Journal of Cellular Biochemistry 103:1732–1746 (2008)

Sphingosine 1-Phosphate Induces EGFR Expression Via Akt/NF-κB and ERK/AP-1 Pathways in Rat Vascular Smooth Muscle Cells

Hsi-Lung Hsieh,[1] Chi-Chin Sun,[2] Chou-Bing Wu,[3] Cheng-Ying Wu,[1] Wei-Hsuan Tung,[1] Hui-Hsin Wang,[1] and Chuen-Mao Yang[1]*

[1]Department of Physiology and Pharmacology, Chang Gung University, Tao-Yuan, Taiwan
[2]Department of Ophthalmology, Chang Gung Memorial Hospital, Keelung, Taiwan
[3]Division of Orthodontics, Department of Dentistry, Chang Gung University, Tao-Yuan, Taiwan

Abstract Sphingosine 1-phosphate (S1P) has been shown to regulate expression of several genes in vascular smooth muscle cells (VSMCs) and contributes to arteriosclerosis. However, the mechanisms regulating epidermal growth factor receptor (EGFR) expression by S1P in aortic VSMCs remain unclear. Western blotting and RT-PCR analyses showed that S1P induced EGFR mRNA and protein expression in a time- and concentration-dependent manner, which was attenuated by inhibitors of MEK1/2 (U0126) and phosphatidylinositide 3-kinase (PI3K; wortmannin), and transfection with dominant negative mutants of ERK and Akt, respectively. These results suggested that S1P-induced EGFR expression was mediated through p42/p44 MAPK and PI3K/Akt pathways in VSMCs. In accordance with these findings, S1P stimulated phosphorylation of p42/p44 MAPK and Akt which was attenuated by U0126 and wortmannin, respectively. Furthermore, S1P-induced EGFR upregulation was blocked by a selective NF-κB inhibitor helenalin. Immunofluorescent staining and reporter gene assay revealed that S1P-induced activation of NF-κB was blocked by wortmannin, but not by U0126, suggesting that activation of NF-κB was mediated through PI3K/Akt. Moreover, S1P-induced EGFR expression was inhibited by an AP-1 inhibitor curcumin and tanshinone IIA. S1P-stimulated AP-1 subunits (c-Jun and c-Fos mRNA) expression was attenuated by U0126 and wortmannin, suggesting that MEK and PI3K/ERK cascade linking to AP-1 was involved in EGFR expression. Upregulation of EGFR by S1P may exert a phenotype modulation of VSMCs. This hypothesis was supported by pretreatment with AG1478 or transfection with shRNA of EGFR that attenuated EGF-stimulated proliferation of VSMCs pretreated with S1P, determined by XTT assay. These results demonstrated that in VSMCs, activation of Akt/NF-κB and ERK/AP-1 pathways independently regulated S1P-induced EGFR expression in VSMCs. Understanding the mechanisms involved in S1P-induced EGFR expression on VSMCs may provide potential therapeutic targets in the treatment of arteriosclerosis. J. Cell. Biochem. 103: 1732–1746, 2008.

Key words: vascular smooth muscle cells; sphingosine 1-phosphate; EGF receptor; NF-κB; AP-1

Epidermal growth factor receptor (EGFR; 170 kDa) activated by EGF and other ligands triggers signaling cascades leading to normal cell proliferation, target gene expression, and neoplastic growth [Wahl and Carpenter, 1987; Kudlow et al., 1988; Carpenter and Cohen, 1990]. Over-expression and aberrant activation of the EGFR signal pathway are relative to cell proliferation, migration, invasion, and angiogenesis [Adamson and Wiley, 1997; Dancey and Freidlin, 2003]. Moreover, several lines of evidence have demonstrated that EGFR expression is regulated through transcription level in various cell types [Kudlow et al., 1988; Merlino, 1990]. Many transcription factors have been identified to regulate the EGFR promoter leading to upregulation of EGFR, including activator protein (AP-1), specificity protein 1 (Sp1), and early growth response-1 (Egr-1) [Kageyama et al., 1988; Johnson et al., 2000;

Hsi-Lung Hsieh and Chi-Chin Sun have equal contribution.

Grant sponsor: National Science Council, Taiwan; Grant number: NSC94-2320-B-182-003; Grant sponsor: Chang Gung Medical Research Foundation; Grant number: CMRPD150311.

*Correspondence to: Chuen-Mao Yang, PhD, Department of Pharmacology, Chang Gung University, 259 Wen-Hwa 1st Road, Kwei-San, Tao-Yuan, Taiwan.
E-mail: chuenmao@mail.cgu.edu.tw

Received 26 March 2007; Accepted 8 August 2007
DOI 10.1002/jcb.21563

Nishi et al., 2002]. Furthermore, previous studies have confirmed EGFR as a major therapeutic target for the treatment of cancers [Dancey and Freidlin, 2003]. Since the level of EGFR expression plays a critical role in both physiological and pathological growth and development, it may also play an important role in the development of vascular pathological responses (i.e., atherosclerosis). Thus, EGFR may be an attractive target to be approached in vascular smooth muscle cells (VSMCs) for the development of atherosclerosis therapeutics.

Sphingosine 1-phosphate (S1P) is an important bioactive lipid mediator that exerts a wide range of physiological activities. Many reports have shown that sphingolipids exert as signaling molecules in several inflammatory diseases such as atherosclerosis [Kluk and Hla, 2001; Spiegel et al., 2002; Tamama and Okajima, 2002]. In particular, metabolites of sphingolipid such as S1P, has emerged as a new class of lipid messengers that regulate cell proliferation, differentiation, and migration [Spiegel and Merrill, 1996; Liu et al., 2000; Yatomi et al., 2000; Radeff-Huang et al., 2004]. It has been indicated that extracellular actions of S1P are mediated by endothelial differentiation gene (EDG) receptors, a family of G protein-coupled receptors (GPCRs), which form a large family of at least eight members [Spiegel and Milstien, 2000; Hla et al., 2001; Payne et al., 2002]. Previous reports have shown that rat VSMCs expresses high levels of EDG3 and EDG5 mRNA and low levels of EDG1 mRNA [Kluk and Hla, 2001; Tamama et al., 2001]. Activation of different EDG receptors by S1P has been shown to stimulate cell proliferation and migration in various cell types [Hobson et al., 2001; Radeff-Huang et al., 2004]. In VSMCs as well, S1P induces pleiotropic actions such as contraction of the cells, expression of vascular adhesion molecules (VCAM), stimulation of DNA synthesis, and cell migration [Lee et al., 1999; Hobson et al., 2001; Tamama et al., 2001]. Moreover, S1P also exerted as an important stimulus for EGFR and PDGFβR activation [Tanimoto et al., 2004] and COX-2 expression [Hsieh et al., 2006] in VSMCs that may have significant implications in the vascular injury and inflammatory responses. The expression of EGFR appears to be mediated by a number of mitogen-activated protein kinases (MAPKs), PI3K/Akt, and transcription factors such as AP-1 in various cell types [Johnson et al., 2000; Pyne and Pyne, 2000; Lee et al., 2001; Singleton et al., 2005]. However, the signaling mechanisms underlying S1P-induced EGFR expression in VSMCs remain unknown.

Previous reports have shown that extracellular stimuli elicit a broad spectrum of biological responses through activation of MAPK cascades, including p42/p44 MAPK, p38 MAPK, and c-Jun N-terminal kinase (JNK) [Marshall, 1994]. Although the bioactive phospholipid metabolites including lysophosphatidic acid (LPA) and S1P have been reported to activate these MAPKs [Pyne and Pyne, 2000; Pitson et al., 2003], the relationship between the activation of these pathways and expression of EGFR is not completely understood in VSMCs. Recently, we have shown that S1P-stimulated p42/p44 MAPK phosphorylation leads to COX-2 expression in rat VSMCs [Hsieh et al., 2006]. Another report has further shown that curcumin inhibits EGFR expression by reducing the *trans*-activation activity of Egr-1 through interruption of ERK signal pathway in human colon cancer cells [Chen et al., 2006]. However, whether the activation of these MAPK pathways by S1P leading to EGFR expression was not determined in VSMCs.

In addition to MAPKs, S1P has been shown to activate NF-κB in many cell types [Shatrov et al., 1997; Siehler et al., 2001]. The transcription factor NF-κB plays an important role in regulating the expression of a variety of genes involved in immune and inflammatory responses, cell proliferation, and apoptosis [Barnes and Karin, 1997; Smith et al., 2000; Osawa et al., 2001]. Moreover, NF-κB exerts a positive regulator in the expression of several genes involved in chronic inflammatory diseases [Barnes and Karin, 1997]. Several reports have demonstrated that regulation of EGFR promoter activity and upregulation of EGFR expression are mediated through a NF-κB-dependent manner in nasopharyngeal carcinoma [Nishi et al., 2003; Tao et al., 2004]. Recently, we have also demonstrated that S1P stimulates NF-κB activation and results in COX-2 expression in rat VSMCs [Hsieh et al., 2006]. However, the molecular mechanisms of induction of EGFR by S1P mediated through NF-κB activation are not completely defined in VSMCs.

In this study, we investigated the molecular mechanisms underlying S1P-induced EGFR expression in rat VSMCs. These findings suggested that S1P induced EGFR expression

at the transcriptional and translational levels, which mediated through activation of p42/p44 MAPK, AP-1, PI3K/Akt, and NF-κB-signaling pathways in rat primary cultured VSMCs.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), Lipofectamine Plus reagent, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). Hybond C membrane and ECL Western blotting detection system were from Amersham Biosciences (Buckinghamshire, UK). Anti-NF-κB (p65 subunit), p42 MAPK, and Akt antibodies were from Santa Cruz (Santa Cruz, CA). PhosphoPlus p42/p44 MAPK and phosphoPlus Akt antibody kits were from Cell Signaling (Beverly, MA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from Biogenesis (New Fields, UK). Anti-EGFR monoclonal antibody was from BD Transduction Laboratories (San Diego, CA). S1P, LY294002, wortmannin, U0126, curcumin, tanshinone IIA, and helenalin were from Biomol (Plymouth Meetings, PA). Bicinchoninic acid (BCA) protein assay kit was from Pierce (Rockford, IL). Sphingosine, enzymes, XTT assay kit, and other chemicals were from Sigma (St. Louis, MO).

Rat VSMCs Culture

VSMCs were isolated from Sprague-Dawley rats and cultured as previously described [Hsieh et al., 2006]. The cells were plated onto (1 ml/well) 12-well culture plates and (10 ml/dish) 10 cm-dishes for Western blotting and RT-PCR analyses, respectively. The medium was changed after 24 h and then every 3 days. VSMCs were identified by their characteristic "hill" and "valley" growth pattern, the absence of factor VII antigen and presence of SMC-specific α-actin [Yang et al., 2005].

Preparation of Cell Extracts and Western Blotting Analysis

VSMCs were plated on 12-well culture plates and made quiescent at confluence by incubation in serum-free DMEM for 24 h. Growth-arrested cells were incubated with or without S1P at 37°C for various times. The cell lysates were collected and the protein concentration was determined by the BCA reagents according to the instructions of the manufacturer. Samples from these cell lysates (30 μg protein) were denatured and subjected to SDS–PAGE using a 10% (w/v) running gel. The phosphorylation of Akt, p42/p44 MAPK, and expression of EGFR were identified and quantified by Western blotting analysis using anti-phospho-Akt and anti-phospho-p42/44 MAPK, and EGFR antibodies according to our previous study [Hsieh et al., 2006]. Briefly, membranes were then incubated overnight at 4°C with the anti-EGFR, anti-phospho-Akt, or anti-phospho-MAPK polyclonal antibody used at a dilution of 1:1,000 in TTBS. Membranes were washed with TTBS four times for 5 min each, incubated with a 1:2,000 dilution of anti-rabbit horseradish peroxidase antibody for 1 h. Following each incubation, the membrane was washed extensively with TTBS. The immunoreactive bands detected by ECL reagents were developed by Hyperfilm-ECL.

Total RNA Extraction and RT-PCR Analysis

In accordance with the previous study, total RNA was extracted from VSMCs [Hsieh et al., 2006]. The cDNA obtained from 0.5 μg total RNA was used as a template for PCR amplification. Oligonucleotide primers were designed based on Genbank entries for rat *EGFR* [Lin et al., 1996], *c-fos*, *c-jun* [Clerk et al., 2002], and *β-actin*. The following primers were used for amplification reaction:For *EGFR*,

forward primer 5′-(TGGTGCCGGGTCTGATGATG)-3′;
reverse primer 5′-(GCAATGCGGTTCTGATACTG)-3′;

For *c-fos*,

forward primer 5′-(AGACGAAGGAAGACGTGTAAGCACTGCAGCT)-3′;
reverse primer 5′-(AAGGAGAATCCGAAGGGAAAGGAATAAGATG)-3′;

For *c-jun*,

forward primer 5′-(ATGACTGCAAAGATGGAAACG)-3′;
reverse primer 5′-(TATTCTGGCTATGCAGTTCAG)-3′;

For *β-actin*,

forward primer 5′-(GAACCCTAAGGCCAACCGTG)-3′;

reverse primer 5′-(TGGCATAGAGGTCTTTACGG)-3′.

The amplification was performed in 30 cycles at 94°C, 30 s; 55°C, 30 s; 72°C, 1 min. PCR fragments were analyzed as previously described [Hsieh et al., 2006]. These primer sets specifically recognize only the genes of interest as indicated by amplification of a single band of the expected size (502 bp for *EGFR*, 600 bp for *c-fos*, 375 bp for *c-jun*, and 514 bp for *β-actin*) and direct sequence analysis of the PCR product.

Plasmids and Transfection

The plasmids encoding dominant negative mutants of Akt and ERK were kindly provided by Drs. R. D. Ye (Department of Pharmacology, University of Illinois at Chicago) and K. L. Guan (Department of Biological Chemistry, University of Michigan), respectively. The plasmid encoding short hairpin RNA of EGFR (EGFR shRNA) was kindly provided by Dr. C. P. Tseng (Department of Medical Biotechnology and Laboratory Science, University of Chang Gung, Tao-Yuan, Taiwan). All plasmids were prepared by using QIAGEN plasmid DNA preparation kits. VSMCs were plated at 3×10^5 cells/ml (2 ml/well) in 6-well culture plates for 24 h, reaching about 80% confluence. Cells were washed once with PBS and serum-free DMEM, and 0.8 ml of serum-free OPTI-MEM I medium was added to each well. The DNA PLUS-Lipofectamine reagent complex was prepared according to instructions of the manufacturer, as previously described [Hsieh et al., 2006].

Immunofluorescent Staining

VSMCs were plated on 6-well culture plates with coverslips. Cells were cultured in serum-free DMEM for 24 h and treated with (2×10^{-5} mol/L) S1P. Cells were fixed and permeabilized as described previously [Hsieh et al., 2006]. The staining was performed by incubating with 10% normal goat serum in PBS for 30 min followed by incubating with primary anti-EGFR or NF-κB p65 antibody (1:100 dilution) for 1 h in PBS with 1% BSA, washing three times with PBS, incubating for 1 h with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (1:100 dilution) in PBS with 1% BSA, washing three times with PBS, and finally mounting with aqueous mounting medium. The images were observed under a fluorescence microscope (ZEISS, Axiovert 200 M).

Transient Transfection and Reporter Gene Assay

The κB promoter construct pNFκB-luciferase reporter construct was transfected into VSMCs using the Lipofectamine reagent according to the instructions of manufacture. To assess promoter activity, cells were collected and disrupted by sonication in lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM EDTA, 1% Triton X-100, and 10% glycerol). After centrifugation, aliquots of the supernatants were tested for luciferase activity using the luciferase assay system (Promega, Madison, WI). Firefly luciferase activities were standardized to β-galactosidase activity.

Cell Proliferation Assay

VSMCs (2×10^4 cells per well) were seeded in 12-well culture plates and incubated at 37°C in DMEM containing 10% FBS. After 72 h incubation, cells were transfected with empty vector (pTOPO-U6) or EGFR shRNA (0.7 μg per well) for 4 h by Metafectene™ transfection reagent according to the manufacturer's instructions (Biontex), and then incubated 20 h in DMEM containing 10% FBS. Cells were growth-arrested by incubation in serum-free DMEM for 24 h and then incubated with or without S1P for 8 h before treatment with EGF for 48 h. Cell proliferation was assessed by the ability of metabolically active cells to reduce tetrazolium salt (XTT) to colored formazan compounds. Cells were exposed to XTT (1 mg/ml, 4 h) and the absorbance of the samples was measured with an ELISA reader (wavelength 450 nm, reference wavelength 620 nm).

Statistical Analysis

Data are expressed as mean ± SEM using GraphPad Prism Program (GraphPad, San Diego, CA). Quantitative data were analyzed using ANOVA followed by Tukey's honestly significant difference tests between individual groups. A value of $P<0.05$ was considered significant.

RESULTS

S1P Induces *EGFR* Gene Expression

First, to determine the effect of S1P on the EGFR protein and mRNA expression, VSMCs were treated with 5–20 μM S1P for the indicated times. S1P induced EGFR protein

expression in a time- and concentration-dependent manner in VSMCs (Fig. 1A). A significant increase in EGFR expression was observed by 4 h, a maximal response peaked at 8 h, and sustained for 16 h. In addition, the extent of EGFR expression induced by S1P was dependent on the concentrations of S1P in VSMCs (Fig. 1A). A maximal effect was obtained with S1P at a concentration of 20 μM. To confirm the specificity of S1P and eliminate the possibility of the breakdown of S1P to sphingosine in the EGFR induction, the sphingosine, another bioactive lipid, was used. As shown in Figure 1B, treatment of cells with (1, 10, and 100 μM) sphingosine for various times had no effect on the EGFR protein expression in VSMCs. This result confirmed the specificity of S1P in this response. Moreover, S1P-induced EGFR expression was further revealed by immunofluorescent staining in VSMCs (Fig. 1C).

To examine whether the increase in EGFR protein by S1P resulted from the increase of *EGFR* mRNA expression, RT-PCR analysis showed that S1P (20 μM) time-dependently induced *EGFR* mRNA expression in VSMCs (Fig. 1D). These results showed that a significant increase in *EGFR* mRNA was observed at 30 min, reached a peak level by 4 h, and maintained at least for 6 h. These results suggested that S1P induced EGFR expression through increasing mRNA and protein levels.

Induction of EGFR by S1P Is Mediated Through p42/p44 MAPK

We have previously shown that S1P stimulates a time- and concentration-dependent phosphorylation of p42/p44 MAPK in VSMCs [Hsieh et al., 2006]. Therefore, to determine whether S1P-induced EGFR expression was mediated through p42/p44 MAPK activation, VSMCs were pretreated with a selective MEK inhibitor U0126 (0.01, 0.1, and 1 μM) for 1 h and then stimulated with (20 μM) S1P for 8 h. As shown in Figure 2A, pretreatment of VSMCs with U0126 (0.1 and 1 μM) significantly inhibited EGFR expression by 90% in response to S1P ($n = 3$, $P < 0.01$). Moreover, pretreatment of VSMCs with a MEK1/2 inhibitor U0126 (1 μM) also significantly inhibited the S1P-induced EGFR mRNA expression ($n = 3$; Fig. 2B). These

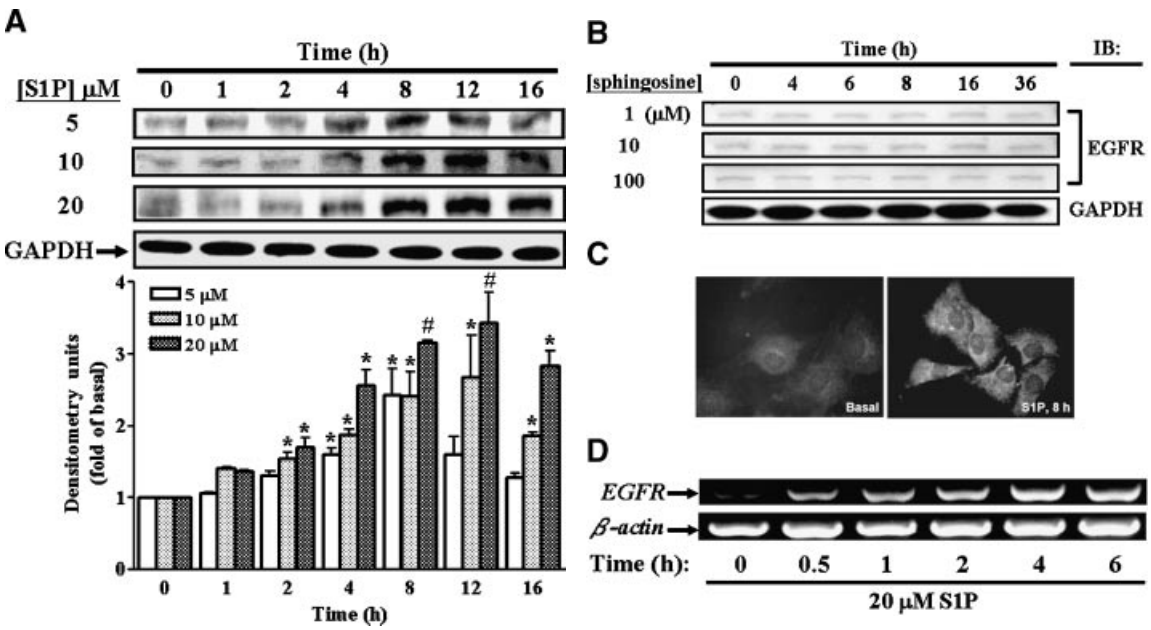

Fig. 1. S1P induces EGFR expression in VSMCs. **A**: Cells were incubated with various concentrations of S1P for the indicated times. **B**: Cells were incubated with sphingosine (1, 10, and 100 μM) for various times. The whole cell lysates were subjected to 10% SDS–PAGE and transferred to nitrocellulose membrane. Western blot analysis was performed using an antiserum reactive with EGFR antibody as described in the section ''Materials and Methods''. Data are expressed as mean ± SEM of four independent experiments. $^{*}P < 0.05$; $^{\#}P < 0.01$ versus vehicle only. **C**: S1P-induced EGFR expression was detected by immunofluorescent staining after 8 h incubation of (**left**) without or (**right**) with S1P (20 μM). Individual cells were imaged as described in ''Materials and Methods''. **D**: Time dependence of S1P-induced EGFR mRNA expression. VSMCs were treated with 20 μM S1P for various times. Total RNA was collected and analyzed by RT-PCR as described in ''Materials and Methods''. The figure represents one of the three individual experiments.

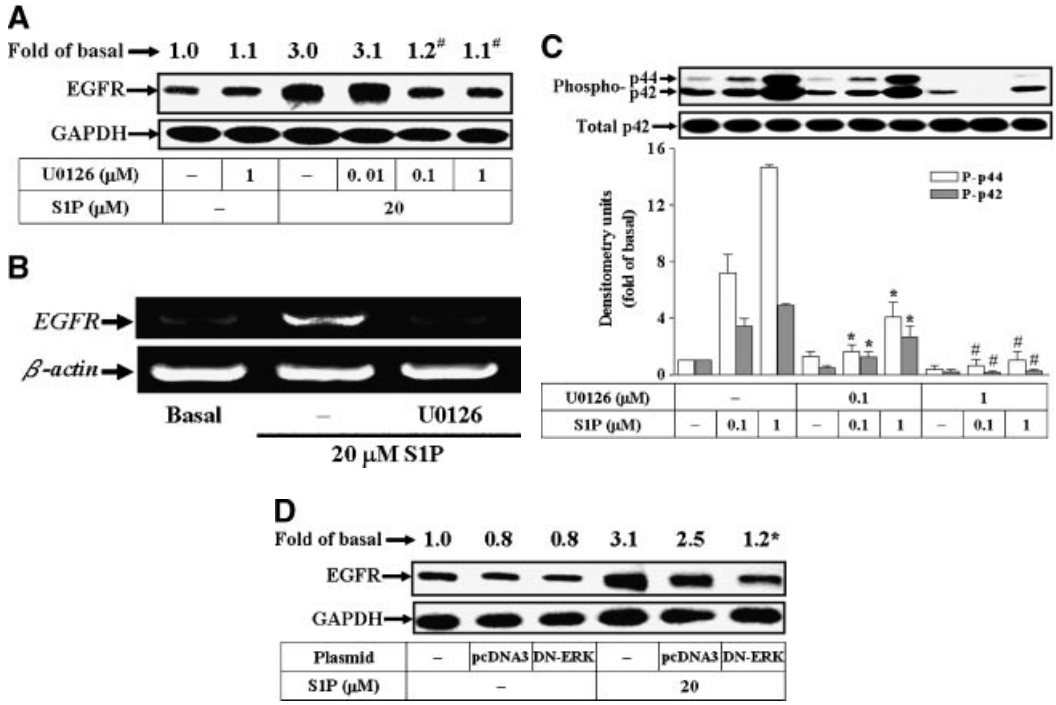

Fig. 2. Involvement of p42/p44 MAPK in S1P-induced EGFR expression in VSMCs. **A**: Effect of U0126 on S1P-induced EGFR expression in VSMCs. For EGFR protein expression, cells were pretreated with U0126 (0.01, 0.1, and 1 μM) for 1 h and then incubated with S1P for 8 h. **B**: For EGFR mRNA expression, cells were treated with S1P for 1 h in the absence or presence of U0126 (1 μM). **C**: Inhibition of S1P-stimulated p42/p44 MAPK phosphorylation by U0126, cells were pretreated with various concentrations of U0126 (0.1 and 1 μM) for 1 h and then stimulated with S1P for 3 min. **D**: Cells were transfected with plasmids encoding pcDNA3 or dominant negative mutant of ERK2 (DN-ERK2), and then stimulated with vehicle or S1P for 8 h. After incubation, the EGFR protein, mRNA expression, and p42/p44 MAPK phosphorylation were analyzed as described in the section ''Materials and Methods''. Membranes were stripped and re-probed with total p42 MAPK (C) or GAPDH (A and D) as an internal control, respectively. Data are expressed as mean ± SEM of three independent experiments. $^{*}P < 0.05$; $^{\#}P < 0.01$ versus S1P alone.

results demonstrated that S1P-induced EGFR expression may be mediated through a p42/p44 MAPK in VSMCs. To ensure whether S1P-stimulated p42/p44 MAPK phosphorylation linked to EGFR expression, pretreatment of VSMCs with U0126 significantly attenuated S1P-stimulated p42/p44 MAPK phosphorylation ($n = 3$, $P < 0.05$, as compared with control) in a concentration-dependent manner (Fig. 2C). The phosphorylation of p42/p44 MAPK by S1P (1 μM) was almost completely inhibited when cells were preincubated with 1 μM U0126. Moreover, pretreatment of U0126 (0.1 and 1 μM) also attenuated S1P (20 μM)-stimulated p42/p44 MAPK phosphorylation (data not shown). To further confirm whether phosphorylation of p42/p44 MAPK (ERK1/2) was directly involved in S1P-induced EGFR expression, VSMCs were transfected with a dominant negative ERK mutant (DN-ERK). Transfection of VSMCs with DN-ERK significantly inhibited S1P-indcuced EGFR expression (Fig. 2D). These results suggested a link between activation of p42/p44 MAPK and induction of EGFR in VSMCs.

S1P Induces EGFR Expression Via PI3K/Akt Cascade

In addition, PI3K/Akt pathway has been shown to be activated by S1P in VSMCs [Tanimoto et al., 2004; Hsieh et al., 2006] that participates in induction of various inflammatory genes such as COX-2 [Jang et al., 2005; Sheu et al., 2005; Hsieh et al., 2006]. Thus, to determine whether PI3K/Akt pathway was involved in S1P-induced EGFR expression, a selective PI3K inhibitor wortmannin [Arcaro and Wymann, 1993] was used. As shown in Figure 3A, pretreatment of VSMCs with wortmannin (0.3 and 3 μM) significantly attenuated S1P-induced EGFR expression ($P < 0.05$, $n = 3$). These results suggested that S1P-stimulated

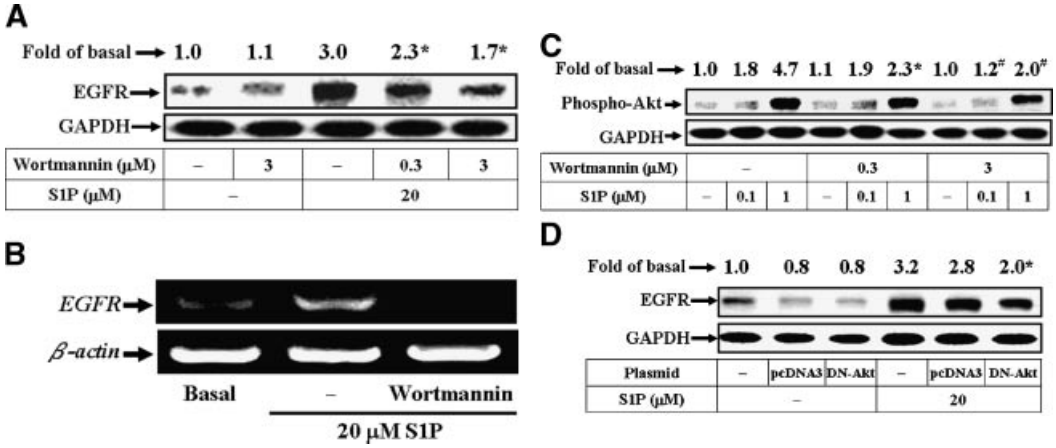

Fig. 3. S1P induces EGFR expression through PI3K/Akt cascade in VSMCs. **A**: For EGFR protein expression, cells were pretreated wortmannin (0.3 and 3 μM) for 1 h and then incubated with S1P for 8 h. **B**: For mRNA expression, cells were pretreated with wortmannin (3 μM) for 1 h and then treated with S1P for 1 h. **C**: Inhibition of S1P-stimulated Akt phosphorylation by wortmannin, cells were pretreated with wortmannin (0.3 and 3 μM) for 1 h and then stimulated with S1P (0.1 and 1 μM) for 3 min. **D**: Cells were transfected with plasmids encoding pcDNA3 or dominant negative Akt (DN-Akt), and then stimulated with S1P for 8 h. After incubation, EGFR protein (A and D), mRNA (B) expression, and phospho-Akt (p-Akt) (C) were determined as described in the section ''Materials and Methods''. Data are expressed as mean ± SEM of three independent experiments. $^{*}P < 0.05$; $^{\#}P < 0.01$ versus S1P alone.

EGFR expression was mediated through PI3K in VSMCs. Correspondingly, S1P-induced EGFR mRNA upregulation was also significantly inhibited by pretreatment of VSMCs with 3 μM wortmannin (Fig. 3B). These results suggested that S1P-induced EGFR expression was, at least in part, mediated through the activation of PI3K pathway in VSMCs. To corroborate the role of PI3K/Akt in EGFR expression, we determined if Akt phosphorylation (Ser^{473}) was stimulated by S1P, which is a prerequisite for the catalytic activity of Akt (Downward, 1998). Our previous report has shown that S1P-stimulated Akt phosphorylation reaches a peak at 5 min and slowly declined thereafter [Hsieh et al., 2006]. Moreover, S1P (1 μM)-stimulated Akt phosphorylation was concentration-dependently inhibited by wortmannin (0.3 and 3 μM) (Fig. 3C). In addition, pretreatment of wortmannin (0.3 and 3 μM) also attenuated S1P (20 μM)-stimulated Akt phosphorylation (data not shown). To further confirm whether Akt was essentially required for S1P-induced EGFR expression, VSMCs were transfected with a dominant negative plasmid of Akt (DN-Akt). The results showed that transfection with DN-Akt significantly attenuated S1P-induced EGFR expression revealed by Western blotting (Fig. 3D). These data indicated that S1P-induced EGFR expression was mediated through PI3K/Akt cascade in VSMCs.

NF-κB Activation Is Required for S1P-Induced EGFR Expression

Several lines of evidence have demonstrated that NF-κB is activated by a variety of GPCR agonists such as BK [Hsieh et al., 2004] and S1P [Hsieh et al., 2006]. To investigate whether NF-κB was involved in S1P-induced EGFR expression in VSMCs, a selective NF-κB inhibitor, helenalin, was used, which is shown to inhibit NF-κB activation by specific and irreversible alkylation of the p65 subunit [Lyss et al., 1998]. As shown in Figure 4A, pretreatment of VSMCs with helenalin (0.1, 1, 10 μM) significantly inhibited S1P-induced EGFR protein expression in a concentration-dependent manner determined by Western blotting ($n = 3$, $P < 0.01$). In addition, S1P-induced EGFR mRNA expression was inhibited by pretreatment with 1 μM helenalin (Fig. 4B). The nuclear translocation of NF-κB is often served as a fundamental index of transcriptional activation. Thus, we further determined whether NF-κB translocation was involved in S1P-induced EGFR expression in VSMCs. The translocation of NF-κB was determined by immunofluorescence staining using a NF-κB (p65 subunit) monoclonal antibody. The staining images showed that S1P stimulated NF-κB (p65) translocation in VSMCs (Fig. 4C, inserted panel). Next, to differentiate whether MAPK and Akt

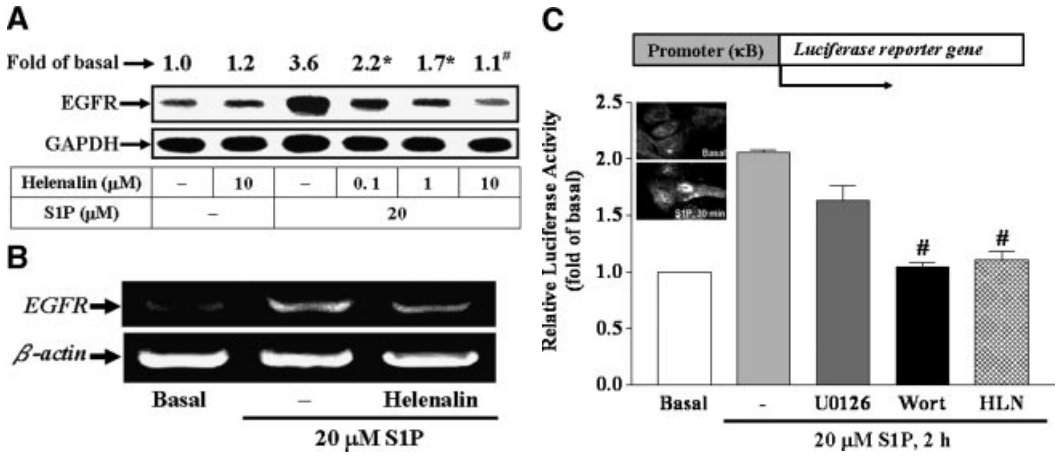

Fig. 4. Activation of NF-κB is required for S1P-induced EGFR expression in VSMCs. **A**: Cells were treated with S1P for 8 h in the absence or presence of helenalin (0.1, 1, and 10 μM). **B**: VSMCs were pretreated with helenalin (1 μM) for 1 h and then treated with S1P for 1 h. After incubation, EGFR protein (A) and mRNA (B) expression were determined as described under ''Materials and Methods''. (**C**, **Inserted panels**) Nuclear translocation of NF-κB induced by S1P, VSMCs were treated with (**lower**) or without (**upper**) S1P for 30 min. The immunofluorescence staining was performed as described in ''Materials and Methods''. C: S1P induces NF-κB activity in VSMCs, cells were transfected with a reporter gene construct (pNFκB-Luc) and then were stimulated with S1P (20 μM) for 2 h in the absence or presence of U0126 (1 μM), wortmannin (Wort, 3 μM), or helenalin (HLN, 1 μM). Luciferase activity was determined in the cell lysates as described under ''Materials and Methods''. Data are expressed as mean ± SEM of three independent experiments. $^{*}P < 0.05$; $^{\#}P < 0.01$ versus S1P alone.

phosphorylation was associated with NF-κB activation, the activation of NF-κB was assessed following S1P stimulation in the presence of inhibitors of MEK1/2 (U0126), PI3K (wortmannin), and NF-κB (helenalin), respectively. Using a promoter-luciferase reporter gene construct contains NF-κB-binding sites (pNFκB-Luc) to determine the activity of NF-κB stimulated by S1P. The activity of NF-κB stimulated by S1P was significantly blocked with pretreatment of wortmannin (Wort; 3 μM) and helenalin (HLN; 1 μM), but not by U0126 (Fig. 4C, $n = 3$, $P < 0.01$). These results confirmed that S1P-induced NF-κB activation was mediated through PI3K/Akt required for upregulation of EGFR in VSMCs.

S1P Upregulates EGFR Expression Via AP-1

In addition to NF-κB, EGFR promoter has been shown to contain several binding site for various transcription factors including AP-1 [Johnson et al., 2000]. To determine whether S1P regulated EGFR expression mediated through transcription factor AP-1, VSMCs were pretreated with two AP-1 inhibitors curcumin (1, 10, and 100 μM) or tanshinone IIA (TAS IIA; 1 and 10 μM), and then stimulated with S1P (20 μM) for 8 h. As shown in Figure 5A, pretreatment of VSMCs with curcumin or tanshinone IIA both significantly inhibited S1P-induced EGFR expression in a concentration-dependent manner. Moreover, we determined whether S1P stimulated AP-1 (containing early mediated gene c-Fos and c-Jun subunits) expression, c-fos and c-jun mRNA expression was determined by RT-PCR. These results showed that stimulation of VSMCs with 20 μM S1P induced *c-fos* and *c-jun* gene expression in a time-dependent manner. The expression of *c-fos* and *c-jun* by S1P reached a peak at 30 min and declined to basal level within 2 h (Fig. 5B). To further investigate whether PI3K/Akt and MEK/ERK cascades were involved in S1P-induced AP-1 expression, VSMCs were pretreated with U0126 (1 μM), wortmannin (3 μM), or curcumin (10 μM) and then stimulated by S1P. As shown in Figure 5C, induction of AP-1 expression by S1P was also inhibited by pretreatment with U0126, wortmannin, and curcumin. These results indicated that S1P-induced EGFR expression was mediated through PI3K and MEK leading to p42/p44 MAPK activation and transcription factor AP-1 expression.

The Sequential Phosphorylation of MEK/ERK and PI3K/Akt Stimulated by S1P

To examine whether the effect of S1P on EGFR expression, Akt and p42/p44 MAPK activation were mediated through the activation of a GPCR receptor coupled to a pertussis toxin (PTX)-sensitive G protein, cells were

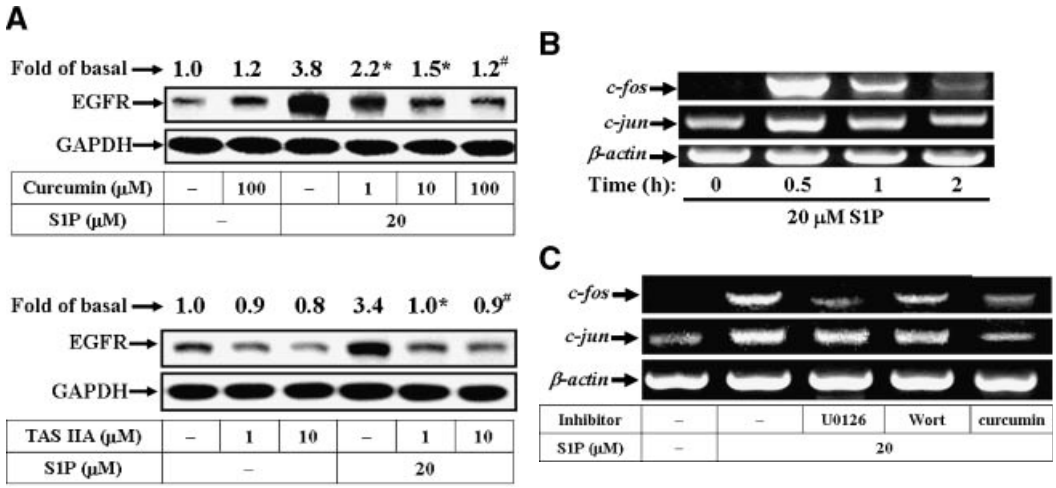

Fig. 5. Expression of AP-1 is essential for S1P-induced EGFR expression in VSMCs. **A**: Cells were treated with S1P for 8 h in the absence or presence of curcumin (**upper**; 1, 10, and 100 μM) and tanshinone IIA (**lower**; TAS IIA, 1, and 10 μM). **B**: Time dependence of S1P-induced AP-1 (c-fos and c-jun) mRNA expression, cells were treated with S1P for various times. **C**: Cells were pretreated with U0126 (1 μM), wortmannin (Wort; 3 μM), or curcumin (10 μM) for 1 h and then treated with S1P for 30 min. After incubation, EGFR protein (A) and AP-1 mRNA (B and C) expression were determined as described in the section ''Materials and Methods''. Data are expressed as mean ± SEM of three independent experiments. $^{*}P < 0.05$; $^{\#}P < 0.01$ versus S1P alone.

pretreated with PTX (10 and 100 ng/ml) for 24 h and then stimulated with S1P. As shown in Figure 6A, pretreatment of RBA-1 with PTX significantly attenuated the S1P-induced expression of EGFR. Moreover, S1P-stimulated phosphorylation of p42/p44 MAPK and Akt were significantly inhibited by pretreatment of PTX (Fig. 6B). These results suggested that the effect of S1P on these responses was mediated through a PTX-sensitive GPCR in VSMCs. We further determine whether MEK/ERK cross-talked with PI3K/Akt stimulated by S1P, cells were pretreated with wortmannin and U0126 for 1 h, respectively, and then stimulated with S1P for 3 min. The data showed that S1P-stimulated p42/p44 MAPK phosphorylation was attenuated by pretreatment of VSMCs with wortmannin (Fig. 6C). In contrast, S1P-stimulated Akt phosphorylation was unaffected by pretreatment with U0126 (Fig. 6D). These results implied that PI3K may be an upstream component of S1P-stimulated p42/p44 MAPK and Akt phosphorylation in VSMCs.

Upregulation of EGFR by S1P Enhances Cell Proliferation

To further demonstrate whether S1P-induced upregulation of EGFR influenced on VSMCs proliferation, cells were pretreated with AG1478 or transfected with EGFR shRNA, incubated with S1P following by EGF, and then measured cell proliferation by XTT assay. We first checked the expression of total EGFR on VSMCs after transfection with EGFR shRNA for 48 h. As shown in Figure 7A, the total EGFR has been knocked down by transfection with EGFR shRNA, and not affected by transfection with the empty vector (pTOPO-U6; as a control). Moreover, the upregulation of EGFR by S1P has also been knocked down by transfection with EGFR shRNA in VSMCs. In our study, we found that upregulation of EGFR by S1P (20 μM) can enhance EGF-induced VSMC proliferation (Fig. 7B). The enhancements of cell proliferation induced by EGF (5 ng/ml) were attenuated by pretreatment with EGFR kinase inhibitor AG1478 (10 μM) after challenged with or without S1P-stimulated for 8 h. Moreover, this enhanced response was also completely inhibited by transfection with EGFR shRNA. These results suggested that upregulation of EGFR by S1P exerted as a phenotype modulation in cell proliferation of VSMCs.

DISCUSSION

Several lines of evidence have shown that EGFR plays an important role in cell proliferation and in cancer cell transformation [Wahl and Carpenter, 1987]. High levels of the EGFR have been detected in various human cancers, including glioblastomas and ovarian cervical

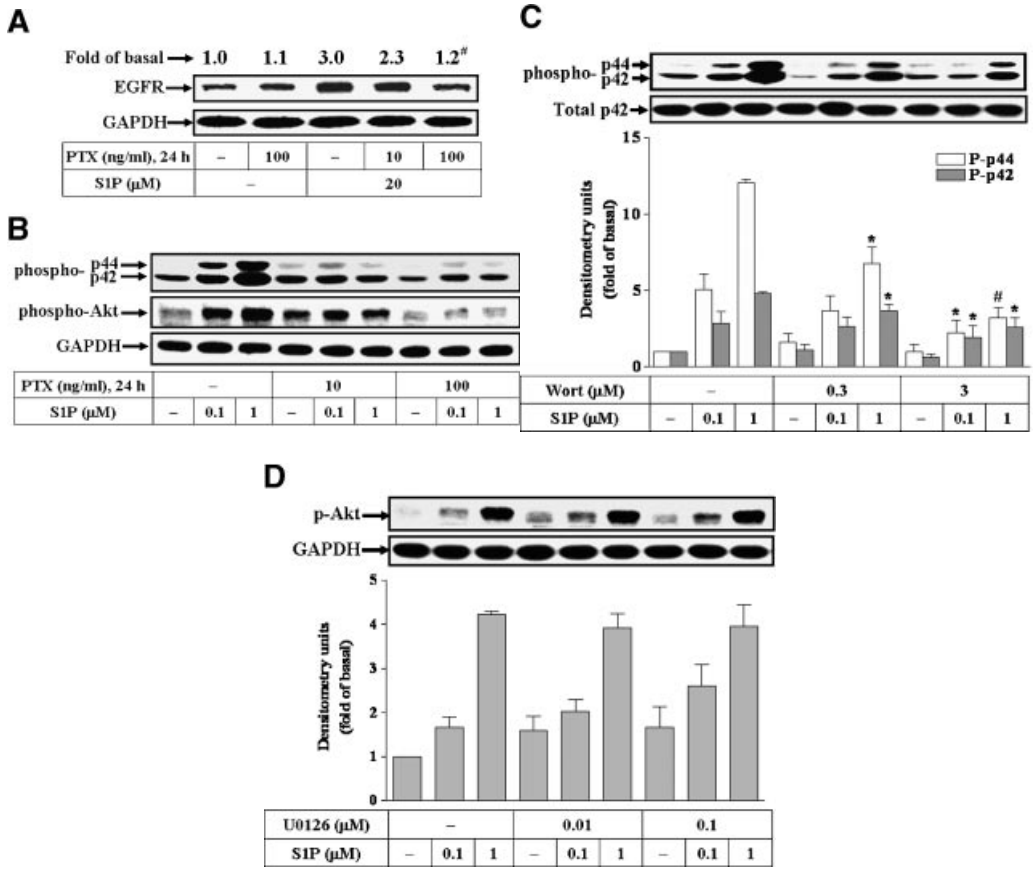

Fig. 6. Effects of pertussis toxin, wortmannin, and U0126 on p42/p44 MAPK and Akt phosphorylation stimulated by S1P in VSMCs. **A**: Cells were pretreated with or without pertussis toxin (PTX, 10 and 100 ng/ml) for 24 h before S1P-stimulated for 8 h. After incubation, the EGFR was determined as described in the section ''Materials and Methods''. **B**: Cells were treated with S1P for 3 min in the absence or presence of PTX (10 and 100 ng/ml), (**C**) wortmannin (Wort, 0.3 and 3 μM), and (**D**) U0126 (0.01 and 0.1 μM). After incubation, the phospho-p42/p44 MAPK and phospho-Akt (p-Akt) were determined as described in the section ''Materials and Methods''. Data are expressed as mean ± SEM of three independent experiments. $^{*}P < 0.05$; $^{\#}P < 0.01$ versus S1P alone.

and kidney tumors [Libermann et al., 1985; Kohler et al., 1989; Yoshida et al., 1994]. Moreover, EGFR exerts a wide range of biological activities in different tissues, blood vessels in particular, including proliferation, development, tumors, and inflammation [Wahl and Carpenter, 1987; Woodworth et al., 2005; Ying and Sanders, 2005]. S1P is elevated in the region during vascular injuries and inflammation [Takuwa et al., 2002; Peng et al., 2004]. Our pervious study has demonstrated that S1P induces COX-2 expression mediated through p42/p44 MAPK and PI3K/Akt activation in VSMCs [Hsieh et al., 2006]. However, little was known about the molecular mechanisms underlying S1P-induced EGFR expression in these cells. In this study, we have applied Western blot analysis, selective pharmacological inhibitors, transfection with dominant negative mutants and shRNA, immunofluorescent staining, gene promoter assay, and XTT assay kit to characterize the mechanisms underlying S1P-induced EGFR expression and cell proliferation. Our results demonstrated that in VSMCs, S1P-induced EGFR expression was mediated through activation of p42/p44 MAPK regulating by MEK and PI3K linking to AP-1. In addition, EGFR induction by S1P was also regulated via PI3K/Akt cascade linking to NF-κB activation. This upregulation of EGFR by S1P exerted as a phenotype modulation in cell proliferation of VSMCs (Fig. 7).

First, we excluded the interference of other bioavtive lipids such as sphingosine which may be the breakdown of S1P on EGFR expression (Fig. 1B). Treatment of VSMCs with sphingosine has no effect on EGFR expression, confirming the specificity of S1P in the induction of EGFR in

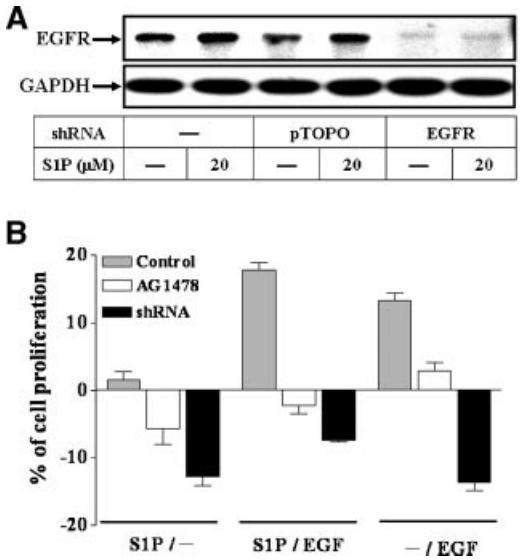

Fig. 7. S1P enhances VSMCs cell proliferation through increasing EGFR expression. VSMCs were transfected with EGFR shRNA or pretreated with AG1478 (10 μM) for 1 h before treatment of S1P for 8 h, and then cells were treated with or without EGF (5 ng/ml) for 48 h. **A**: The expression of total EGFR was determined after transfection of empty vector (pTOPO) or EGFR shRNA and then treated with S1P for 8 h. Representative images are shown for 48 h. The figure represents one of the three individual experiments. **B**: The percentages of cell proliferation were measured by XTT assay as described in the section ''Materials and Methods''. Data are expressed as mean ± SEM of three independent experiments.

VSMCs. Next, activation of p42/p44 MAPK might be implicated in expression of inflammatory genes in vascular injury and inflammation [Yang et al., 2000, 2005; Kim et al., 2003]. S1P has been reported to act as an important mediator through activation of p42/p44 MAPK cascade in different cell types [Pyne and Pyne, 2000; Tanimoto et al., 2004]. Our recent report has demonstrated that activation of p42/p44 MAPK leads to S1P-induced COX-2 expression in VSMCs [Hsieh et al., 2006]. Therefore, we first investigated the role of p42/p44 MAPK involved in EGFR expression in VSMCs. In this study, our results demonstrated that activation of p42/p44 MAPK was necessary for S1P-induced EGFR expression in VSMCs, since pretreatment with U0126, a selective inhibitor of MEK1/2 [Favata et al., 1998], significantly attenuated S1P-induced EGFR mRNA and protein expression and p42/p44 MAPK phosphorylation in VSMCs. In addition, transfection with dominant negative mutant of ERK2 evidently reduced S1P-induced EGFR expression, suggesting that activation of p42/p44 MAPK was required for EGFR expression. These results were consistent with the reports that activation of p42/p44 MAPK plays a crucial role in EGFR expression in human colon cancer cells [Chen et al., 2006].

In addition to p42/p44 MAPK, S1P-stimulated activation of PI3K/Akt has been reported in various cell types [Lee et al., 2001; Spiegel et al., 2002; Singleton et al., 2005; Hsieh et al., 2006]. PI3K/Akt has been shown to be implicated in the pathogenesis of inflammatory responses [Kim et al., 2001; Koyasu, 2003] and linked to induction of growth factors expression [Jang et al., 2005; Sheu et al., 2005]. Therefore, we investigated the involvement of PI3K/Akt in S1P-induced EGFR expression using a PI3K inhibitor, wortmannin. Both EGFR mRNA and protein upregulation by S1P were significantly inhibited by wortmannin, suggesting that EGFR induction was mediated through a PI3K-dependent pathway. PI3K is known to initiate a series of events that leads to Akt phosphorylation and translocation to the internal surface of the cell membrane, resulting in activation of Akt [Brazil and Hemmings, 2001]. As expected, we found that S1P stimulated a rapid phosphorylation of Akt in a time- and concentration-dependent manner, which was inhibited by wortmannin in a concentration-dependent manner. These results suggested that PI3K/Akt participated in S1P-induced EGFR expression. This hypothesis was further supported by the results that transfection of VSMCs with a dominant negative plasmid of Akt apparently attenuated the expression of EGFR, indicating the involvement of PI3K/Akt in EGFR expression induced by S1P (Fig. 3D). Moreover, we also determined that S1P-induced EGFR expression via activation of p42/p44 MAPK and Akt by a PTX-sensitive G protein-coupled receptor-dependent manner (Fig. 6A,B). Interestingly, our data showed that S1P-stimulated p42/p44 MAPK phosphorylation was attenuated by wortmannin in VSMCs (Fig. 6C), consistent with previous study that PI3K may be an important mediator in regulating p42/p44 MAPK activity [Moelling et al., 2002]. In contrast, S1P-stimulated Akt phosphorylation was unaffected by U0126 (Fig. 6D). These results implied that S1P-induced EGFR expression may be sequentially mediated through PI3K-p42/p44 MAPK cascade.

It has been well established that vascular responses following exposure to extracellular stimuli are highly dependent on activation of transcription factor NF-κB, which plays an

important role in regulation of gene expression [Barnes and Karin, 1997; Didonato et al., 1997]. In addition, several studies have emerged that PI3K/Akt phosphorylates IκB kinase (IKK) in response to platelet-derived growth factors (PDGF), TNF-α, and BK [Ozes et al., 1999; Romashkova and Makarov, 1999; Xie et al., 2000]. This activated Akt increases transcriptional activation of NF-κB [Kim et al., 2001], which plays a critical role in regulating inducible gene expression. The NF-κB releasing by phosphorylation and degradation of IκB, resulting in activation and translocation of NF-κB into nucleus, is essential for the expression of several genes such as VCAM-1 and COX-2 in VSMCs [Kanellis et al., 2003; Hsieh et al., 2006]. Moreover, a previous study has shown that LMP-1 induces EGFR expression mediated through activation of NF-κB in epithelial cells [Miller et al., 1998]. In this study, induction of EGFR mRNA and protein expression by S1P was significantly inhibited by a specific NF-κB inhibitor helenalin [Lyss et al., 1998], indicating that activation of NF-κB was involved in S1P-induced expression of EGFR. Moreover, the nuclear translocation and activity of NF-κB following S1P exposure were confirmed by immunofluorescent staining and NF-κB promoter assay, respectively. The activation of NF-κB element in promoter by S1P was inhibited by a PI3K inhibitor wortmannin, but not by U0126. These results demonstrated that NF-κB was required for S1P-induced EGFR expression mediated through PI3K/Akt pathway, but not p42/p44 MAPK. Our findings suggested that PI3K/Akt was an important component of the signaling pathway linked to NF-κB activation and EGFR expression induced by S1P, consistent with previous studies that pre-incubation of A549 cells with either wortmannin or LY294002 completely abrogated BK- [Pan et al., 1999] and S1P- [Hsieh et al., 2006] induced NF-κB activation in VSMCs. In contrast, the purified NF-κB can bind to the putative sites, there is no evidence that NF-κB transactivates the EGFR promoter region [Nishi et al., 2003], and wortmannin has no effect on the induction of a NF-κB reporter gene stimulated by TNF-α [Romashkova and Makarov, 1999; Reddy et al., 2000] and COX-2 expression induced by BK via ERK linking to NF-κB activation [Chen et al., 2004]. These differences are still controversial. It is possible that PI3K/Akt affects NF-κB activation in more than one way and that the nature of its effects may vary in a cell-type-specific manner.

Furthermore, the *EGFR* promoter has been shown to contain several regulatory sites of DNA-binding factors such as AP-1, which may play a role in upregulation of EGFR in various cell types [Johnson et al., 2000]. Therefore, to determine whether AP-1 was involved in S1P-induced EGFR expression, two AP-1 inhibitor curcumin and tanshinone IIA (Park et al., 1999) were used. We demonstrated that S1P-induced EGFR expression was inhibited by curcumin and tanshinone IIA (Fig. 5A). Moreover, we also found that S1P-stimulated AP-1 components (c-Fos and c-Jun) expression was significantly attenuated by U0126, wortmannin, and curcumin (Fig. 5). These results further confirmed that MEK- and PI3K-p42/p44 MAPK were important signaling cascades leading to AP-1 and EGFR expression induced by S1P. These results demonstrated that S1P-dependent regulation of the transcriptional activity of EGFR was mediated through p42/p44 MAPK-AP-1 and Akt-NF-κB signaling pathways in VSMCs.

In conclusion, we reported here that S1P exerted its enhancing effects on EGFR gene expression and cell proliferation as a phenotype modulation in rat VSMCs. The p42/p44 MAPK, PI3K/Akt, AP-1, and NF-κB cascades cooperatively mediated these S1P responses. Based on

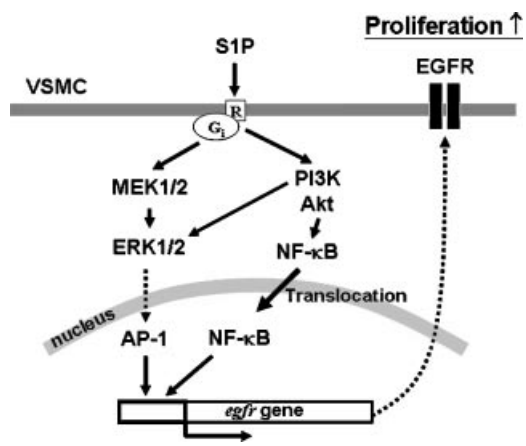

Fig. 8. Schematic representation of signaling pathways involved in S1P-induced EGFR expression in VSMCs. Binding of S1P to PTX-sensitive G protein-coupled receptors results in activation of p42/p44 MAPK, PI3K/Akt, AP-1, and NF-κB pathways. The EGFR transcription is independently regulated by p42/p44 MAPK-AP-1 and Akt-NF-κB pathways. These signaling pathways might enforce each other and contribute to sustained activation of transcription factors required for EGFR expression and then enhance cell proliferation during EGF-stimulation in VSMCs.

the observations from literatures and our findings, Figure 8 depicts a model for the molecular mechanisms underlying S1P-induced EGFR gene expression and then enhanced cell proliferation in VSMCs. These findings concerning S1P-induced EGFR upregulation imply that S1P might play an important role in vascular inflammatory diseases such as atherosclerosis, at least in part, mediated through p42/p44 MAPK, PI3K/Akt, AP-1, and NF-κB-signaling pathways in rat VSMCs. Adding to our recent study [Hsieh et al., 2006], these results indicated a role of VSMCs, in addition to their contractile function, as inflammatory cells involved in the cell proliferation which may contribute to the vascular wall thickness seen in atherosclerosis. Pharmacological approaches suggest that targeting EGFR and their signaling components may yield useful therapeutic targets for atherosclerosis.

ACKNOWLEDGMENTS

The authors appreciate Dr. K.L. Guan (Department of Biological Chemistry, University of Michigan, MI), Dr. R.D. Ye (Department of Pharmacology, University of Illinois at Chicago), and Dr. C.P. Tseng (Department of Medical Biotechnology and Laboratory Science, University of Chang Gung) for providing dominant negative mutants of ERK2 (ERK2 K52R), Akt, and EGFR shRNA, respectively. The authors also thank Ms. Yu-Ching Cheng for her technical assistance in this study.

REFERENCES

Adamson ED, Wiley ML. 1997. The *EGFR* gene family in embryonic cell activities. Curr Top Dev Biol 35:71–120.

Arcaro A, Wymann MP. 1993. Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: The role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. Biochem J 296:297–301.

Barnes PJ, Karin M. 1997. Nuclear factor-κB: A pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 336:1066–1071.

Brazil DP, Hemmings BA. 2001. Ten years of protein kinase B signalling: A hard Akt to follow. Trends Biochem Sci 26:657–664.

Carpenter G, Cohen S. 1990. Epidermal growth factor. J Biol Chem 265:7709–7712.

Chen BC, Yu CC, Lei HC, Chang MS, Hsu MJ, Huang CL, Chen MC, Sheu JR, Chen TF, Chen TL, Inoue H, Lin CH. 2004. Bradykinin B_2 receptor mediates NF-κB activation and cyclooxygenase-2 expression via the Ras/Raf-1/ERK pathway in human airway epithelial cells. J Immunol 173:5219–5228.

Chen A, Xu J, Johnson AC. 2006. Curcumin inhibits human colon cancer cell growth by suppressing gene expression of epidermal growth factor receptor through reducing the activity of the transcription factor Egr-1. Oncogene 25:278–287.

Clerk A, Kemp TJ, Harrison JG, Mullen AJ, Barton PJ, Sugden PH. 2002. Up-regulation of c-jun mRNA in cardiac myocytes requires the extracellular signal-regulated kinase cascade, but c-Jun N-terminal kinases are required for efficient up-regulation of c-Jun protein. Biochem J 368:101–110.

Dancey JE, Freidlin B. 2003. Targeting epidermal growth factor receptor—Are we missing the mark? Lancet 362: 62–64.

Didonato J, Hayakawa A, Rothwarf DM, Zandi E, Karin M. 1997. A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. Nature 388:548–554.

Downward J. 1998. Mechanisms and consequences of activation of protein kinase B/Akt. Curr Opin Cell Biol 10:262–267.

Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM. 1998. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem 273: 18623–18632.

Hla T, Lee MJ, Ancellin N, Paik JH, Kluk MJ. 2001. Lysophospholipids-receptor revelations. Science 294: 1875–1878.

Hobson JP, Rosenfeldt HM, Barak LS, Olivera A, Poulton S, Caron MG, Milstien S, Spiegel S. 2001. Role of the sphingosine-1-phosphate receptor EDG-1 in PDGF-induced cell motility. Science 291:1800–1803.

Hsieh HL, Yen MH, Jou MJ, Yang CM. 2004. Intracellular signalings underlying bradykinin-induced matrix metalloproteinase-9 expression in rat brain astrocyte-1. Cell Signal 16:1163–1176.

Hsieh HL, Wu CB, Sun CC, Liao CH, Lau YT, Yang CM. 2006. Sphingosine-1-phosphate induces COX-2 expression via PI3K/Akt and p42/p44 MAPK pathways in rat vascular smooth muscle cells. J Cell Physiol 207:757–766.

Jang BC, Paik JH, Kim SP, Shin DH, Song DK, Park JG, Suh MH, Park JW, Suh SI. 2005. Catalase induced expression of inflammatory mediators via activation of NF-κB, PI3K/AKT, p70S6K, and JNKs in BV2 microglia. Cell Signal 17:625–633.

Johnson AC, Murphy BA, Matelis CM, Rubinstein Y, Piebenga EC, Akers LM, Neta G, Vinson C, Birrer M. 2000. Activator protein-1 mediates induced but not basal epidermal growth factor receptor gene expression. Mol Med 6:17–27.

Kageyama R, Merlino GT, Pastan I. 1988. Epidermal growth factor (EGF) receptor gene transcription. Requirement for Sp1 and an EGF receptor-specific factor. J Biol Chem 263:6329–6336.

Kanellis J, Watanabe S, Li JH, Kang DH, Li P, Nakagawa T, Wamsley A, Sheikh-Hamad D, Lan HY, Feng L, Johnson RJ. 2003. Uric acid stimulates monocyte chemoattractant protein-1 production in vascular smooth muscle cells via mitogen-activated protein kinase and cyclooxygenase-2. Hypertension 41:1287–1293.

Kim D, Kim S, Koh H, Yoon SO, Chung AS, Cho KS, Chung J. 2001. Akt/PKB promotes cancer cell invasion via

increased motility and metalloproteinase production. FASEB J 15:1953–1962.
Kim JI, Jo EJ, Lee HY, Cha MS, Min JK, Choi CH, Lee YM, Choi YA, Baek SH, Ryu SH, Lee KS, Kwak JY, Bae YS. 2003. Sphingosine 1-phosphate in amniotic fluid modulates cyclooxygenase-2 expression in human amnion-derived WISH cells. J Biol Chem 278:31731–31736.
Kluk MJ, Hla T. 2001. Role of the sphingosine 1-phosphate receptor EDG-1 in vascular smooth muscle cell proliferation and migration. Circ Res 89:496–502.
Kohler M, Janz I, Wintzer HO, Wagner E, Bauknecht T. 1989. The expression of EGF receptors, EGF-like factors and c-myc in ovarian and cervical carcinomas and their potential clinical significance. Anticancer Res 9:1537–1547.
Koyasu S. 2003. The role of PI3K in immune cells. Nat Immunol 4:313–319.
Kudlow JE, Bjorge JD, Kobrin MS, Paterson AJ. 1988. Regulation of EGF receptor and transforming growth factor-α expression. Adv Exp Med Biol 234:105–126.
Lee MJ, Thangada S, Claffey KP, Ancellin N, Liu CH, Kluk M, Volpi M, Sha'afi RI, Hla T. 1999. Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. Cell 99:301–312.
Lee MJ, Thangada S, Paik JH, Sapkota GP, Ancellin N, Chae SS, Wu M, Morales-Ruiz M, Sessa WC, Alessi DR, Hla T. 2001. Akt-mediated phosphorylation of the G protein-coupled receptor EDG-1 is required for endothelial cell chemotaxis. Mol Cell 8:693–704.
Libermann TA, Nusbaum HR, Razon N, Kris R, Lax I, Soreq H, Whittle N, Waterfield MD, Ullrich A, Schlessinger J. 1985. Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. Nature 313:144–147.
Lin F, Zhao L, Wise GE. 1996. In vivo and in vitro effects of epidermal growth factor on its receptor gene expression in rat dental follicle cells. Arch Oral Biol 41:485–491.
Liu Y, Wada R, Yamashita T, Mi Y, Deng CX, Hobson JP, Rosenfeldt HM, Nava VE, Chae SS, Lee MJ, Liu CH, Hla T, Spiegel S, Proia RL. 2000. Edg-1, the G protein coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. J Clin Invest 106:951–961.
Lyss G, Knorre A, Schmidt TJ, Pahl HL, Merfort I. 1998. The anti-inflammatory sesquiterpene lactone helenalin inhibits the transcription factor NF-κB by directly targeting p65. J Biol Chem 273:33508–33516.
Marshall CJ. 1994. MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. Curr Opin Genet Dev 4:82–89.
Merlino GT. 1990. Epidermal growth factor receptor regulation and function. Semin Cancer Biol 1:277–284.
Miller WE, Cheshire JL, Baldwin AS Jr, Raab-Traub N. 1998. The NPC derived C15 LMP1 protein confers enhanced activation of NF-κB and induction of the EGFR in epithelial cells. Oncogene 16:1869–1877.
Moelling K, Schad K, Bosse M, Zimmermann S, Schweneker M. 2002. Regulation of Raf-Akt Cross-talk. J Biol Chem 277:31099–31106.
Nishi H, Nishi KH, Johnson AC. 2002. Early Growth Response-1 gene mediates up-regulation of epidermal growth factor receptor expression during hypoxia. Cancer Res 62:827–834.
Nishi H, Neta G, Nishi KH, Akers LM, Rikiyama T, Proctor KN, Murphy BA, Johnson AC. 2003. Analysis of the epidermal growth factor receptor promoter: The effect of nuclear factor-κB. Int J Mol Med 11:49–55.
Osawa Y, Banno Y, Nagaki M, Brenner DA, Naiki T, Nozawa Y, Nakashima S, Moriwaki H. 2001. TNF-alpha-induced sphingosine 1-phosphate inhibits apoptosis through a phosphatidylinositol 3-kinase/Akt pathway in human hepatocytes. J Immunol 167:173–180.
Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB. 1999. NF-κB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature 401:82–85.
Pan ZK, Christiansen SC, Ptasznik A, Zuraw BL. 1999. Requirement of phosphatidylinositol 3-kinase activity for bradykinin stimulation of NF-κB activation in cultured human epithelial cells. J Biol Chem 274:9918–9922.
Park S, Song JS, Lee DK, Yang CH. 1999. Suppression of AP-1 activity by tanshinone and cancer cell growth inhibition. Bull Korean Chem Soc 20:925–928.
Payne SG, Milstien S, Spiegel S. 2002. Sphingosine-1-phosphate dual messenger functions. FEBS Lett 531:54–57.
Peng X, Hassoun PM, Sammani S, McVerry BJ, Burne MJ, Rabb H, Pearse D, Tuder RM, Garcia JG. 2004. Protective effects of sphingosine 1-phosphate in murine endotoxin-induced inflammatory lung injury. Am J Respir Crit Care Med 169:1245–1251.
Pitson SM, Moretti PA, Zebol JR, Lynn HE, Xia P, Vadas MA, Wattenberg BW. 2003. Activation of sphingosine kinase 1 by ERK1/2-mediated phosphorylation. EMBO J 22:5491–5500.
Pyne S, Pyne NJ. 2000. Spingosine-1-phosphate signaling in mammalian cells. Biochem J 349:385–402.
Radeff-Huang J, Seasholtz TM, Matteo RG, Brown JH. 2004. G protein mediated signaling pathways in lysophospholipid induced cell proliferation and survival. J Cell Biochem 92:949–966.
Reddy SA, Huang JH, Liao WS. 2000. Phosphatidylinositol 3-kinase as a mediator of TNF-induced NF-κB activation. J. Immunol 164:1355–1363.
Romashkova J, Makarov SS. 1999. NF-κB is a target of AKT in anti-apoptotic PDGF signalling. Nature 401:86–90.
Shatrov VA, Lehmann V, Chouaib S. 1997. Sphingosine-1-phosphate mobilizes intracellular calcium and activates transcription factor NF-κB in U937 cells. Biochem Biophys Res Commun 234:121–124.
Sheu ML, Chao KF, Sung YJ, Lin WW, Lin-Shiau SY, Liu SH. 2005. Activation of phosphoinositide 3-kinase in response to inflammation and nitric oxide leads to the up-regulation of cyclooxygenase-2 expression and subsequent cell proliferation in mesangial cells. Cell Signal 17:975–984.
Siehler S, Wang Y, Fan X, Windh RT, Manning DR. 2001. Sphingosine 1-phosphate activates nuclear factor-κB through Edg receptors. Activation through Edg-3 and Edg-5, but not Edg-1, in human embryonic kidney 293 cells. J Biol Chem 276:48733–48739.
Singleton PA, Dudek SM, Chiang ET, Garcia JG. 2005. Regulation of sphingosine 1-phosphate-induced endothelial cytoskeletal rearrangement and barrier enhancement by S1P1 receptor, PI3 kinase, Tiam1/Rac1, and alpha-actinin. FASEB J 19:1646–1656.
Smith WL, DeWitt DL, Garavito RM. 2000. Cyclooxygenases: Structural, cellular, and molecular biology. Annu Rev Biochem 69:145–182.

Spiegel S, Merrill AH Jr. 1996. Sphingolipid metabolism and cell growth regulation. FASEB J 10:1388–1397.

Spiegel S, Milstien S. 2000. Functions of a new family of sphingosine-1-phosphate receptors. Biochim Biophys Acta 1484:107–116.

Spiegel S, English D, Milstien S. 2002. Sphingosine 1-phosphate signaling: Providing cells with a sense of direction. Trends Cell Biol 12:236–242.

Takuwa Y, Takuwa N, Sugimoto N. 2002. The Edg family G protein-coupled receptors for lysophospholipids: Their signaling properties and biological activities. J Biochem 131:767–771.

Tamama K, Okajima F. 2002. Sphingosine 1-phosphate signaling in atherosclerosis and vascular biology. Curr Opin Lipidol 13:489–495.

Tamama K, Kon J, Sato K, Tomura H, Kuwabara A, Kimura T, Kanda T, Ohta H, Ui M, Kobayashi I, Okajima F. 2001. Extracellular mechanism through the Edg family of receptors might be responsible for sphingosine-1-phosphate-induced regulation of DNA synthesis and migration of rat aortic smooth-muscle cells. Biochem J 353:139–146.

Tanimoto T, Lungu AO, Berk BC. 2004. Sphingosine 1-phosphate transactivates the platelet-derived growth factor beta receptor and epidermal growth factor receptor in vascular smooth muscle cells. Circ Res 94:1050–1058.

Tao YG, Tan YN, Liu YP, Song X, Zeng L, Gu HH, Tang M, Li W, Yi W, Cao Y. 2004. Epstein-Barr virus latent membrane protein 1 modulates epidermal growth factor receptor promoter activity in a nuclear factor kappa B-dependent manner. Cell Signal 16:781–790.

Wahl MI, Carpenter G. 1987. Role of growth factors and their receptors in the control of normal cell proliferation and cancer. Clin Physiol Biochem 5:130–139.

Woodworth CD, Michael E, Marker D, Allen S, Smith L, Nees M. 2005. Inhibition of the epidermal growth factor receptor increases expression of genes that stimulate inflammation, apoptosis, and cell attachment. Mol Cancer Ther 4:650–658.

Xie P, Browning DD, Hay H, Mackman N, Ye RD. 2000. Activation of NF-κB by bradykinin through a Gα(q)-and Gβγ-dependent pathway that involves phosphoinositide 3-kinase and Akt. J Biol Chem 275:24907–24914.

Yang CM, Chiu CT, Wang CC, Chien CS, Hsiao LD, Lin CC, Tu MT, Pan SL. 2000. Activation of mitogen-activated protein kinase by oxidized low-density lipoprotein in canine cultured vascular smooth muscle cells. Cell Signal 12:205–214.

Yang CM, Lin MI, Hsieh HL, Sun CC, Ma YH, Hsiao LD. 2005. Bradykinin-induced p42/p44 MAPK phosphorylation and cell proliferation via Src, EGF receptors, and PI3-K/Akt in vascular smooth muscle cells. J Cell Physiol 203:538–546.

Yatomi Y, Ohmori T, Rile G, Kazama F, Okamoto H, Sano T, Satoh K, Kume S, Tigyi G, Igarashi Y, Ozaki Y. 2000. Sphingosine 1-phosphate as a major bioactive lysophospholipid that is released from platelets and interacts with endothelial cells. Blood 96:3431–3438.

Ying WZ, Sanders PW. 2005. Enhanced expression of EGF receptor in a model of salt-sensitive hypertension. Am J Physiol Renal Physiol 289:F314–F321.

Yoshida K, Tosaka A, Takeuchi S, Kobayashi N. 1994. Epidermal growth factor receptor content in human renal cell carcinomas. Cancer 73:1913–1918.