin cytoskeletal remodeling	Myocyte hypertrophy	Myocyte hypertrophy
Cell–cell/cell–matrix adhesion	Focal adhesion	Fibronectin matrix assembly
	Platelet aggregation	Platelet aggregation
	Leukocyte–endothelial interactions	Leukocyte–endothelial interactions
Chemotaxis/kinesis	Tumor cells (transcellular)	Neutrophils (inhibition)
	Endothelial cells	Endothelial cells
Secretion	Neurotransmitter release	Protein growth factors
	Protein growth factors	
Altered electrical excitability; ion conductance	Neuroblasts	Ventricular myocytes
	Smooth muscle cells	
	Cerebrovascular myocytes	
Intracellular signaling		TNFα, adhesion
		PDGF, proliferation

1995; Tigyi *et al.*, 1995, 1996a,b; Postma *et al.*, 1996; Sakano *et al.*, 1996; Durante *et al.*, 1997; Kawa *et al.*, 1997; Oral *et al.*, 1997; Seewald *et al.*, 1997; Yatomi *et al.*, 1997; MacDonell *et al.*, 1998; Mathes *et al.*, 1998; Rodriguez-Fernandez and Rozengurt, 1998; Titievsky *et al.*, 1998; Xia *et al.*, 1998; Brocklyn *et al.*, 1999; Dubin *et al.*, 1999; Kranenburg *et al.*, 1999; Lee *et al.*, 1999a; Panetti *et al.*, 1999; Rizza *et al.*, 1999; Shahrestanifar *et al.*, 1999; Siess *et al.*, 1999; Zhang *et al.*, 1999). The third are linked to activation of ion fluxes that include Ca^{2+}, K^+, and Cl^-, which lead to contraction, secretion, and altered excitability in different cell types (Jalink *et al.*, 1994; Watsky, 1995; Postma *et al.*, 1996). Stimulation of proliferation of many different types of cells by both LPA and S1P, through a pertussis toxin-inhibitable mechanism, is the defining activity of PLGFs/LPLs. A complex set of LPL-initiated signaling pathways results in increases in intranuclear levels of the Ras-dependent ternary complex factor (TCF) and the Rho-dependent serum response factor (SRF), which together bind to and transcriptionally activate the serum response element (SRE) in promoters of many immediate-early response genes critical to cellular proliferation (Hill and Treisman, 1995; Hill *et al.*, 1995; Fromm *et al.*, 1997). It is presumed that Edg R transduction of LPL signals to SRE requires association with both $G_i/_o$ to recruit Ras and with $G_{12}/_{13}$ to engage Rho. Confirming evidence comes from the individual abilities of pertussis toxin inactivation of G_i and of *Clostridium botulinum* C3 ADP-ribosyltransferase inactivation of Rho to inhibit proliferative effects of LPLs and to exert greater suppression when applied in combination.

In addition to direct nuclear stimulation of cellular proliferation, LPLs also have indirect effects, which include increased secretion of autocrine protein growth factors, heightened expression of receptors for protein growth factors, and enhanced expression of plasma membrane-localized protein growth factors such as the heparin-binding epidermal growth factor (EGF)-like growth factor, which acts on EGF receptors of neighboring cells by a juxtacrine mechanism (Piazza *et al.*, 1995; Goetzl *et al.*, 1999b,d). In a few types of cells, where LPA elicits an increase in intracellular concentration of cyclic AMP, there is suppression of cellular proliferation (Tigyi *et al.*, 1994). The mechanisms whereby LPLs improve cellular survival are not fully understood, but include reduction in apoptosis. In the few types of cells for which mechanisms of suppression of apoptosis by LPLs have been elucidated, studies have detected both alterations in intracellular levels of effector proteins of the Bcl family and selective inhibition of activity of specific caspases. In T lymphocytes, the protective effects of LPLs are associated with increases in protective Bcl-2, decreases in apoptosis-promoting Bax, and inhibition of caspases 3, 6, and 7, but not 8 (Cuvillier *et al.*, 1996, 1998; Goetzl *et al.*, 1999a).

Cytoskeleton-based functional responses to LPLs include alterations in cellular morphology during differentiation, as typified by LPA-induced rounding of stellate periventricular neurons, and retraction of neurites evoked by LPA and S1P in postmitotic neurons (Table 2) (Tigyi and Miledi, 1992; Hecht *et al.*, 1996; Postma *et al.*, 1996; Tigyi *et al.*, 1996a,b; Brocklyn *et al.*, 1999; Kranenburg *et al.*, 1999). In such responses, stress fiber formation reflects changes in the state of the microfilament network. Activation of Rho-mediated signaling is responsible for the rearrangements of the actin cytoskeleton and actin-associated signaling molecules causing a loss of the differentiated phenotype. However, Rho-mediated signals also inhibit the signal transduction program of neuronal differentiation by inhibiting neurite outgrowth and the cessation of cell proliferation in response to treatments, which induce neuronal differentiation (Jalink *et al.*, 1994; Kozma *et al.*, 1997; Sebok *et al.*, 1999). Similarly to LPA, S1P affects vascular differentiation of endothelial cells, which involves the Rho-dependent formation of adherens junctions (Lee *et al.*, 1999b). LPA and S1P evoke focal adhesion kinase activity, activate cell surface adhesive proteins, and initiate assembly of a fibronectin matrix on cells (Ridley and Hall, 1992; Seufferlein *et al.*, 1996; Rodriguez-Fernandez and Rozengurt, 1998; Sakai *et al.*, 1998; Zhang *et al.*, 1999). Integrated expression of these responses mediates PLGF/LPL-elicited platelet aggregation and endothelial interactions with platelets and leukocytes. These and other related events are crucial to cellular chemotactic, secretory, and contractile responses to LPA and S1P. The principal current obstacle to better understanding of the mechanisms underlying cellular effects of LPLs is the absence of bioavailable and potent pharmacological agonists and antagonists specific for Edg Rs and other LPL Rs.

Regulatory molecules: Inhibitors and enhancers

A wide range of analogs and other variants of LPA and S1P have been synthesized in studies of the structural determinants of activity of the parent compounds (Bittman *et al.*, 1996; Liliom *et al.*, 1996; Lynch *et al.*, 1997; Fischer *et al.*, 1998), but most have the same undesirable physicochemical properties as

LPA and S1P, and none is a significantly more potent agonist or full antagonist of mammalian receptors. Limited applications of antireceptor antibodies, biochemical inhibitors of characteristic signaling pathways, and genetic approaches – such as overexpression of one receptor or antisense ablation of one receptor, have been useful in the early phases of research, but have not delineated use of individual receptors by cells nor been helpful for *in vivo* investigations.

Bioassays used

The basic bioassays have been summarized succinctly in published works (Spiegel and Merrill, 1996; Tigyi *et al.*, 1999).

IN VIVO BIOLOGICAL ACTIVITIES OF LIGANDS IN ANIMAL MODELS

Normal physiological roles

LPA and/or fluid-phase lysophospholipid precursors of LPA are elevated in at least four different clinical settings (**Table 3**): (1) acute lung diseases, such as *adult respiratory distress syndrome* (ARDS) and acute inflammatory exacerbations of chronic lung diseases, such as *asthma*, (2) surface epithelial cell injury, as in transcorneal freezing or cutaneous *burns*, (3) certain malignancies of which *ovarian cancer* is the most extensively analyzed, and (4) in CSF following *subarachnoid hemorrhage* (Yakubu *et al.*, 1997). Of these conditions, animal models have been established or adapted for studies of the roles of lysophospholipids in lung and ocular tissue trauma and inflammation.

Initiation of lung inflammation in guinea pigs by intratracheal administration of LPS induced secretion of type II secretory phospholipase A_2 ($sPLA_2$) into bronchoalveolar fluid and accompanying 3- to 10-fold increases in the concentrations of palmitic acid, total free fatty acids (FFAs), and lyso-phosphatidylcholine (lyso-PC) (Chilton *et al.*, 1996; Arbibe *et al.*, 1998). A specific inhibitor of $sPLA_2$ reduced by a mean of 60% the increases in levels of FFAs and lyso-PC evoked by LPS. Similar increases in the concentrations of FFAs and lyso-PC were attained by administration of guinea pig recombinant $sPLA_2$, in parallel with major decreases in surfactant content of phospholipids (Arbibe *et al.*, 1998). The capacity of lysophospholipase D in lung tissues to convert lyso-PC to LPA is suggested to be one source of increased LPA in pulmonary secretions of injured or inflamed lungs, but was not demonstrated directly. LPA and its active variants cyclic PA and alkenyl-GP and lysophosphatidylserine were identified at biologically active concentrations in aqueous humor and lacrimal gland fluid from rabbit eyes (Liliom *et al.*, 1998). The concentrations of LPA and its homologs were increased after corneal injury to levels which stimulated proliferation of keratinocytes isolated from uninjured rabbit corneas, suggesting a role in normal *wound healing*.

Knockout mouse phenotypes

These are limited to a receptor knockout (see chapter on Lysophospholipid growth factor receptors).

Interactions with cytokine network

LPLs modify secretion of some cytokine growth factors as noted above.

Table 3 Pathophysiological contributions

Model or disease	Site	LPL	Contribution
Rabbit corneal injury	Aqueous humor	LPA, LPA isomers	*Wound healing*
Human lung injury	BAL	LPA, LPC, other PLs	Wound healing
Human *atherosclerosis*	Vascular lesions	LPA	Platelet/endothelial activation
Human *ovarian cancer*	Tumor/*metastases*	LPA	*Tumor growth*, spread
			Increased vascular permeability

BAL, bronchoalveolar lavage fluid.

PATHOPHYSIOLOGICAL ROLES IN NORMAL HUMANS AND DISEASE STATES AND DIAGNOSTIC UTILITY

Normal levels and effects

The plasma concentrations of LPA and S1P are mid-nanomolar and up to micromolar, respectively, and serum concentrations of both are micromolar.

Role in experiments of nature and disease states

Elevated concentrations of LPA, lyso-PC, and some other phospholipids have been detected in lesional fluids of several inflammatory and neoplastic diseases (Table 3). However, only in ovarian carcinoma and possibly other gynecologic cancers have tissue and plasma levels of LPA been increased so consistently as to suggest pathogenetic roles and even utility as a biochemical marker of these malignancies (Xu *et al.*, 1998). The ability of LPA to stimulate increased expression by *ovarian cancer* cells of adhesive proteins characteristic of the neoplastic state, such as vascular endothelial growth factor (VEGF) (Hu *et al.*, 2000), transcellular migration (Imamura *et al.*, 1993), and proliferation (Goetzl *et al.*, 1999e), without effects on normal ovarian surface epithelial cells, has contributed to the authenticity of suggestions that plasma levels of LPA represent a useful marker for even early stages of ovarian cancer. Any role of LPA in ovarian and other cancers remains to be proven in further analyses of mechanisms and much larger clinical studies, involving the effects of different forms of treatment. However, it appears certain that ovarian cancer cells do produce LPA in amounts far exceeding those of any other form of cancer so far examined *in vitro*. Further, ovarian cancer cells express Edg Rs for LPA qualitatively different from those detected on normal ovarian cells (see chapter on Lysophospholipid growth factor receptors).

References

An, S., Dickens, M. A., Bleu, T., Hallmark, O. G., and Goetzl, E. J. (1997a). Molecular cloning of the human Edg2 protein and its identification as a functional cellular receptor for lysophosphatidic acid. *Biochem. Biophys. Res. Commun.* **321**, 619–622.

An, S., Bleu, T., Huang, W., Hallmark, O. G., Coughlin, S. R., and Goetzl, E. J. (1997b). Identification of cDNAs encoding two G protein-coupled receptors for lysosphingolipids. *FEBS Lett.* **417**, 279–282.

An, S., Goetzl, E. J., and Lee, H. (1998a). Signaling mechanisms and molecular characteristics of G protein-coupled receptors for lysophosphatidic acid and sphingosine 1-phosphate. *J. Cell. Biochem. Suppl.* **30/31**, 147–157.

An, S., Bleu, T., Hallmark, O. G., and Goetzl, E. J. (1998b). Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. *J. Biol. Chem.* **273**, 7906–7910.

Arbibe, L., Koumanov, K., Vial, D., Rougeot, C., Faure, G., Havet, N., Longacre, S., Vargaftig, B. B., Bereziat, G., Voelker, D. R., Wolf, C., and Touqui, L. (1998). Generation of lyso-phospholipids from surfactant in acute lung injury is mediated by type-II phospholipase A2 and inhibited by a direct surfactant protein a-phospholipase A2 protein interaction. *J. Clin. Invest.* **102**, 1152–1160.

Baker, R., and Chang, H.-Y. (2000). A metabolic path for the degradation of lysophosphatidic acid, an inhibitor of lysophosphatidylcholine lysophospholipase, in neuronal nuclei of cerebral cortex. *Biochem. Biophys. Acta* (in press).

Bandoh, K., Aoki, J., Hosono, H., Kobayashi, S., Kobayashi, T., Murakami-Murofushi, K., Tsujimoto, M., Arai, H., and Inoue, K. (1999). Molecular cloning and characterization of a novel human G-protein-coupled receptor, edg7, for lysophosphatidic acid. *J. Biol. Chem.* **274**, 27776–27785.

Bittman, R., Swords, B., Liliom, K., and Tigyi, G. (1996). Inhibitors of lipid phosphatidate receptors: N-palmitol-serine and N-palmitoyl-tyrosine phosphoric acids. *J. Lipid Res.* **37**, 391–398.

Bornfeldt, K. E., Graves, L. M., Raines, E. W., Igarashi, Y., Wayman, G., Yamamura, S., Yatomi, Y., Sidhu, J. S., Krebs, E. G., Hakomori, S.-I., and Ross, R. (1995). Sphingosine-1-phosphate inhibits PDGF-induced chemotaxis of human arterial smooth muscle cells: spatial and temporal modulation of PDGF chemotactic signal transduction. *J. Cell. Biol.* **130**, 193–206.

Brindley, D. N., and Waggoner, D. W. (1998). Mammalian lipid phosphate phospholydrolases. *J. Biol. Chem.* **273**, 24281–24284.

Brocklyn, J. R. V., Tu, Z., Edsall, L. C., Schmidt, R. R., and Spiegel, S. (1999). Sphingosine 1-phosphate-induced cell rounding and neurite retraction are mediated by the G protein-coupled receptor H218. *J. Biol. Chem.* **274**, 4626–4632.

Chilton, F. H., Averill, F. J., Hubbard, W. C., Fonteh, A. N., Triggiani, M., and Liu, M. C. (1996). Antigen-induced generation of lyso-phospholipids in human airways. *J. Exp. Med.* **183**, 2235–2245.

Cuvillier, O., Pirianov, G., Kleuser, B., Vanek, P. G., Coso, O. A., Gutkind, J. S., and Spiegel, S. (1996). Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. *Nature* **381**, 800–803.

Cuvillier, O., Rosenthal, D. S., Smulson, M. E., and Spiegel, S. (1998). Sphingosine 1-phosphate inhibits activation of caspases that cleave poly (ADP-ribose) polymerase and lamins during Fas- and ceramide-mediated apoptosis in Jurkat T lymphocytes. *J. Biol. Chem.* **273**, 2910–2916.

Dixon, R. J., Young, K., and Brunskill, N. J. (1999). Lysophosphatidic acid-induced calcium mobilization and proliferation in kidney proximal tubular cells. *Am. Phys. Soc.* **276**, F191–F198.

Dubin, A. E., Bahnson, T., Weiner, J. A., Fukushima, N., and Chun, J. (1999). Lysophosphatidic acid stimulates neurotransmitter-like conductance changes that precede GABA and L-glutamate in early, presumptive cortical neuroblasts. *J. Neurosci.* **19**, 1371–1381.

Durante, W., Liao, L., Peyton, K. J., and Schafer, A. I. (1997). Lysophosphatidylcholine regulates cationic amino acid transport and metabolism in vascular smooth muscle cells. *J. Biol. Chem.* **272**, 30154–30159.

Eberhardt, C., Gray, P. W., and Tjoelker, L. W. (1997). Human lysophosphatidic acid acyltransferase. *J. Biol. Chem.* **272**, 20299–20305.

Eichholtz, T., Jalink, K., Fahrenfort, I., and Moolenaar, W. H. (1993). The bioactive phospholipid lysophosphatidic acid is released from activated platelets. *Biochem. J.* **291**, 677–680.

Fischer, D. J., Liliom, K., Guo, Z., Nusser, N., Virag, T., Murakami-Murofushi, K., Kobayashi, S., Erickson, J. R., Sun, G., Miller, D. D., and Tigyi, G. (1998). Naturally occurring analogs of lysophosphatidic acid elicit different cellular responses through selective activation of multiple receptor subtypes. *Mol. Pharmacol.* **54**, 979–988.

Fourcade, O., Simon, M. F., Viode, C., Rugani, N., Leballe, F., Ragab, A., Fournie, B., Sarda, L., and Chap, H. (1995). Secretory phospholipase A2 generates the novel lipid mediator lysophosphatidic acid in membrane microvesicles shed from activated cells. *Cell* **80**, 919–927.

Fromm, C., Coso, O. A., Montaner, S., Xu, N., and Gutkind, J. S. (1997). The small GTP-binding protein rho links G protein-coupled receptors and G alpha12 to the serum response element and to cellular transformation. *Proc. Natl Acad. Sci. USA* **94**, 10098–10103.

Gabr, Y. (1956). Observations on a substance in human plasma which gives a slow contraction of ileum *in vitro*. *Br. J. Pharmacol.* **11**, 93–98.

Gaits, F., Fourcade, O., Balle, F. L., Gueguen, G., Gaige, B., Gassama-Diagne, A., Fauvel, J., Salles, J.-P., Mauco, G., Simon, M.-F., and Chap, H. (1997). Lysophosphatidic acid as a phospholipid mediator: pathways of synthesis. *FEBS Lett.* **410**, 54–58.

Gerrard, J. M., and Robinson, P. (1984). Identification of the molecular species of lysophosphatidic acid produced when platelets are stimulated by thrombin. *Biochem. Biophys. Acta* **705**, 487–492.

Goetzl, E. J., and An, S. (1998). Diversity of cellular receptors and functions for the lysophospholipid growth factors lysophosphatidic acid and sphingosine 1-phosphate. *FASEB J.* **12**, 1589–1598.

Goetzl, E. J., and Lynch, K. (ed) (2000). Lysophospholipids and eicosanoids in cancer and in cardiovascular and neurodegenerative diseases. *Ann. NY Acad. Sci.* Conference, New York.

Goetzl, E. J., Kong, Y., and Mei, B. (1999a). Lysophosphatidic acid and sphingosine 1-phosphate protection of T cells from apoptosis in association with suppression of Bax. *J. Immunol.* **162**, 2049–2056.

Goetzl, E. J., Kong, Y., and Kenney, J. S. (1999b). Lysophospholipid enhancement of human T cell sensitivity to diphtheria toxin by increased expression of heparin-binding epidermal growth factor. *Proc. Assoc. Am. Phys.* **111**, 259–269.

Goetzl, E. J., Lee, H., Azumi, T., Stossel, T. P., and Karliner, J. S. (1999c). Gelsolin (GS) binding of lysophosphatidic acid (LPA) and enhancement of presentation of LPA to rat cardiac myocytes (RCMs). *FASEB J.* **13**, A1423.

Goetzl, E. J., Dolezalova, H., Kong, Y., and Zeng, L. (1999d). Dual mechanisms for lysophospholipid induction of proliferation of human breast carcinoma cells. *Cancer Res.* **59**, 4732–4737.

Goetzl, E. J., Dolezalova, H., Kong, Y., Hu, Y.-L., Jaffe, R. B., Kalli, K. R., and Conover, C. A. (1999e). Distinctive expression and functions of the type 4 endothelial differentiation gene-encoded G protein-coupled receptor for lysophosphatidic acid in ovarian cancer. *Cancer Res.* **59**, 5370–5375.

Gomez-Munoz, A., Duffy, P. A., Martin, A., O'Brien, L., Byun, H.-S., Bittman, R., and Brindley, D. (1995). Short-chain ceramide-1-phosphates are novel stimulators of DNA synthesis and cell division: antagonism by cell-permeable ceramides. *Mol. Pharmacol.* **47**, 883–889.

Guo, Z., Liliom, K., Fischer, D. J., Bathurst, I. C., Tomei, L. D., Kiefer, M. C., and Tigyi, G. (1996). Molecular cloning of a high-affinity receptor for the growth factor-like lipid mediator lysophosphatidic acid from Xenopus oocytes. *Proc. Natl Acad. Sci. USA* **93**, 14367–14372.

Hannun, Y. A. (1994). The sphingomyelin cycle and the second messenger function of ceramide. *J. Biol. Chem.* **269**, 3125–3128.

Hecht, J. H., Weiner, J. A., Post, S. R., and Chun, J. (1996). Ventricular zone gene-1 (vzg-1) encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex. *J. Cell. Biol.* **135**, 1071–1083.

Heringdorf, D. M. Z., Koppen, C. J. V., and Jakobs, K. H. (1997). Molecular diversity of sphingolipid signalling. *FEBS Lett.* **410**, 34–38.

Herrlich, A., Daub, H., Knebel, A., Herrlich, P., Ullrich, A., Schultz, G., and Gudermann, T. (1998). Ligand-independent activation of platelet-derived growth factor receptor is a necessary intermediate in lysophosphatidic, acid-stimulated mitogenic activity in L cells. *Proc. Natl Acad. Sci. USA* **95**, 8985–8990.

Higgs, H. N., Han, M. H., Johnson, G. E., and Glomset, J. A. (1998). Cloning of a phosphatidic acid-preferring phospholipase A1 from bovine testis. *J. Biol. Chem.* **273**, 5468–5477.

Hill, C. S., and Treisman, R. (1995). Differential activation of c-fos promoter elements by serum, lysophosphatidic acid, G proteins and polypeptide growth factors. *EMBO J.* **14**, 5037–5047.

Hill, C. S., Wynne, J., and Treisman, R. (1995). The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. *Cell* **81**, 1159–1170.

Himmel, H. M., Heringdorf, D. M. S., Windorfer, B., van Koppen, C. J., Ravens, U., and Jakobs, K. H. (1998). Guanine nucleotide-sensitive inhibition of L-type Ca^{2+} current by lysophingolipids in RINm5F insulinoma cells. *Mol. Pharmacol.* **53**, 862–869.

Hisano, N., Yatomi, Y., Satoh, K., Akimoto, S., Mitsumata, M., Fujino, M. A., and Ozaki, Y. (1999). Induction and suppression of endothelial cell apoptosis by sphingolipids: a possible *in vitro* model for cell–cell interactions between platelets and endothelial cells. *Blood* **93**, 4293–4299.

Holtsberg, F. W., Steiner, M. R., Keller, J. N., Mark, R. J., Mattson, M. P., and Steiner, S. M. (1998). Lysophosphatidic acid induces necrosis and apoptosis in hippocampal neurons. *J. Neurochem.* **70**, 66–76.

Hu, Y. L., Goetzl, E. J., Mills, G. B., Ferrara, N., and Jaffe, R. B. (2000). Induction of VEGF expression by LPA in normal and neoplastic human ovarian epithelial cells (in press).

Imamura, F., Horai, T., Mukai, M., Shinkai, K., Sawada, M., and Akedo, H. (1993). Induction of *in vitro* tumor cell invasion of cellular monolayers by lysophosphatidic acid or phospholipase D. *Biochem. Biophys. Res. Commun.* **193**, 497–503.

Inoue, C. N., Ko, Y. H., Guggino, W. B., Forster, H. G., and Epstein, M. (1997). Lysophosphatidic acid and platelet-derived growth factor synergistically stimulate growth of cultured rat mesangial cells. *Proc. Soc. Exp. Biol. Med.* **216**, 370–379.

Jalink, K., van Corvem, E. J., Hengeveld, T., Morii, N., Narumiya, S., and Moolenaar, W. H. (1994). Inhibition of lysophosphatidate- and thrombin-induced retraction and neuronal cell rounding by ADP-ribosylation of the small GTP-binding protein Rho. *J. Cell. Biol.* **126**, 801–810.

Kai, M., Wada, I., Imai, S.-I., Sakane, F., and Kanoh, H. (1997). Cloning and characterization of two human isozymes

of Mg^{2+}-independent phosphatidic acid phosphatase. *J. Biol. Chem.* **272**, 24572–24578.

Kawa, S., Kimura, S., Hakomori, S.-I., and Igarashi, Y. (1997). Inhibition of chemotactic motility and trans-endothelial migration of human neutrophils by sphingosine 1-phosphate. *FEBS Lett.* **420**, 196–200.

Kawasawa, Y., Kume, K., and Shimizu, T. (1998). Cloning and characterization of a mouse homolog of *Xenopus* PSP24 LPA receptor. *FASEB J.* **12**, 1460.

Kobayashi, T., Tanaka-Ishii, R., Taguchi, R., Ikezawa, H., and Murakami-Murofushi, K. (1999). Existence of a bioactive phospholipid, cyclic phosphatidic acid, in human serum. *Life Sci.* **56**, 245–253.

Koh, J. S., Lieberthal, W., Heydrick, S., and Levine, J. S. (1998). Lysophosphatidic acid is a major serum noncytokine survival factor for murine macrophages which acts via the phosphatidylinositol 3-kinase signaling pathway. *J. Clin. Invest.* **102**, 716–727.

Kohama, T., Olivera, A., Edsall, L., Nagiec, M. M., Dickson, R., and Spiegel, S. (1998). Molecular cloning and functional characterization of murine sphingosine kinase. *J. Biol. Chem.* **273**, 23722–23728.

Kolodney, M. S., and Elson, E. L. (1993). Correlation of myosin light chain phosphorylation with isometric contraction of fibroblasts. *J. Biol. Chem.* **268**, 23850–23855.

Kozma, R., Sarner, S., Ahmed, S., and Lim, L. (1997). Rho family GTPases and neuronal growth cone remodeling: relationship between increased complexity induced by Cdc42Hs, Rac1 and acetylcholine and collapse induced by RhoA and lysophosphatidic acid. *Mol. Cell. Biol.* **17**, 1201–1211.

Kranenburg, O., Poland, M., Horck, F. P. G., Drechsel, D., Hall, A., and Moolevaar, W. H. (1999). Activation of rhoA by lysophosphatidic acid and Ga12/13 subunits in neuronal cells: induction of neurite retraction. *Mol. Biol. Cell* **10**, 1851–1857.

Lapetina, E. G., Billah, M. M., and Cautrecasas, P. (1981a). The phosphatidylinositol cycle and the regulation of arachidonic acid production. *Nature* **292**, 367–369.

Lapetina, E. G., Billah, M. M., and Cautrecases, P. (1981b). Lysophosphatidic acid potentiates thrombin-induced production of arachidonate metabolites in platelets. *J. Biol. Chem.* **256**, 11984–11987.

Lee, H., Goetzl, E. J., and An, S. (1999a). Lysophosphatidic acid and sphingosine 1-phosphate stimulate endothelial cell wound healing. *Am. J. Physiol. (Cell Physiol.)* **238**, C612–C618.

Lee, M.-J., Thangada, S., Claffey, K. P., Ancellin, N., Liu, C. H., Kluk, M., Volpi, M., Sha'afi, R. I., and Hla, T. (1999b). Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. *Cell* **99**, 301–312.

Levine, J. S., Koh, J. S., Triaca, V., and Lieberthal, W. (1997). Lysophosphatidic acid: a novel growth and survival factor for renal proximal tubular cells. *Am. Physiol. Soc.* **273**, F575–F585.

Liliom, K., Bittman, R., Swords, B., and Tigyi, G. (1996). N-Palmitoyl-serine and N-palmitoyl-tyrosine phosphoric acids are selective competitive antagonists of the lysophosphatidic acid receptors. *Mol. Pharmacol.* **50**, 616–623.

Liliom, K., Guan, Z., Tseng, J. L., Desiderio, D. M., Tigyi, G., and Watsky, M. (1998). Growth factor-like phospholipids generated following corneal injury. *Am. J. Physiol. (Cell Physiol.)* **274**, C1065–C1073.

Lynch, K. R., Hopper, D. W., Carlisle, S. J., Catalano, J. G., Zhang, M., and Macdonald, T. L. (1997). Structure/activity relationships in lysophosphatidic acid: the 2-hydroxyl moiety. *Mol. Pharmacol.* **52**, 75–81.

MacDonell, K. L., Severson, D. L., and Giles, W. R. (1998). Depression of excitability by sphingosine 1-phosphate in rat ventricular myocytes. *Am. J. Physiol.* **275**, H2291–H2299.

Mandala, S. M., Thornton, R., Tu, Z., Kurtz, M. B., Nickels, J., Broach, J., Menzeleev, R., and Spiegel, S. (1998). Sphingoid base 1-phosphate phosphatase: a key regulator of sphingolipid metabolism and stress response. *Proc. Natl Acad. Sci. USA* **95**, 150–155.

Mathes, C., Fleig, A., and Penner, R. (1998). Calcium release-activated calcium current (ICRAC) is a direct target for sphingosine. *J. Biol. Chem.* **273**, 25020–25030.

Melendez, A., Floto, R. A., Gillooly, D. J., Harnett, M. M., and Allen, J. M. (1998). FcgRI coupling to phospholipase D initiates sphingosine kinase-mediated calcium mobilization and vesicular trafficking. *J. Biol. Chem.* **273**, 9393–9402.

Moolenaar, W. H. (1995). Lysophosphatidic acid signalling. *Cell Biol.* **7**, 203–210.

Moolenaar, W. H., Kranenburg, O., Postma, F. R., and Zondag, G. (1997). Lysophosphatidic acid: G protein signaling and cellular responses. *Curr. Opin. Cell Biol.* **9**, 168–173.

Oral, H. II, Dorn, G. W. II, and Mann, D. L. (1997). Sphingosine mediates the immediate negative inotropic effects of tumor necrosis factor-α in the adult mammalian cardiac myocyte. *J. Biol. Chem.* **272**, 4836–4842.

Panetti, T. S., Peyruchaud, O., and Mosher, D. F. (2000). Lysophosphatidic acid and sphingosine-1-phosphate stimulate endothelial cell migration: involvement of rho, ras and PI3-kinase. *Atheroscler. Thromb. Vasc. Biol.* (in press).

Pebay, A., Torrens, Y., Toutant, M., Cordier, J., Glowinski, J., and Tence, M. (1999). Pleiotropic effects of lysophosphatidic acid on striatal astrocytes. *GLIA* **28**, 1–9.

Piazza, G. A., Ritter, J. L., and Baracka, C. A. (1995). Lysophosphatidic acid induction of transforming growth factors a and b: modulation of proliferation and differentiation in cultured human keratinocytes and mouse skin. *Exp. Cell Res.* **216**, 51–64.

Postma, F. R., Jalink, K., Hengeveld, T., and Moolenaar, W. H. (1996). Sphingosine-1-phosphate rapidly induces rho-dependent neurite retraction: action through a specific cell surface receptor. *EMBO J.* **15**, 2388–2395.

Pyne, S., Rakhit, S., Conway, A.-M., McKie, A., Darroch, P., Tate, R., and Pyne, N. (1999). Extracellular actions of sphingosine 1-phosphate through endothelial differentiation gene products in mammalian cells: role in regulating proliferation and apoptosis. *Biochem. Soc. Trans.* **27**, 12–17.

Rani, C. S. S., Wang, F., Fuior, E., Berger, A., Wu, J., Sturgill, T. W., Beitner-Johnson, D., LeRoith, D., Varticovski, L., and Spiegel, S. (1997). Divergence in signal transduction pathways of platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptors: involvement of sphingosine 1-phosphate in PDGF but not EGF signaling. *J. Biol. Chem.* **272**, 10777–10783.

Ridley, A. J., and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70**, 389–399.

Rizza, C., Leitinger, N., Yue, J., Fischer, D. J., Wang, D.-A., Shih, P. T., Lee, H., Tigyi, G., and Berliner, J. A. (1999). Lysophosphatidic acid as a regulator of endothelial/leukocyte interaction. *Lab. Invest.* **79**, 1227–1235.

Roberts, R., Sciorra, V. A., and Morris, A. J. (1998). Human type 2 phosphatidic acid phosphohydrolases. *J. Biol. Chem.* **273**, 22059–22067.

Rodriguez-Fernandez, J. L., and Rozengurt, E. (1998). Bombesin, vasopressin, lysophosphatidic acid, and sphingosylphosphorylcholine induce focal adhesion kinase activation in intact swiss 3T3 cells. *J. Biol. Chem.* **273**, 19321–19328.

Sakai, T., Peyruchaud, O., Fassler, R., and Mosher, D. F. (1998). Restoration of b1A integrins is required for lysophosphatidic acid-induced migration of b1-null mouse fibroblastic cells. *J. Biol. Chem.* **273**, 19378–19382.

Sakano, S., Takemura, H., Yamada, K., Imoto, K., Kaneko, M., and Ohshika, H. (1996). Ca2+ mobilizing action of sphingosine in Jurkat human leukemia T cells. *J. Biol. Chem.* **271**, 11148–11155.

Sato, K., Tomura, H., Igarashi, Y., Ui, M., and Okajima, F. (1999). Possible involvement of cell surface receptors in sphingosine 1-phosphate-induced activation of extracellular signal-regulated kinase in C6 glioma cells. *Mol. Pharmacol.* **55**, 126–133.

Schissel, S. L., Keesler, G. A., Schuchman, E. H., Williams, K. J., and Tabas, I. (1998). The cellular trafficking and zinc dependence of secretory and lysosomal sphingomyelinase, two products of the acid sphingomyelinase gene. *J. Biol. Chem.* **273**, 18250–18259.

Schmidt, A., Wolde, M., Thiele, C., Fest, W., Kratzin, H., Podtelejnikov, A. V., Witke, W, Huttner, W. B., and Soling, H. -D. (1999). Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid. *Nature* **401**, 133–141.

Schumacher, K. A., Classen, H. G., and Spath, M. (1979). Vasoactive lysophospholipids. *Thromb. Haemost.* **42**, 631–640.

Sebok, A., Nusser, N., Debreceni, B., Guo, Z., Santos, M. F., Szeberenyi, J., and Tigyi, G. (1999). Different roles for RhoA during neurite initiation, elongation, and regeneration in PC12 cells. *J. Neurochem.* **73**, 949–960.

Seewald, S., Sachinidis, A., Dusing, R., Ko, Y., Seul, C., Epping, P., and Vetter, H. (1997). Lysophosphatidic acid and intracellular signalling in vascular smooth muscle cells. *Atherosclerosis* **130**, 121–131.

Seufferlein, T., Withers, D. J., Mann, D., and Rozengurt, E. (1996). Dissociation of mitogen-activated protein kinase activation from p125 focal adhesion kinase tyrosine phosphorylation in Swiss 3T3 cells stimulated by bombesin, lysophosphatidic acid, and platelet-derived growth factor. *Mol. Biol. Cell* **7**, 1865–1875.

Shahrestanifar, M., Fan, X., and Manning, D. R. (1999). Lysophosphatidic acid activates NF-kB in fibroblasts. *J. Biol. Chem.* **274**, 3828–3833.

Siddiqui, R., and English, D. (1996). Phosphatidic acid binding to human neutrophils: effects on tyrosine kinase regulated intracellular Ca2+ mobilization. *Cell Signal.* **8**, 349–354.

Siddiqui, R., and English, D. (1997). Phosphatidic acid elicits calcium mobilization and actin polymerization through a tyrosine kinase-dependent process in human neutrophils: a mechanism for induction of chemotaxis. *Biochim. Biophys. Acta* **139**, 81–95.

Siess, W., Zangl, K. J., Essler, M., Bauer, M., Brandl, R., Corrinth, C., Bittman, R., Tigyi, G., and Aepfelbacher, M. (1999). Lysophosphatidic acid mediates the rapid activation of platelets and endothelial cells by mildly oxidized low density lipoprotein and accumulates in human atherosclerotic lesions. *Proc. Natl Acad. Sci. USA* **96**, 6931–6936.

Spiegel, S., and Merrill, J. A. H. (1996). Sphingolipid metabolism and cell growth regulation. *FASEB J.* **10**, 1388–1398.

Spiegel, S., and Milstien, S. (1995). Sphingolipid metabolites: members of a new class of lipid second messengers. *J. Membr. Biol.* **146**, 225–237.

Thumser, A. E., Voysey, J. E., and Wilton, D. C. (1994). The binding of lysophospholipids to rat liver fatty acid-binding protein and albumin. *Biochem. J.* **301**, 801–806.

Tigyi, G., and Miledi, R. (1992). Lysophosphatidates bound to serum albumin activate membrane currents in Xenopus oocytes and neurite retraction in PC12 pheochromocytoma cells. *J. Biol. Chem.* **267**, 21360–21367.

Tigyi, G., Dyer, D. L., and Miledi, R. (1994). Lysophosphatidic acid possesses dual action in cell proliferation. *Proc. Natl Acad. Sci. USA* **91**, 1908–1912.

Tigyi, G., Hong, L., Yakubu, M., Parfenova, H., Shibata, M., and Leffler, C. W. (1995). Lysophosphatidic acid alters cerebrovascular reactivity in piglets. *Am. J. Physiol.* **268**, H2048–H2055.

Tigyi, G., Fischer, D., Yang, C., Dyer, D., Sebok, A., and Mildei, R. (1996a). Lysophosphatidic acid-induced neurite retraction in PC12 cells: control by phosphoinositide-Ca^{2+} signaling and Rho. *J. Neurochem.* **66**, 537–548.

Tigyi, G., Fischer, D., Dyer, D., Sebok, A., and Miledi, R. (1996b). Lysophosphatidic acid-induced neurite retraction in PC12 cells: neurite protective effects of cyclic AMP signaling. *J. Neurochem.* **66**, 549–558.

Tigyi, G. J., Liliom, K., Fischer, D. J., and Guo, Z. (1999). "Phospholipid Growth Factors: Identification and Mechanism of Action." CRC Press, Washington, DC.

Titievsky, A., Titievskaya, I., Pasternack, M., Kaila, K., and Tornquist, K. (1998). Sphingosine inhibits voltage-operated calcium channels in GH4C1 cells. *J. Biol. Chem.* **273**, 242–247.

Tokumura, A. (1995). A family of phospholipid autocoids: occurrence, metabolism and bioactions. *Prog. Lipid Res.* **34**, 151–184.

Tokumura, A., Fukuzawa, K., and Tsukatami, H. (1978). Lysophosphatidic acids with smooth muscle activities. *Lipids* **13**, 468–472.

Tokumura, A., Iimori, M., Nishioka, Y., Kitahara, M., Sakashita, M., and Tanaka, S. (1994). Lysophosphatidic acids induce proliferation of cultured vascular smooth muscle cells from rat aorta. *Am. Physiol. Soc.* **267**, C204–C210.

Tokumura, A., Nishioka, Y., Yoshimoto, O., Shinomiya, J., and Fukuzawa, K. (1999). Substrate specificity of lysophospholipase D which produces bioactive lysophosphatidic acids in rat plasma. *Biochim. Biophys. Acta* **1437**, 235–245.

Tomiuk, S., Hofmann, K., Nix, M., Zumbansen, M., and Stoffel, W. (1998). Cloned mammalian neutral sphingomyelinase: functions in sphingolipid signaling? *Proc. Natl Acad. Sci. USA* **95**, 3638–3643.

Van Corven, E. J., Groenink, A., Jalink, K., Eichholtz, T., and Moolenaar, W. H. (1989). Lysophosphatidate-induced cell proliferation: identification and dissection of signaling pathways mediated by G proteins. *Cell* **59**, 45–54.

Van Corven, E. J., Hordijk, P. L., Medema, R. H., Bos, J. L., and Moolenaar, W. H. (1993). Pertussis toxin-sensitive activation of p21ras by G protein-coupled receptor agonists in fibroblasts. *Proc. Natl Acad. Sci. USA* **90**, 1257–1261.

Vogt, W. (1949). Sum einfluss biogener Stoffe auf die Darmmotilitat. *Arch. Exp. Path. Pharmak.* **206**, 1–11.

Vogt, W. (1957). The chemical nature of Darmstoff. *J. Physiol.* **137**, 154–167.

Waggoner, D. W., Gomez-Munoz, A., Dewald, J., and Brindley, D. N. (1996). Phosphatidate phosphohydrolase catalyzes the hydrolysis of ceramide 1-phosphate, lysophosphatidate, and sphingosine 1-phosphate. *J. Biol. Chem.* **271**, 16506–16509.

Wang, A., Loo, R., Chen, Z., and Dennis, E. A. (1997). Regiospecificity and catalytic triad of lysophospholipase I. *J. Biol. Chem.* **272**, 22030–22036.

Watsky, M. A. (1995). Lysophosphatidic acid, serum and hyperosmolarity activate Cl^- currents in corneal keratocytes. *Am. J. Physiol.* **269**, C1385–1393.

Wu, J., Spiegel, S., and Sturgill, T. W. (1995). Sphingosine 1-phosphate rapidly activates the mitogen-activated protein kinase pathway by a G protein-dependent mechanism. *J. Biol. Chem.* **270**, 11484–11488.

Xia, P., Gamble, J. R., Rye, K.-A., Wang, L., Hii, C. S. T., Cockerill, P., Khew-Goodall, Y., Bert, A. G., Barter, P. J., and Vadas, M. A. (1998). Tumor necrosis factor-a induces adhesion molecule expression through the sphingosine kinase pathway. *Proc. Natl Acad. Sci. USA* **95**, 14196–14201.

Xu, Y., Shen, Z., Wiper, D. W., Wu, M., Morton, R. E., Elson, P., Kenedy, A. W., Belinson, J., Markman, M., and Casey, G. (1998). Lysophosphatidic acid as a potential biomarker for ovarian and other gynecologic cancers. *JAMA* **280**, 719–723.

Yakubu, M. A., Liliom, K., Tigyi, G. J., and Leffler, C. W. (1997). Role of lysophosphatidic acid in endothelin-1- and hematoma-induced alteration of cerebral microcirculation. *Am. J. Physiol. (Regul. Integr. Comp. Physiol.)* **273**, R703–709.

Yatomi, Y., Ruan, F., Hakomori, S. I., and Igarashi, Y. (1995). Sphingosine-1-phosphate: a platelet-activating sphingolipid released from agonist-stimulated human platelets. *Blood* **86**, 193–202.

Yatomi, Y., Yamamura, S., Ruan, F., and Igarashi, Y. (1997). Sphingosine 1-phosphate induces platelet activation through an extracellular action and shares a platelet surface receptor with lysophosphatidic acid. *J. Biol. Chem.* **272**, 5291–5297.

Zhang, Q., Peyruchaud, O., French, K. J., Magnusson, M. K., and Mosher, D. F. (1999). Sphingosine 1-phosphate stimulates fibronectin matrix assembly through a rho-dependent signal pathway. *Blood* **93**, 2984–2990.

Zondag, G. C. M., Postma, F. R., Etten, I. V., Verlaan, I., and Moolenaar, W. H. (1998). Sphingosine 1-phosphate signalling through the G-protein-coupled receptor edg-1. *Biochem. J.* **330**, 605–609.

ACKNOWLEDGEMENTS

We are grateful to Dr Songzhu An for critical reading of this chapter and to Bethann Easterly for textual production.

ELSEVIER

Prostaglandins & other Lipid Mediators 64 (2001) 11–20

PROSTAGLANDINS & other LIPID MEDIATORS

Pleiotypic mechanisms of cellular responses to biologically active lysophospholipids

Edward J. Goetzl*

Departments of Medicine and Microbiology-Immunology, University of California Medical Center, San Francisco, CA 94143, USA

Abstract

The activities of cell-derived lysophospholipid (LPL) growth factors on cellular proliferation and a range of proliferation-independent functions are regulated at multiple levels. This section focuses first on the capacity of the actin-severing protein gelsolin to bind lysophosphatidic acid (LPA), but not sphingosine 1-phosphate (S1P), and either sequester LPA or present it to responsive cells. Expression of members of the family of endothelial differentiation gene-encoded G protein-coupled receptors (Edg Rs) for LPLs is controlled developmentally and by cell-activating stimuli. Edg R transduction of cellular effects of LPLs involves both direct actions on target cells and induction of generation of proteins with relevant actions capable of amplifying or diminishing primary direct effects of LPLs. These general mechanisms are evident in Edg R mediation of proliferation, cytokine secretion and suppression of apoptosis. The availability of functionally-active anti-Edg R antibodies and Edg R-specific pharmacological probes, establishment of Edg R transgenes and gene knockouts, and identification of natural genetic anomalies of LPL metabolism and recognition by Edg Rs will permit elucidation of the *in vivo* activities of LPA and S1P normally and in disease states.

Keywords: Growth factors; Cytokines; Gelsolin; Apoptosis; Receptors

1. Introduction

There have been astounding advances in our understanding of the biological roles of two major subfamilies of lysophospholipid (LPL) mediators in the past four years. Biologically active LPLs are generated by many types of mammalian cells and they have protean

* Tel.: +1-415-476-5339; fax: +1-415-476-6915.
E-mail address: egoetzl@itsa.ucsf.edu (E.J. Goetzl).

0090-6980/01/$ – see front matter
PII: S0090-6980(01)00104-6

capacities to affect growth and functions of diverse cells in multiple organ systems [1–5]. Lysophosphatidic acid (LPA) is the most prominent member of the lysoglycerol-containing phospholipid subfamily, which predominates quantitatively among lipid structural components of cellular membranes. Sphingosine-1-phosphate (S1P) is a highly-active lysosphingophospholipid, which is structurally and functionally related to LPA. The subfamily of cellular lysosphingophospholipids are quantitatively diminutive in contrast to the subfamily of lysoglycerophospholipids, but exhibit great structural complexity and have a range of biological effects similar to those of lysoglycerophospholipids. Alkenyl- and cyclic-variants of LPA, and sphingosylphosphorylcholine are other naturally-occurring LPLs which exhibit many cellular effects. The LPLs of both subfamilies are related also by being products of metabolism of cellular membrane phospholipids, increasing in concentration transiently in relation to cellular responses, requiring carrier proteins for cellular presentation, moving and interacting with proteins in cellular membranes, and potently influencing cells through one or more subfamilies of G protein-coupled receptors (GPCRs) [6–10]. Our knowledge of every aspect of the cellular generation, recognition and effects of LPLs has progressed recently through discoveries of distinctive biosynthetic and metabolic pathways and the definition of structures and signaling pathways of the many members of a novel subfamily of endothelial differentiation gene (edg)-encoded GPCRs (Edg Rs) dedicated to LPLs.

2. Diversity of lysophospholipid cellular sources and binding proteins

The nature and amounts of LPLs produced by multi-enzymatic pathways are determined by the origin, basic type, proliferative state, and level of functional activation of the cells [1–5]. Platelets, macrophages, other leukocytes, some epithelial cells and some tumor cells are major sources of both LPA and S1P. Although fibroblasts responding to protein growth factors produce LPA and S1P, only small amounts appear to be released into extracellular fluids. LPA also is synthesized at interfaces between cellular plasma membranes and extracellular fluid both by secretory phospholipase A2 hydrolysis of PA exposed on microvesicles shed from activated platelets and leukocytes, and by phospholipase D cleavage of leukocyte-surface lysophosphatidylcholine. LPL generation from non-malignant cells is initiated by physical perturbation or by stimulation of plasma membrane receptors or other surface proteins, which activate critical controlling enzymes such as phospholipases and sphingosine kinase. In contrast, tumor cells may secrete LPLs spontaneously, which was described first for LPA production by ovarian cancers. LPA and S1P were identified recently in mildly-oxidized low density lipoproteins of the lipid-rich core in atherosclerotic plaques, where they may contribute to proliferation of vascular smooth muscle cells, as well as platelet and endothelial activation. Characterization of cellular production of LPLs under physiological conditions is limited to only a few examples.

Platelet adhesion to vascular surfaces and homotypic aggregation induced by thrombin result in generation of both LPA and S1P in quantities sufficient to raise effective LPL concentrations up to 1 to 5 μM [11,12]. Alpha$_2$-adrenergic stimulation of adipocytes provokes release of LPA at concentrations capable of inducing proliferation, spreading and maturation of preadipocytes [13]. The elevated level of production of LPA by ovarian

carcinoma cells leads to concentrations in ascites of up to 20 μM and even up to 10-fold higher than normal in plasma of patients, in direct relationships with the stage of disease and tumor burden [14].

The critical functional role of fluid-phase protein-binding for solubility, sequestration and cellular delivery of LPLs has been demonstrated in many model systems [1–5]. Most secreted LPA and S1P are bound by serum albumin, which is a high-capacity and low-affinity (Kd = 360 nM for LPA) reservoir capable of delivering LPLs to all known target cells. Two quite independent investigations recently documented the capacity of LPA to interact functionally with plasma gelsolin [15,16]. The results of one showed that LPA suppresses the actin-severing activity of gelsolin in a manner similar to L-alpha-phosphatidylinositol-4,5-diphosphate (PIP2) by apparently binding to the same two sites [15] and the other that high-affinity binding of LPA by normal plasma concentrations of gelsolin reduced its cellular effects, whereas much lower concentrations of gelsolin led to more efficient cellular delivery of LPA than serum albumin [16]. Equilibrium binding of ^{3}H-LPA by human purified plasma gelsolin and human recombinant gelsolin were characterized by a valence of up to 1.8 and respective mean Kd values of 6.2 nM and 32 nM, as contrasted with 357 nM for fatty acid-free bovine serum albumin.

Characteristics of the binding curves and gelsolin concentration-dependence of effects on cellular activities of LPA suggested binding site cooperativity, such that gelsolin levels of or lower than 10% of that in normal plasma delivered LPA to cells very effectively, whereas higher concentrations reduced LPA activity relative to that seen with serum albumin [16]. Initial studies of the functional consequences of binding of LPA by gelsolin were conducted in rat cardiac myocytes (RCMs), which were washed thoroughly prior to stimulation to remove any endogenous gelsolin. In the absence of protein, nuclear signaling in RCMs transfected with an SRE-luciferase reporter was observed luminometrically only with 10^{-6} M LPA, whereas there were significant reports at 10^{-7} M and 10^{-6} M LPA with 100 μg/ml of fatty acid-free BSA or 30 μg/ml gelsolin, and 10^{-9} M to 10^{-6} M LPA with 3 μg/ml gelsolin. Stimulation of RCM hypertrophy, as assessed by increased incorporation of ^{3}H-leucine into proteins, similarly was stimulated significantly more by 10^{-8} M to 10^{-6} M LPA in 3 μg/ml and 10 μg/ml of gelsolin than 100 μg/ml of fatty acid-free serum albumin or protein-free medium.

That gelsolin is the active protein was supported by finding that human recombinant gelsolin and human purified plasma gelsolin exhibited similar concentration-activity relationships for cellular signaling and functional effects [16]. The role of gelsolin was confirmed further by demonstrating inhibition by anti-gelsolin antibody of signaling of cardiac myocytes by LPA with gelsolin, but not with serum albumin. The lack of alteration of LPA-like cellular effects of anti-Edg-4 R antibody and of S1P by gelsolin showed that gelsolin acted directly on LPA, rather than on an element of Edg R mediation or cellular transduction. The possibility of LPA binding to PIP2 sites of gelsolin was validated by characteristics of competitive interactions between LPA and both PIP2 and gelsolin substituent peptides constituting the known PIP2 binding sites in relation to cellular responses, as well as fluid-phase binding [17].

Although our understanding of the role of gelsolin as a carrier and cellular presenter of LPA is still developing, it is possible to propose a tentative model (Fig. 1). For cells which

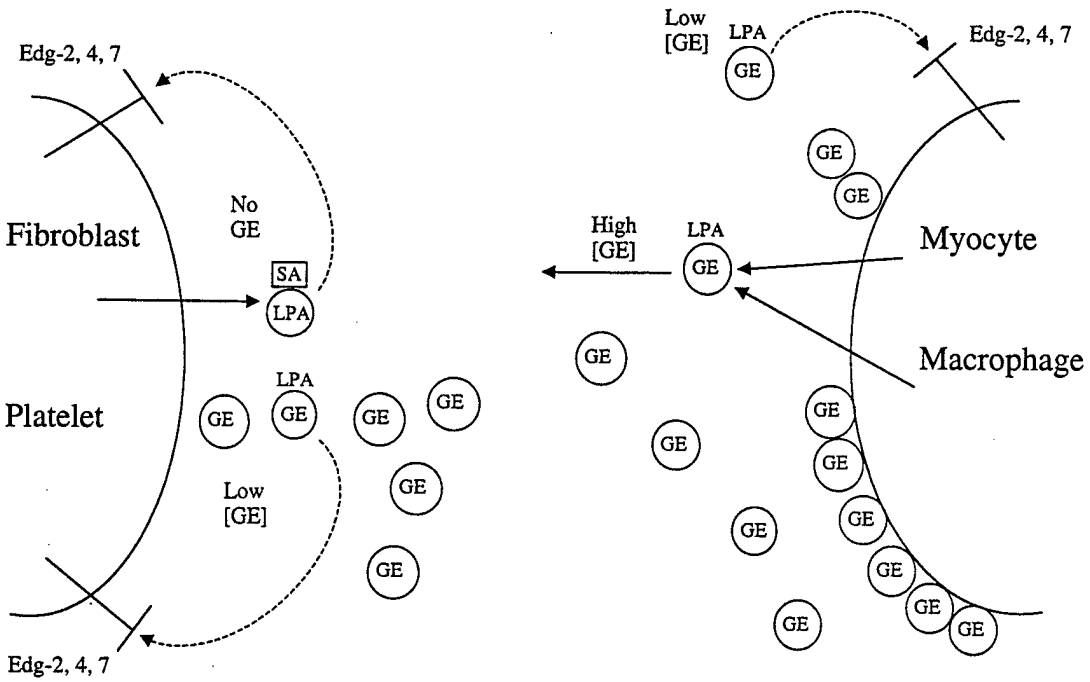

Fig. 1. Trapping, sequestration and cellular presentation of LPA by secreted gelsolin. SA = serum albumin, GE = gelsolin, T = Edg R for LPA; solid line = release or diffusion and dashed line = receptor stimulation.

produce gelsolin in functionally-relevant quantities, such as myocytes and macrophages, high local concentrations of gelsolin would minimize autocrine actions of LPA, as well as actions of LPA from other sources by sequestration and diffusional dissipation. At lower concentrations, gelsolin may present less avidly-bound LPA to Edg Rs. For cells which secrete little or no gelsolin and generate high or low concentrations of LPA, such as platelets and fibroblasts respectively, serum albumin may be the principal carrier and cellular presenter or this function may be shared with low levels of gelsolin from other cells. Other proteins capable of binding S1P and other bioactive lysosphingolipids may exist and could have functions analogous to gelsolin for LPA.

3. Cellular G protein-coupled receptors for lysophospholipids

As the structures of Edg Rs and other GPCRs for LPLs of both major families have been reviewed recently and will be discussed in several other sections of this volume, the present comments will focus on unique features of cell- and tissue-specific distribution of Edg Rs, and results of early analyses of developmental and activation-induced regulation of expression of Edg Rs. The initial realization that many different types of mammalian cells express two or more types of Edg Rs prompted searches for Edg R-null cells, which could be used for investigations of the biochemical and cellular properties of individual Edg Rs introduced by transfection. These surveys and related studies led to three observations of novel biolog-

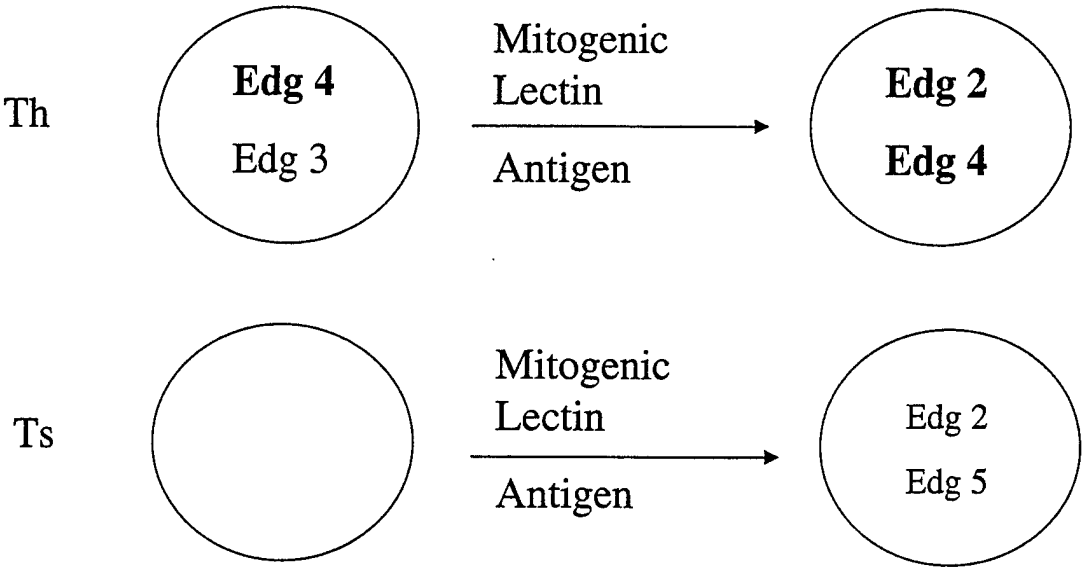

Fig. 2. Effects of activation of T helper lymphocyte on expression of Edg receptors. Th = helper T cell, Ts = suppressor T cell. The weight of characters depicting each Edg receptor signifies its relative frequency of representation.

ical properties of Edg Rs. The **first** was that differentiation and maturation of some types of hematopoietic and immune cells resulted in substantial modification of their complement of Edg Rs. Induction of differentiation of human promyelocytic leukemia cells of the HL-60 line by three distinct stimuli resulted in suppression of expression of functional Edg-3 Rs, as shown by a striking reduction in S1P-evoked increases in $[Ca^{++}]i$ [18]. Mouse early thymocytes express Edg-3 and -5 Rs, detected by reverse transcription-polymerase chain reaction (RT-PCR) semi-quantification of mRNA and by Western blots, which nearly disappear after exposure of the thymocytes to antigen and antigen-presenting cells or other stimuli of differentiation. Edg-4 and -6 become the predominant LPL receptors on mature T helper cells after the earliest subdivision into T helper/inducer and T suppressor/cytotoxic sets, with only minor residual representation of Edg-3 on T helper/inducer cells and no detectable Edg Rs on T suppressor/cytotoxic cells, as determined from analyses of human blood T cells (Fig. 2) [19,20]. Mitogen- or antigen-elicited activation of human blood-derived T helper cells results in loss of Edg-3 R, a slight decrease in Edg-4 and the appearance of a high level of Edg-2 R, according to results of RT-PCR analyses and Western blots [20]. These alterations in profiles of Edg Rs are accompanied by the expected changes in responses to LPA and S1P.

The **second** observation was that malignant transformation of some types of cells results in the appearance and often predominance of one or more Edg Rs not expressed by the equivalent non-malignant cell. Numerous human ovarian cancer cell lines express high levels of Edg-4 R not detectable in either primary cultures of human normal ovarian surface epithelial cells or immortalized lines of human normal ovarian surface epithelial cells [21]. In other tumors, malignant transformation results in quantitative changes in levels of

expression of more than one Edg R. For example, several lines of human breast cancer cells have higher levels of Edg-3 and -5, and a lower level of Edg-2 than human normal breast epithelial cells in primary cultures [22]. It remains to be determined if such differences contribute in any way to pathogenesis or even may serve as functional markers of some aspect of cancer biology.

The **third** was an apparent lack of variants of Edg Rs attributable to mutations or alternative splicing, compared to other families of GPCRs of similar size and complexity, such as those for some neuropeptides and for prostanoids. One alternative form of human Edg-4 R was discovered in ovarian cancer cells and found to result from loss of one G, which caused a frame-shift disrupting the termination codon and leading to carboxyl-terminal elongation by 33 amino acids [9]. As extracellular and cytoplasmic loops of Edg-4 are small, it is assumed that many transductional factors dock on the cytoplasmic carboxyl-terminal tail, as for other structurally similar GPCRs. Thus it was not surprising to discover that the longer Edg-4 R is a modest gain-of-function mutant.

4. Multiplicity of cellular functional responses to lysophospholipids: shared mechanisms

LPLs influence nearly every element of cellular function from proliferation to synthetic activities to distinctive effector functions to apoptosis. Two central concepts which have emerged from studies of cellular functional responses to LPLs are: 1) Edg R mediation of an effect of an LPL often involves direct and indirect mechanisms, and 2) Different Edg Rs specific for the same LPL may transduce distinct or even opposite effects (Fig. 3, left side). Edg R transduction of LPL stimulation of proliferation of breast and ovarian cancer cells involves first direct signaling through the SRE in promoters of many immediate-early growth-related genes, and then stimulation of production, secretion, and effects of several different endogenously-derived protein growth factors. These and related earlier observations in keratinocytes suggested the first distinctive biological aspect of Edg R mediation of growth (Fig. 3, left side). LPL-stimulated breast and ovarian cancer cells both report great increases in SRE-luciferase activity, which attains a plateau within 4–5 hr. LPL-stimulated breast and ovarian cancer cells then secrete higher levels of type 2 insulin-like growth factor (IGF-II), which reaches a peak after 36–48 hr [21,22]. With the same time-course, LPL-stimulated ovarian cancer cells, but not breast cancer cells, secrete much higher levels of vascular endothelial growth factor (VEGF) which mediates altered permeability as well as cell proliferation [23]. That this indirect later pathway dominates after 24 hr of LPL stimulation was demonstrated by achieving over 90% suppression of S1P-elicited breast cancer cell proliferation with a neutralizing antibody to the type 1 receptor for IGF-II (IGFR 1). Similar recruitment of endogenous protein growth factors has been demonstrated in human T lymphoblastoma cells, where both LPA and S1P elicit increases in membrane-associated heparin-binding epidermal growth factor-like growth factor through a ras-dependent mechanism [24].

Some lines of human ovarian cancer cells have high levels of Edg-2, as well as of the more distinctive Edg-4 type of LPA receptor. It had been shown earlier that LPA could suppress

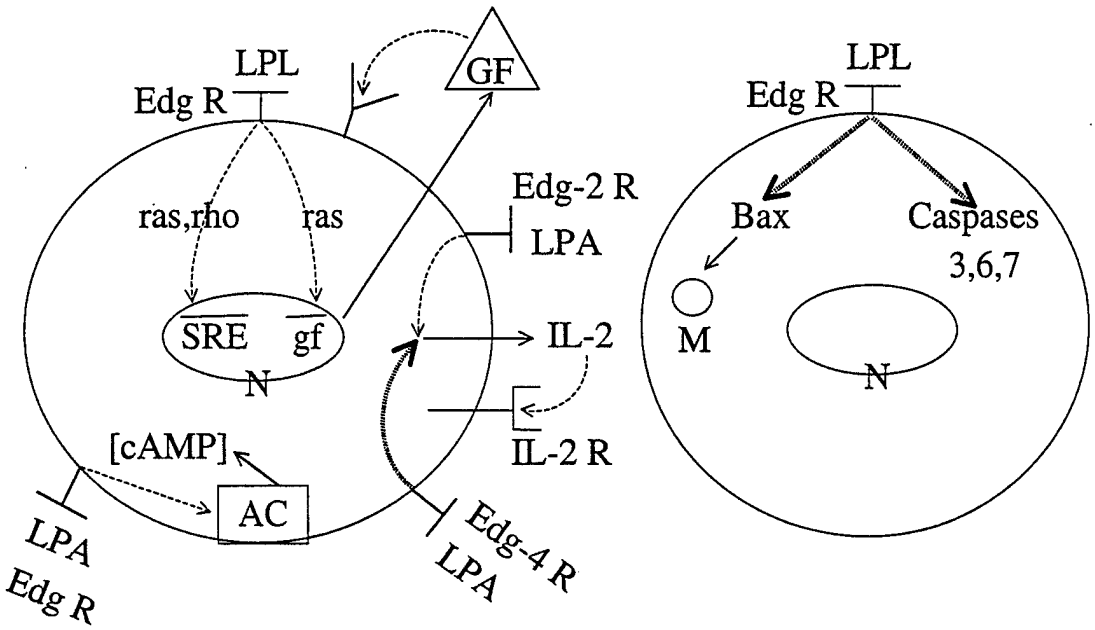

Fig. 3. Edg receptor transduction of effects of LPLs on proliferation and apoptosis. SRE = serum response element in the promoter of immediate-early growth-related response genes, gf = growth factor-encoding gene, GF = protein growth factor, IL-2 = interleukin-2, AC = adenylyl cyclase, [cAMP] = intracellular concentration of cyclic AMP, N = nucleus, M = mitochondria. Solid line = production or secretion, dashed line = stimulation, wavy or double dotted line = inhibition or suppression.

proliferation of some types of cells in association with increases in [cAMP]i [25]. On this background, the second distinctive biological aspect of Edg R signaling of growth was recognized from observations that the proliferative responses of high Edg-2 R-bearing ovarian cancer cells to LPA were uniformly less than those of low Edg-2 R-bearing lines [26]. That this differential responsiveness was attributable to transduction of a proliferation-inhibiting signal from LPA-occupied Edg-2 Rs was suggested by the stimulatory effect of anti-Edg-4 R monoclonal antibody on proliferation of ovarian cancer cells and by the suppressive effect of anti-Edg-2 R monoclonal antibody. Similar opposing activities of the two LPA Rs also was observed recently in human blood T helper cells stimulated to produce IL-2 by activation of their T cell receptor with adherent anti-CD3 plus anti-CD28 antibodies [20]. Freshly isolated T helper cells express predominantly Edg-4 R and either LPA or anti-Edg-4 R antibody decreases secretion of IL-2 [20]. In contrast, mitogen-activated T helper cells express more Edg-2 R than Edg-4 R and in them LPA or anti-Edg-2 R antibody increases secretion of IL-2 [27]. The biochemical and cellular mechanisms accounting for opposite effects of Edg-2 and Edg-4 Rs are not yet known.

The Edg Rs mediate at least two highly specific actions which suppress apoptosis initiated by diverse stimuli (Fig. 3, right side). The first is reduction in the cellular concentration of the pro-apoptotic protein Bax, which was quantified in a human T lymphoblastoma cell line by Western blots developed with two antibodies of different specificities [28]. Results of recent studies also have revealed Edg R transduction of decreases in apoptosis-associated

insertion of Bax into mitochondria of T lymphoblastoma cells. A second mechanism of inhibition of apoptosis identified in a line of leukemic human T cells is suppression of the activities of death caspases 3, 6 and 7, but not 8 [29]. As for the mechanisms of Edg R effects on cellular proliferation, both anti-apoptotic actions result from alteration in the levels or activities of critical control or effector proteins.

5. Specificity of cellular responses and adaptation to lysophospholipids

Edg R mediation of a particular response of any type of cell to an LPL is determined by many factors, of which the most important appear to be the natural range of functions of that cell, the density of expression of each type of Edg R, and the efficiency of coupling of cellular signaling pathways to each type of Edg R. For an LPL to stimulate chemotactic migration of a cell, for example, the target cell must be capable of migration, the density of the cognate Edg R must be sufficient to permit detection of a concentration gradient across the diameter of the cell, and the Edg R must transduce all signals which are integrated in the constitution of chemotaxis. These signals clearly require coupling of the engaged Edg Rs to multiple G proteins, involve changes in $[Ca^{++}]i$ and [cAMP]i, and would alter functions of the cell as diverse as adhesiveness, cytoskeletal structure, and secretion of proteases necessary for transmigration of basement membranes and other tissue structures. As efficiency of coupling of each Edg R to any G protein differs, this may be another example of an advantage of expression of several Edg Rs for the same LPL by one type of cell. Each transductional mechanism and component of the integrated response also is anticipated to be affected by signals from receptors for other co-elicited mediators on the responding cells. Thus specificity is determined by numerous factors external to the LPL-Edg R system, as well as those constituting the basic response mechanisms. Most of the basic and external determinants of specificity predicted to act in the LPL-Edg R system have not been elucidated thus far.

Many other aspects of Edg R mediation of cellular responses to LPLs also have not been approached experimentally. Essentially nothing is known of mechanisms of hypersensitization, accommodation and desensitization or of interactions with other growth factor and mediator systems. Ongoing studies have just begun to approach even the most straightforward elements of Edg R control of cellular responses, such as relationships between receptor density and response magnitude or cross-talk between different Edg Rs.

6. Future directions of research in lysophospholipid biology

Many of the remaining questions regarding Edg R mediation of LPL effects in complex tissues and organ systems may only be addressed definitively by genetic approaches, which modify levels of production of LPLs, LPL cellular transport systems and extracellular carrier proteins, and receptor expression and signaling. Several approaches have defined initial pharmacophores for each LPL mediator, but products of these efforts and several other potential synthetic agonists and antagonists lack sufficient bioavailability, specificity and

potency for meaningful in vivo or even in vitro studies. Such early results illustrate the difficulties of drug development when each physiological and pathological setting is characterized by a different mixture of bioactive LPLs and LPL receptors. Further development of LPL medicinal chemistry is expected to provide the necessary tools for assigning LPL signaling events to specific receptors. It is hoped also that emerging sets of Edg R-neutralizing antibodies will both represent early tools for initial cellular and animal studies, and facilitate standardization of assays needed for drug discovery.

Acknowledgement

Supported by grant HL31809 from the National Institutes of Health, CRP 1PF0265 from the California Dept. of Health Services, and the Department of the Army.

References

[1] Spiegel S, Milstein S. Sphingolipid metabolites: members of a new class of lipid second messengers. J Membrane Biol 1995;146:225.
[2] Moolenaar WH, Kranenburg O, Postma FR, Zondag G. Lysophosphatidic acid: G protein signaling and cellular responses. Curr Opin Cell Biol 1997;9:168.
[3] Goetzl EJ, An S. Diversity of cellular receptors and functions for the lysophospholipid growth factors lysophosphatidic acid and sphingosine 1-phosphate. FASEB J 1998;12:1589.
[4] An S, Goetzl EJ, Lee H. Signaling mechanisms and molecular characteristics of G protein-coupled receptors for lysophosphatidic acid and sphingosine 1-phosphate. J Cell Biochem Suppls 1998;30/31:147.
[5] Lynch KR, Im D-S. Life on the edg. Trends Pharmacol Sci 2000;20:473.
[6] Lee M-J, Van Brocklyn JR, Thangada S, Liu CH, Hand AR, Menzeleev RS, Spiegel S, Hla T. Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. Science 1998;279:1552.
[7] Hecht JH, Weiner JA, Post SR, Chun J. Ventricular zone gene-1 (vzg-1) encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex. J Cell Biol 1996;135:1071.
[8] An S, Bleu T, Huang W, Hallmark OG, Coughlin SR, Goetzl EJ. Identification of cDNAs encoding two G protein-coupled receptors for lysosphingolipids. FEBS Letters 1997;417:279.
[9] An S, Bleu T, Hallmark OG, Goetzl EJ. Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. J Biol Chem 1998;273:7906.
[10] Okamoto H, Takuwa N, Gonda K, et al. Edg-1 is a functional sphingosine 1-phosphate receptor that is linked via a Gi/o to multiple signaling pathways, including phospholipase C activation, Ca2+ mobilization, ras-mitogen-activated protein kinase activation, and adenylate cyclase inhibition. J Biol Chem 1998;273: 27104.
[11] Eicholtz T, Jalink K, Fahrenfort I, Moolenaar WH. The bioactive lysophospholipid lysophosphatidic acid is released from activated platelets. Biochem J 1993;291:677.
[12] Yatomi Y, Ruan FQ, Hakomori SI, Igarashi Y. Sphingosine-1-phosphate: A platelet-activating sphingolipid released from agonist-stimulated human platelets. Blood 1995;86:193.
[13] Valet P, Pages C, Jeanneton O, et al. Alpha2-adrenergic receptor-mediated release of lysophosphatidic acid by adipocytes. J Clin Invest 1998;101:1431.
[14] Xu Y, Shen Z, Wiper DW, et al. Lysophosphatidic acid as a potential biomarker in ovarian and other gynecological tumors. J Am Med Assoc 1998;280:719.
[15] Meerschaert K, DeCorte V, DeVille Y, Vandekerckhove K, Gettemans J. Gelsolin and functionally similar actin-binding proteins are regulated by lysophosphatidic acid. EMBO J 1998;17:5923.

[16] Goetzl EJ, Lee H, Azuma T, Stossel TP, Turck CW, Karliner JS. Gelsolin binding and cellular presentation of lysophosphatidic acid. J Biol Chem, 2000;275:14573.
[17] Dolezalova H, Shankar G, Solow-Cordero DE, Kong Y, Lee H, Goetzl EJ. Cellular presentation of lysophosphatidic acid (LPA) by the human plasma gelsolin (GE) substituent peptide GE135–169. FASEB J, 2000;14:A1464.
[18] Sato K, Murata N, Kon J, et al. Downregulation of mRNA expression of Edg-3, a putative sphingosine 1-phosphate receptor coupled to Ca2+ signaling, during differentiation of HL-60 leukemia cells. Biochem Biophys Res Commun 1998;253:253.
[19] Graler MH, Bernhardt G, Lipp M. Edg6, a novel G protein-coupled receptor related to receptors for bioactive lysophospholipids, is specifically expressed in lymphoid tissue. Genomics 1998;53:164.
[20] Goetzl EJ, Kong Y, Voice J. Differential constitutive expression of functional receptors for lysophosphatidic acid by human blood lymphocytes. J Immunol, 2000;164:4996.
[21] Goetzl EJ, Dolezalova H, Kong Y, Hu Y-L, Jaffe RB, Kalli KR, Conover CA. Distinctive expression and functions of the type 4 (mutant) endothelial gene-encoded G protein-coupled receptor for lysophosphatidic acid in ovarian cancer. Cancer Res 1999;59:5370.
[22] Goetzl EJ, Dolezalova H, Kong Y, Zeng L. Dual mechanisms for lysophospholipid induction of proliferation of human breast carcinoma cells. Cancer Res 1999;59:4732.
[23] Hu Y-L, Goetzl EJ, Mills GB, Ferrara N, Jaffe N & RB. Induction of VEGF expression by LPA in normal and neoplastic human ovarian epithelial cells. Clin Cancer Res, 1999; J National Cancer Institute 2001 in press.
[24] Goetzl EJ, Kong Y, Kenney JS. Lysophospholipid enhancement of human T cell sensitivity to Diphtheria toxin by increased expression of heparin-binding epidermal growth factor. Proc Assoc American Physicians 1999;111:259.
[25] Tigyi G, Dyer DL, Miledi R. Lysophosphatidic acid possesses dual action in cell proliferation. Proc Natl Acad Sci USA 1994;91:1908.
[26] Furui T, LaPushin R, Mao M, Kahn H, Watt SR, Watt M-AV, Lu Y, Fang X, Tsutsui S, Siddik ZH, Bast RC, Mills GB. Overexpression of Edg-2/vzg-1 induces apoptosis and anoikis in ovarian cancer cells in a lysophosphatidic acid independent manner. Clin Cancer Res, in press.
[27] Goetzl EJ, Lee H, Dolezalova H, Kalli KR, Conover CA, Hu Y-L, Azuma T, Stossel TP, Karliner JS, Jaffe RB. Mechanisms of lysolipid phosphate effects on cellular survival and proliferation. Ann NY Acad Sci 2000;905:177.
[28] Goetzl EJ, Kong Y, Mei B. Lysophosphatidic acid and sphingosine 1-phosphate protection of T cells from apoptosis in association with suppression of Bax. J Immunol 1999;162:2049–56.
[29] Cuvillier O, Rosenthal DS, Smulson ME, Spiegel S. Sphingosine 1-phosphate inhibits activation of caspases that cleave poly (ADP-ribose) polymerase and lamins during Fas- and ceramide-mediated apoptosis in Jurkat T lymphocytes. J Biol Chem 1998;273:2910–6.

Review Article
TheScientificWorldJOURNAL (2002) 2, 324–338
ISSN 1537-744X; DOI 10.1100/tsw.2002.124

TheScientificWorld
www.thescientificworld.com

Lysophospholipid Growth Factors and Their G Protein-Coupled Receptors in Immunity, Coronary Artery Disease, and Cancer

Edward J. Goetzl*[,1], Markus Graeler[1], Mei-Chuan Huang[1], and Geetha Shankar[2]

[1]*Departments of Medicine and Microbiology, University of California, UB8B, 533 Parnassus at 4th, San Francisco, CA 94143-0711;* [2]*Ceretek, LLC, Suite 200, 1501 Harbor Bay Parkway, Alameda, CA 94502*

Received December 21, 2001; Revised January 7, 2002; Accepted January 7, 2002; Published February 6, 2002

The physiological lysophospholipids (LPLs), exemplified by lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), are omnific mediators of normal cellular proliferation, survival, and functions. Although both LPA and S1P attain micromolar concentrations in many biological fluids, numerous aspects of their biosynthesis, transport, and metabolic degradation are unknown. Eight members of a new subfamily of G protein-coupled LPA/S1P receptors, originally termed Edg Rs, bind either LPA or S1P with high affinity and transduce a series of growth-related and/or cytoskeleton-based functional responses. The most critical areas of LPL biology and pathobiology are neural development and neurodegeneration, immunity, atherosclerosis and myocardial injury, and cancer. Data from analyses of T cells established two basic points: (1) the plasticity and adaptability of expression of LPA/S1P Rs by some cells as a function of activation, and (2) the role of opposing signals from two different receptors for the same ligand as a mechanism for fine control of effects of LPLs. In the heart, LPLs may promote coronary atherosclerosis, but are effectively cytoprotective for hypoxic cardiac myocytes and those exposed to oxygen free radicals. The findings of production of LPA by some types of tumor cells, overexpression of selected sets of LPA receptors by the same tumor cells, and augmentation of the effects of protein growth factors by LPA have suggested pathogenetic roles for the LPLs in cancer. The breadth of physiologic and pathologic activities of LPLs emphasizes the importance of developing bioavailable nonlipid agonists and antagonists of the LPA/S1P receptors for diverse therapeutic applications.

KEY WORDS: lysophosphatidic acid, sphingosine 1-phosphate, serum response element, T cells, chemotaxis, cytokines, hypoxia, apoptosis, ovarian cancer, cardioprotection

DOMAINS: growth and growth factors, transmembrane signaling, hematology, cardiovascular biology, biochemistry, cell biology, pharmacology, immunology, inflammation, cell death, cancer

*Corresponding author. Emails: Edward J. Goetzl, egoetzl@itsa.ucsf.edu; Markus Graeler, graeler@itsa.ucsf.edu; Mei-Chuan Huang, mhuang@itsa.ucsf.edu; Geetha Shankar, gshankar@ceretek.com

INTRODUCTION

Lysophosphatidic acid (LPA), sphingosine 1-phosphate (S1P), and other lysophospholipids (LPLs) are amphipathic products of stimulated cells, which are present in many mammalian physiological fluids at up to micromolar concentrations and have diverse effects on cellular survival, proliferation, and functions[1,2,3,4,5]. The LPLs were discovered first chemically in the mid- to late-1800s, among other organic solvent-extractable components of cells and tissues. Glyceryl-LPLs, exemplified by LPA, are members of the phospholipid family that predominates quantitatively among lipid-structural constituents of cellular membranes. Sphingosyl-LPLs were named for their shared sphingoid backbone and represent a quantitatively diminutive family compared to glyceryl-LPLs. However, as shown by the protean biological activities of S1P, sphingosyl-LPLs are one of the most structurally and functionally complex classes of biological mediators. As for our evolving understanding of some other bioactive lipids, the appreciation of crucial roles of LPLs in cellular biology derives from recent observations of their rapid generation from cell membrane precursors, often transient early increases in concentration in relation to cellular responses, and potent effects on critical activities of cells both as intracellular messengers and extracellular mediators.

The current intense focus on LPLs was initiated in part by the discovery that many of their effects as extracellular mediators are transduced by a novel family of highly specific G protein-coupled receptors (GPCRs). GPCRs for LPLs[2,4,5,6] were identified first as immediate-early expression elements of responses of endothelial cells to differentiating stimuli, and thus were designated tentatively the endothelial differentiation gene-encoded receptors (Edg Rs). A recent nomenclature subcommittee of the International Union of Pharmacological Scientists (IUPHAR) has recommended replacing these designations by terms based on ligand specificity and order of discovery (see Table 1). The consideration of LPLs and their G protein-coupled receptors (GPCRs) in this review will be directed principally to descriptions of their distinctive effects as mediators of cellular differentiation, survival, proliferation, and cytoskeleton-based functions in several mammalian systems. There will also be discussions of new findings that provide compelling support for roles of LPLs in several human diseases. Most prominent among these are autoimmunity, coronary artery disease, and some forms of cancer.

BIOSYNTHESIS OF LPLs

The series of enzymes responsible for separate production of LPA and S1P, which are to be secreted after cellular stimulation, consists of different phospholipases, sphingolipases, and highly specialized lipid kinases (Fig. 1). A different array of intracellular enzymes participates in *de novo* synthesis of precursors to be stored in cellular membranes as the sources of LPA and S1P released from stimulated cells (Fig. 1). Phospholipid-rich plasma membrane microvesicles are released from activated platelets, leukocytes, epithelial cells, and some tumors. After sphingomyelinase conditions these membrane microvesicles, phospholipase C (PLC)- and/or PLD-dependent mechanisms liberate phosphatidic acid, which is converted to LPA by secretory PLA2 and possibly other phospholipases[7,8]. Analogously, much of the secreted S1P is generated from cell membrane stores of sphingolipids by the sequential actions of sphingomyelinases, ceramidase, and sphingosine kinase (SK) (Fig. 1)[9]. A novel pathway for generation of extracellular S1P has been described very recently. In some circumstances, SK is secreted and converts extracellular sphingosine to S1P[10]. The relative importance of this source of S1P has not yet been evaluated systematically. PLA2 and SK are the dominant rate-controlling enzymes in their respective synthetic pathways[7,9]. However, concurrent degradative activities of a series of lysophospholipases, lysolipid phosphatases, and acyltransferases, and a S1P-specific lyase contribute significantly to the courses of appearance and net maximal concentrations of

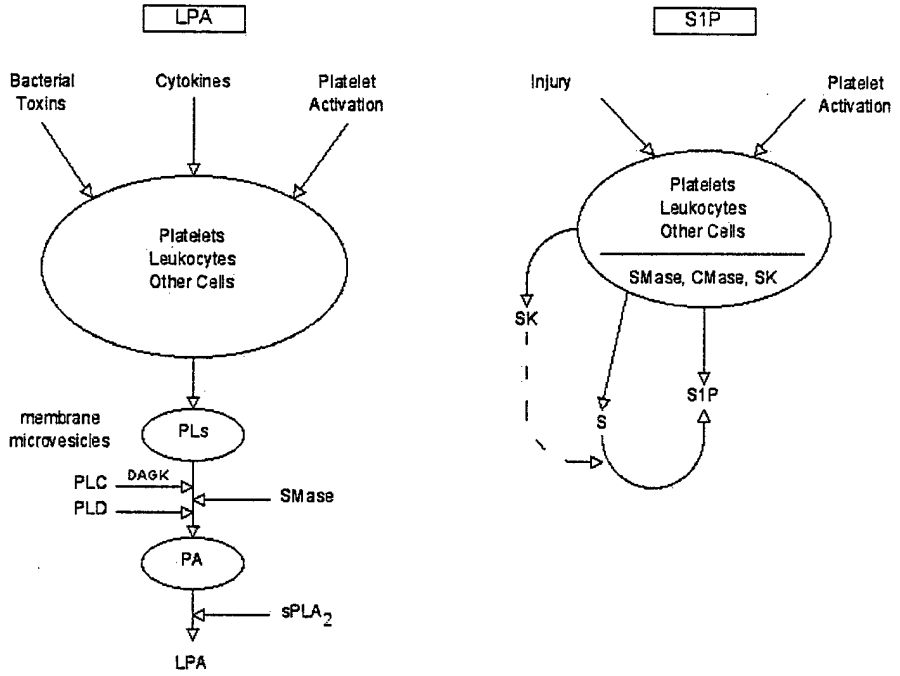

FIGURE 1. Cellular generation and release of lysophospholipid mediators. PL = phospholipid, PLC = phospholipase C, PLD = phospholipase D, DAGK = diacylglycerol kinase, sPLA2 = secretory phospholipase A2, PA = phosphatidic acid, SMase = sphingomyelinase, CMase = ceramidase, S = sphingosine, SK = sphingosine kinase.

LPA and S1P attained in any reaction[6]. The dependence of tissue and fluid concentrations of LPA and S1P on multiple LPL-generating and LPL-metabolizing enzymes suggests that many different genetic defects will alter their respective bioavailable levels, with functional significance for numerous organ systems.

CELLULAR SOURCES, TRANSPORT, AND DELIVERY OF LPLs

LPA was detected by its vasoactivity in incubated normal plasma in 1954, tentatively identified in 1960, and definitively characterized structurally and as a serum vasoactive and platelet-active factor in 1978–1979[11,12]. The serum concentration of LPA is micromolar, in contrast to nanomolar levels in fresh plasma, confirming that platelets are a major source of LPA[13]. Macrophages, some other types of leukocytes, epithelial cells, ovarian cancers, and some other tumors also produce LPA, resulting in high intracellular concentrations and plasma levels up to 50 μM in some inflammatory and neoplastic diseases. The interactions of LPA with various intracellular lipid-binding proteins have not been studied to date. Extracellular LPA is bound by serum albumin with an apparent Kd of 350 nM, which enhances cellular delivery and effective potency[14]. Recent analyses have shown high-affinity binding of LPA to plasma gelsolin, with an apparent Kd of 6 to 7 nM, which delivers LPA to some types of cells more efficiently and with more potent activity than serum albumin[14]. LPA binds to the two L-α-phosphatidylinositol 4,5-bisphosphate (PIP_2) sites of gelsolin in competition with PIP_2. Cellular delivery of LPA by gelsolin is most effective at concentrations of 1% to up to 10% of those in normal plasma. At concentrations over 10% of those of normal plasma, gelsolin traps LPA and prevents access to

cells with an efficiency that may explain the lack of effect of plasma LPA on endothelial cells normally. Thus plasma gelsolin may carry LPA in an inactive state. LPA may then be delivered to cells in an active state when gelsolin levels drop in plasma and tissue fluids as a result of dilution and binding to actin released from injured cells. This possibility is supported by findings of gelsolin concentrations optimal for cellular delivery of LPA in fluids of burned tissues and airway secretions of inflammatory lung diseases. Sphingosyl-LPL mediators, such as S1P, are formed during turnover and degradation of membrane sphingolipids by diverse sphingomyelinases and downstream enzymes in numerous types of cells[1]. Plasma and serum concentrations of S1P are in the micromolar range, with extensive binding normally to serum albumin and possibly other proteins, but not gelsolin.

CELLULAR RECEPTORS FOR LPLs

Two distinct subfamilies of GPCRs bind LPA and S1P specifically and transduce diverse cellular signals by associating with one or more G proteins (Table 1). Based on amino acid sequence identities, S1P1 (Edg-1), S1P2 (Edg-5), S1P3 (Edg-3), and S1P5 (Edg-8) belong to one structural cluster, and LPA1 (Edg-2), LPA2 (Edg-4), and LPA3 (Edg–7) are members of a second structural cluster[5]. Members of both subfamilies range in size from 351 to 400 amino acids, and are encoded by genes in chromosomes 1, 9, or 19 (Table 1). The amino acid sequence of S1P4 (Edg-6) lies between those of the two major clusters by amino acid sequence identity[17]. LPA/S1P Rs (Edg Rs) share other structural features, which as yet have not been linked clearly to function. The N-linked glycosylation sites of the amino-terminus and multiple potential sites of phosphorylation in intracellular regions, typical of all GPCRs, are preserved in the LPA/S1P receptors (Edg Rs). In contrast, the disulfide bond most often formed between cysteines in the first and second extracellular loops of other GPCRs is observed between the second and third extracellular loops of most LPA/S1P receptors (Edg Rs). In LPA2 (Edg-4 R), an alanine replaces the proline typical of the seventh transmembrane domain sequence NPXXY of other GPCRs. An Src homology-2 (SH2) segment exists in the intracellular face of S1P2 (Edg-5), but has not been shown to express characteristic functions. The mRNAs encoding some of the LPA/S1P Rs (Edg Rs) have the AU-rich sequence AUUUA in their 3'-untranslated region, which is an mRNA-stabilizing structure typical of growth-related immediate-early genes.

TABLE 1
S1P and LPA Subfamily of G Protein-Coupled Receptors: Structure and Signaling Mechanisms

Human Receptor Nomenclature	Ligand	Kd (nM)	Chromosomal Location	Protein Size (# of AAs)	Gα Subunit Coupling	Signaling Mechanisms		
						CAMP	Ca	ERK
$S1P_1$ (Edg 1)	S1P	8	1p21.1-3	381	i/o	↓	↑/-	↑
$S1P_2$ (Edg 5)	S1P	27	19p13.2	354	i/o, q, 12/13	↑	-/↑	↑
$S1P_3$ (Edg 3)	S1P	23	9q22.1-2	378	i/o, q, 12/13	↑	↑	↑
$S1P_5$ (Edg 8)	S1P	2	19	400	i/o	↓	-	↓
$S1P_4$ (Edg 6)	S1P	15	19p13.3	384	12/13	↓	-	↑
LPA_1 (Edg 2)	LPA	N/A	9q31.3-34.1	364	i/o, 12/13	↓	↑	↑/↓[a]
LPA_2 (Edg 4)	LPA	73	19p12	351 (382)[b]	q, i	-/↓	↑	↑
LPA_3 (Edg 7)	LPA	206	1p22.3-31.1	353	i/o, q	-/↓	↑	↑

Note: ↑: Stimulation; ↓: inhibition; -: no change
[a]Although LPA1 receptors have been shown to stimulate ERK in some cells, many reports also suggest that it is a negative regulator of cell growth.
[b]The number of amino acids (AAs) in the wild type is 351 and in the mutant is 382.

TABLE 2
Human Edg Receptor Distribution and Their Pathophysiological Roles

Receptor	Major Tissue Distribution	K/O Phenotype	Expression in Disease States
$S1P_1$ (Edg 1)	Endothelial cells, CNS neurons, many other cell types	Loss of vascular integrity; embryonic lethal due to intraplacental hemorrhage	
$S1P_2$ (Edg 5)	Brain, heart	Increased neuronal excitability; myoclonic seizures in some neonates	
$S1P_3$ (Edg 3)	Heart, many other cell types	No apparent phenotype; loss of S1P-mediated signaling in $Edg3^{-/-}$ embryonic fibroblasts	
$S1P_5$ (Edg 8)	Brain, spleen		
$S1P_4$ (Edg 6)	Spleen, leukocytes, thymus, lungs		
LPA_1 (Edg 2)	Brain (periventricular neurons and non-neuronal white matter), heart, placenta, GI tract	Craniofacial abnormalities; defective suckling behavior; 50% neonatal survival	*Decreased* in MS lesions; *decreased* in cancers, including ovarian cancer
LPA_2 (Edg 4)	Leukocytes		*Increased* in many cancers, including ovarian, prostate, cervical and uterine
LPA_3 (Edg 7)	Prostate, heart, brain, kidneys, testes		*Increased* in certain cancers, including ovarian and prostate cancers

Note: CNS = central nervous system, GI = gastrointestinal, MS = multiple sclerosis.

The amphipathic nature of LPA has prevented completion of accurate binding studies until recently, when it was discovered that sodium vanadate would suppress unspecific binding and provide data similar to that from analyses of S1P binding. The Kd values for Edg Rs range from 2 nM to over 200 nM (Table 1). Each Edg R exhibits a unique pattern of coupling to Gα proteins, which explains in large part the observed differences in utilization of signal transduction mechanisms (Table 1). For example, all Edg Rs that couple to Gi/o signal decreases in [cAMP]i, except for S1P2 (Edg-5), where the pattern is more complex. Similarly, all Edg Rs coupled to Gi/o and/or Gq exhibit an increase in $[Ca^{++}]i$, except for S1P5 (Edg-8). Distinctive patterns of tissue distribution of each LPA/S1P receptor (Edg R) have been mapped principally by semiquantitative PCR techniques, Northern blots, *in situ* hybridization, and Western blots with monoclonal antibodies to multiple substituent peptides (Table 2). Although most LPA/S1P receptors are widely represented in multiple tissues, each is differently distributed in the nervous, endocrine, cardiovascular, and immune systems at sites of their principal effects.

Several GPCRs in the orphan pool, which are not meaningfully homologous with the original Edg subfamily, recently have been identified as specific receptors for biologically active phospholipids (PLs). Although S1P1, S1P2, and S1P3 bind sphingosylphosphorylcholine (SPC) with low affinity, the first authentic SPC receptor was found to be the GPCR previously designated GPR68, or the ovarian cancer G protein-coupled receptor 1 (OGR1). The binding of SPC to OGR1 is high affinity, stereospecific for D-erythro-SPC, and stimulatory for a transient increase in $[Ca^{++}]i$[16]. Although the expression of OGR1 is highest in spleen, blood leukocytes, liver, lungs, small intestines, and testes, little is known of its native physiological functions. That there is such high representation of OGR1 in lymphocytes is of great interest from the perspective of the immune connections of two other newly recognized PL GPCRs. The lymphocyte GPCR, termed G2A, is a specific high-affinity receptor for lysophosphatidylcholine (LPC)[17]. G2A signaling through increases in $[Ca^{++}]i$ and ERK MAP kinase activity enhances T cell migration. The exact immune roles of increases in G2A expression by lymphocytes after prolonged

mitogenic or stress stimulation are unknown, but late negative regulation of immune responses seems likely. This possibility is reinforced by the findings that mice, which genetically lack G2A, develop late-onset autoimmunity characterized by lymphoid hyperplasia, expanded sets of B cells and T cells, antinuclear autoantibodies, and immune complex nephritis. The glycosphingolipid D-galactosyl-β-1,1'-sphingosine, or psychosine, which has diverse effects on cell migration and functions normally and in globoid cell leukodystrophy, is the specific ligand for the GPCR designated TDAG8[18]. TDAG8 is encoded by the T cell death-associated gene 8, which is up-regulated during T cell apoptosis and also expressed by several subsets of mononuclear phagocytes. TDAG8 and OGR1 show 41% amino acid sequence identity, and have substantial homology with G2A. A fourth PL GPCR, GPR4, has approximately 50% homology with OGR1, binds SPC with high-affinity, and also recognizes LPC with a relatively lower affinity. GPR4 and OGR1 have different tissue distributions. GPR4 also is highly expressed in lung tissues, and found in substantial amounts in liver, kidney, ovary, and lymph nodes[19]. Thus this newest subfamily of GPCRs for PLs is heavily expressed by different immune cells and may transduce important immunoregulatory effects of several complex PLs.

IN VITRO CELLULAR EFFECTS OF LPLs: GENERAL CONSIDERATIONS

The principal biological activities of LPA and S1P are as extracellular mediators, which have two basic types of effects (Table 3)[5]. The first are growth-related and include proliferation, differentiation, enhanced survival, and decreased sensitivity to apoptosis of diverse types of cells. The second are cytoskeleton-based functional effects, which include shape change, altered adherence, chemotaxis, contraction, and secretion. Stimulation of proliferation of many different types of cells by both LPA and S1P, through a Pertussis toxin-inhibitable mechanism, is the defining activity of LPLs. A complex set of LPL-initiated signaling pathways results in increases in intranuclear levels of the Ras-dependent ternary complex factor (TCF) and the Rho-dependent serum response factor (SRF), which together bind to and transcriptionally activate the serum response element (SRE) in promoters of many immediate-early response genes critical to cellular proliferation. It is presumed that Edg R transduction of LPL signals to SRE requires association with both Gi/o to recruit Ras and with G12/13 to engage Rho. Confirming evidence comes from the individual abilities of Pertussis toxin inactivation of Gi and of Clostridium botulinum C3 ADP-ribosyl-transferase inactivation of Rho to inhibit proliferative effects of LPLs, and from their greater suppressive effect when applied in combination. In addition to direct nuclear stimulation of cellular proliferation, LPLs also have indirect effects which include increased secretion of autocrine protein growth factors, heightened expression of receptors for protein growth factors, and enhanced expression of plasma membrane-localized protein growth factors such as the heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF), which acts on EGF receptors of neighboring cells by a juxtacrine mechanism. In a few types of cells, where LPA elicits an increase in intracellular concentration of cyclic AMP, there is suppression of cellular proliferation[20]. The mechanisms whereby LPLs improve cellular survival are not fully understood, but include reduction in apoptosis. In the few types of cells for which mechanisms of suppression of apoptosis by LPLs have been elucidated, studies have detected both alterations in intracellular levels of effector proteins of the Bcl family and selective inhibition of activity of specific caspases. In T lymphocytes, the protective effects of LPLs are associated with increases in protective Bcl-2, decreases in apoptosis-promoting Bax, and inhibition of caspases 3, 6, and 7, but not 8[21,22].

Cytoskeleton-based functional responses to LPLs include alterations in cellular morphology during differentiation, as typified by LPA-induced rounding of stellate periventricular neurons, and in postmitotic cells, as for the neurite retraction evoked by LPA and S1P (Table 3)[5]. In such responses, stress fiber formation reflects changes in the state of the microfilament network. LPA and S1P evoke focal adhesion kinase activity, activate cell-surface adhesive proteins, and initiate

TABLE 3
Biological Activities of LPA and S1P

		LPA	S1P
a.	Growth-Related Effects		
	Stimulate Cellular Proliferation	Fibroblasts	Fibroblasts
		Renal tubular cells	Monocytes
		Mesangial cells	
		Smooth muscle cells (vascular)	
		Keratinocytes	
		T cells	T cells
		Cancer cells	Cancer cells
	Increase Cellular Survival	Macrophages	
		B lymphocytes	
	Suppress Apoptosis	Renal tubular cells	Fibroblasts
		Cardiac myocytes	Endothelial cells
		T cells	T cells
		Monocytes	Monocytes
			Oocytes
b.	Cytoskeleton-Based Responses:		
	Cell Morphology	Neurite retraction	Neurite retraction
		Actin cytoskeletal remodeling	
		Myocyte hypertrophy	Myocyte hypertrophy
	Cell-cell/Cell-matrix adhesion	Focal adhesion	Fibronectin matrix assembly
		Platelet aggregation	Platelet aggregation
		Leuk.-Endothelial Interactions	Leuk.-Endothelial Interactions
	Chemotaxis/kinesis	Tumor cells (transcellular)	Neutrophils (inhibition)
		Endothelial cells	Endothelial cells
	Secretion	Neurotransmitter release	
		Protein growth factors	Protein growth factors
	Altered electrical excitability; Ion conductance	Neuroblasts	Ventricular myocytes
		Smooth muscles	
		Cerebrovascular myocytes	
	Intracellular signaling		TNF-alpha, adhesion PDGF, proliferation

Note: Leuk = leukocyte, TNF = tumor necrosis factor, PDGF = platelet-derived growth factor.

assembly of a fibronectin matrix on cells. Integrated expression of these responses mediates LPL-elicited platelet aggregation and endothelial interactions with platelets and leukocytes. These and other related events are crucial to cellular chemotactic, secretory, and contractile responses to LPA and S1P. The chemotactic response of several cell types is affected oppositely by S1P, depending on which S1P receptor is the predominant transducer. S1P1 and S1P3 signal chemotactic responses and amplify those from other growth factors, whereas S1P2 signals inhibition of growth factor-evoked chemotaxis. The principal difference in signaling pathways, to which the opposite responses are attributable, is stimulation of the small GTPase Rac by S1P1 and S1P3, in contrast to inhibition of Rac by S1P2.

The principal current obstacle to better understanding the mechanisms underlying the cellular effects of LPLs is the absence of bioavailable and potent pharmacological agonists and antagonists specific for each LPA/S1P (Edg) R. A wide range of analogues and other variants of LPA and S1P have been synthesized for studies of the structural determinants of activity of the parent compounds[6], but most have the same undesirable physicochemical properties as LPA and S1P, and none is a significantly more potent agonist or full antagonist of mammalian receptors. Limited applications of antireceptor antibodies, biochemical inhibitors of characteristic signaling pathways, and genetic approaches, such as overexpression of one receptor or antisense

ablation of one receptor, have been useful in the early phases of research, but have not delineated use of individual receptors by most native cells nor been helpful for *in vivo* investigations.

The capacity of S1P to act as a potent intracellular messenger was suggested initially by its compartmentalized generation and localized high concentration in cells responding to protein growth factors[23]. That inhibitors of the sphingosine kinase (SK), which controls synthesis of S1P, suppressed transduction of signals only from some receptors for protein growth factors selectively, and that exogenous S1P reversed this suppression, supported a role for S1P as an intracellular messenger. Similar, but less convincing, data implicate LPA as an intracellular messenger in other instances of cellular signaling.

EFFECTS OF LPLs ON SURVIVAL, PROLIFERATION, AND FUNCTIONAL ACTIVATION OF T CELLS

Most of the initial studies designed to elucidate the contributions of LPLs in immunity and inflammation have focused on T cells because of their central role in adaptive immunity, chronic inflammation, allergy, and autoimmunity. In addition, T cells are a valuable model for analyses of the proliferative responses evoked and regulated by LPLs. LPLs enhance T cell proliferation and some other responses by the same four often-interactive mechanisms observed in other types of cells. The first is Ras- and Rho-dependent enhancement of serum response element (SRE) activity in promoters of immediate-early growth-related genes. The second is induction of T cell production and secretion of one or more T cell–active polypeptide growth factors. The third is sensitization of T cells to the effects of T cell–selective growth factors. The fourth mechanism, which has been appreciated increasingly as studies of LPLs are extended to a wider range of cell types, is inhibition of proliferation. This phenomenon was observed first in some myelocytes in which LPA increased intracellular concentration of cyclic AMP ([cAMP]i). The results of recent studies of the roles of LPLs in survival and apoptosis of T cells and some other immune cells have revealed alterations in the concentration, localization, or activity of functionally relevant cellular proteins critical to endogenous control of proliferation.

In the initial studies, LPA and S1P had striking effects on T cell susceptibility to apoptosis due to alterations in cellular levels of proteins of the Bcl-2 superfamily and of the caspase array[22]. LPA and S1P also increased T cell sensitivity to diphtheria toxin (DT) as a result of enhanced T cell expression of the receptor for DT, which is HB-EGF[24]. As for many types of T cell tumor lines, cultured Tsup-1 cells of a human $CD4^{+}8^{+}3^{low}$ lymphoblastoma line resembling "double-positive" thymocytes express Edg-2, -3, -4, and –5 Rs, but not Edg-1 R, as determined by both RT-PCR analyses and Western blots. Tsup-1 cell apoptosis was induced by antibodies to CD2, CD3 plus CD28 in combination, Fas, and by cell-permeant ceramide, and was assessed by morphological characteristics, increases in end labeling of free 3'-OH groups of DNA, and release of radioactively labeled fragments of DNA. At 10^{-10} to 10^{-7} M, both LPA and S1P protected Tsup-1 cells from apoptosis evoked by antibodies to surface proteins[22]. In contrast, S1P but not LPA suppressed apoptosis elicited by C6-ceramide. The failure of LPA to prevent ceramide-induced apoptosis of Tsup-1 cells was partially due to suppression by ceramide of the expression of Edg-2 and –4 Rs, but not Edg-3 and –5 Rs. At 10^{-9} to 10^{-7} M, both LPA and S1P suppressed Tsup-1 cell content of the apoptosis-promoting protein Bax, without altering levels of Bcl-2 or Bcl-xL. That LPA and S1P suppression of Bax was mediated by LPA/S1P Rs (Edg Rs) was shown by selective antisense-mediated reduction in expression of LPA Rs and S1P Rs separately to levels which prevented depression of Bax by LPA and S1P, respectively, and concomitantly blocked suppression of apoptosis by each LPL. At higher levels of S1P, but not LPA, prevention of Tsup-1 cell apoptosis correlated best with inhibition of activity of caspases 3, 6, and 7.

Other investigations of the effects of LPA and S1P on T cell survival revealed striking sensitization of Tsup-1 cells to the action of DT. HB-EGF is a plasma membrane protein of T

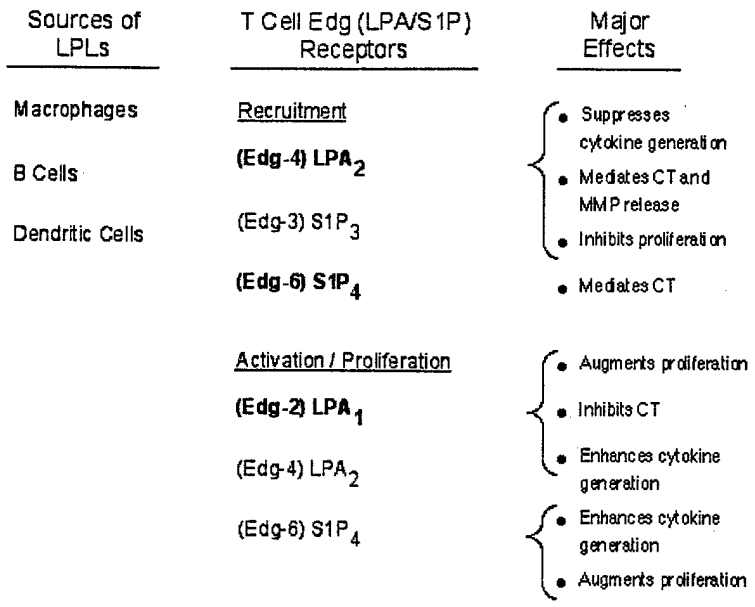

FIGURE 2. LPLs in human T cell–dependent immune responses. CT = chemotaxis, MMP = matrix metalloproteinase.

cells, which binds to EGF Rs and matrix proteoglycans, and is the cellular receptor for DT. Under conditions which enhanced sensitivity to DT, LPA and S1P increased Tsup-1 cell expression of HB-EGF[24]. The involvement of increased levels of HB-EGF in LPL enhancement of Tsup-1 cell sensitivity to DT was confirmed by HB-EGF neutralizing antibody blockade of the DT-sensitizing activity of LPA and S1P. Specific inhibitors of pathways of signaling characteristic of Edg Rs reduced LPA and S1P stimulation of both expression of HB-EGF and increased sensitivity to DT. Reductions in expression of LPA and S1P (Edg) Rs by the same antisense strategies used in the earlier studies of LPL protection from apoptosis supported the central roles of these receptors in mediating LPL enhancement of expression of T cell HB-EGF. Thus LPL stimulation of T cell proliferation is augmented by both suppression of apoptosis and enhancement in expression of endogenous protein growth factors.

In contrast to most human T cell tumors and some sets of mouse splenic T cells, which express all of the LPA/S1P (Edg) Rs albeit at substantially differing levels, human blood T cells bear a very restricted representation of LPA and S1P receptors (Fig. 2). Freshly isolated normal human blood $CD4^+$ T cells (helper/inducer or Th cells) showed predominantly Edg-4 Rs (LPA2) and Edg-6 Rs (S1P4), with traces of Edg-3 (S1P3) in some individuals[25]. $CD8^+$ T cells (suppressor/cytotoxic or Ts cells) from the blood of the same normal subjects had no detectable LPA/S1P (Edg) Rs. After activation with a mitogenic lectin or, to a lesser extent, with a combination of anti-CD3 plus anti-CD28 monoclonal antibodies (MoAbs) capable of stimulating T cell antigen receptors, the pattern of expression of LPA/S1P Rs changes rapidly and significantly (Fig. 2)[26]. The level of LPA2 (Edg-4) Rs in Th cells decreases by approximately 50%, that of S1P4 (Edg-6) Rs also decreases, and S1P3 (Edg-3) Rs disappear, as assessed by semi-quantification of mRNA and by Western blots. There is concurrent appearance of LPA1 (Edg-2) Rs in Th cells at a level less than or equal to that of LPA2 (Edg-4) Rs. Barely detectable levels of LPA1 (Edg-2) and S1P2 (Edg-5) Rs develop in Ts cells. Thus T cell expression of Edg-type LPA/S1P Rs is highly flexible, in relation to the source and state of functional activation of the T cells.

Equally impressive modifications in human blood Th cell functional responses to LPLs are observed after exposure to mitogens (Fig. 2). The responses to LPA have been examined in detail, but those evoked by S1P are still under investigation. Prior to mitogen stimulation, LPA elicits Th cell migration through a basement membrane-like Matrigel barrier by enhancing chemokinetic mobility and augmenting secretion of matrix metalloproteinases (MMPs) of the gelatinase subfamily. This response is presumed to be transduced by the Edg-4 (LPA2) R, as agonist-like mouse anti-LPA2 (Edg-4) R IgG MoAbs elicit migration of freshly isolated Th cells similarly to LPA. LPA has an inhibitory effect on IL-2 generation and secretion by freshly isolated Th cells incubated with a combination of adherent anti-CD3 plus anti-CD28 MoAbs, which again is reproduced by agonist-like mouse anti-LPA2 (Edg-4 R) IgG MoAbs. S1P effects resemble those of LPA in the freshly isolated Th cells, but results have not delineated the respective roles of Edg-3 (S1P3) and Edg-6 (S1P4) Rs (Fig. 2). The function-directed activities of LPA are reversed after mitogenic activation of human blood Th cells. An apparent transductional dominance of the newly induced Edg-2 (LPA1) Rs over the remaining Edg-4 (LPA2) Rs results in a lack of direct stimulation of Th cell migration and instead promotes significant LPA inhibition of Th cell chemotactic responses to chemokine stimulation. This new predominance of Edg-2 R signals over those from Edg-4 Rs results in a striking net enhancement of Th cell generation and secretion of IL-2 and of Th cell proliferation (Fig. 2). The present data for S1P effects on mitogen-activated Th cells, which are presumed to be mediated by Edg-6 Rs, are limited to augmentation of proliferation and modest enhancement of IL-2 secretion.

As the activated human blood Th cells express LPA1 and LPA2 Rs codominantly, but no subset of native T cells bears LPA1 R alone, two sets of Jurkat T cells were generated which expressed predominantly LPA1 or LPA2 Rs by transfection of a series of plasmids encoding sense and antisense messages for these Edg Rs followed by hygromycin selection to stabilize lines, as assessed by Western blots[27]. In the LPA2 (Edg-4) R-dominant Jurkat T cells, LPA evoked migration and suppressed IL-2 production, whereas in LPA1 (Edg-2) R-dominant Jurkat T cells, LPA did not evoke migration, suppressed chemokine-elicited migration, and enhanced IL-2 generation. Thus the effects of LPA in LPA2 (Edg-4) R-only and LPA1 (Edg-2) R-only Jurkat T cells exactly mimicked those observed respectively in freshly isolated human blood Th cells and later in mitogen-activated Th cells (Fig. 2). When viewed in terms of the recruitment and activation stages of Th cell involvement in immune responses, the first effects of LPA and probably S1P are to facilitate Th cell movement to the site of an immune response, while suppressing other reactions, such as cytokine secretion, that might be injurious to normal cells in the path of their migration. After their arrival at the site of an immune response, further Th cell migration capable of reducing the population of necessary effector Th cells is suppressed by LPA and effector reactions including cytokine generation are augmented by LPA (Fig. 2). These opposing effects of LPA transduced by Edg-2 and Edg-4 Rs suggest one advantage of multiple Edg Rs specific for the same ligand in precise regulation of complex cellular responses. Similar opposing functions of Edg-2 and Edg-4 Rs also have been observed in some human ovarian cancer cells (OCCs).

EFFECTS OF LPLs ON HUMAN OVARIAN CANCER CELLS

As high levels of LPA in plasma and ascitic fluid of patients with ovarian cancer correlate with a poor prognosis, it was considered important to investigate the expression and functions of LPA/S1P (Edg) Rs in human OCCs as compared to nonmalignant ovarian surface epithelial cells (OSE). Analyses of mRNA encoding all LPA/S1P Rs showed that LPA2 (Edg-4) and LPA3 (Edg-7) Rs are the predominant Edg Rs in OCCs and ovarian cancer tissues, and are expressed at far higher levels in almost all human ovarian cancer tissue samples than in matching adjacent noninvolved ovarian tissues (Fig. 3)[28]. In contrast, mRNA encoding LPA1 (Edg-2) Rs is more abundant in OSE than OCCs, and in matching adjacent noninvolved ovarian tissues than in human

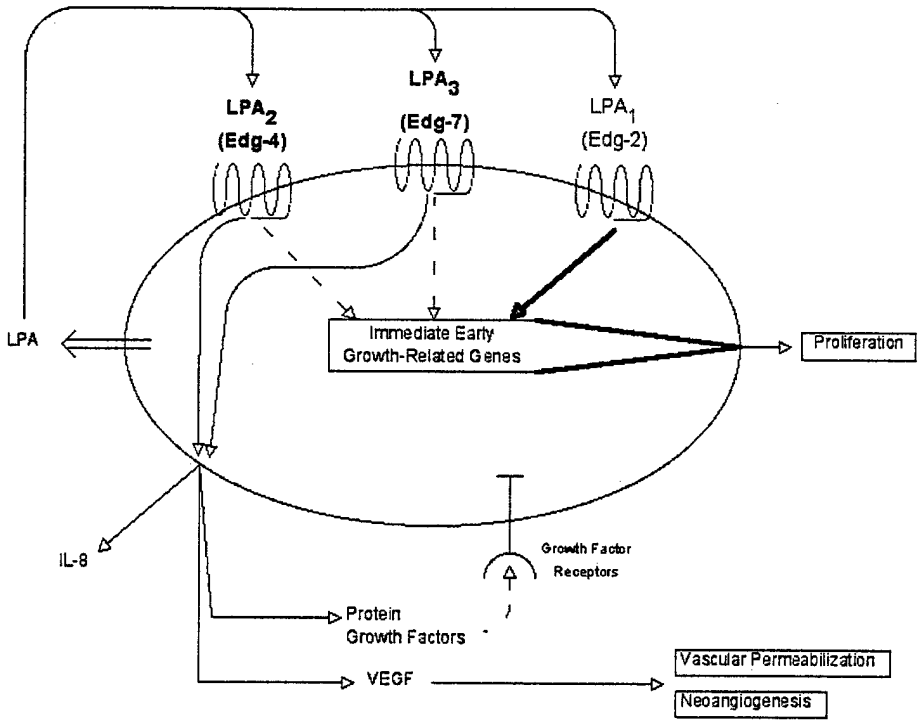

FIGURE 3. LPA and LPA receptors in ovarian cancer. Dashed line with arrow = stimulation, heavy solid line with arrow = inhibition, IL-8 = interleukin-8, VEGF = vascular endothelial growth factor.

human ovarian cancer tissue samples. S1P3 (Edg-3) and S1P2 (Edg-5) R mRNA also were consistently higher in OSE cells than in OCCs. Edg-1 R was expressed at similarly low levels in all lines of ovarian cells. Western blots supported the findings of higher levels of LPA2 (Edg-4) and LPA3 Rs in OCCs than nonmalignant ovarian epithelial cells, and higher levels of LPA1, S1P2, and S1P3 Rs in OSE cells than OCCs (Fig. 3). Thus it was expected that OCCs would be more responsive functionally to LPA and normal ovarian cells to S1P.

LPA stimulated proliferation of the OV202 primary line of OCCs, but not IOSE 29 cells, as assessed by increases in uptake of ^{3}H-thymidine and cell counts (Fig. 3)[28]. At 10^{-8} M, LPA evoked significant mean increases in uptake of ^{3}H-thymidine by OV202 cells of 1.7-fold and 2.1-fold, respectively, after 3 and 5 days of stimulation. SRE-luciferase activity of OV202 OCC transfectants, which represents one index of immediate-early gene responses to Edg R signaling, was increased significantly by 10^{-9} to 10^{-6} M LPA up to a mean maximum of threefold, whereas there was no response of OSE cell transfectants. In contrast, as predicted from the expression profile of Edg Rs, the SRE-luciferase responses to S1P were greater for OSE cells than OV202 cells. OV202 OCC generation of type II insulin-like growth factor (IGF-II), which is a potent mitogen for OCCs, was increased significantly by 10^{-8} and 10^{-7} M LPA and S1P to maximal levels of approximately tenfold higher than medium alone. LPA also may promote ovarian tumor growth by increasing angiogenesis through stimulation of secretion of vascular endothelial growth factor (VEGF), which is the same protein as vascular permeability factor (VPF). LPA increased secretion of VEGF/VPF by the OVCAR-3 line of human OCCs, up to a mean maximum of fourfold, through a transcriptional activation mechanism, without influencing VEGF/ VPF secretion by OSE cells (Fig. 3)[29]. Pharmacological inhibitors of Edg R transduction suppressed similar LPL stimulation of OCC proliferation, IGF-II generation, and VEGF production and secretion. The capacity of some

OCCs to secrete functionally relevant amounts of LPA suggests that the LPL-Edg R axis may be an autocrine growth and angiogenesis system in ovarian cancer (Fig. 3). The up-regulation of VEGF/VPF also may contribute to the ascites, which is so characteristic of the local peritoneal invasion by ovarian cancer. LPA2 (Edg-4) R and LPA3 (Edg-7) R may be markers for malignant transformation of ovarian epithelial cells, as well as transducers of proliferation by direct nuclear signaling and enhancement of secretion of protein GFs.

IN VIVO BIOLOGICAL ACTIVITIES OF LPLs AND RELATED PLs IN ANIMAL MODELS

LPA, S1P, fluid-phase precursors and homologues of LPA and S1P, and phospholipid ligands of other GPCRs are elevated in at least three different clinical settings: (1) acute lung diseases, such as adult respiratory distress syndrome (ARDS), and acute inflammatory exacerbations of chronic lung diseases, such as asthma, (2) surface epithelial cell injury, as in transcorneal freezing or cutaneous burns, and (3) certain malignancies of which ovarian cancer has been analyzed the most extensively. Of these conditions, animal models have been established or adapted for studies of the roles of LPLs and related phospholipids in lung and ocular tissue trauma and inflammation. An LPA R-dependent mouse model of human ovarian cancer is being developed most recently for investigations of the roles of LPA in the pathobiology of ovarian cancer and of its susceptibility to LPA R antagonist–based therapeutic agents.

Initiation of lung inflammation in guinea pigs by intratracheal administration of lipopolysaccharide induced secretion of LPA-generating type II secretory phospholipase A2 (sPLA2) into bronchoalveolar fluid and accompanying three- to tenfold increases in the concentrations of palmitic acid, total free fatty acids (FFAs), and lyso-phosphatidylcholine (lyso-PC)[30,31]. A specific inhibitor of sPLA2 reduced by a mean of 60% the increases in levels of FFAs and lyso-PC evoked by lipopolysaccharide. Similar increases in the concentrations of FFAs and lyso-PC were attained by administration of guinea pig recombinant sPLA2, in parallel with major decreases in surfactant content of phospholipids. The capacity of lysophospholipase D in lung tissues to convert lyso-PC to LPA is suggested to be one source of increased LPA in pulmonary secretions of injured or inflamed lungs, but was not demonstrated directly. LPA and its active variants cyclic PA and alkenyl-GP were identified at biologically active concentrations in aqueous humor and lacrimal gland fluid from rabbit eyes[32]. The concentrations of LPA and its homologues were increased after corneal injury to levels that stimulated proliferation of keratinocytes isolated from uninjured rabbit corneas.

The phenotypes of mice with a genetic deletion of one LPA/S1P R have revealed LPL functions in some instances (Table 2). The most dramatic abnormalities were seen in S1P1 (Edg-1) R K/O mice, in whom angiogenesis was normal, but vascular integrity was not established due to defective circumvascular migration and adherence of smooth muscle cells and pericytes[33]. The resultant intraplacental hemorrhages led to embryonic lethality in 100% of homozygous S1P1 K/O mice. In contrast, S1P2 K/O mice have a more subtle neural phenotype, characterized by abnormal neuronal excitability and often-fatal seizures. Very modest phenotypic alterations of the facial structures and resultant suckling problems were seen in LPA1 (Edg-2) R K/O mice. Their nervous system appears to be normal functionally, in part due to compensatory increases in neural expression of LPA2 (Edg-4) Rs. No abnormalities were reported for the S1P3 (Edg-3) R K/O mice[34].

PROTECTION OF CARDIOMYOCYTES FROM HYPOXIA AND OXYGEN FREE RADICALS

S1P and perhaps LPA are vital mediators of cardiovascular organogenesis and functions. S1P1 (Edg-1) Rs have a critical role in development of vascular integrity during embryogenesis. S1P directs endothelial cell migration and intercellular connections, and both S1P and LPA evoke platelet aggregation and elicit proliferation of vascular smooth muscle cells. S1P and LPA alter regional blood flow, including that in the coronary circulation, and modify cardiac inotropy and chronotropy. The S1P and LPA released from mildly oxidized low-density lipoproteins thus may promote atherosclerosis by multiple mechanisms.

Initial profiling of LPL GPCRs in rodent cardiomyocytes and myocardial tissues revealed expression of LPA1 (Edg-2), LPA2 (Edg-4), S1P2 (Edg-5), and S1P3 (Edg-3), but only a low level of S1P1 (Edg-1) and no S1P4 (Edg-6) by Western blots developed with mouse anti-LPL receptor peptide MoAbs[14]. In cultured layers of rat cardiac myocytes, both LPA and S1P evoked serum response element (SRE) transcriptional activity, as assessed by luciferase reporter assays, and hypertrophy, as quantified by increased incorporation of [^{3}H]leucine into proteins. The SRE responses to LPA and S1P were inhibited by both Pertussis toxin and by the C3 exoenzyme inactivator of Rho, and were protein kinase C (PKC) and PI-3 kinase-dependent. S1P was more potent and effective overall than LPA. Gelsolin derived from the cardiomyocytes may serve to regulate the local availability and presentation of LPA. S1P and SPC, which is in plasma at concentrations similar to S1P, also exert a broad range of effects on cardiac myocyte ion channels and electrical activity[6]. S1P and LPA enhance survival of many different types of cells, including endothelial cells, through their respective GPCRs by changing levels of one or more apoptosis-controlling proteins of the Bcl superfamily and by inhibiting activation and/or activities of several caspases.

The capacity of functional LPA/S1P Rs to mediate cardiocytoprotection against injury inflicted by both hypoxia and oxygen free radicals is a new concept and has been examined only recently in several systems. Under normoxic conditions, where neonatal rat cardiac myocytes show a mean viability of nearly 90% after 18–20 h of culture, severe hypoxia reduced viability to 60%, and this was prevented by preincubation with S1P or LPA[35]. In perfused intact mouse hearts, preinfusion of only 10 nM S1P or the S1P-elevating ganglioside GM-1 protected cardiac myocytes from ischemic damage, as shown by improved hemodynamics, decreased release of creatine kinase, and reduced infarct size[36]. S1P and LPA also protect cardiac myocytes and some other cells from injury by oxygen free radicals in several settings.

PATHOPHYSIOLOGICAL ROLES OF LPLs IN HUMAN DISEASE STATES: OVERVIEW

Elevated concentrations of LPA, lyso-PC, and some other phospholipids have been detected in lesional fluids of several inflammatory and neoplastic diseases. However, only in ovarian carcinoma and possibly other gynecologic cancers have tissue and plasma levels of LPA been increased so consistently as to suggest major pathogenetic roles and possible utility as a biochemical marker of these malignancies[37]. The ability of LPA to stimulate increased expression of tumor cell adhesive proteins and of tumor cell–derived protein growth factors characteristic of the neoplastic state, such as VEGF, transcellular migration, and proliferation, without effects on normal ovarian surface epithelial cells, has contributed to the authenticity of suggestions that plasma levels of LPA represent a useful and functional marker for even early stages of ovarian cancer. Any role of LPA in ovarian and other cancers remains to be proven in further analyses of mechanisms and by large-scale clinical studies of LPA receptor antagonists, involving the effects of different forms of treatment. However, it appears certain that OCCs

produce LPA in amounts far exceeding those of any other form of cancer so far examined *in vitro*. Further, OCCs express Edg Rs for LPA qualitatively different from those detected on normal ovarian cells. Although similar quantification of plasma S1P in coronary artery disease has been suggested as an indicator of platelet activation, and thus of severity of activity in chronic ischemic heart disease, no clinical data support this indication.

REFERENCES

1. Spiegel, S. and Milstien, S. (1995) Sphingolipid metabolites: members of a new class of lipid second messengers. *J. Membrane Biol.* **146,** 225–237.
2. Moolenaar, W.H., Kranenburg, O., Postma, F.R., and Zondag, G.C. (1997) Lysophosphatidic acid: G-protein signaling and cellular responses. *Curr. Opin. Cell Biol.* **9,** 168–173.
3. Goetzl, E.J. and An, S. (1998) Diversity of cellular receptors and functions for the lysophospholipid growth factors lysophosphatidic acid and sphingosine 1-phosphate. *FASEB J.* **12,** 1589–1598.
4. Chun, J., Contos, J.J., and Munroe, D. (1999) A growing family of receptor genes for lysophosphatidic acid (LPA) and other lysophospholipids (LPs). *Cell Biochem. Biophys.* **30,** 213–242.
5. Goetzl, E.J. and Lynch, K.R. (2000) Lysophospholipids and eicosanoids in biology and pathophysiology. *Ann. N. Y. Acad. Sci.* **905,** 1–357.
6. Goetzl. E.J., Hla, T., and Tigyi, G., Eds. Lysophospholipids and related bioactive lipids in health and disease. *Biochim. Biophys. Acta (suppl.)* in press.
7. Fourcade, O., Simon, M.F., Viode, C., Rugani, N., Leballe, F., Ragab, A., Fournie, B., Sarda, L., and Chap, H. (1995) Secretory phospholipase A2 generates the novel lipid mediator lysophosphatidic acid in membrane microvesicles shed from activated cells. *Cell* **80,** 919–927.
8. Valet, P., Pages, C., Jeanneton, O., Daviaud, D., Barbe, P., Record, M., Saulnier-Blache, J.S., and Lafontan, M. (1998) Alpha2-adrenergic receptor-mediated release of lysophosphatidic acid by adipocytes. A paracrine signal for preadipocyte growth. *J. Clin. Invest.* **101,** 1431–1438.
9. Nava, V.E., Lacana, E., Poulton, S., Liu, H., Sugiura, M., Kono, K., Milstien, S., Kohama, T., and Spiegel, S. (2000) Functional characterization of human sphingosine kinase-1. *FEBS Lett.* **473,** 81–84.
10. Ancellin, N., Colmont, C., Su, J., Li, Q., et al. (2002) Extracellular export of sphingosine kinase-1 enzyme: sphingosine 1-phosphate generation and the induction of angiogenic vascular maturation. *J. Biol. Chem.* in press.
11. Tokumura, A., Fukuzawa, K., and Tsukatani, H. (1978) Effects of synthetic and natural lysophosphatidic acids on the arterial blood pressure of different animal species. *Lipids* **13,** 572–574.
12. Schumacher, K.A., Classen, H.G., and Spath, M. (1979) Platelet aggregation evoked *in vitro* and *in vivo* by phosphatidic acids and lysoderivatives: identity with substances in aged serum (DAS). *Thromb. Haemost.* **42,** 631–640.
13. Eichholtz, T., Jalink, K., Fahrenfort, I., and Moolenaar, W.H. (1993) The bioactive phospholipid lysophosphatidic acid is released from activated platelets. *Biochem. J.* **291(3),** 677–680.
14. Goetzl, E.J., Lee, H., Azuma, T., Stossel, T.P., Turck, C.W., and Karliner, J.S. (2000) Gelsolin binding and cellular presentation of lysophosphatidic acid. *J. Biol. Chem.* **275,** 14573–14578.
15. Graler, M.H., Bernhardt, G., and Lipp, M. (1998) EDG6, a novel G-protein-coupled receptor related to receptors for bioactive lysophospholipids, is specifically expressed in lymphoid tissue. *Genomics* **53,** 164–169.
16. Xu, Y., Zhu, K., Hong, G., Wu, W., Baudhuin, L.M., Xiao, Y., and Damron, D.S. (2000) Sphingosyl-phosphorylcholine is a ligand for ovarian cancer G-protein-coupled receptor 1. *Nat. Cell Biol.* **2,** 261–267.
17. Kabarowski, J.H., Zhu, K., Le, L.Q., Witte, O.N., and Xu, Y. (2001) Lysophosphatidylcholine as a ligand for the immunoregulatory receptor G2A. *Science* **293,** 702–705.
18. Im, D.S., Heise, C.E., Nguyen, T., O'Dowd, B.F., and Lynch, K.R. (2001) Identification of a molecular target of psychosine and its role in globoid cell formation. *J. Cell Biol.* **153,** 429–434.
19. Zhu, K., Baudhuin, L.M., Hong, G., et al. (2001) Sphingosylphosphorylcholine and lysophosphatidyl choline are ligands for the G protein-coupled receptor GPR4. *J. Biol. Chem.* **276,** 41325–41335.
20. Tigyi, G., Dyer, D.L., and Miledi, R. (1994) Lysophosphatidic acid possesses dual action in cell proliferation. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 1908–1912.
21. Cuvillier, O., Rosenthal, D.S., Smulson, M.E., and Spiegel, S. (1998) Sphingosine 1-phosphate inhibits activation of caspases that cleave poly (ADP-ribose) polymerase and lamins during Fas- and ceramide-mediated apoptosis in Jurkat T lymphocytes. *J. Biol. Chem.* **273,** 2910–2916.
22. Goetzl, E.J., Kong, Y., and Mei, B. (1999) Lysophosphatidic acid and sphingosine 1-phosphate protection of T cells from apoptosis in association with suppression of Bax. *J. Immunol.* **162,** 2049–2050.
23. Spiegel, S. and Milstien, S. (2000) Sphingosine-1-phosphate: signaling inside and out. *FEBS Lett.* **476,** 55–57.
24. Goetzl, E.J., Kong, Y., and Kenney, J.S. (1999) Lysophospholipid enhancement of human T cell sensitivity to diphtheria toxin by increased expression of heparin-binding epidermal growth factor. *Proc. Assoc. Am. Physicians* **111,** 259–269.

25. Goetzl, E.J., Kong, Y., and Voice, J.K. (2000) Cutting edge: differential constitutive expression of functional receptors for lysophosphatidic acid by human blood lymphocytes. *J. Immunol.* **164,** 4996–4999.
26. Zheng, Y., Voice, J.K., Kong, Y., and Goetzl, E.J. (2000) Altered expression and functional profile of lysophosphatidic acid receptors in mitogen-activated human blood T lymphocytes. *FASEB J.* **14,** 2387–2389.
27. Zheng, Y., Kong, Y., and Goetzl, E.J. (2001) Lysophosphatidic acid receptor-selective effects on Jurkat T cell migration through a Matrigel model basement membrane. *J. Immunol.* **166,** 2317–2322.
28. Goetzl, E.J., Dolezalova, H., Kong, Y., Hu, Y.L., Jaffe, R.B., Kalli, K.R., and Conover, C.A. (1999) Distinctive expression and functions of the type 4 endothelial differentiation gene-encoded G protein-coupled receptor for lysophosphatidic acid in ovarian cancer. *Cancer Res.* **59,** 5370–5375.
29. Hu, Y.L., Tee, M.K., Goetzl, E.J., Auersperg, N., Mills, G.B., Ferrara, N., and Jaffe, R.B. (2001) Lysophosphatidic acid induction of vascular endothelial growth factor expression in human ovarian cancer cells. *J. Natl. Cancer Inst.* **93,** 762–768.
30. Chilton, F.H., Averill, F.J., Hubbard, W.C., Fonteh, A.N., Triggiani, M., and Liu, M.C. (1996) Antigen-induced generation of lyso-phospholipids in human airways. *J. Exp. Med.* **183,** 2235–2245.
31. Arbibe, L., Koumanov, K., Vial, D., Rougeot, C., Faure, G., Havet, N., Longacre, S., Vargaftig, B.B., Bereziat, G., Voelker, D.R., Wolf, C., and Touqui, L. (1998) Generation of lyso-phospholipids from surfactant in acute lung injury is mediated by type-II phospholipase A2 and inhibited by a direct surfactant protein A-phospholipase A2 protein interaction. *J. Clin. Invest.* **102,** 1152–1160.
32. Liliom, K., Guan, Z., Tseng, J.L., Desiderio, D.M., Tigyi, G., and Watsky, M.A. (1998). Growth factor-like phospholipids generated after corneal injury. *Am. J. Physiol.* **274,** C1065–1074.
33. Liu, Y., Wada, R., Yamashita, T., Mi, Y., Deng, C.X., Hobson, J.P., Rosenfeldt, H.M., Nava, V.E., Chae, S.S., Lee, M.J., Liu, C.H., Hla, T., Spiegel, S., and Proia, R.L. (2000) Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *J. Clin. Invest.* **106,** 951–961.
34. Ishii, I., Friedman, B., Ye, X., Kawamura, S., McGiffert, C., Contos, J.J., Kingsbury, M.A., Zhang, G., Heller Brown, J., and Chun, J. (2001) Selective loss of sphingosine 1-phosphate signaling with no obvious phenotypic abnormality in mice lacking its G protein-coupled receptor, LP(B3)/EDG-3. *J. Biol. Chem.* **276,** 33697–33704.
35. Karliner, J.S., Honbo, N., Summers, K., Gray, M.O., and Goetzl, E.J. (2001) The lysophospholipids sphingosine-1-phosphate and lysophosphatidic acid enhance survival during hypoxia in neonatal rat cardiac myocytes. *J. Mol. Cell. Cardiol.* **33,** 1713–1717.
36. Jin, Z.Q., Zhou, Hui-Zhong, Zhu P, Honbo N., et al. (2002) Cardioprotection mediated by sphingosine 1-phosphate and the ganglioside GM-1in wild-type and epsilon PKC knockout mouse hearts. In press.
37. Mills, G.B., May, C., Hill, M., Campbell, S., Shaw, P., and Marks, A. (1990) Ascitic fluid from human ovarian cancer patients contains growth factors necessary for intraperitoneal growth of human ovarian adenocarcinoma cells. *J. Clin. Invest.* **86,** 851–855.

ADDITIONAL REFERENCES (SYNOPSIS)

Tokumura, A. (1995) A family of phospholipid autacoids: occurrence, metabolism and bioactions. *Prog. Lipid Res.* **34,** 151–184.

Gaits, F., Fourcade, O., Le Balle, F., Gueguen, G., Gaige, B., Gassama-Diagne, A., Fauvel, J., Salles, J.P., Mauco, G., Simon, M.F., and Chap, H. (1997) Lysophosphatidic acid as a phospholipid mediator: pathways of synthesis. *FEBS Lett.* **410,** 54–58.

This article should be referenced as follows:

Goetzl, E.J., Graeler, M., Huang, M-C., and Shankar, G. (2002) Lysophospholipid growth factors and their G protein-coupled receptors in immunity, coronary artery disease, and cancer. *TheScientificWorldJOURNAL* **2,** 324–338.

Handling Editor:

Charles N. Serhan, Principal Editor for *Inflammation* – a domain of TheScientificWorld.

BIOSKETCH

Edward J. Goetzl is Robert L. Kroc Professor of Medicine and Professor of Microbiology/Immunology, University of California, San Francisco. Prof. Goetzl conducts research in the areas of immune effector mechanisms, inflammation, G protein-coupled receptors, lipid mediators, neuropeptides, neuroimmunology, and ovarian cancer. Additional research activities include elucidation of roles of lysophospholipid and neuropeptide mediators and their G protein-coupled receptors in T cell development and homing, T cell-dependent immunity and hypersensitivity, cardiac myocyte resistance to apoptosis, and induction of gender-specific cancers.

0031-6997/02/5402-265–269$7.00
PHARMACOLOGICAL REVIEWS

Pharmacol Rev 54:265–269, 2002

Vol. 54, No. 2
20204/989285
Printed in U.S.A

International Union of Pharmacology. XXXIV. Lysophospholipid Receptor Nomenclature

JEROLD CHUN, EDWARD J. GOETZL, TIMOTHY HLA, YASUYUKI IGARASHI, KEVIN R. LYNCH, WOUTER MOOLENAAR, SUSAN PYNE, AND GABOR TIGYI

Merck Research Laboratories, La Jolla, California (J.C.); Department of Medicine, University of California, San Francisco, California (E.J.G.); Department of Physiology, University of Connecticut, Farmington, Connecticut (T.L.H.); Department of Biomembrane and Biofunctional Chemisty, Hokkaido University, Sapporo, Japan (Y.I.); Department of Pharmacology, University of Virginia, Charlottesville, Virginia (K.R.L.); Division of Cellular Biochemistry, Netherlands Cancer Institute, Amsterdam, The Netherlands (W.M.); Department of Physiology and Pharmacology, University of Strathclyde, Glasgow, Scotland (S.P.); and Department of Physiology, University of Tennessee, Memphis, Tennessee (G.T.)

This paper is available online at http://pharmrev.aspetjournals.org

***Abstract*—The lysophospholipids, lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), are now recognized as important extracellular signaling molecules. These lipid mediators are pleiotropic; among the most common cellular responses are mitogenesis, cell survival (anti-apoptosis), inhibition of adenylyl cyclase and calcium mobilization. Physiologic events associated with these mediators include platelet aggregation, vasopressor activity, wound healing, immune modulation, and angiogenesis. Many of the actions of LPA and S1P are mediated through a set of eight G protein-coupled receptors. Five of these are S1P-prefering while the remaining three are LPA receptors. These receptors are expressed widely and in aggregate signal through a variety of heterotrimeric G proteins. The lysophospholipid receptor family is referred to commonly as the "Edg" group (e.g., Edg-1, Edg-2, etc.). Herein, the molecular pharmacology of the lysophospholipid receptors is reviewed briefly, and a rational nomenclature for LPA and S1P receptors that is consistent with the International Union of Pharmacology guidelines is proposed.**

I. Introduction

Lysophosphatidic acid (LPA[1]) and sphingosine 1-phosphate (S1P) are now known to be pleiotropic extracellular signaling molecules. The first report of lysophosphatidic acid-evoked responses from tissue described its activity as a vasopressor (Tokumura et al., 1978). Subsequently, LPA was found to be proliferative for cultured cells (van Corven et al., 1989)—an observa-

Address correspondence to: Dr. Kevin R. Lynch, Subcommittee Chair, Pharmacology Box 800735, University of Virginia Health System, 1300 Jefferson Park Avenue, Charlottesville, VA 22908.

[1] Abbreviations: LPA, lysophosphatidic acid; S1P, sphingosine 1-phosphate; LPL, lysophospholipid; GPCR, G protein-coupled receptor; Edg, endothelial differentiation gene; NC-IUPHAR, International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification; SPC, sphingosylphosphorylcholine; LPC, lysophosphatidylcholine.

Composition of the NC-IUPHAR Subcommittee on Lysophospholipid Receptors: Jerold Chun, Merck Research Laboratories, 505 Coast Boulevard South, La Jolla, CA 92037; Edward J. Goetzl, Department of Medicine, University of California, U88B Box 0711, 533 Parnassus Ave., San Francisco, CA 94143-0711; Timothy Hla, Center for Vascular Biology, Department of Physiology, University of Connecticut, 263 Farmington Ave., Farmington, CT 06030; Yasuyuki Igarashi, Department of Biomembrane and Biofunctional Chemistry, Hokkaido University, Nishi 6, Kita 12, Kita-ku, Sapporo 060-0812, Japan; Kevin R. Lynch (Chair), Department of Pharmacology, Box 800735, University of Virginia, 1300 Jefferson Park Ave., Charlottesville, VA 22908-0735; Wouter H. Moolenaar, Division of Cellular Biochemistry, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands; Susan Pyne, Department of Physiology and Pharmacology, University of Strathclyde, 27 Taylor St., Glasgow G4 ONR, Scotland; and Gabor Tigyi, Department of Physiology, University of Tennessee, 894 Union Ave., Memphis, TN 38163.

tion that spurred much subsequent investigation. S1P has also been shown to be proliferative for cultured cells (Zhang et al., 1991). Investigations into numerous aspects of lysophospholipid (LPL) signaling led to the realization that many LPL-evoked events are mediated by heterotrimeric G proteins, thus predicting the existence of LPL G protein-coupled receptors (GPCRs).

II. Discovery of Lysophospholipid Receptors

The receptor cluster that contains the eight LPL receptors has the colloquial name "Edg" (an acronym for endothelial differentiation gene). The name Edg (or EDG) was coined in 1990 by Hla and Maciag to describe a set of immediate early response gene products cloned from human umbilical vein endothelial cells; Edg-1 was found to be a rhodopsin family GPCR (Hla and Maciag, 1990). A GPCR that was 50% identical was reported in 1993 ("AGR16"; Okazaki et al., 1993) and independently the following year ("H218"; MacLennan et al., 1994). The Edg name appeared again in 1995 (Edg-2) to describe a third, more distantly related, GPCR (Masana et al., 1995). The orthologous (species homolog) mouse GPCR was then described ("rec1.3"; Macrae et al., 1996). The other five members of the cluster were named in order of their appearance as Edg-3, Edg-4, Edg-6, Edg-7, and Edg-8 and eventually AGR16/H218 became commonly known as Edg-5.

A seminal event in the LPL field occurred when Chun and colleagues discovered that mouse Edg-2 (they called it "vzg-1") is an LPA receptor (Hecht et al., 1996). This was followed quickly by three reports that Edg-3 and Edg-1 are S1P-preferring receptors (An et al., 1997; Lee et al., 1998; Zondag et al., 1998). Within several years, reports from a number of groups established that there are eight Edg receptor genes in the human genome. Five of these encode S1P receptors (Edg-1, -3, -5, -6, and -8) whereas the remaining three encode LPA receptors (Edg-2, -4, and -7). The S1P receptors share about 50% identical amino acids whereas the LPA receptors have about 55% sequence identity. The subclusters are about 35% identical. A maximum parsimony tree showing graphically the relationship among the LPL receptors and the distantly related platelet-activating factor receptor is shown as the Fig. 1. Different aspects of LPL biochemistry, physiology, and cell biology are discussed in a variety of recent review articles (Hla, 2001; Tigyi, 2002) (also for review, see Chun et al., 1999; Moolenaar, 1999; Pyne and Pyne, 2000; Fukushima et al., 2001; Hla et al., 2001).

III. Receptor Nomenclature

Although the Edg acronym proved prescient for Edg-1 (Hla et al., 2001), this name has little relevance to the other seven receptors in the family. The names AGR16, H218, vzg-1, rec1.3, etc. are trivial also. Furthermore, the Edg name has been applied to unrelated proteins

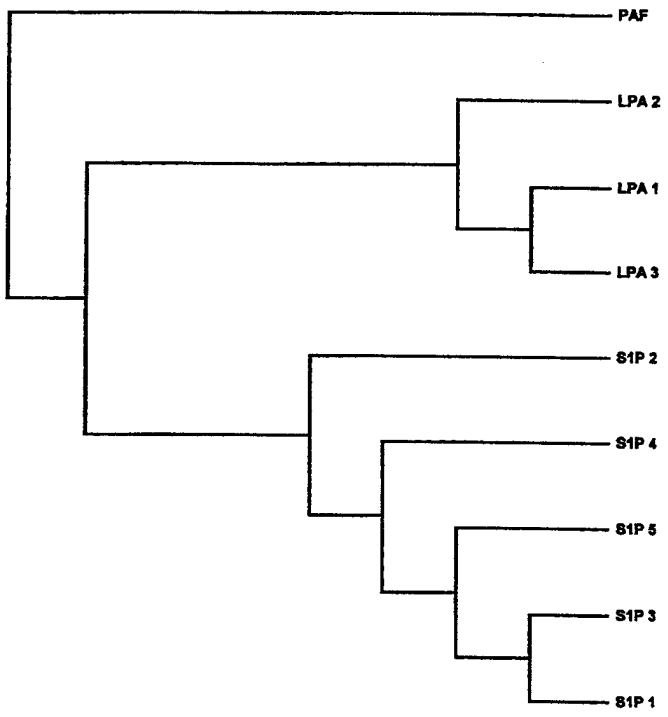

FIG 1. The single maximum parsimony tree of the human LPL receptor amino acid sequences and the human platelet-activating factor receptor sequence. This unrooted tree was built with PROTPARS (available at www.evolution.genetics.washington.edu) in PHYLIP package (5 runs, 10–23 jumbles per run).

and thus is a possible source of confusion (Hla et al., 1997). A rational, alternate nomenclature has been proposed (Chun et al., 1999), but this scheme has not been embraced, and it is not consistent with IUPHAR guidelines (Humphrey et al., 2000; Ruffolo et al., 2000). According to those guidelines, a receptor is to be named with the abbreviation for the natural agonist with the highest potency, followed by a subscripted arabic number. Because the NC-IUPHAR Subcommittee on Lysophospholipid Receptors recommends that sphingosine 1-phosphate be abbreviated S1P (rather than SPP or Sph-P), Edg-1 becomes $S1P_1$ and Edg-2 becomes LPA_1. The subcommittee decided further that the order of numbering is to reflect the chronology of the publication of receptor sequence (regardless of whether the ligand was known at that time), thus Edg-5/H218/AGR16 becomes $S1P_2$, etc. Table 1 lists recommended LPL receptor names, the IUPHAR Receptor Code (Humphrey et al., 2000), and previous LPL receptor names. LPL recep-

TABLE 1
LPL receptors

Agonist Ligand	IUPHAR Nomenclature	Receptor Code	Edg Name	Previous Names
LPA	LPA_1	2.1:LPL:2:LPA1:	Edg-2	Ip_{A1}, vzg-1, rec1.3
LPA	LPA_2	2.1:LPL:4:LPA2:	Edg-4	Ip_{A2}
LPA	LPA_3	2.1:LPL:7:LPA3:	Edg-7	Ip_{A3}
S1P	$S1P_1$	2.1:LPL:1:S1P1:	Edg-1	Ip_{B1}
S1P	$S1P_2$	2.1:LPL:3:S1P2:	Edg-5	Ip_{B2}, AGR16, H218
S1P	$S1P_3$	2.1:LPL:5:S1P3:	Edg-3	Ip_{B3}
S1P	$S1P_4$	2.1:LPL:6:S1P4:	Edg-6	Ip_{B4}
S1P	$S1P_5$	2.1:LPL:8:S1P5:	Edg-8	Ip_{B5}, nrg-1

tor splice variants, such as that described for the human LPA_3 receptor (Fitzgerald et al., 2000), are not named individually since they have not been shown to have distinct pharmacologic properties. The recommended receptor nomenclature is flexible in that it readily accommodates additional LPL GPCRs, regardless of their similarity to the Edg cluster. Additional LPA receptors (Guo et al., 1996) as well as sphingosylphosphorylcholine (SPC) (Xu et al., 2000) and lysophosphatidylcholine (LPC) (Kabarowski et al., 2001) receptors have been suggested. However, until data verifying these identifications is published independently, the NC-IUPHAR Subcommittee on Lysophospholipid Receptors has decided against including these putative LPL receptors at this juncture.

IV. Nonhuman Lysophospholipid Receptors

Current evidence suggests the existence of an orthologous set of eight LPL receptor genes in rodents; presumably this will hold true for all mammals. An analysis of the nearly complete *Takifugu rubripes* (Japanese puffer fish) genome reveals at least ten LPL receptor-like genes—four LPA receptor-like and six S1P receptor-like (K. R. Lynch, unpublished observation). There is little, if any, evidence for LPL receptors in any nonvertebrate species. The IUPHAR nomenclature should be applied to mammalian receptors only because the nonmammalian LPL receptors are so distant as to make identification of orthologs problematic. For example, the zebrafish gene, *miles apart* (Mil)—a mutation of which results in cardiac bifida (Kupperman et al., 2000)—has been suggested to be orthologous to the human $S1P_2$ receptor because of their 60% identical amino acids. However, this is an insufficient basis to assume the correspondence in function between Mil and $S1P_2$ that is predicted for orthologous genes—and the deletion of the $S1P_2$ gene in mice does not result in detectable abnormalities in cardiac development (MacLennan et al., 2001). The exception to the rule is the LPA_1 receptor, which shows a high degree of sequence conservation among chicken, fish, amphibians (*Xenopus*), and mammals. Remarkably, the LPA_1 receptor from all these species shares >90% identical amino acids, with most of the mismatch at the amino-terminal regions. The other LPL receptors—and GPCRs in general—share only 40 to 70% identical amino acids when sequences from disparate vertebrate species are compared. The extraordinary sequence conservation of the LPA_1 receptor leads one to wonder whether this protein is serving some function beyond binding LPA and signaling heterotrimeric G proteins.

V. Lysophospholipid Receptor Ligands

A synthetic chemistry focused on LPL receptors is currently underway, but there are very few defining ligands at present. For both LPA and S1P, the addition of a head group (e.g., choline) to form a phosphate diester or the replacement of the phosphate with an alcohol result in decreases in potency of several log orders (for review, see Lynch and Macdonald, 2001). Conversely, the degree of saturation of the alkyl moiety of either ligand has little effect on potency, that is dihydro S1P is equipotent to S1P and 16:0 LPA is equipotent to 18:1 LPA—with the important exception of the LPA_3 receptor, which has a pronounced preference for unsaturated LPAs (Bandoh et al., 1999; Im et al., 2000b). Recently, two LPA receptor antagonists have been described. The first is di-octyl glycerol pyrophosphate, which is a competitive antagonist of the LPA_3 receptor with a reported K_i of 100 nM (Fisher et al., 2001). The second, which is an *N*-oleoyl ethanolamide phosphate that is substituted at the second carbon with a benzyl-4-oxybenzyl moiety, is a competitive antagonist of the LPA_1 and LPA_3 receptors (K_i values 125 and 430 nM, respectively). The opposite enantiomer (*R*) of the latter compound is about 10-fold more potent in blocking the LPA_3 receptor, but is an agonist at the LPA_1 site (Heise et al., 2001).

There are currently no available S1P receptor selective agonists or antagonists, but the availability of a radioligand binding assay allows the measurement of ligand affinities at recombinant S1P receptors. Although no one group has yet reported comparative binding analyses of all five S1P receptors, the reported K_D values from different laboratories (obtained by equilibrium binding methods) are in the range of 1 to 60 nM, with the $S1P_4$ receptor having the lowest affinity for S1P (van Brocklyn et al., 2000). A model of the $S1P_1$ receptor ligand binding domain has been proposed (Parrill et al., 2000) with a specific emphasis on the Arg-Glu-Gly motif that is present at the exofacial aspect of the third transmembrane-spanning region of all S1P receptors. An obvious prediction is that side chains of the arginine and glutamate residues interact with the vicinal phosphate and amino groups on S1P. This prediction was tested by mutating the Arg-Glu-Gly motif to that conserved among LPA receptors (Arg-Gln-Gly), and the ligand selectivity of the mutant receptors ($S1P_1$ and LPA_1) was found to switch in concert with the mutations (Wang et al., 2001). Thus the hydrophilic "head group" of the lysophospholipids is thought to interact with the amino-terminal aspect of the third transmembrane region. However, the areas of the receptor protein that interact with the hydrophobic "tail" of the LPL ligands are uncertain at present.

VI. Lysophospholipid Receptor Gene Knockouts

The function of the LPL receptors has been explored by germ line ablation of the individual receptor genes. In addition to the zebrafish *miles apart* mutation mentioned above, the LPA_1, $S1P_1$, $S1P_2$, and $S1P_3$ receptor genes in mice have been "knocked out". The $S1P_3$ receptor −/− mice are without obvious phenotype (Ishii et al.,

2001) whereas some 20% of $S1P_2$ receptor −/− mice were reported to experience at least one epileptic seizure between 3 and 6 weeks of age (MacLennan et al., 2001). The $S1P_1$ receptor −/− mice die about gestational age E13 from failure of the vasculature to become invested with smooth muscle (Liu et al., 2000). Mice lacking a functional LPA_1 receptor gene are born but have a defect in their suckling behavior—apparently because of a defect in olfaction—that in turn results in increased neonatal mortality and stunted growth of survivors (Contos et al., 2000). Analyses of the phenotypes of mice with the remaining four LPL receptor genes ablated are not yet published but are underway in several laboratories.

VII. Lysophospholipid Receptor Expression

Finally, the expression of each of the LPL receptor genes has been examined in rodents and humans. The general lack of high quality antibodies and high affinity radiolabeled ligands necessitates inferring receptor expression by measurement of accumulation of the cognate mRNAs. The LPA_1 receptor mRNA is restricted largely (in rodents) to myelinating glia including Schwann cells, but before birth the LPA_1 mRNA is abundant in developing cortical neurons (Hecht et al., 1996; Weiner et al., 1998). However, the human LPA_1 receptor mRNA is reported to be found in the extract of many tissues including heart, brain, colon, small intestine, and prostate but not in extracts of liver, lung, thymus, or leukocytes (An et al., 1998). LPA_2 receptor expression is most prominent in leukocytes (An et al., 1998) whereas the LPA_3 receptor mRNA is found in extracts of kidney, lung, heart, pancreas, and prostate (Bandoh et al., 1999; Im et al., 2000b). In mouse, all three LPA receptor type mRNAs are prominent in testes extracts (Contos and Chun, 2001). Among the S1P receptors, the $S1P_1$ receptor type is expressed ubiquitously as is the $S1P_3$ receptor (Yamaguchi et al., 1996). The $S1P_2$ receptor is expressed in embryonic brain and postnatally in brain, heart, lung, stomach, intestine, and adrenal gland (Okazaki et al., 1993; MacLennan et al., 1994). The $S1P_4$ receptor is unusual in that its expression is confined to lymphoid tissue (Gräler et al., 1998). Finally, the $S1P_5$ receptor mRNA is found in white matter and spleen in rats (Im et al., 2000a).

VIII. Conclusion

Although there is little likelihood that additional Edg cluster receptor genes will be found, their identification represents only the end of the beginning. The importance of the LPL mediators is evidenced by the steady increase of publications focused on these molecules. The application of selective ligands under development that mimic or block LPLs at their receptors will surely reveal a rich pathophysiology controlled by this signaling system.

References

An S, Bleu T, Hallmark OG, and Goetzl EJ (1998) Characterization or a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. *J Biol Chem* **273**:7907–7910.

An S, Bleu T, Huang W, Hallmark OG, Coughlin SR, and Goetzl EJ (1997) Identification of cDNAs encoding two G protein-coupled receptors for lysophospholipids. *FEBS Lett* **417**:279–282.

Bandoh K, Aoki J, Hosono H, Kobayashi S, Kobayashi T, Murakami-Murofushi K, Tsujimoto M, Arai H, and Inoue K (1999) Molecular cloning and characterization of a novel human G protein-coupled receptor, EDG7, for lysophosphatidic acid. *J Biol Chem* **274**:27776–27785.

Chun J, Contos JJA, and Munroe D (1999) A growing family of receptor genes for lysophosphatidic acid (LPA) and other lysophospholipids (LPs). *Cell Biochem Biophys* **30**:213–242.

Contos JJA and Chun J (2001) The mouse *lp$_{A3}$/jEdg7* lysophosphatidic acid receptor gene: genomic structure, chromosomal localization and expression pattern. *Gene (Amst)* **267**:243–253.

Contos JJA, Fukushima N, Weiner JA, Kaushai D, and Chun J (2000) Requirement for the lpA1 lysophosphatidic acid receptor gene in normal suckling behavior. *Proc Natl Acad Sci USA* **97**:13384–13389.

Fisher DJ, Nusser N, Virag T, Yokoyama K, Wang D, Baker DL, Bautista D, Parrill AL, and Tigyi G (2001) Short-chain phosphatidates are subtype-selective antagonists of lysophosphatidic acid receptors. *Mol Pharmacol* **60**:776–784.

Fitzgerald LR, Dytko GM, Sarau HM, Mannan IJ, Ellis C, Lane PA, Tan KB, Murdoch PR, Wilson S, Bergsma DJ, et al. (2000) Identification of an EDG7 variant, HOFNH30, a G protein-coupled receptor for LPA. *Biochem Biophys Res Comm* **273**:805–810.

Fukushima N, Ishii I, Contos JJA, Weiner JA, and Chun J (2001) Lysophospholipid receptors. *Annu Rev Pharmacol Toxicol* **41**:507–534.

Gräler MH, Bernhardt G, and Lipp M (1998) EDG6, a novel G protein-coupled receptor related to receptors for bioactive lysophospholipids, is specifically expressed in lymphoid tissue. *Genomics* **53**:164–169.

Guo Z, Liliom K, Fisher DJ, Bathurst IC, Tomei ID, Kiefer MC, and Tigyi G (1996) Molecular cloning of a high affinity receptor for the growth factor-like mediator lysophosphatidic acid from *Xenopus* oocytes. *Proc Natl Acad Sci USA* **93**:14367–14372.

Hecht JH, Weiner JA, Post SR, and Chun J (1996) Ventricular zone gene-1 (*vzg-1*) encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex. *J Cell Biol* **135**:1071–1083.

Heise CE, Santos WL, Schreihofer AM, Heasley BH, Mukhin YV, Macdonald TL, and Lynch KR (2001) Activity of 2-substituted LPA analogs at LPA receptors: discovery of a LPA1/LPA3 receptor antagonist. *Mol Pharmacol* **60**:1173–1180.

Hla T (2001) Sphingosine 1-phosphate receptors. *Prostaglandins Other Lipid Mediat* **64**:135–142.

Hla T, Lee MJ, Ancellin N, Paik JK, and Kluk MJ (2001) Lysophospholipids—receptor revelations. *Science (Wash DC)* **294**:1875–1878.

Hla T and Maciag TJ (1990) An abundant transcript induced in differentiating human endothelial cells encodes a polypeptide with structural similarities to G protein coupled receptors. *J Biol Chem* **265**:9308–9313.

Hla T, Zimrin AB, Evans M, Ballas K, and Maciag T (1997) The immediate-early gene product MAD-3/EDG-3/IkappaB alpha is an endogenous modulator of fibroblast growth factor-1 (FGF-1) dependent human endothelial cell growth. *FEBS Lett* **414**:419–424.

Humphrey PPA, Barnard EA, Bonner TI, Catterall WA, Dollery CT, Fredholm BB, Godfraind T, Harmar AJ, Langer SZ, Lauder V, et al. (2000) The IUPHAR receptor code, in *The IUPHAR Compendium of Receptor Characterization and Classification*, 2nd ed, pp 9–23, IUPHAR Media, London.

Im DS, Heise CE, Ancellin N, O'Dowd BF, Shei GJ, Heavens RP, Rigby MR, Hla T, Mandala S, McAllister G, et al. (2000a) Characterization of a novel sphingosine 1-phosphate receptor, Edg-8. *J Biol Chem* **275**:14281–14286.

Im DS, Heise CE, Harding MA, George SR, O'Dowd BF, Theodorescu D, and Lynch KR (2000b) Molecular cloning and characterization of a lysophosphatidic acid receptor, Edg-7, expressed in prostate. *Mol Pharmacol* **57**:753–759.

Ishii I, Friedman B, Ye X, Kawamura S, McGiffert C, Contos JJ, Kingsbury MA, Zhang G, Heller Brown J, and Chun J (2001) Selective loss of sphingosine 1-phosphate signaling with no obvious phenotypic abnormality in mice lacking its G protein-coupled receptor, LP(B3)/EDG-3. *J Biol Chem* **276**:33697–33704.

Kabarowski JH, Zhu K, Le LQ, Witte ON, and Xu Y (2001) Lysophosphatidylcholine as a ligand for the immunoregulatory receptor G2A. *Science (Wash DC)* **293**:702–705.

Kupperman E, An S, Osborne H, Waldron S, and Stainier DYR (2000) A sphingosine 1-phosphate receptor regulates cell migration during vertebrate heart development. *Nature (Lond)* **406**:192–195.

Lee MJ, van Brocklyn JR, Thangada S, Liu CH, Hand AR, Menzeleev R, Spiegel S, and Hla T (1998) Sphingosine 1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science (Wash DC)* **279**:1552–1555.

Liu Y, Wada R, Yamashita T, Mi Y, Deng CX, Hobson JP, Rosenfeldt HM, Nava VE, Chae SS, Lee MJ, et al. (2000) Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *J Clin Investig* **106**:951–961.

Lynch KR and Macdonald TL (2001) Structure activity relationships of lysophospholipid mediators. *Prostglandins Other Lipid Mediat* **64**:33–45.

MacLennan AJ, Browe CS, Gaskin AA, Lado DC, and Shaw G (1994) Cloning and characterization of a putative G protein coupled receptor potentially involved in development. *Mol Cell Neurosci* **5**:201–209.

MacLennan AJ, Carney PR, Zhu WJ, Chaves AH, Garcia J, Grimes JR, Anderson KJ, Roper SN, and Lee N (2001) An essential role for the H218/AGR16/Edg-5/LP_{B2} sphingosine 1-phosphate receptor in neuronal excitability. *Eur J Neurosci* **14**:203–209.

Macrae AD, Premont RT, Jaber M, Peterson AS, and Lefkowitz RJ (1996) Cloning,

characterization and chromosomal localization of rec1.3, a member of the G-protein-coupled receptor family highly expressed in brain. *Mol Brain Res* **42**:245–254.

Masana MI, Brown RC, Pu H, Gurney ME, and Dubocovich ML (1995) Cloning and characterization of a new member of the G protein-coupled receptor EDG family. *Receptors Channels* **3**:255–262.

Moolenaar WH (1999) Bioactive lysophospholipids and their G protein-coupled receptors. *Exp Cell Res* **253**:230–238.

Okazaki H, Ishizaka N, Sakurai T, Kurokawa K, Goto K, Kumada M, and Takuwa Y (1993) Molecular cloning of a novel putative G protein coupled receptor expressed in the cardiovascular system. *Biochem Biophys Res Comm* **190**:1104–1109.

Parrill AL, Wang DA, Bautista DL, van Brocklyn JR, Lorincz Z, Fischer DJ, Baker DL, Liliom K, Spiegel S, and Tigyi GJ (2000) Identification of Edg1 receptor residues that recognize sphingosine 1-phosphate. *J Biol Chem* **275**:39379–39384.

Pyne S and Pyne N (2000) Sphingosine 1-phosphate signalling in mammalian cells. *Biochem J* **349**:385–402.

Ruffolo RR, Humphrey PPA, Watson SP, and Spedding M (2000) Revised NC-IUPHAR recommendations for nomenclature of receptors, in *The IUPHAR Compendium of Receptor Characterization and Classification*, 2nd ed, pp 7–8, IUPHAR Media, London.

Tigyi G (2002) *Biochim Biophys Acta*, in press.

Tokumura A, Fukuzawa K, Akamatsu Y, Yamada S, Suzuki T, and Tsukatani H (1978) Identification of vasopressor phospholipid in crude soybean lecithin. *Lipids* **13**:468–472.

van Brocklyn JR, Gräler MH, Bernhardt G, Hobson JP, Lipp M, and Spiegel S (2000) Sphingosine 1-phosphate is a ligand for the G protein-coupled receptor EDG-6. *Blood* **95**:2624–2629.

van Corven EJ, Groenink A, Jalink K, Eichholtz T, and Moolenaar WH (1989) Lysophosphatidate-induced cell proliferation: identification and dissection of signaling pathways mediated by G proteins. *Cell* **59**:45–54.

Wang DA, Lorincz Z, Bautista DL, Liliom K, Tigyi G, and Parrill AL (2001) A single amino acid determines lysophospholipid specificity of the S1P1 (EDG1) and LPA1 (EDG2) phospholipid growth factor receptors. *J Biol Chem* **276**:49213–49220.

Weiner JA, Hecht JH, and Chun J (1998) Lysophosphatidic acid receptor gene *vzg-1/lp$_{A1}$/edg-2* is expressed by mature oligodendrocytes during myelination in the postnatal murine brain. *J Comp Neurol* **398**:587–598.

Xu Y, Zhu K, Hong G, Wu W, Baudhuin LM, Xiao YJ, and Damron DS (2000) Sphingophosphorylcholine is a ligand for ovarian cancer G protein coupled receptor 1. *Nat Cell Biol* **2**:261–267.

Yamaguchi F, Tokuda M, Hatase O, and Brenner S (1996) Molecular cloning of the novel human G protein-coupled receptor (GPCR) gene mapped on chromosome 9. *Biochem Biophys Res Comm* **227**:608–614.

Zhang H, Desai NN, Olivera A, Seki T, Brooker G, and Spiegel S (1991) Sphingosine 1-phosphate, a novel lipid, involved in cellular proliferation. *J Cell Biol* **114**:155–167.

Zondag GCM, Postma FR, van Ettern I, Verlaan I, and Moolenaar WH (1998) Sphingosine 1-phosphate signalling through the G protein coupled receptor, Edg-1. *Biochem J* **330**:605–609.

ELSEVIER

Biochimica et Biophysica Acta 1582 (2002) 161–167

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Review

Lysophospholipid mediators of immunity and neoplasia

Mei-Chuan Huang [a], Markus Graeler [a], Geetha Shankar [b], Juliet Spencer [b], Edward J. Goetzl [a,*]

[a] *UB8B, UC Box 0711, Departments of Medicine and Microbiology–Immunology, University of California Medical Center, 533 Parnassus at 4th Avenue, San Francisco, CA 94143-0711, USA*
[b] *Ceretek, LLC, Alameda, CA 94502, USA*

Received 30 January 2002; accepted 30 January 2002

Abstract

Lysophosphatidic acid (LPA), sphingosine 1-phosphate (S1P) and some other structurally related lysophospholipids are active growth factors and stimuli for diverse cellular functions. LPA and S1P promote early T cell migration to tissue sites of immune responses and regulate T cell proliferation and secretion of numerous cytokines. Edg-4 (LPA_2) LPA receptors, which are constitutively expressed by helper T cells, and Edg-2 (LPA_1) LPA receptors, which are expressed only by activated helper T cells, transduce opposite effects of LPA on some T cell responses. A similar mechanism is observed for fine regulation of Edg R-mediated effects of LPA on ovarian cancer cells. Edg-4 (LPA_2) R transduces proliferative responses, recruitment of autocrine protein growth factors, and migration of ovarian cancer cells, whereas Edg-2 (LPA_1) R transduces inhibition of Edg-4 (LPA_2) R-mediated responses and concurrently elicits apoptosis and anoikis of ovarian cancer cells. Edg-4 (LPA_2) R is a distinctive functional marker for ovarian carcinoma, and is expressed both as the wild-type and a carboxyl-terminally extended gain-of-function mutant. Newly discovered non-lipid agonists and antagonists for individual Edg receptors will permit more sophisticated analyses of their respective contributions in human biology and pathophysiology, and may represent novel therapeutic modalities in immune disorders and cancer.

Keywords: T cell; G protein-coupled receptor; Cytokine; Growth factor; Serum response element; Ovarian cancer

1. Introduction

It has been known for decades that biologically active lysophospholipids (LPLs) are generated by many types of mammalian cells and have diverse effects on growth and functions of most cells in multiple organ systems [1–4]. Lysophosphatidic acid (LPA) is the most prominent member of the lysoglycerol-containing phospholipid subfamily, which predominates quantitatively among lipid structural components of cellular membranes. Sphingosine-1-phosphate (S1P) is a highly active lysosphingophospholipid, which is structurally and functionally related to LPA. The subfamily of cellular lysosphingophospholipids are quantitatively diminutive in contrast to the subfamily of lysoglycerophospholipids, but exhibit great structural complexity and have a range of biological effects similar to those of lysoglycerophospholipids. Alkenyl- and cyclic-variants of LPA, sphingosylphosphorylcholine, and lysophosphatidylcholine are other naturally occurring LPLs which elicit many cellular responses. These LPL mediators also are related by being products of metabolism of cellular membrane phospholipid components, increasing in concentration transiently in relation to cellular responses, requiring carrier proteins for extracellular transport and cellular presentation, moving and interacting with proteins in cellular membranes, and potently influencing cells through one or more subfamilies of G protein-coupled receptors (GPCRs) [5,6]. Eicosanoid mediators derived from arachidonic acid, such as prostaglandin E2 (PGE2) and leukotriene B4 (LTB4), resemble both major subtypes of LPLs in being products of metabolism of cellular membrane phospholipids, increasing in extracellular concentration during activation of many types of cells, and transducing cellular signals through distinct subfamilies of GPCRs [7]. However, the PGs and LTs are principally mediators of a range of cellular functions other than proliferation, whereas the LPLs are active growth factors, extracellular mediators of some other cellular activities and, in a few instances, intracellular messengers.

Numerous important aspects of cellular responses to LPLs as intracellular messengers and extracellular mediators

* Corresponding author. Tel.: +1-415-476-5339; fax: +1-415-476-6915.
E-mail address: egoetzl@itsa.ucsf.edu (E.J. Goetzl).

1388-1981/02/$ - see front matter
PII: S1388-1981(02)00151-8

have been reviewed recently [8] and will be described in several sections of the present volume. Our knowledge of every aspect of the cellular recognition and effects of LPLs has progressed recently through the definition of structures and signaling pathways of the many members of a novel subfamily of endothelial differentiation gene (edg)-encoded GPCRs (Edg Rs) dedicated to LPLs. As for PG and LT GPCRs, each Edg R couples to two or more different types of G proteins resulting in complex patterns of signal transduction. This section will focus on sources and effects of LPLs in immune cells and cancer cells, and on the regulation of expression and signal transduction of Edg Rs in T cells and ovarian cancer cells.

2. Generation of lysophospholipid mediators by immune cells: similarities to cellular specificities of production of PGs and LTs in the immune system

The nature and amounts of LPLs produced by multienzymatic pathways of the major types of immune cells are presently under investigation. In cells of other systems, the synthesis and secretion of LPA and S1P are determined by the origin, basic type, proliferative state, and level of functional activation of the cells. Platelets, some epithelial cells, and some tumor cells are proven major sources of both LPA and S1P. Although fibroblasts responding to protein growth factors produce LPA and S1P, only small amounts appear to be released into extracellular fluids. Mononuclear phagocytes may generate LPLs, but assignment of the source of these products has been complicated by the frequent association of other cells or fragments of cells with monocytes and macrophages. Examples are the rosetting of platelets around monocytes and the presence of fragments of platelets and other leukocytes in monocytes and macrophages. This is an especially important problem because large quantities of LPA and probably S1P are synthesized by cellular fragments at interfaces between the plasma membranes and extracellular fluid. LPA is generated at such membrane sites both by secretory phospholipase A2 hydrolysis of PA exposed on the microvesicles from activated platelets and leukocytes, and by phospholipase D cleavage of leukocyte-surface lysophosphatidyl-choline [9,10]. LPL generation from non-malignant cells is initiated by physical perturbation or by stimulation of plasma membrane receptors or other surface proteins, which activate critical controlling enzymes such as phospholipases and sphingosine kinase. In contrast, tumor cells may secrete LPLs spontaneously, which was described first for LPA production by ovarian cancers [11].

Characterization of cellular production of LPLs under physiological conditions is limited to only a few examples. Platelet adhesion to vascular surfaces and homotypic aggregation induced by thrombin result in generation of both LPA and S1P in quantities sufficient to raise effective LPL concentrations up to 1–5 μM [12,13]. Alpha$_2$-adrenergic stimulation of adipocytes provokes release of LPA at concentrations capable of inducing proliferation, spreading and maturation of preadipocytes [14]. The elevated level of production of LPA by ovarian carcinoma cells leads to concentrations in ascites of up to 20 μM and even up to 10-fold higher than normal in plasma of patients, in direct

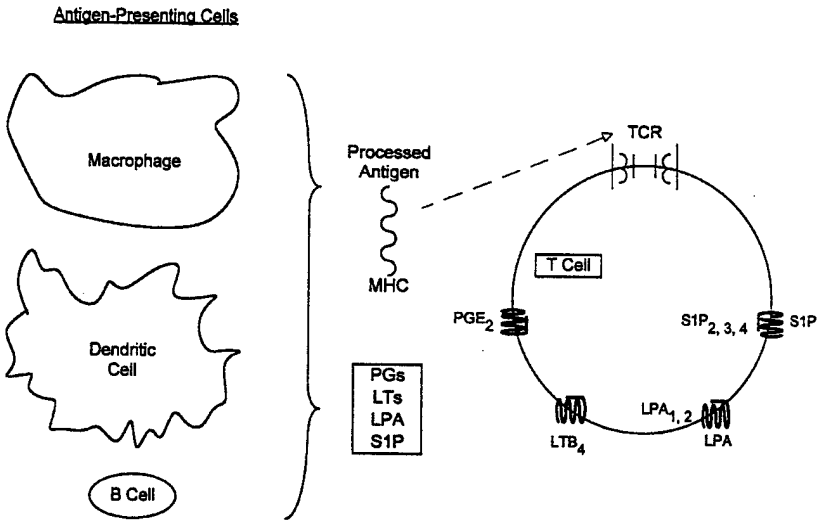

Fig. 1. Lipid mediators in T cell-dependent immune responses. Two shared functions of the different antigen-presenting cells (APCs) depicted (left) are: (a) antigen processing and presentation to T cell receptors (TCRs) in association with APC major histocompatibility complexes (MHCs), and (b) generation and secretion of diverse lipid mediators, including the eicosanoid subfamilies of prostaglandins (PGs) and leukotrienes (LTs), and the lysophospholipids (LPLs) lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P). T cells (right) express G protein-coupled receptors (GPCRs) for PGE2, LTB4, LPA and S1P.

relationships with the stage of disease and tumor burden [11]. The early data from incomplete analyses of subtypes of immune cells suggest that mononuclear phagocytes, some types of dendritic cells, and B cells generate LPA and S1P, whereas there is little or no detectable output from T cells. This pattern follows exactly what has been observed for the generation of eicosanoid mediators by immune cells (Fig. 1). The antigen-presenting cells (APC), including macrophages, dendritic cells, and B cells, which process antigens for presentation to the T cell receptor in the context of specific major histocompatibility complex (MHC) antigens appear to produce LPA and S1P, as well as an array of eicosanoids, such as prostaglandin E2 (PGE2) and leukotriene B4 (LTB4). In contrast, the T cells secrete little or none of any of these lipid mediators. T cells, especially those of the helper subset (Th), express high levels of GPCRs specific for each of these lipid mediators (Fig. 1). Thus, APCs may signal T cells not only through antigen-dependent mechanisms involving the TCR and a variety of co-receptors, but also by secreting lipid mediators recognized by T cell GPCRs capable of altering a wide range of T cell functions critical for immune recruitment and activation.

3. T cell recognition and effects of lipid mediators

The responses of T cells of diverse subsets to PGs and LTs have been reviewed extensively [7], so that the present focus will be on LPA and S1P. Human T cell lymphoblastomas and some sets of mouse splenic T cells express all of the Edg Rs albeit at substantially differing levels [15]. In contrast, human blood T cells bear a very restricted representation of Edg Rs for LPA and S1P (Fig. 2). Freshly isolated normal human blood Th cells showed predominantly Edg-4 Rs (LPA2) and Edg-6 Rs (S1P4), with traces of Edg-3 (S1P3) in some individuals [16]. Suppressor T cells (Ts) from blood of the same normal subjects had no detectable Edg Rs. After activation with a mitogenic lectin or, to a lesser extent, with a combination of anti-CD3 plus anti-CD28 monoclonal antibodies, the pattern of expression of Edg Rs changes rapidly and significantly [17] (Fig. 2). The level of Edg-4 Rs in Th cells decreases by approximately 50%, that of Edg-6 Rs also decreases, and Edg-3 Rs disappear, as assessed by semi-quantitative RT-PCR and Western blots. There is a concurrent appearance of Edg-2 Rs in Th cells at a level less than or equal to that of Edg-4 Rs. Barely detectable levels of Edg-2 and Edg-5 Rs develop in Ts cells. Thus, T cell expression of Edg LPA and S1P Rs is highly flexible, in relation to the source and state of functional activation of the T cells.

Equally impressive modifications in Th cell functional responses to LPLs are observed after exposure to mitogens [17] (Fig. 2). The responses to LPA have been examined in detail, but those evoked by S1P are still under investigation. Prior to mitogen stimulation, LPA elicits Th cell migration through a connective tissue-like barrier by enhancing chemokinetic mobility and augmenting secretion of matrix metalloproteinases (MMPs) of the gelatinase subfamily. This response is presumed to be transduced by the Edg-4 (LPA2) R, as agonist-like mouse monoclonal anti-Edg-4 R IgG antibodies elicit migration of freshly isolated Th cells similarly to LPA. LPA has an inhibitory effect on IL-2

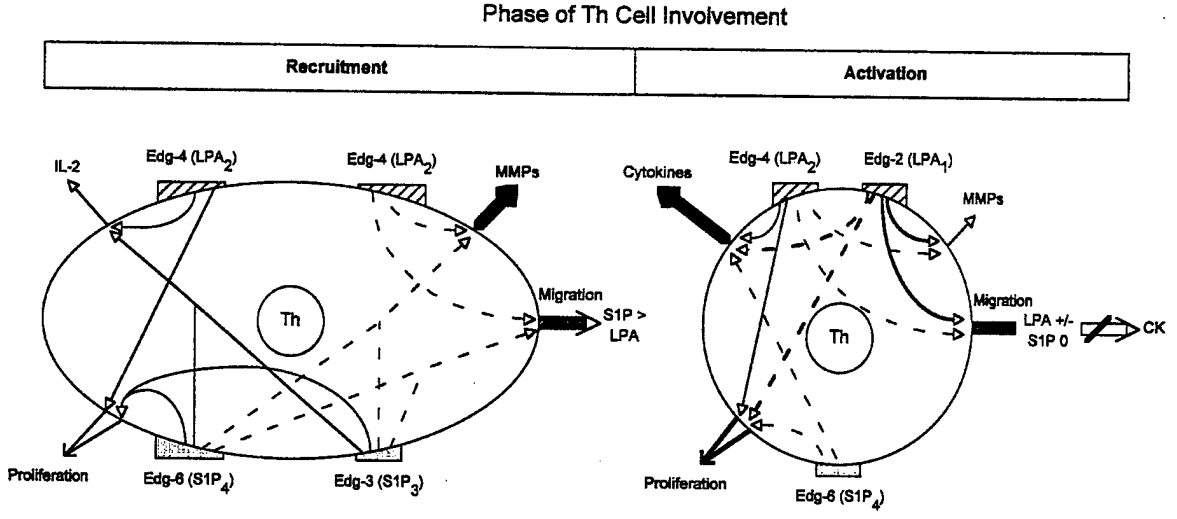

Fig. 2. Edg receptor transduction of effects of LPLs on helper T cell (Th cell) recruitment (left side) to sites of immune responses and functional activation (right side). The terms for Edg Rs recommended by the nomenclature subcommittee of the IUPHAR are shown in parentheses. The width of the bar depicting each Edg R represents its relative frequency of expression and the weight of each line portrays the magnitude of stimulation (dashed lines) or inhibition (solid lines). MMP = matrix metalloproteinase, CK = chemokine.

generation and secretion by freshly isolated Th cells incubated with a combination of adherent anti-CD3 plus anti-CD28 monoclonal antibodies, which again is reproduced by agonist-like mouse monoclonal anti-Edg-4 R IgG antibodies [16]. S1P effects resemble those of LPA in the freshly isolated Th cells, but results have not delineated the respective roles of Edg-3 and Edg-6 Rs (Fig. 2). The function-directed activities of LPA are reversed after mitogenic activation of Th cells. An apparent transductional dominance of the newly induced Edg-2 Rs over the remaining Edg-4 Rs results in a lack of direct stimulation of Th cell migration, but significant inhibition of Th cell chemotactic responses to chemokine stimulation [16,17]. This new predominance of Edg-2 R signals over those from Edg-4 Rs results in striking net enhancement of Th cell generation and secretion of IL-2 and of Th cell proliferation (Fig. 2). The present data for S1P effects on mitogen-activated Th cells, which are presumed to be mediated by Edg-6 Rs, are limited to an unexplained minor enhancement of IL-2 secretion.

As the activated Th cells express Edg-2 and Edg-4 Rs co-dominantly, but no subset of native T cells expresses Edg-2 R alone, a set of Jurkat T cells were generated by selection-stabilized transfection of different series of plasmids encoding sense and antisense messages for Edg Rs to attain lines which expressed predominantly Edg-2 or Edg-4 Rs, as assessed by Western blots [18]. In the Edg-4 R-dominant Jurkat T cells, LPA evoked migration and suppressed IL-2 production, whereas in Edg-2 R-dominant Jurkat T cells, LPA did not evoke migration, suppressed chemokine-elicited migration, and enhanced IL-2 generation. Thus, the effects of LPA in Edg-4 R-only and Edg-2 R-only Jurkat T cells exactly mimicked those observed, respectively, in freshly isolated Th cells and mitogen-activated Th cells (Fig. 2). When viewed in terms of the recruitment and activation stages of Th cell involvement in immune responses, the first effects of LPA and probably S1P are to facilitate Th cell movement to the site of an immune response, while suppressing other reactions such as cytokine secretion, which might be injurious to normal cells in the path of their migration. After their arrival at the site of an immune response, further Th cell migration capable of reducing the population of necessary effector Th cells is suppressed by LPA, and effector reactions including cytokine generation are augmented by LPA (Fig. 2). These opposing effects of LPA transduced by Edg-2 and Edg-4 Rs suggest one advantage of multiple Edg Rs specific for the same ligand in precise regulation of complex cellular responses. Similar opposing functions of Edg-2 and Edg-4 Rs have also been observed in some tumor cells.

4. Edg R expression and effects in ovarian cancer

The findings of much higher concentrations of LPA in plasma of patients with ovarian cancer than in plasma of most patients with cancer of other types and all normal subjects motivated a series of related investigations [11]. It was next demonstrated that cultured human ovarian cancer cells (OCCs), but not breast cancer cells or a variety of other human tumor cells, produce high levels of LPA. Semi-quantitative RT-PCR analyses and Western blots of Edg Rs in many lines of human OCCs revealed expression of Edg-4 R (LPA2) in all but one and Edg-7 R (LPA3) in 75% [19]. Identical studies of normal ovarian surface epithelial cells (OSEs) and those immortalized by introduction of SV40 virus T antigen (IOSEs) showed no Edg-4 R, and only low levels of Edg-7 R. The most inconsistent pattern was for Edg-2 R (LPA1), which had a wide range of expression in OCCs from about 60% to 220% of the mean for OSEs and IOSEs. Most S1P Rs were found in OCCs with no distinctive pattern, but the levels generally were lower than in OSEs and IOSEs.

Edg R probe analyses of arrays of paired samples of nucleic acids from ovarian tumor tissues and adjacent normal tissues of the same patient were performed using the Cancer Profiling Array (Clontech, Palo Alto, CA). The arrays contained cDNA pairs from 13 different tumors and paired normal tissues, including breast, uterus, colon, stomach, ovary, lung, kidney, rectum, thyroid gland, prostate, small intestine, pancreas, and cervix. Edg-4 R and Edg-7 R expression were upregulated in tumor tissues of >80% of cancer patient samples tested relative to the corresponding level in adjacent normal tissue. The mean ratios of expression for both Edg LPA receptors in tumor to normal tissues were 2.0 or higher in the majority of all tumors and were highest in ovarian tumors, where many ratios were at or over 3.0. In contrast, there was no significant elevation of expression of Edg-4 or Edg-7 Rs in stomach and kidney cancer. There were also no significant changes in expression of Edg S1P receptors. As for the results with ovarian cell cultures, tissue ratios for Edg-2 R also showed the widest range but overall mean expression in ovarian cancers was decreased by nearly 60%, as compared with the matched normal samples.

LPA stimulated proliferation of OCCs, but not IOSEs, which was reproduced by agonist-like mouse monoclonal anti-Edg-4 R antibody, as assessed by increased uptake of ^{3}H-thymidine [19]. Increases in proliferation attributable to Edg-4 and/or Edg-7 R signaling were confirmed by responses in OCCs transfected with a serum response element (SRE)-luciferase reporter plasmid [19] (Fig. 3). The SRE reporter responses were suppressed by MEK kinase inhibitors and by Pertussis toxin. That the greatest proliferative responses were seen after 4–5 days of stimulation suggested involvement of autocrine protein growth factors released by the stimulated OCCs. LPA was shown to evoke release of type II insulin-like growth factor (IGF-II) in amounts capable of eliciting OCC proliferative responses and SRE reporter responses. Further, two types of antibodies which neutralize IGF-II or block the type 1 IGF receptor suppressed the proliferative responses of OCCs

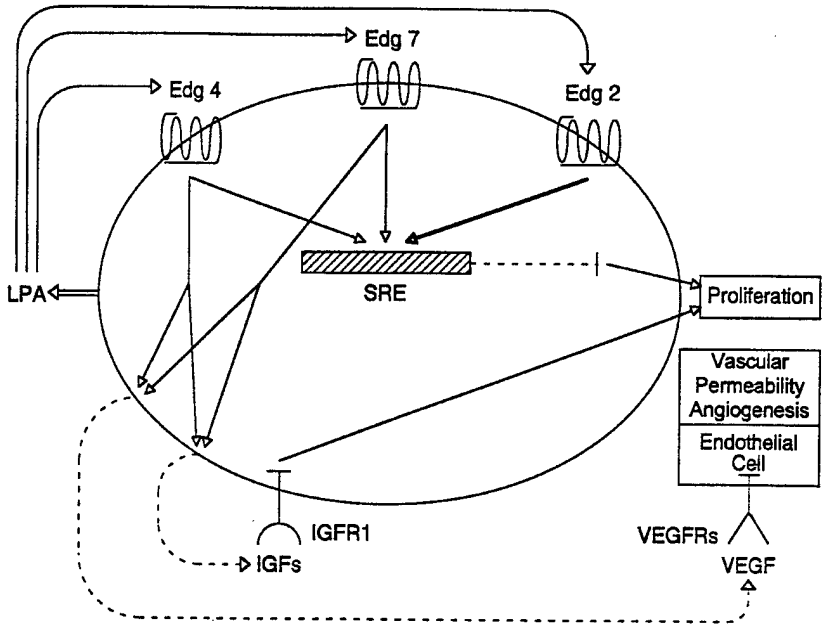

Fig. 3. Pathogenetic roles of lysophospholipid growth factors and Edg receptors in ovarian cancer. SRE = serum response element, IGFs = insulin-like growth factors, IGFR1 = type 1 receptor for IGFs, single solid line = stimulation, bold line = inhibition, double solid line and dashed line = secretion.

[19] (Fig. 3). LPA also evoked the release of vascular endothelial growth factor (VEGF) by OCCs by a stimulatory action on transcription [20]. Thus, Edg Rs transduce three different effects of LPA on OCCs (Fig. 3). Proliferation is stimulated directly through an SRE-dependent mechanism and indirectly through augmented release of IGFs. LPA also promotes angiogenesis and enhanced vascular permeability in ovarian cancer by amplifying secretion of VEGF.

5. A functionally distinctive mutant form of Edg-4 R in OCCs

In addition to the altered pattern of expression of Edg Rs and recruitment of autocrine protein growth factors in ovarian cancer, which both augment the proliferative and transforming responses to LPA, some OCCs have a gain-in-function mutant of Edg-4 R [21]. Although studies of the frequency of expression of this mutant Edg-4 R and its level of representation relative to wild-type Edg-4 R are still in progress, it now appears that 60–70% of ovarian cancers express mutant Edg-4 R at a level far lower than that of the wild-type Edg-4 R. A loss of one G in the sequence encoding the carboxy-terminal tail of Edg-4 R results in a frame-shift disrupting the termination codon and leading to carboxy-terminal elongation by 33 amino acids [21]. Since the extracellular and cytoplasmic loops of Edg-4 Rs are small, it is assumed that many transduction factors dock on the intracellular tail, as has been demonstrated for other structurally similar GPCRs. Matched sets of Edg-4 R transfectants, each member of which expresses an identical level of either mutant Edg-4 R or wild-type Edg-4 R in the same endogenous Edg R-null cell line, have been examined for their respective responses to LPA. In the endogenous Edg R-null HTC4 rat hepatoma cell line, human wild-type Edg-4 R and mutant Edg-4 R transduce LPA-evoked Ca^{2+} responses which are similar in magnitude and time-course. In contrast, several integrated functional responses of HTC4–mutant Edg-4 R transfectants differ substantially from those of HTC4–wild-type Edg-4 R transfectants. The production of VEGF by HTC4–mutant Edg-4 R cells is significantly higher than that by HTC4–wild-type Edg-4 R cells after 48 and 72 h of incubation, but not after 24 h, without exogenous LPA (Fig. 4). The addition of LPA at a high concentration further enhances production of VEGF by HTC4–mutant Edg-4 R cells, but not by HTC4–wild-type Edg-4 R cells, after 72 h. The differences between levels of secretion of immunodetectable VEGF by the two cell lines are highly significant and predict major functional distinctions as well. The Edg-4 R signaling mechanisms remain to be elucidated. That the difference in VEGF secretion between the two types of HTC4 cells increases with incubation time suggests the possibility of a positive feedback loop, in which mutant Edg-4 R may elicit greater secretion of LPA by HTC4 cells than the wild-type Edg-4 R in addition to greater signaling of VEGF production. Future studies will be designed to delineate the respective contributions of higher LPA output and more efficient signal

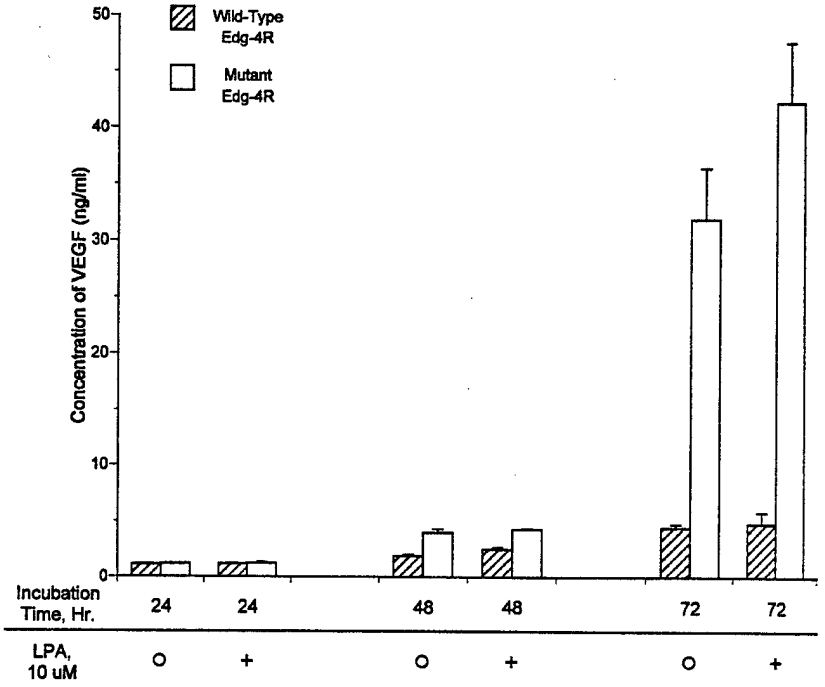

Fig. 4. Edg-4 receptor subtype-dependence of stimulation of VEGF secretion by HTC4 cell transfectants. Each bar symbol depicts the mean ± S.D. of the results of two studies.

transduction to the greater VEGF production by HTC4–mutant Edg-4 R cells, and to the other increased functions of these cells.

6. Regulation by Edg-2 Rs of the OCC responses to LPA

The findings of opposing T cell effects of LPA transduced by Edg-4 and Edg-2 Rs indicated the possibility of a similar antagonistic relationship for some responses of OCCs to LPA. This hypothesis was assessed in two different types of OCCs, the line of A2780 cells which express Edg-4 and -7 but only a very low level of Edg-2 Rs and OV202 primary OCCs, which naturally co-express Edg-4 and -7 Rs at levels similar to those of A2780 cells but also have a high level of Edg-2 Rs. LPA-evoked proliferation of the A2780 OCCs was significantly higher than that of OV202 OCCs, as quantified by uptake of ^{3}H-thymidine (Table 1). The SRE-luciferase signals elicited by LPA, which reflect proliferative responses, also were greater in A2780 than OV202 OCCs. To more directly implicate a suppressive influence of Edg-2 Rs, HTC4–wild-type Edg-4 R cells were transfected with human Edg-2 Rs and the proliferative and SRE-luciferase responses to LPA compared for the two types of cells (Table 1). The introduction of Edg-2 Rs significantly suppressed both ^{3}H-thymidine uptake and the SRE-luciferase signal. Thus, Edg-2 and Edg-4 Rs may transduce opposing effects of LPA in OCCs as well as in T cells. The results of other investigators indicate that Edg-2 Rs, but not Edg-4 Rs, also decrease the viability

Table 1
Edg-2 R mediation of effects of LPA on proliferation and SRE activation of ovarian cancer cells

	Native OCCs		Transfectants	
	Edg-4/-7 Rs	Edg-4/-7 Rs + Edg-2 Rs	Edg-4 Rs alone	Edg-4 Rs + Edg-2 Rs
	(all with 10 nM LPA)			
Proliferation (mean CPM; $n=3$)	5679	2345	4133	1853
SRE-luciferase activity (mean RLUs) ($n=3$)	12,367	4672	9527	3088

CPM = net increase in counts/min of ^{3}H-thymidine uptake after 4 days, corrected for uptake by cells without LPA; RLU = net increase in relative light units from luciferase generated after 24 h of LPA stimulation, corrected for that observed without LPA. The native OCCs are A2780 (left column) and OV202 (right column), and the transfected cell lines are rat HTC4 cells.

of OCCs by the two mechanisms of apoptosis and anoikis [22].

Acknowledgements

The authors are grateful to Yvonne Kong for expert laboratory studies and to Robert Chan for preparation of graphics.

The research described was supported by grant HL-31809 from the National Institutes of Health and grant CRP 1 PF0265 from the California Department of Health Services.

References

[1] S. Spiegel, S. Milstien, J. Membr. Biol. 146 (1995) 225–237.
[2] W.H. Moolenaar, O. Kranenburg, F.R. Postma, G.C. Zondag, Curr. Opin. Cell Biol. 9 (1997) 168–173.
[3] E.J. Goetzl, S. An, FASEB J. 12 (1998) 1589–1598.
[4] S. An, E.J. Goetzl, H. Lee, J. Cell. Biochem. Suppl. 30–31 (1998) 147–157.
[5] K.R. Lynch, D.S. Im, Trends Pharmacol. Sci. 20 (1999) 473–475.
[6] E.J. Goetzl, Prostaglandins 64 (2001) 11–20.
[7] E.J. Goetzl, S. An, W.L. Smith, FASEB J. 9 (1995) 1051–1058.
[8] E.J. Goetzl, K.R. Lynch, Ann. N. Y. Acad. Sci. 905 (2000) 1–357.
[9] O. Fourcade, M.F. Simon, C. Viode, N. Rugani, F. Leballe, A. Ragab, B. Fournie, L. Sarda, H. Chap, Cell 80 (1995) 919–927.
[10] J.H. Exton, Ann. N. Y. Acad. Sci. 905 (2000) 61–68.
[11] Y. Xu, Z. Shen, D.W. Wiper, M. Wu, R.E. Morton, P. Elson, A.W. Kennedy, J. Belinson, M. Markman, G. Casey, JAMA, J. Am. Med. Assoc. 280 (1998) 719–723.
[12] T. Eichholtz, K. Jalink, I. Fahrenfort, W.H. Moolenaar, Biochem. J. 291 (Pt. 3) (1993) 677–680.
[13] Y. Yatomi, F. Ruan, S. Hakomori, Y. Igarashi, Blood 86 (1995) 193–202.
[14] P. Valet, C. Pages, O. Jeanneton, D. Daviaud, P. Barbe, M. Record, J.S. Saulnier-Blache, M. Lafontan, J. Clin. Invest. 101 (1998) 1431–1438.
[15] E.J. Goetzl, Y. Kong, B. Mei, J. Immunol. 162 (1999) 2049–2056.
[16] E.J. Goetzl, Y. Kong, J.K. Voice, J. Immunol. 164 (2000) 4996–4999.
[17] Y. Zheng, J.K. Voice, Y. Kong, E.J. Goetzl, FASEB J. 14 (2000) 2387–2389.
[18] Y. Zheng, Y. Kong, E.J. Goetzl, J. Immunol. 166 (2001) 2317–2322.
[19] E.J. Goetzl, H. Dolezalova, Y. Kong, Y.L. Hu, R.B. Jaffe, K.R. Kalli, C.A. Conover, Cancer Res. 59 (1999) 5370–5375.
[20] Y.L. Hu, M.K. Tee, E.J. Goetzl, N. Auersperg, G.B. Mills, N. Ferrara, R.B. Jaffe, J. Natl. Cancer Inst. 93 (2001) 762–768.
[21] S. An, T. Bleu, O.G. Hallmark, E.J. Goetzl, J. Biol. Chem. 273 (1998) 7906–7910.
[22] T. Furui, R. Lapushin, M. Mao, H. Khan, S.R. Watt, M.A. Watt, Y. Lu, X. Fang, S. Tsutsui, Z.H. Siddik, R.C. Bast, G.B. Mills, Clin. Cancer Res. 5 (1999) 4308–4318.

683.9

Role of Ser/Thr Protein Kinases in Melanocortin 3-Receptor Signaling

James M Wachira, Cleo A Hughes-Darden, Christopher V Taylor, T Joan Robinson. Dept of Biology, Morgan State University, 1700 E. Cold spring Lane, Baltimore, MD 21251

The melanocortin-3 receptor, MC3-R, is implicated in autonomic functions including some cardiovascular effects of melanocortins. The aim of this study is to characterize the signal transduction pathways that mediate neural melanocortin effects. Metabolism of intracellular Ca^{2+} was analyzed by dual fluorescence imaging of Fura-2AM loaded MC3-R CAD neuronal cells. An increase in intracellular Ca^{2+} was observed upon treatment with γ-2-melanocyte stimulating hormone (γ-2-MSH). A MC3-R-GFP fusion protein localized mainly to the plasma membrane in the perikarya and to neurites in differentiated CAD cells. Treatment with γ-2-MSH led to a punctate appearance and co-immunoprecipation of the receptor fusion protein with protein kinase C-γ (PKC-γ). Differentiation of some neuronal cells has been reported to be associated with a reciprocal change in the expression levels of protein kinase C and protein kinase A (PKA). Induction of CAD cell differentiation with serum deprivation was associated with down-regulation of PKA, the related protein kinase B (PKB/Akt1) and the atypical PKC-ζ that was less pronounced in MC3-R transfected cells. The levels of PKC-α, PKC-γ and PKC-β were unchanged. These studies indicate a role for PKC isozymes in γ-2-MSH/MC3-R receptor signaling and in neuronal cell differentiation.

Supported by NIH RIMI grant 5P20RR11606-05 and MARC grant GM02977-17

683.10

The p23 Co-chaperone Regulates Human Aryl Hydrocarbon (Dioxin) Receptor Signaling in a Yeast Model System

Marc B. Cox[1], Charles Albert Miller[2]. [1]Molecular and Cellular Biology Program, Tulane University Health Sciences Center, New Orleans, LA, [2]Environmental Health Sciences, Tulane University Health Sciences Center, 1430 Tulane Avenue, New Orleans, LA 70112

The aryl hydrocarbon receptor is a transcription factor that binds toxic ligands and requires the action of Hsp90 and cofactors for proper function. Aryl hydrocarbon receptor exists in a cytoplasmic complex with Hsp90, co-chaperone proteins, and p23 prior to ligand exposure. Whether p23 has a role in aryl hydrocarbon receptor signaling is unknown. In vitro studies suggest that p23 enhances the stability of aryl hydrocarbon receptor-Hsp90 complexes following ligand binding. This stabilization may involve p23's ATP-dependent interaction with the nucleotide-binding site of Hsp90. We developed a yeast model system that expressed functional human aryl hydrocarbon receptor and Arnt proteins to test whether p23 has a role in signaling. Deletion of the SBA1 gene (yeast p23 homolog) reduced ligand-mediated signaling in reporter gene assays. Furthermore, expression of human p23 fully restored aryl hydrocarbon signaling in the sba1 mutant. We investigated the role of p23 in a temperature sensitive hsp90 mutant strain that was defective in aryl hydrocarbon receptor signaling. The mutation in this strain maps to the ATP p23 binding pocket of hsp90, suggesting that the aryl hydrocarbon receptor signaling defect and temperature sensitivity may be due to impaired p23-Hsp90 interactions. In support of this hypothesis, we found that p23 overexpression suppressed both temperature sensitivity and aryl hydrocarbon receptor signaling defects in this hsp90 mutant.

683.11

Interactions between plasma membrane phospholipid scramblase 1 (PLSCR1) and the epidermal growth factor receptor (EGFR)

Jun Sun[1], Meera Nanjundan[1], Linda J Pike[2], Therese Wiedmer[1], Peter J Sims[1]. [1]Dept. of Molecular & Experimental Medicine, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, [2]Dept. of Biochemistry & Molecular Biophysics, Washington University, St. Louis, MI

We have identified physical and functional interactions between the EGFR and PLSCR1, an endofacial plasma membrane protein proposed to affect phospholipid organization. Stimulation of KB cells with EGF transiently elevated the Tyr-phosphorylation of PLSCR1, peaking at 5 minutes. Abl inhibitor STI571 partially reduced phosphorylation of PLSCR1, a known substrate of c-Abl, but did not substantially affect its EGF-dependent phosphorylation, suggesting PLSCR1 is also a substrate of EGFR tyrosine kinase, or another EGF-activated kinase. Physical association of EGFR with PLSCR1, coinciding with increased phosphorylation, was confirmed by Western blotting, following immunoprecipitation of either EGFR or PLSCR1. Furthermore, EGFR and PLSCR1 were both found to partition into membrane lipid rafts. Immunofluorescence studies indicated that both EGFR and PLSCR1 underwent rapid endocytosis following EGF stimulation. Whereas subsequent ubiquitination and degradation of EGFR was confirmed, no ubiquitination of PLSCR1 was detected, with subsequent redistribution of PLSCR1 antigen suggesting its recycling to the plasma membrane. [Supported by NIH (HL36946, HL61200, HL63819) & Leukemia & Lymphoma Society (5071-02)].

683.12

Bidirectional Regulation of Expression of the G protein – coupled Edg-5 Receptor for Sphingosine 1-Phosphate (S1P) in Human MDA-MB-453 Breast Cancer Cells (BCCs).

Hana Dolezalova, Edward J Goetzl. Medicine, UC San Francisco, 533 Parnassus Ave. Room UB8B. San Francisco, CA 94143-0711

BCCs express endothelial differentiation gene - encoded receptors (Edg Rs) Edg-3 and Edg-5 for the S1P growth factor, which elicits increases in [Ca++]i and stimulates serum response element (SRE) transcriptional activity in BCCs transiently transfected with pSRE-luciferase plasmid. Incubation of BCCs with 1-100 nM 1, 25α-dihydroxyvitamin D3 (VD3) for 24 and 48 hours suppressed expression of Edg-3 and Edg-5 receptors, as assessed by real-time PCR, by a maximum of 60% of control (mean, n=3). Pretreatment of BCCs with 1-100 nM VD3 inhibited S1P-elicited increases in [Ca++]i in parallel with reduced levels of Edg-3 and Edg-5 Rs. Incubation of BCCs with 10 uM retinoic acid (RA) for 24-72 hours enhanced expression of Edg-5 up to a maximum of 10-fold, as assessed by real-time PCR, and inversely reduced expression of Edg-3 by up to 35% of control. The functional significance of the opposing effects of VD3 and RA on Edg-5 Rs is uncertain at this time. However, similar suppression of cellular levels and signaling of the predominant Edg-3 Rs by both VD3 and RA suggest that this is the primary mechanism for their inhibition of S1P contributions to proliferation of BCCs.

683.13

Stearate Inhibits Epithelial Growth Factor Receptor Trafficking in Human Breast Cancer Cells

Chuanyu Li, Hong Zhao, Jeffrey Carroll, Robert W Hardy. Department of Pathology, University of Alabama at Birmingham, 701 South 19th St., Birmingham, Alabama 35294

It is unclear whether epithelial growth factor receptor (EGFR) endosomal trafficking plays a role in generating biological responses. We reported that stearate inhibits proliferation of Hs578T cells. Our hypothesis is that stearate inhibits EGF induced cell proliferation by altering intracellular trafficking of the EGFR. Fluorescence–activated cell sorting (FACS) analysis, confocal microscopy, immunoprecipitation and immunoblot experiments were used to address this hypothesis. FACS analysis indicated that there was no difference in EGF-induced internalization of EGFR between control and stearate treated cells (37±3.13% vs. 41±1.75%, $p>0.05$, n=3). Confocal microscopy indicated that in control cells but not stearate treated cells, EGFR was translocated to a perinuclear location. Immunoprecipitation and immunoblot experiments indicated that stearate dissociates annexin II from EGFR while MAP kinase tyrosine phosphorylation in response to EGF is unaltered. FACS analysis indicates that stearate inhibits the G0 G1 to S transition. Thus, stearate inhibits EGF-induced EGFR intracellular trafficking but not internalization possibly via inhibition of EGFR association with annexin II. RWH, NIH-RO1-CA81236

683.14

Connective Tissue Growth Factor induces adhesion, contraction and cytoskeletal rearrangement in human mesangial cells: the role of Erk and Rho Kinases.

John Kenneth Crean[1], Darren Finlay[2], Catherine Godson[1], Finian Martin[2], Hugh R Brady[1]. [1]Department of Medicine and Therapeutics, Conway Institute of Biomolecular and Biomedical Research and Dublin Molecular Medicine Centre, 41 Eccles Street, Dublin, County Dublin D7 Ireland, [2]Department of Pharmacology, Conway Institute of Biomolecular and Biomedical Research and Dublin Molecular Medicine Centre, Dublin, County Dublin Ireland

Connective Tissue Growth Factor (CTGF) is a member of a family of immediate-early genes that co-ordinate complex biological processes during differentiation and tissue repair. We have investigated the role of CTGF in integrin-mediated adhesion, adhesive signaling and the production of matrix components in mesangial cells. Cultured human mesangial cells were stimulated with rhCTGF. Cytoskeletal assembly was assessed by staining for F-actin with rhodamine-phalloidin. Contraction, adhesion, chemotaxis and proliferation were measured by standard assays. Cell