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Award Number: DAMD17-99-1-9108

TITLE: Lysophospholipid Receptors and Effects in Breast Cancer

PRINCIPAL INVESTIGATOR: Edward Goetzl, M.D.

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

REPORT DATE: July 2000

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
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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 2000	Annual (1 Jul 99 - 30 Jun 00)

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

5. FUNDING NUMBERS
DAMD17-99-1-9108

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

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California
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)*

Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are widely-distributed growth factors generated enzymatically from membrane precursors in activated cells, which attain micromolar concentrations in plasma and tissue fluids. 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-2 to -5, and low levels of Edg-7, but no Edg-1, -6 or -8. BCCS have higher levels of Edg-3 and -5 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 autocrine protein growth factors, such as the type II insulin-like growth factor. 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 R may become a diagnostic index of breast cancer and Edg R-directed antagonists may suppress growth and dissemination of breast cancers.

14. SUBJECT TERMS Breast Cancer

15. NUMBER OF PAGES 25

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

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Edward J. Doty 07/21/00

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(5) INTRODUCTION:

A subfamily of at least eight structurally homologous G protein-coupled receptors for the growth factors lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are encoded by endothelial differentiation genes (Edg Rs). Edg-1, -3, -5, -6 and -8 Rs are specific for S1P and Edg-2, -4 and -7 are specific for LPA. Edg Rs transduce two distinct types of cellular responses to LPA and S1P. 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. The second are growth-independent and encompass a cluster of cytoskeleton-dependent functions, including adhesion, migration, contraction and secretion. Preliminary studies showed that human breast cancer cells (BCCs) of several cultured lines express high levels of some Edg Rs, which transduce LPA and S1P stimulation of proliferation by: i. activation of serum-response element (SRE)-driven transcription of growth genes, ii. enhancement of BCC generation of insulin-like growth factor II (IGF-II), and iii. increased BCC-surface expression of type I receptors for IGF-II (IGF-I-Rs) and heparin-binding epidermal growth factor-like growth factor (HB-EGF), which augments proliferation by interacting with both EGF Rs and proteoglycans of cells and tissues. The emphasis of several specific aims was modified in this first year to address suggestions of the reviewers that we: a) define how Edg R expression and functions in malignant BCCs differ from those in normal breast epithelial cells and b) attempt to understand the role of Edg Rs in uncontrolled growth of BCCs.

(6) BODY:

a) Hypothesis and specific aims- The central hypothesis is that S1P and LPA stimulate proliferation and malignant functions of BCCs by several distinct Edg R-dependent mechanisms, including direct transcriptional activation of immediate-early growth-related genes with controlling SRE sequences and upregulation of autocrine protein growth factor circuits. Further, it is suggested that functionally-significant differences in these pathways will be found which distinguish between BCCs and non-malignant breast epithelial cells. Current specific aims are:

i. Culture normal human breast epithelial cells in medium similar to that supporting BCCs for a time sufficient to complete studies of expression and functions of Edg Rs, and of regulation of expression of Edg Rs in comparison with regulation in BCCs.

ii. Apply "real-time" Taq-Man PCR methodology to quantification of Edg Rs in estrogen receptor (ER)-positive and ER-negative BCCs and normal breast epithelial cells.

iii. Investigate mechanisms of Edg R-dependent stimulation of BCC and normal breast epithelial cell proliferation by S1P and LPA using assays based on transfection with SRE-luciferase and Ca++-sensitive reporters.

iv. Elucidate pathways by which Edg Rs of BCCs and normal breast epithelial cells transduce S1P and LPA signals enhancing expression of HB-EGF and IGF-I Rs, and generation and secretion of IGF-II.

v. Determine the capacity of normal breast epithelial cells and BCCs to secrete gelsolin at concentrations capable of stabilizing and delivering LPA to Edg-2, -4 and -7 Rs.

b) Analyses of Edg Rs in BCCs and normal breast epithelial cells- The application of semi-quantitative PCR and radio-PCR to BCCs of one ER-positive and one ER-negative line demonstrated a similar rank-order of relative frequency of expression of Edg Rs with Edg-3>> Edg-4> Edg-5>/= Edg-2> Edg-7, and no detectable Edg-1, -6 or -8 in either line. These initial observations were confirmed recently and mRNAs encoding the Edg Rs were more accurately quantified with a "real-time" Taq-Man PCR method using probes and primers determined to have optimal sensitivity for this subfamily of GPCRs. 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.

c) Regulation of expression of the predominant Edg-3 R of BCCs- The active metabolite of vitamin D, 1-, 25-dihydroxy-vitamin D3 (DHVD), suppresses the level of expression of Edg-3 Rs in BCCs with both time- and DHVD concentration-dependence. The optimal time of exposure was 24-48 h and the most effective concentration of DHVD was 10-8 M, which together resulted in mean maximal suppression of 68%, as contrasted with 26% for Edg-5 Rs, 15% for Edg-2 s, and consistent 10-15% enhancement of the level of mRNA for Edg-4 Rs. The slight suppression by DHVD of mRNA encoding Edg-3 Rs in normal breast epithelial cells was not significant under any conditions, including 10-10 M to 10-7 M DHVD and 12 h to 72 h incubation. The principal difference so far then is a much greater susceptibility of Edg-3 Rs in BCCs than in normal breast epithelial cells to suppression by DHVD. The transcriptional mechanism(s) by which DHVD suppresses Edg-3 Rs are under investigation.

d) Biochemical pathways transducing Edg R-dependent functional responses of BCCs to S1P and LPA- Functional studies to date have been completed with ER-positive and ER-negative BCCs, but not normal breast epithelial cells. Proliferation of BCCs, as assessed simply by cell counts after 72 h, was increased significantly by 10-8 M to 10-6 M LPA and S1P and maximally by up to three- to four-fold by 10-6 M LPA and S1P. There were no significant differences between LPA and S1P nor between ER-positive and ER-negative BCCs. The direct nuclear pathway for stimulation was confirmed using BCCs transfected with the SRE-luciferase reporter, in which 10-9 M S1P and LPA induced 5- to 20-fold increases in signal, further increases were LPA/S1P concentration-dependent, and mean maximal increases at 10-7 M to 10-6 M were up to 85-fold. Preincubation of BCCs with a range of microbial toxins and pharmacological inhibitors selective for

individual defined pathways demonstrated a dependence of SRE-luciferase signals on Gi alpha, Ras/MEK and Rho family GTPases.

e) Enhancement of secretion of IGF-II to functionally-relevant concentrations by S1P and LPA- 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 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. 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.

f) Binding of LPA and delivery to BCCs by gelsolin plasma protein- Gelsolin is a cytosolic and plasma protein, which severs actin through a mechanism inhibited by either PIP2 or LPA binding to the same two sites of gelsolin. Gelsolin binds LPA with a higher affinity (mean Kd= 6 nM) than serum albumin (Kd= 360 nM), the usual protein employed for LPA delivery in laboratory studies. At concentrations lower than 10% of that in plasma, gelsolin significantly enhances the functional effectiveness and intensity of biochemical signaling of LPA. At concentrations greater than 10% of that in normal plasma, gelsolin sequesters LPA extensively and prevents cellular stimulation. The structural determinants of gelsolin binding of LPA and delivery to human BCCs has been examined with a series of synthetic substituent peptides of gelsolin. Synthetic gelsolin peptide (GE 135-169), which contains both LPA-/PIP2-binding sites of intact gelsolin protein, and its constituent pieces GP1 (135-149) and GP2 (150-169), which each has one of the two LPA-/PIP2-binding sites, were examined in parallel with human recombinant intact gelsolin for effects on BCC responses to LPA in the SRE-luciferase assay. GE 135-169 enhanced significantly the SRE-luciferase signals elicited by 10-9 M to 10-7 M LPA from MDA-MB-453 human breast cancer cells. Maximal enhancement was observed at 0.01-0.1 uM recombinant intact gelsolin 0.3-3 uM GE 135-169, as compared to 0.5-2 uM for fatty acid-free serum albumin. The same concentrations of gelsolin protein and GE 135-169 enhanced LPA-, but not S1P-, induced increases in [Ca++]i in the MDA-MB-453 cells, as assessed by quantification of Fluo-4 AM signals in a fluorometric imaging plate reader. The constituent GP1 and GP2 peptides were less potent than GE 135-169, but at high concentrations blocked the LPA-binding activity of GE 135-169. Future studies also will examine the possibility that BCCs secrete gelsolin.

(7) APPENDICES:

(1) Key Research Accomplishments-

* Application of "real-time" Taq-Man PCR to quantification of Edg Rs in normal human breast epithelial cells and BCCs.
* Demonstration that levels of Edg-3 and Edg-5 are higher in BCCs than in normal breast epithelial cells, and that Edg-3 S1P R is the predominant Edg in BCCs.
* Documentation that S1P and LPA stimulate secretion of IGF-II by BCCs, and that this IGF-II activity contributes substantially to the enhancement f proliferation by S1P/LPA.
* Discovery that 1-,25-dihydroxy-vitamin D3 selectively suppresses expression of Edg-3 Rs in BCCs by transcriptional mechanisms.
* Finding that the plasma protein gelsolin presents LPA, but not S1P, to BCCs more potently than serum albumin.

(2) Publications-

i. 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.

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

iii. 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.

iv. Dolezalova, H., Cunningham, M.D., Solow-Cordero, D.E., Kong, Y., Lee, H., and Goetzl, E.J. Cellular presentation of lysophosphatidic acid (LPA) by the human plasma gelsolin (GE) substituent peptide GE 135-169. FASEB J. 14: A1464, 2000.

[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.

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[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.

DAMD17-99-1-9108

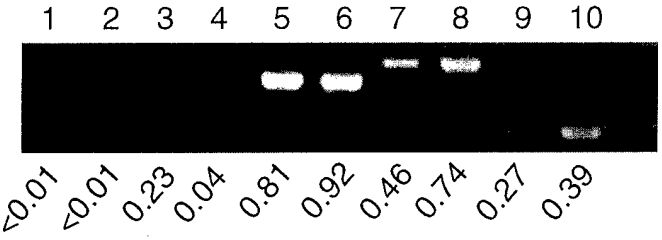

Fig. 1. RT-PCR semiquantification of mRNA encoding Edg Rs in MCF-7 and MDA-MB-453 cells. The volume of cDNA mixture from each type of BCC was selected to equalize the level of amplified G3PDH cDNA product. *Lanes 1, 3, 5, 7,* and *9* are from MDA-MB-453 cells, and *Lanes 2, 4, 6, 8,* and *10* are from 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

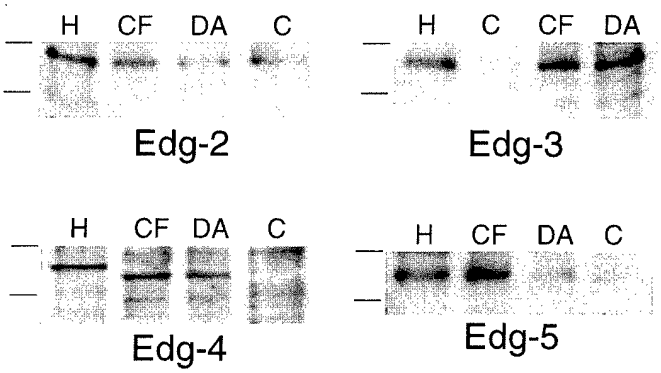

Fig. 2. Western blot analysis of the expression of Edg-2, Edg-3, Edg-4, and Edg-5 Rs by MCF-7 and MDA-MB-453 BCCs. The four samples analyzed for content of each Edg R are: *H,* 3 μg of protein extracted from HTC4 rat liver cells that were stably transfected with the respective Edg Rs; *C,* 10 μg of protein 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.

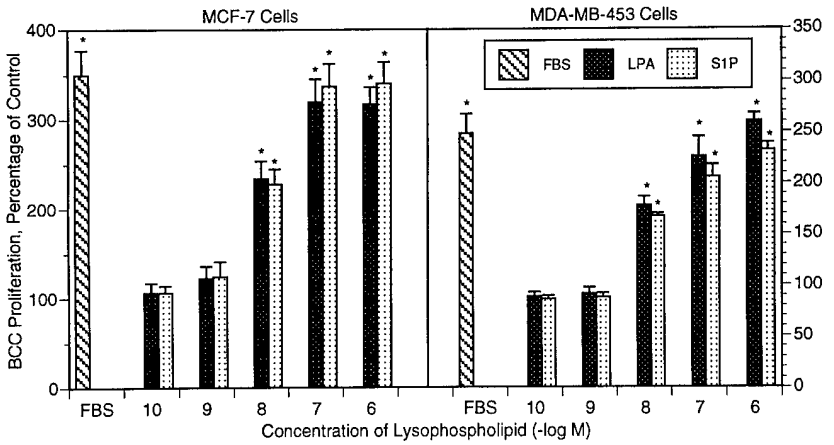

Fig. 3. Stimulation of proliferation of MCF-7 and MDA-MB-453 BCCs by LPA and S1P. *Columns,* means of the results of three studies performed in duplicate; *bars,* SD. FBS is the 2% FBS-positive control. The serum-free medium alone controls (100%) were 1.5, 1.4, and 1.5 × 10^4/well in the three studies 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

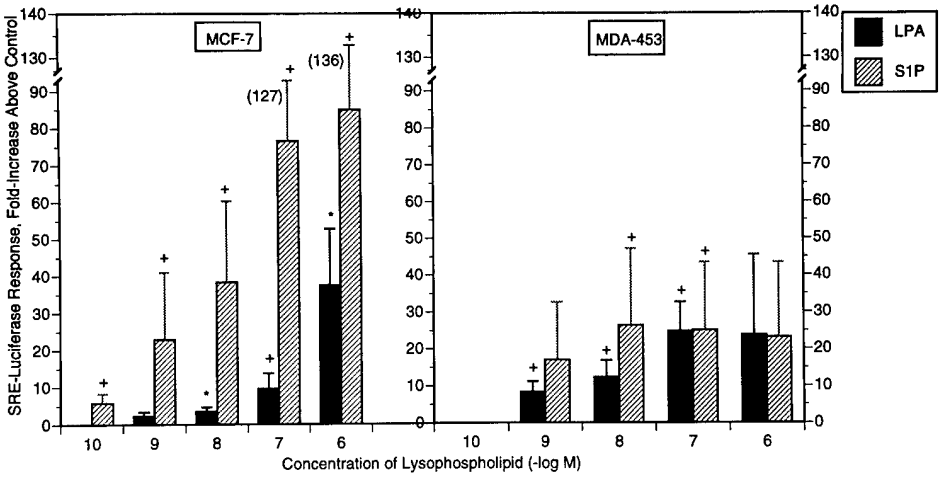

Fig. 4. SRE reporter assay of LPA and S1P stimulation of human BCCs. *Columns,* means of the results of three studies performed in duplicate; *bars,* SD. The medium alone control values were 1272, 957, and 352 luminometer units for MCF-7 BCCs and 269, 715, and 1401 for MDA-MB-453 BCCs. The statistical 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

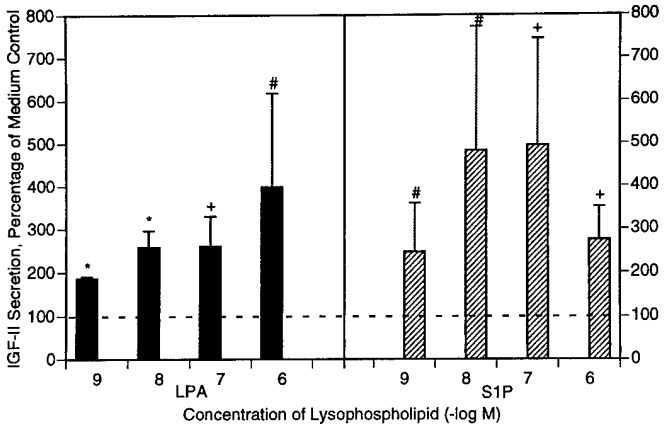

Fig. 5. Stimulation by LPA and S1P of MCF-7 BCC secretion of IGF-II. *Columns*, means of the results of three studies; *bars*, SD. Secretion of IGF-II in medium alone was 1.7, 3.0, and 4.1 ng/ml in the three studies. The statistical methods and symbols are the same as in Fig. 4, except that # = $P =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

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

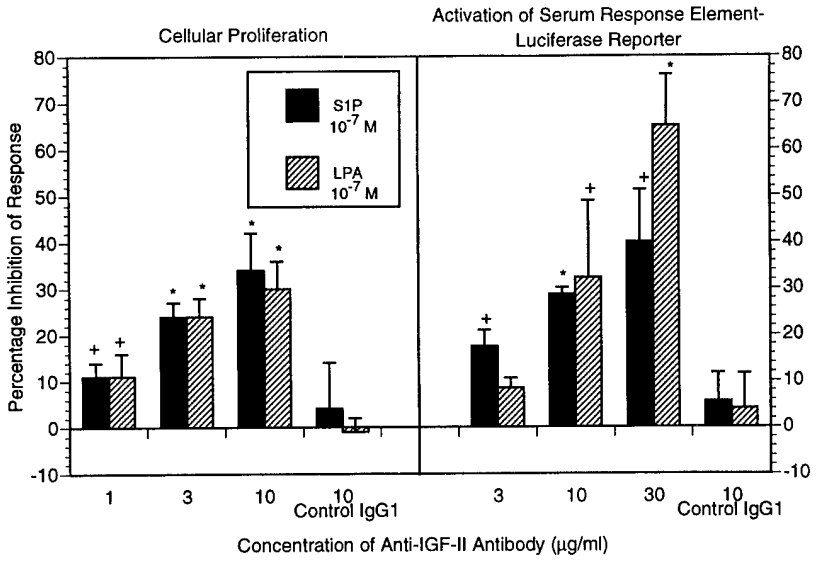

Fig. 6. Suppression of MCF-7 BCC responses to LPA and S1P by a neutralizing anti-IGF-II mouse monoclonal antibody. *Columns*, means of the results of three studies performed in duplicate; bars, SD. The control (0% inhibition) responses to 10^{-7} M LPA and S1P are shown in Figs. 3 and 4. The statistical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

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ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

Volume 905

LYSOPHOSPHOLIPIDS AND EICOSANOIDS IN BIOLOGY AND PATHOPHYSIOLOGY

Edited by Edward J. Goetzl and Kevin R. Lynch

The New York Academy of Sciences
New York, New York
2000

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.

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

Volume 905

LYSOPHOSPHOLIPIDS AND EICOSANOIDS IN BIOLOGY AND PATHOPHYSIOLOGY

Edited by Edward J. Goetzl and Kevin R. Lynch

The New York Academy of Sciences
New York, New York
2000

DAMD17-99-1-9108

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

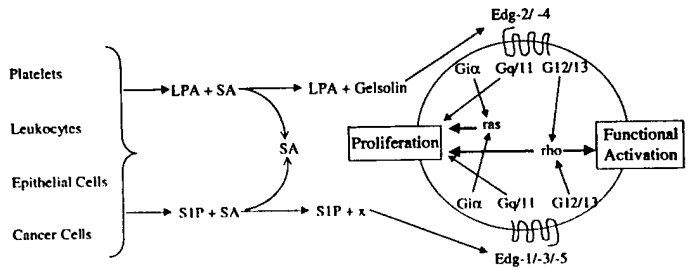

FIGURE 1. Generation, transport, and effects of lysolipid phosphate mediators. SA = serum albumin; x = postulated high-affinity S1P-binding protein.

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 (*edg*s) 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 increases 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).

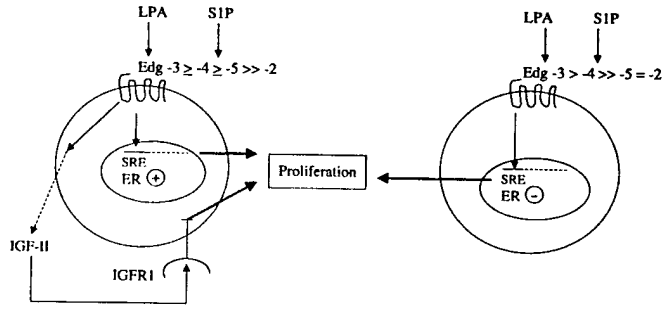

FIGURE 2. Lysolipid phosphate effects on human breast cancer cells. ER = estrogen receptor; SRE = serum response element; IGF-II = type II insulin-like growth factor; IGFR1 = type 1 insulin-like growth factor receptor.

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

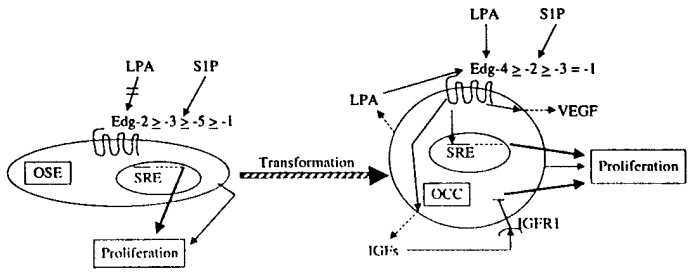

FIGURE 3. Lysolipid phosphate effects on human ovarian cancer cells. OCC = ovarian cancer cell; OSE = normal ovarian surface epithelial cell; SRE = serum response element; IGFs = a mixture of types I and II insulin-like growth factors; IGFR1 = type 1 insulin-like growth factor receptor.

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.

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CHARACTERIZATION, REGULATION AND PRE-PURIFICATION OF HUMAN NEUTROPHIL PHOSPHOLIPASE D. J.M. Horn, J.A. Lehman, G. Alter, J. Horwitz and J. Gomez-Cambronero. Departments of Physiology, Anatomy and Biochemistry, Wright State University School of Medicine, Dayton, Ohio 45435 and MCP•Hahnemann School of Medicine.

Utilizing the transphosphatidylation reaction catalyzed by phospholipase D (PLD) in the presence of a primary alcohol and the short-chain phospholipid PC8, we characterized the enzyme from human neutrophils and DMSO-treated HL-60 cells that express the neutrophilic phenotype. A pH optimum of 7.8-8.0 was determined. PIP_2, EDTA/EGTA, and ATP were found to enhance basal PLD activity *in vitro*. Inhibitory elements were: oleate, Triton X-100, n-octyl-β-glucopyranoside, divalent cations, GTPγS and H_2O_2. The apparent K_m for PC8 was 0.06 mM and the V_{max} was 22.8 pmol x mg^{-1} x hr^{-1}. Analysis by Western blotting and immunoprecipitation revealed that neutrophil PLD is recognized by an anti-pan PLD antibody but only minimally by anti-PLD1 or anti-PLD2 antibodies. Further, neutrophil PLD is tyrosine phosphorylated upon cell stimulation with GM-CSF and fMet-Leu-Phe. Partial pre-purification using column liquid chromatography was performed after cell subfractionation. Based on the enzyme's regulatory and inhibitory factors, its location away from the cell membrane and its molecular weight, these data indicate an enzyme isoform that might be different from the mammalian PLD1/2 forms described earlier. The present results lay the foundation for further purification of this granulocyte PLD isoform. [Supported by NIH HL056653 and AHA 9806283].

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PHOSPHOLIPASE D AS PART OF AN OSMOTIC SENSOR IN LEISHMANIA DONOVANI. J. Gomez-Cambronero, J.A. Lehman, J.M. Horn, F. Yu and J.J. Blum. Depts. Physiology and Anatomy, Wright State University School of Medicine, Dayton, OH, and Cell Biology, Duke University Medical Center, Durham, NC.

We report here that the phospholipase D isoform PLD1 is present in the parasitic protozoan *Leishmania donovani*. Activity is dependent on primary alcohol, Ca^{2+} and Mg^{2+} ions, and its optimum pH is 8.0, with a small peak also at pH 5.5. An exposure of promastigotes from both logarithmic and stationary phase cultures to hypotonicity (163 mOsm) causes an increase of PLD activity that reaches its peak level about 5 minutes after the change in osmolality. Exposure to hypertonicity (617 mOsm) also causes an increase in PLD activity but to a lesser extent. Cells grown under hypo- or hyperosmotic conditions have higher PLD activity levels than cells grown under iso-osmotic conditions, but the effects of acute osmotic stress are appreciably larger than the effects of long term exposure. Since the increase in PLD activity, in response to acute hypo-osmotic stress is slower than the rapid change in shape or the release of amino acids, which are largely complete in one minute, PLD might represent the response rather than the consequence of cell volume variations. It seems likely that, in addition to its role in the response to osmotic stress, PLD in *L. donovani* could also play an important role in lipid turnover, such as the release of lipophosphoglycan from the membrane during the infective phase. [Supported by grants from NIH HL056653 and AHA 9806283].

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A NEW INHIBITOR OF PHOSPHOLIPASE D (PLD) HINTS TO AN ACTIVATION MECHANISM BY PROTEOLYSIS. B.J. Andrews, K. Bond, J.M. Horn, J.A. Lehman, A.L. Dugan, and J. Gomez-Cambronero. Departments of Physiology and Biophysics and Anatomy, Wright State University School of Medicine, Dayton, Ohio 45435.

While conducting a purification protocol of PLD from human granulocytes, we observed that the enzyme activity was inhibited by a protease inhibitor cocktail (PIC) in a dose-dependent manner. Of the six inhibitors that make up the cocktail, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), a serine esterase inhibitor, was found to be the sole inhibitor of PLD, while $AEBSNH_2$, an inactive structural analogue, showed no effect. 1 mM AEBSF caused a 50-70% loss of neutrophil PLD activity *in vitro* and 20-30% loss of purified plant PLD activity. When lysates were incubated with AEBSF for short (<1 min) times before the assay, 50% inhibition was achieved at concentrations of 50-100 μM. Although AEBSF failed to inhibit PMA-stimulated PLD activity in intact neutrophils that were washed after treatment, activity was inhibited when it was present in the assay mixture of anti-PLD immunoprecipitates. Finally, AEBSF did not have an effect on other neutrophil signal transduction events, particularly, tyrosine phosphorylation or p42MAPK activation. We suggest that a proteolytic-related pathway could exist for regulation of PLD. An AEBSF-sensitive serine protease could lay upstream of PLD that, when active, increases PLD activity. [Supported by NIH HL056653, AHA 9806283, and STREAMS NIH HL07805.]

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INFLUENCE OF PROSTAGLANDIN E2 ON FETAL PERFUSION PRESSURE IN HUMAN PLACENTAL COTYLEDON. B.V. Rama Sastry and M.E. Hemontolor. Vanderbilt University School of Medicine, Nashville, TN 37232-6125.

Tobacco use during pregnancy increases spontaneous abortions and preterm births. Nicotine in tobacco crosses human placenta and part of it is metabolized to cotinine. Both nicotine and cotinine activate placental phospholipase A2 forming increased formation of PGE2. In order to verify whether nicotine and cotinine influence fetal blood pressure, we studied their influence in presence PGE2 in perfused human placental cotyledon. PGE2 (150 ng/ml) increased fetal perfusion pressure (mm Hg) from 70.6 ± 3.5 to 122 ± 5. Cotinine (2000ng/ml) or nicotine (2000ng/ml) did not alter fetal pressure. Cotinine did not influence the effect of PGE2 while nicotine blocked the effect of PGE2 on fetal pressure. Nicotine releases placental acetylcholine which dilates the placental fetal blood vessels. Cotinine does not influence the release of acetylcholine in placenta. Therefore, accumulation of cotinine in fetal circulation increases the formation of PGE2 and contributes for the disturbance of placental blood flow and spontaneous abortions in tobacco smokers.

[Supported by the Study Center for Anesthesia Toxicology of Vanderbilt University and The Smokeless Tobacco Research Council.]

LIPID AND LIPID SECOND MESSENGERS: PHOSPHOLIPIDS (879-880)

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INCORPORATION OF [^{14}C]-ETHANOL INTO CELLULAR LIPIDS AND PLATELET ACTIVATING FACTOR IN ENDOTHELIAL CELLS
R.M. Magai, S.D. Shukla. Department of Pharmacology, School of Medicine, University of Missouri, Columbia, MO 65212.

It is unknown what effect ethanol has on phospholipid pools in vascular endothelial cells. We investigated the metabolic fate of [^{14}C]-ethanol in calf pulmonary artery endothelial (CPAE) cells as well as the effect of ethanol on the fate of [^{3}H]-acetate in the cells under unstimulated and stimulated conditions. The cells were incubated in [^{14}C]-ethanol for 8 hours, incubated -/+ 10 μM ATP for 10 minutes, and their lipids analyzed. The [^{14}C]-ethanol was incorporated into various endothelial cell phospholipids: phosphatidylcholine, phosphatidylethanol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, sphingomyelin (Sph), as well as into neutral lipids. This incorporation was reduced in the presence of pyrazole or cyanamide, inhibitors of ethanol metabolism. Exogenously supplied [^{3}H]-acetate was also incorporated into several CPAE cell phospholipids. However, the pattern of distribution was different from that of [^{14}C]-ethanol. Most of the [^{3}H]-acetate went into neutral lipids, whereas the [^{14}C]-ethanol was mostly incorporated in phosphatidylcholine. Interestingly, it was also noted that a small percentage of [^{14}C]-ethanol and [^{3}H]-acetate were incorporated into the PAF fraction. Methanolic hydrolysis of the total cell lipids showed that there was also difference in the distribution of the [^{14}C]-ethanol and the [^{3}H]-acetate between the water-soluble and fatty acyl moieties. Stimulation of the cells with ATP increased incorporation of [^{3}H]-acetate into both PAF and Sph, whereas ATP stimulation had no effect on the incorporation of [^{14}C]-ethanol. The data demonstrate that ethanol not only affects but also contributes to the phospholipid pools and to the formation of lipid mediators in endothelial cells which may be crucial in the responses of the endothelium.

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CELLULAR PRESENTATION OF LYSOPHOSPHATIDIC ACID (LPA) BY THE HUMAN PLASMA GELSOLIN (GE) SUBSTITUENT PEPTIDE GE135-169. H. Dolezalova, M.D. Cunningham, D.E. Solow-Cordero, Y. Kong, H. Lee and E.J. Goetzl. University of California, San Francisco, CA 94143 and Ceretek, LLC, South San Francisco, CA 94080.

Gelsolin is a cytosolic and plasma protein, which severs actin in a process inhibited by binding of either L-α-phosphatidylinositol-4, 5-bisphosphate (PIP_2) or LPA to the same two sites on GE. GE binds LPA with a higher affinity (Kd=6nM) than serum albumin (SA) (Kd=360nM) and, at concentrations 10% or less of that in plasma, GE augments cellular growth factor activity of LPA. Synthetic GE 135-169, which constitutes the PIP_2/LPA-binding sites, enhanced significantly 10^{-9}M to 10^{-7}M LPA stimulation of nuclear signals from MDA-MB-453 human breast cancer cells transfected with an LPA-activatable serum response element (SRE)-luciferase reporter. Maximal enhancement was at 0.3-3μM for GE 135-169, 0.01-0.1μM for GE and 0.5-2μM for SA. The same concentrations of GE 135-169, GE and serum albumin enhanced LPA-induced increases in $[Ca^{++}]i$ significantly in A431 human squamous carcinoma cells, assessed by quantification of Fluo-4 AM signals in a Fluorometric Imaging Plate Reader. GE 135-169 thus enhances LPA effects on cells with the same activity as intact GE, but with approximately 1/30 the potency, suggesting that LPA binding is a primary determinant of the specificity of cellular presentation by GE.