Lysophosphatidic Acid-induced Squamous Cell Carcinoma Cell Proliferation and Motility Involves Epidermal Growth Factor Receptor Signal Transactivation

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ABSTRACT

Transactivation of the epidermal growth factor receptor (EGFR) represents the paradigm for cross-talk between G protein-coupled receptors (GPCRs) and receptor tyrosine kinase signaling pathways. In a variety of squamous cell carcinoma cell lines of the head and neck, we found that treatment with the GPCR agonist lysophosphatidic acid (LPA), bradykinin, thrombin, and carbachol results in rapid tyrosine phosphorylation of the EGFR. In these tumor cells, signal transactivation of the EGFR and the oncoprotein HER2/neu is critically dependent on metalloprotease activity. Using the metalloprotease inhibitor batimastat, the EGFR-specific tyrosinophosphatase AG1478, and a dominant-negative EGFR mutant, we show that in HNSCC cell lines, EGFR tyrosine phosphorylation, recruitment of the adaptor proteins SHC and Gab1, and activation of the ERK/mitogen-activated protein kinase pathway in response to LPA depend both on metalloprotease function and EGFR tyrosine kinase activity. Most importantly, critical characteristics of HNSCC cell lines such as DNA synthesis, cell cycle progression and tumor cell migration are stimulated by LPA and can be abrogated by interfering with EGFR signal transmission. Together, our results demonstrate the importance of a mechanism that promotes head and neck cancer cell proliferation and motility by GPCR ligands involving EGFR transactivation. Our findings suggest that highly abundant GPCR ligands such as LPA may function as tumor promoters and determinants of HNSCC progression.

INTRODUCTION

GPCRs1 are the largest family of cell surface receptors involved in the regulation of numerous physiological functions, and there is increasing evidence for a direct correlation between aberrant GPCR signaling and the development and progression of hyperproliferative disorders such as cancer (1). A myriad of extracellular agonists have been demonstrated to act through GPCRs including biogenic amines, peptide and glycoprotein hormones, neuropeptides, serine proteases, neurotransmitters, eicosanoids, and phospholipids such as sphingosine-1-phosphate and LPA.

Various cellular responses to LPA have been documented including rapid cytoskeletal rearrangements, stimulation of cell proliferation, suppression of apoptosis (2), and induction of tumor cell invasion (3). Moreover, LPA represents the major mitogenic activity in serum, and platelet-derived LPA is an important mediator in wound healing and tissue regeneration (2). LPA levels are elevated in plasma and ascites of ovarian cancer patients (4), and it is likely to play a prominent role in the pathology of other types of human cancer.

We and others have demonstrated that many signaling events elicited by GPCR ligands are dependent on the function of the EGFR in a variety of nontransformed cell systems (5). The EGFR together with its relatives HER2/neu, HER3, and HER4 belong to a family of four closely related RTKs, which may form active homo- or heterodimers upon interaction with agonistic ligands such as EGF, HB-EGF, and TGF-α. These growth factors are synthesized as transmembrane precursors that are released from the cell surface by proteolytic cleavage and subsequently activate RTKs of the EGFR family by autocrine or paracrine stimulation. The EGFR has been shown to be instrumental in the activation of MAPK pathways, induction of gene expression (6, 7), stimulation of DNA synthesis (8), and regulation of cell cycle progression (9) by agonist-treated GPCRs. Recently, we discovered a new mechanistic concept for EGFR transactivation by which GPCR stimulation leads to metalloprotease-dependent cleavage of membrane-anchored proHB-EGF in COS-7 and HEK-293 cells (10). According to this model, GPCR-induced proHB-EGF shedding results in the release of the mature growth factor from the cell surface, which subsequently activates the EGFR. Limited understanding, however, is available of the pathophysiological role of this TMPS mechanism of EGFR transactivation in human cancer.

HNSCC is one of the most common malignancies and a major cause of cancer-related mortality worldwide. The 5-year survival rate for this disease is ~50%. Overexpression and autocrine activation of the EGFR is detected in 90% of HNSCC (11), and high expression levels of the EGFR and HER2/neu have been proposed as prognostic markers that correlate with poor clinical outcome (12). Therefore, these RTKs serve as molecular targets for recently developed, target-directed HNSCC therapies (13). Interestingly, overexpression of the EGFR and TGF-α have been connected to high levels of activated ERK/MAPK in HNSCC tumors (14), which led to considerable interest in understanding the EGFR-directed mitogenic signaling pathways in this type of cancer.

MATERIALS AND METHODS

Reagents, Antibodies, and Plasmids. AG1478 was obtained from Alexis Biochemicals. Bradykinin, asiansomycin, and fibronectin were from Calbiochem. Batimastat (BB94) was kindly provided by Dr. K. Maskos (Max-Planck-Institute of Biochemistry, Martinsried, Germany). LPA and all other chemicals were purchased from Sigma. Antibodies used were sheep polyclonal anti-EGFR (Upstate Biotechnology); rabbit polyclonal anti-Erk2 antibody, rabbit polyclonal anti-p38 antibody, and mouse monoclonal anti-SHC antibody (Santa Cruz Biotechnology); rabbit polyclonal anti-phospho-p44/p42 (Thr-202/Tyr-204) MAPK antibody and rabbit polyclonal anti-phospho-p38 (Thr-180/Tyr-182) MAPK antibody (New England Biolabs); mouse monoclonal anti-phosphotyrosine antibody 4G10 (UBI); mouse monoclonal anti-HA antibody (Babco); and mouse monoclonal anti-Pan ERK1/2 antibody (BD Transduction Laboratories). Mouse monoclonal anti-EGFR antibody 108.1, rabbit polyclonal anti-SHC antibody, rabbit polyclonal anti-Gab1 antibody (7), and rabbit polyclonal anti-HER2/neu antibody (8) have been described before. The constructs pcDNA3-HA-ERK2, pRKS-HER-CDS3, and pRKS-βPDGFR-CDS04 were used in this study (7).

Cell Culture and Transfections. All HNSCC cell lines were obtained from the American Type Culture Collection and cultured as recommended. SCC-9 cells in 6-well dishes were transfected transiently at 70% confluency with a total of 2 μg of DNA by using a modified calcium phosphate precipitation method as described previously (10). One day after transfections, the
cells were serum starved for 24 h. Transfection efficiency was typically ~50%, as determined by LacZ staining after transfection of a LacZ-containing expression plasmid. Cell lysis, immunoprecipitation, and immunoblotting was performed as described previously (7).

**MAPK Assay.** HA-ERK2 or endogenous ERK2 was immunoprecipitated from lysates obtained from 6-well dishes using 0.5 μg of anti-HA antibody or 0.4 μg of anti-ERK2 antibody, respectively. Precipitates were washed three times with 50 mM Hepes, pH 7.5, 150 mM NaCl, 10% Glycerol; 0.1% Triton-X-100 and washed once with kinase buffer [20 mM Hepes (pH 7.5), 10 mM MgCl₂, 1 mM DTT, and 200 μM sodium orthovanadate]. Kinase reactions were performed in 30 μl of kinase buffer supplemented with 0.5 mg/ml myelin basic protein, 50 μM ATP, and 1 μCi of [γ-32P]ATP for 10 min at room temperature. Reactions were stopped by the addition of 30 μl of Laemmli buffer and subjected to gel electrophoresis on 15% gels. Labeled MBP was quantitated using a phosphorimager (Fuji).  

**Incorporation of [3H]Thymidine into DNA.** SCC-9, SCC-25, SCC-4, and SCC-15 cells were seeded into 12-well plates (2.5 × 10⁴, 6 × 10⁴, 2 × 10⁵, and 3 × 10⁵ cells/well, respectively). Upon serum deprivation for 48 h, cells were subjected to 20 min preincubation with either DMSO (control), 250 nM AG1478, or 5 or 10 μM batimastat before ligand treatment. After 18 h incubation, cells were pulse-labeled with [3H]thymidine (1 μCi/ml) for 4 h, and thymidine incorporation was measured by trichloroacetic acid precipitation and subsequent liquid scintillation counting.

**Distribution of Cell Cycle Phases.** SCC-25 cells were seeded into 6-well plates (1.5 × 10⁵ cells/well). Upon serum deprivation for 48 h, cells were subjected to 20 min preincubation with either DMSO (control) or batimastat before ligand treatment. After 18 h incubation, cells were collected and incubated in hypotonic buffer containing 0.1% sodium acetate, 0.1% Triton X-100, and 20 μg/ml propidium iodide for 2 h on ice. Samples were analyzed on a Becton Dickinson FACScalibur flow cytometer.

**In Vitro Wound Closure Assay.** The assay was performed as described previously (3) with some modifications. Confluent monolayers of SCC-9 cells were wounded with a uniform scratch, the medium was removed, and cells previously (3) with some modifications. Confluent monolayers of SCC-9 cells were wounded with a uniform scratch, the medium was removed, and cells were permitted to migrate for 36 h. After incubation, the cells that had migrated to the lower surface were fixed and stained with 0.1% basic protein, 50 μg/ml propidium iodide for 2 h on ice. Absorbance at 570 nm was measured in a micro-plate reader.

**Statistical Analysis.** Student’s t test was used to compare data between two groups. Values are expressed as mean ± SD of at least triplicate samples. *P* < 0.05 was considered statistically significant.

**RESULTS**

**GPCR Agonists Stimulate EGFR Tyrosine Phosphorylation via a Metalloprotease-dependent Pathway in HNSCC.** GPCR-induced EGFR transactivation was shown previously to couple G-protein activation to the Ras-MAPK pathway in nontransformed cell lines such as Rat-1 fibroblasts, COS-7, HEK-293, and vascular smooth muscle cells (5). In contrast, little is known about the molecular mechanisms and the involvement of EGFR transactivation in cancer cell pathophysiology. To investigate the functional role of EGFR transactivation in squamous cell carcinoma, we screened the HNSCC cell lines SCC-4, SCC-9, SCC-15, SCC-25, FaDu, and Detroit-562 for their responsiveness to the GPCR ligands LPA (10 μM), thrombin (1 unit/ml), carbachol (1 mM), and bradykinin (5 μM) at physiological concentrations. After stimulation for 3 min, cell lysates were subjected to immunoprecipitation with anti-EGFR antibodies and immunoblotted against phosphoysrin. Although treatment of serum-deprived SCC-4, SCC-9, and FaDu cells with either GPCR agonist or EGF (7.5 ng/ml) resulted in tyrosine phosphorylation of the EGFR (Fig. 1A, representative data shown for SCC-9), transactivation of the EGFR was induced by LPA and thrombin in SCC-25, by LPA and bradykinin in SCC-15, and by LPA in Detroit-562 (Table 1), demonstrating that cross-talk pathways linking GPCR stimulation with EGFR activation are installed both in HNSCC cells that display low (FaDu and SCC-9) and high (SCC-15, SCC-4, and Detroit-562) EGFR expression levels. In addition, these findings suggest a role for the EGFR as a convergence point for signaling by diverse GPCR agonists and demonstrate that HNSCC cells may be targets for stimulation by multiple ligands.

Recently, we have provided evidence that GPCR-induced EGFR transactivation requires metalloprotease activation and cleavage of the membrane-anchored growth factor precursor proHB-EGF in COS-7 and HEK-293 cells (10). To address the question of whether a ligand-dependent mechanism is also involved in EGFR transactivation in HNSCC cells, we examined the effect of the metalloprotease inhibitor batimastat (BB94) on the EGFR phosphoysrin content after stimulation with GPCR ligands as described above. In all cell lines, batimastat (10 μM) completely abrogated the EGFR transactivation signal, whereas this compound did not interfere with EGF-induced responses (Fig. 1B, representative data shown for LPA in SCC-9, SCC-15, and SCC-4). In addition, batimastat drastically reduced basal tyrosine phosphorylation levels of the EGFR in SCC-25 cells during a period of 6 h after serum withdrawal (Fig. 1C), suggesting the critical involvement of metalloproteases in autocrine growth factor precursor shedding in HNSCC, presumably by regulating basal EGFR ligand availability. Together, these experiments demonstrate that a variety of physiologically relevant GPCR agonists are capable of inducing rapid EGFR activation in head and neck cancer cells via a metalloprotease-dependent pathway. Moreover, metalloproteases are required for constitutive EGFR tyrosine phosphorylation in HNSCC. The finding that LPA was the predominant stimulus of EGFR activation among several GPCR ligands in all cell lines examined (Table 1) led us to further focus our investigations on LPA-induced signal transactivation in HNSCC.

**Transactivation of HER2/neu Is Dependent on Metalloprotease Function and EGFR Tyrosine Kinase Activity.** Because the oncoprotein HER2/neu, which serves as a prognostic marker in HNSCC (12), has been reported to be transactivated by agonist-treated GPCRs in Rat-1 fibroblasts (8), we raised the question of whether HER2/neu is activated in response to LPA in this tumor type. The experiment presented in Fig. 1D demonstrates that LPA (20 μM) dramatically increased tyrosine phosphorylation of HER2/neu in SCC-9 cells and that transactivation of HER2/neu was sensitive to batimastat. In addition, tyrosine phosphorylation of HER2/neu after LPA or EGF treatment was abolished by the EGFR inhibitor AG1478 (250 nM). Phosphorylation of HER2/neu, therefore, appears to result from EGFR transphosphorylation. The above results implicate that the regulation of metalloproteases and the intrinsic EGFR tyrosine kinase activity are critical for LPA-induced transactivation of HER2/neu in HNSCC cells.

**EGFR Association and Tyrosine Phosphorylation of SHC and Gab1 upon LPA Treatment Is Metalloprotease Dependent.** One key downstream event in the transmission of mitogenic signals by the activated EGFR is the association and subsequent tyrosine phospho-
were serum starved for 48 h, pretreated with batimastat (BB94, 10 \( \mu \)M) or AG1478 (Fig. 1E). Moreover, batimastat did not alter EGFR-stimulated SHC and Gab1 tyrosine phosphorylation.

Our data demonstrate that LPA mobilizes the docking proteins SHC and Gab1 by activating the EGFR through a metalloprotease-dependent pathway.

**Activation of the ERK/MAPK Pathway by LPA Requires Both EGFR Function and Metalloprotease Activity.** Activation of the ERK/MAPK pathway is a key step in the regulation of important cellular responses such as cell proliferation. We therefore investigated the effect of LPA and EGFR stimulation on MAPK activity in head and neck cancer cells by immunoblotting cell lysates with phospho-specific MAPK antibodies. In time course experiments, LPA (10 \( \mu \)M)- and EGFR (5 ng/ml)-induced ERK/MAPK activation was detectable as early as 3 min after stimulation and peaked within 15 min in both SCC-9 and SCC-25 cells (Fig. 2A, representative data shown for SCC-25). Furthermore, LPA-induced ERK activation was slightly more transient when compared with EGFR stimulation. In contrast to ERK1/2, LPA and EGFR only led to low-level activation of the stress-responsive MAPK p38, whereas anisomycin stimulation (5 \( \mu \)M) served as a positive control. On the basis of these findings, we assessed the functional role of the EGFR in activation of the MAPK ERK2 evoked by LPA in HNSCC cells. Previously, expression of a dominant-negative EGFR mutant has been shown to block EGFR-specific downstream signaling events (7). We therefore coexpressed HA-tagged ERK2 (HA-ERK2) together with the EGFR mutant HER-CD533 in SCC-9 cells. HA-ERK2 activity was measured in vitro with an immunocomplex assay using MBP as a substrate. As shown in Fig. 2B, LPA (10 \( \mu \)M) and EGFR (5 ng/ml) lead to an 8-fold increase of

**Fig. 1.** Effect of metalloprotease inhibition on GPCR-stimulated tyrosine phosphorylation of the EGFR, HER2/neu, and downstream adaptor protein recruitment in head and neck cancer cell lines. A, SCC-9 cells were serum starved for 48 h and treated with 10 \( \mu \)M LPA, 1 unit/ml thrombin (Thr), 1 mM carbachol (Car), 5 \( \mu \)M bradykinin (Bk), or 7.5 ng/ml EGF for 3 min. After lysis, EGFR was immunoprecipitated (IP) using monoclonal anti-EGFR antibody. Tyrosine-phosphorylated EGFR was detected by immunoblotting with monoclonal anti-phosphotyrosine (PY) antibody, followed by reprobing of the same filter with polyclonal anti-HER2/neu antibody (EGFR). B, quiescent SCC-9, SCC-15, and SCC-4 cells were serum starved for 48 h, pretreated with batimastat (BB94, 10 \( \mu \)M) or an equal volume of vehicle (DMSO) for 20 min, and stimulated for 3 min with 10 \( \mu \)M LPA or 7.5 ng/ml EGF. Cell lysates were analyzed as described in A. C, SCC-25 cells were seeded at 3 \( \times \) 10^5 cells in 6-well dishes in standard medium and incubated for 18 h. Cells were washed with PBS and incubated with medium without FCS in the presence of batimastat (BB94, 10 \( \mu \)M) or vehicle (DMSO) for the indicated period of time. Cell lysates were analyzed as described in A. D, quiescent SCC-9 cells were pretreated with batimastat (BB94, 10 \( \mu \)M), AG1478 (250 nM), or an equal volume of vehicle (DMSO) for 20 min and stimulated for 3 min with 20 \( \mu \)M LPA or 10 ng/ml EGF. After cell lysis, HER2/neu was immunoprecipitated using polyclonal anti-HER2/neu antibody. Tyrosine-phosphorylated HER2/neu was detected by immunoblotting with polyclonal anti-HER2/neu antibody. E, SCC-9 cells were serum starved for 48 h, preincubated with inhibitors as described in D, and stimulated for 5 min with 10 \( \mu \)M LPA or 7.5 ng/ml EGF. After cell lysis, SHC and Gab1 were immunoprecipitated using polyclonal anti-SHC and anti-Gab1 antibody, respectively. Tyrosine-phosphorylated proteins were detected by immunoblotting with monoclonal anti-phosphotyrosine antibody, followed by reprobing of the same filter with monoclonal anti-SHC antibody.

rlation of adaptor proteins (16). Furthermore, SHC and Gab1 phosphorylation represent important regulatory steps in mitogenic GPCR signaling (7, 17). To address the role of the EGFR and metalloproteases in LPA-induced adaptor protein recruitment, we immunoprecipitated SHC from SCC-9 lysates and immunoblotted against phosphotyrosine. As demonstrated in Fig. 1E, LPA stimulation lead to increased tyrosine phosphorylation of SHC and two proteins of Mr 170,000 and Mr 116,000, which commonprecipitated with activated SHC. The Mr 170,000 protein showed immunoreactivity with anti-EGFR antibodies (data not shown), whereas in agreement with earlier reports the faint Mr 116,000 band could be identified as the adaptor protein Gab1 (7, 18). Moreover, pretreatment of SCC-9 cells with batimastat or AG1478 completely prevented LPA-induced tyrosine phosphorylation of SHC and of the commonprecipitated EGFR and Gab1. As shown by immunoblot analysis, the increased Gab1 phosphotyrosine content in response to LPA treatment was sensitive to batimastat and AG1478 (Fig. 1E). Moreover, batimastat did not alter EGFR-stimulated SHC and Gab1 tyrosine phosphorylation. Together, our data demonstrate that LPA mobilizes the docking proteins SHC and Gab1 by activating the EGFR through a metalloprotease-dependent pathway.

**Activation of the ERK/MAPK Pathway by LPA Requires Both EGFR Function and Metalloprotease Activity.** Activation of the ERK/MAPK pathway is a key step in the regulation of important cellular responses such as cell proliferation. We therefore investigated the effect of LPA and EGFR stimulation on MAPK activity in head and neck cancer cells by immunoblotting cell lysates with phospho-specific MAPK antibodies. In time course experiments, LPA (10 \( \mu \)M)- and EGFR (5 ng/ml)-induced ERK/MAPK activation was detectable as early as 3 min after stimulation and peaked within 15 min in both SCC-9 and SCC-25 cells (Fig. 2A, representative data shown for SCC-25). Furthermore, LPA-induced ERK activation was slightly more transient when compared with EGFR stimulation. In contrast to ERK1/2, LPA and EGFR only led to low-level activation of the stress-responsive MAPK p38, whereas anisomycin stimulation (5 \( \mu \)M) served as a positive control. On the basis of these findings, we assessed the functional role of the EGFR in activation of the MAPK ERK2 evoked by LPA in HNSCC cells. Previously, expression of a dominant-negative EGFR mutant has been shown to block EGFR-specific downstream signaling events (7). We therefore coexpressed HA-tagged ERK2 (HA-ERK2) together with the EGFR mutant HER-CD533 in SCC-9 cells. HA-ERK2 activity was measured in vitro with an immunocomplex assay using MBP as a substrate. As shown in Fig. 2B, LPA (10 \( \mu \)M) and EGFR (5 ng/ml) lead to an 8-fold increase of

![Fig. 1](cancerres.aacrjournals.org)
HA-ERK2 activity in cells transfected with empty vector, whereas in the presence of different amounts of HER-CD533, activation of HA-ERK2 was inhibited up to 75%. As specificity controls, expression of HER-CD533 did not influence PDGF-stimulated HA-ERK2 activation, and the dominant-negative βPDGFR-CD504 mutant had no significant effect on HA-ERK2 activation by LPA (data not shown).

To extend the results obtained with transfected ERK in SCC-9 cells, we investigated the requirement of EGFR and metalloprotease activation with interfering with EGFR signal transmission.

**Fig. 2.** Inhibition of ERK/MAPK activation by interfering with EGFR signal transmission. A, time course of ERK1/2 and p38/MAPK activation. Quiescent SCC-25 cells were treated with 10 μM LPA, 5 ng/ml EGF, or 5 μg/ml anisomycin (AI) for the indicated times. After lysis, activated ERK1/2 and p38 were detected by immunoblotting of total lysates with polyclonal anti-phospho-ERK (P-ERK) or anti-phospho-p38 (P-p38) antibody. *-, unspecific signal. B, effect of dominant-negative EGFR HER-CD533 on ERK activation. SCC-9 cells were transiently transfected with an expression plasmid encoding HA-ERK2 (250 ng/well). Where indicated, a plasmid encoding HER-CD533 (+, 150 ng/well; ++, 450 ng/well) was cotransfected. After serum starvation for 24 h, cells were treated for 7 min with LPA (10 μM) or EGF (5 ng/ml) and lysed, and HA-ERK2 activity was determined using MBP as substrate as described in "Materials and Methods." Phosphorylated MBP was visualized by autoradiography after gel electrophoresis, and HA-ERK2 was immunoblotted in parallel using monoclonal anti-HA antibody. Quantitative analysis of HA-ERK2 activation from three independent experiments is shown (means; bars, SD). *, P < 0.001 for the difference between control + LPA and HER-CD533 + LPA; **, P < 0.005 for control + EGF versus HER-CD533 + EGF. For expression control of HER-CD533, transfected cells were labeled with [35S]methionine, and lysates were subjected to immunoprecipitation with monoclonal anti-HER-CD533 antibody followed by autoradiography. C, quiescent SCC-9 and SCC-25 cells were preincubated with batimastat (10 μM), AG1478 (250 nM), or vehicle (DMSO) for 20 min and stimulated for 7 min with 10 μM LPA or 5 ng/ml EGF. After cell lysis, endogenous ERK2 was immunoprecipitated using polyclonal anti-ERK2 antibody, ERK2 activity was determined using MBP as substrate, and ERK2 was immunoblotted in parallel using polyclonal anti-HER-CD533 antibody. Quantitative analysis of endogenous ERK2 activation from three independent experiments is shown (means; bars, SD). *, P < 0.005 for the difference between DMSO + LPA versus BB94 + LPA and AG1478 + LPA; **, P < 0.01 for DMSO + EGF versus AG1478 + EGF (SCC-9); ***, P < 0.05 for DMSO + EGF versus AG1478 + EGF (SCC-25).
ities for activation of endogenous ERK/MAPK in response to LPA. AG1478 treatment completely abrogated ERK2 activation upon LPA and EGF stimulation in SCC-9 and SCC-25 cancer cells (Fig. 2C). Furthermore, LPA-triggered ERK2 activation was almost completely inhibited by batimastat, whereas ERK2 activation by exogenous EGF was minimally affected. Taken together, these data demonstrate a critical role for metalloprotease-mediated transactivation of the EGFR in promotion of the ERK/MAPK pathway in HNSCC cells.

Metalloprotease-dependent Transactivation of the EGFR Is Required for LPA-induced DNA Synthesis and S-Phase Progression. Because we have observed that batimastat reduces basal tyrosine phosphorylation levels of the EGFR in SCC-25 cells (Fig. 1C), we next investigated whether metalloprotease or EGFR inhibition influences proliferation of HNSCC cells under normal growth conditions in the presence of 10% FCS. As shown in Fig. 3A, batimastat and AG1478 completely blocked growth of SCC-25 cells, demonstrating
that metalloprotease and EGFR activities are required for growth of HNSCC cells.

For further quantification of mitogenic signaling in response to LPA, we measured the rate of DNA synthesis by an \(^{3}H\)thymidine incorporation assay. In SCC-9 and SCC-25 cells that express low and medium levels of EGFR, respectively, AG1478 blocked DNA synthesis in response to LPA or EGF stimulation (Fig. 3B). Furthermore, batimastat reduced the rate of DNA synthesis by LPA in a dose-dependent fashion up to 45% (10 \(\mu M\) batimastat) in SCC-9, whereas in SCC-25 cells we observed complete inhibition of DNA synthesis by batimastat already at 5 \(\mu M\). This difference in batimastat sensitivity indicates variations in the dependence of HNSCC cells on the TMPS pathway. Interestingly, DNA synthesis induction by exogenous EGF was also reduced by batimastat by 40% in SCC-9 cells and 35% in SCC-25 cells, suggesting that EGFR stimulation results in enhanced shedding of endogenous EGFR ligands. These findings are consistent with reports showing that HNSCC cell lines and tumors are mitogenically stimulated by EGFR-like autocrine systems (19). In the EGFR-overexpressing cell lines SCC-4 and SCC-15, LPA was also able to stimulate thymidine incorporation in a batimastat- and AG1478-dependent manner (Fig. 3B), suggesting that cross-talk pathways between GPCRs and the EGFR are also relevant for head and neck cancer cell proliferation with high EGFR background.

To extend the results on proliferative responses upon GPCR stimulation, we investigated growth factor-induced cell cycle progression of serum-deprived SCC-25 cells by flow cytometric analysis. As shown in Table 2, the accumulation of a S-phase cell population in response to LPA (25 \(\mu M\)) was sensitive to metalloprotease inhibition. Complete abolishment of LPA-induced S-phase progression was observed in the presence of 5 \(\mu M\) batimastat (Fig. 3C). Under these experimental conditions, EGF (50 ng/ml) stimulated S-phase entry was reduced by 50% which, as mentioned above, indicates an involvement of metalloprotease-dependent growth factor precursor cleavage in the EGFR action on these cells. Together, these data emphasize the biological significance of metalloprotease-dependent EGFR signal transactivation in LPA-induced mitogenic signaling of head and neck cancer cells.

**Table 2. LPA-induced S-phase progression is blocked by the metalloprotease inhibitor batimastat in SCC-25 cells**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Batimastat</th>
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<td>−</td>
<td>5(^a)</td>
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<td>65</td>
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<td>71</td>
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<tr>
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<td>6</td>
<td>69</td>
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\(^a\) Flow cytometric analysis of the SCC-25 cell cycle (% of cells). Quiescent SCC-25 cells were preincubated with batimastat or vehicle (DMSO) for 20 min and incubated in the absence or presence of ligands (LPA, 25 \(\mu M\); EGF, 50 ng/ml) for 18 h. Representative data of one of three experiments are shown.

LPA Enhances HNSCC Cell Motility via Transactivation of the EGFR. Besides proliferation, cell motility represents another critical parameter in the pathobiology of cancer. Recent reports demonstrated that EGFR-like ligands such as HB-EGF, TGF-\(\alpha\), and amphiregulin promote invasion of HNSCC cells in vitro (20) and that LPA stimulation is capable of promoting migration of ovarian cancer cells (3). We therefore hypothesized that GPCR stimulation, which leads to EGFR activation, might influence the migratory behavior of head and neck cancer cells:

(a) We investigated the effect of LPA on migration of HNSCC keratinocytes in an in vitro wound closure assay. Migration of cells was studied by scraping a wound into a confluent monolayer of SCC-9 or SCC-25 cells and determining the rate of closure. Both LPA (5 \(\mu M\) or 20 \(\mu M\)) and HB-EGF (20 ng/ml) drastically enhanced closure of the wounded area (Fig. 4A, representative data shown for SCC-9). Furthermore, wound closure in response to LPA was completely blocked by AG1478 or batimastat at selective concentrations. These observations suggested a role of the EGFR transactivation pathway in the regulation of the migratory behavior of head and neck cancer cells.

(b) We assessed the chemotactic motility of HNSCC cells in response to growth factor stimulation in a modified Boyden chamber assay. Similar to the results obtained in the induction of wound closure, chemotactic migration of SCC-9 cells toward fibronectin was strongly potentiated by LPA or EGF stimulation (Fig. 4B). Moreover, inhibition of EGFR or metalloprotease activity prevented LPA-triggered migration of SCC-9 cells.
Together, these data substantiate the importance of EGFR and metalloprotease function in GPCR-stimulated motility of head and neck cancer cells.

**DISCUSSION**

Given the significance of both heptahelical receptor-mediated and direct EGFR signaling in tumor cell biology, we investigated the role of EGFR signal transmission in response to GPCR agonists in head and neck cancer cell proliferation and motility. Our data provide evidence that treatment of HNSCC cells with bradykinin, carbachol, thrombin, or LPA results in rapid EGFR activation. Previously, bradykinin stimulation of HNSCC cell lines has been reported to cause elevation of intracellular calcium levels, whereas calcium influx inhibitors blocked proliferation and migration of head and neck cancer cells (21). Moreover, it has been shown that the thrombin receptor PAR1 is widely expressed in oral squamous cell carcinoma and that thrombin enhances growth of metastatic HNSCC cells (22). The experimental finding that the LPA receptor-EGFR cross-talk is established in all six head and neck cancer cell lines examined in this study (Fig. 1A and Table 1) suggests that the EGFR signal transactivation pathway in response to LPA is a major hallmark of this type of cancer. In light of our findings, the EGFR may function as a central integrator of signaling by diverse, cancer-promoting GPCR ligands in HNSCC. Expression of a variety of pathophysiologically significant GPCRs and the role of the EGFR as a convergence point for heptahelical receptor stimulation provides a rational explanation for the enhanced sensitivity of head and neck cancer cells toward motility- and growth-promoting stimuli. Further investigations are to be conducted to determine whether the observed GPCR expression patterns are a prerequisite to or the consequence of neoplastic transformation in HNSCC.

Our experimental results presented here show that in analogy to COS-7, HEK-293, and Rat-1 (10) in head and neck cancer cells (Fig. 1B), a thus far unidentified, batimastat-sensitive shedding activity is induced upon GPCR stimulation that results in the release of soluble EGFR ligands. Our finding that in SCC-9 HNSCC cells LPA treatment also leads to tyrosine phosphorylation of the oncoprotein HER2/ neu (Fig. 1D) confirms our previous observations in Rat-1 fibroblasts (8) and further expands the significance of the TPMS pathway. A critical role for EGFR-HER2/2/4 heterodimers in the etiology of HNSCC has been suggested recently by the finding that EGFR and HER2/2/4 are specifically coexpressed in neoplastic epithelium of tumors when compared with normal tissue (23). Our findings that transactivation of both the EGFR and HER2/2/4 required metalloprotease activity (Fig. 1, B and D) and that the EGFR-specific inhibitor AG1478 completely prevented tyrosine phosphorylation of HER2/2/4 by LPA (Fig. 1D) establish the LPA receptors and possibly other GPCRs as new upstream regulators of EGFR and HER2/2/4 signals. How much the other EGFR family members HER3 and HER4 contribute to serpine receptor signal transmission in HNSCC remains to be examined.

When we investigated the role of EGFR transactivation in LPA-induced mitogenic signaling, we found that inhibition of EGFR function or metalloprotease activity by small chemical compounds blocked EGFR association and phosphorylation of the tyrosine kinase substrates SHC and Gab1 upon LPA treatment (Fig. 1E). Furthermore, we observed that expression of a dominant-negative EGFR mutant abrogated ERK/MAPK activation by LPA in SCC-9 cells (Fig. 2B). Similarly, endogenous ERK2 activation by LPA was blocked by AG1478 in SCC-9 and SCC-25 (Fig. 2C). These experimental data indicate that the EGFR is instrumental in transducing mitogenic signals in response to LPA in head and neck cancer cells. Interestingly, Albanell et al. (14) have reported that immunostaining of activated ERK1/2 was associated with high EGFR and HER2/neu expression levels in head and neck tumor biopsies. Moreover, anti-EGFR therapy with Cetuximab (C225) resulted in lower ERK activation and decreased keratinocyte proliferation in HNSCC patients (14). Combined with our data, GPCR-induced activation of the EGFR in head and neck cancer cells might lead to enhanced ERK/MAPK activity and proliferation in vivo. Besides the EGFR dependency of MAPK activation by LPA in HNSCC, studies with the metalloprotease inhibitor batimastat suggested the critical involvement of a shedding activity in the stimulation of ERK (Fig. 2C). These observations agree with previous reports on the ligand dependency of ERK activation in vascular smooth muscle cells (6, 24) and MDA-MB-231 cells (25). Our current results further indicate that LPA-induced DNA synthesis and S-phase cell cycle progression requires EGFR and metalloprotease activity in HNSCC cells with both low and high EGFR expression levels (Fig. 3). Because LPA treatment of Detroit-562 cells did not result in further stimulation of cell proliferation,4 EGFR activity may not be significantly enhanced by GPCR ligands in cancer cells with the highest EGFR overexpression.

A further important aspect of our findings is that, in addition to the proliferative responses, EGFR signal transactivation plays a direct role in the regulation of the migratory behavior of head and neck cancer cells. It has been reported before that wound stimuli induce metalloprotease-dependent shedding of EGF-like ligands in keratinocytes (26) and that LPA enhances wound closure and invasion in ovarian cancer cells (3). Interestingly, we show that LPA treatment drastically increased the rate of wound closure and chemotactic migration in an EGFR- and metalloprotease-dependent manner (Fig. 4), providing a mechanistic explanation for GPCR-triggered wound healing and migration via transactivation of the EGFR in HNSCC. In summary, our findings highlight the importance of EGFR signal transactivation in cancer cell proliferation and migration and strongly support a role of diverse GPCRs and their ligands as prognostic determinants for HNSCC.

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* Unpublished observation.
EGFR SIGNAL TRANSCACTIVATION IN HNSCC


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