

Scalarial Inhibition of Epidermal Growth Factor
Receptor-mediated Akt Phosphorylation Is Independent of
Secretory Phospholipase A₂

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The abbreviations used are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; PI3K, phosphatidylinositol 3-kinase; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; PDK-1, 3-phosphoinositide-dependent protein kinase 1; ERK, extracellular signal-regulated kinases; sPLA₂, secretory phospholipase A₂; cPLA₂, cytosolic phospholipase A₂; HMEC, human dermal microvascular endothelial cells.

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ABSTRACT

The marine natural product 12-epi scalaradial (SLD) is a specific secretory phospholipase A₂ (sPLA₂) inhibitor. However, little is known whether this compound has other pharmacological effects. Here, we revealed a novel effect of SLD on epidermal growth factor receptor (EGFR)-mediated Akt phosphorylation. SLD dose- and time-dependently inhibited epidermal growth factor (EGF)-stimulated Akt phosphorylation which is required for Akt activation. SLD also blocked the EGF-stimulated membrane translocation of 3-phosphoinositide-dependent protein kinase 1 and inhibited phosphatidylinositol 3-kinase activity. This inhibition is specific for SLD because other phospholipase inhibitors, including sPLA₂ inhibitor thioetheramide-PC, cytosolic PLA₂ inhibitor arachidonyl trifluoromethyl ketone, cytosolic PLA₂ and Ca²⁺-independent PLA₂ inhibitor methyl arachidonyl fluorophosphonate, phospholipase C inhibitor U73122, and cyclooxygenases inhibitor indomethacin, failed to inhibit EGF-stimulated Akt phosphorylation. What's more, arachidonic acid, the main sPLA₂-catalyzed metabolite, was not able to rescue SLD inhibition of EGF-stimulated Akt phosphorylation. Overexpression of group II A or group X sPLA₂ did not reverse the inhibitory effect of SLD on Akt phosphorylation, either. Our results demonstrate that SLD inhibits EGFR-mediated Akt phosphorylation and this novel effect of SLD is independent of sPLA₂.

Epidermal growth factor (EGF) is a potent mitogen that initiates signaling transduction cascades by binding to EGF receptor (EGFR) (Spaargaren et al., 1991; Carpenter and Cohen, 1990). Following ligand binding, EGFRs oligomerize, become autophosphorylated, and finally are activated (Emlet et al., 1997). EGFR activates two major downstream pathways: phosphatidylinositol 3-kinase (PI3K)/3-phosphoinositide-dependent protein kinase 1 (PDK-1)/Akt (also known as protein kinase B) pathway and Ras/Raf/MEK/extracellular signal-regulated kinases (ERK) signaling pathway (Carpenter and Cohen, 1990; Emlet et al., 1997; Okano et al., 2000). PI3K/Akt pathway plays important roles in a number of cellular events like diabetes, cell proliferation, differentiation, transformation and apoptosis (Dumont et al., 1989; Lou et al., 2002). Aberrant activation of the PI3K/Akt pathway has been widely implicated in many cancers and drugs against this pathway are likely to have broad applications for treating different types of cancer (Luo et al., 2003).

Phospholipase A₂ (PLA₂) enzymes represent a family of esterases catalyzing the hydrolysis of the *sn*-2 position of membrane glycerophospholipids to liberate free fatty acids and lysophospholipids (Kudo and Murakami, 2002). There are four major families of PLA₂s: the 14-kDa secretory PLA₂ (sPLA₂), the Ca²⁺-independent PLA₂ (iPLA₂), the 85-kDa cytosolic PLA₂ (cPLA₂), and platelet-activating factor acetylhydrolases (Kudo and Murakami, 2002; Murakami et al., 2000). To study the role of different PLA₂s in eicosanoid production as well as other pathophysiological functions such as inflammation, considerable effort has been put into developing specific inhibitors for different PLA₂s. These inhibitors included marine natural

products and PLA₂ substrate and product analogs (Hope et al., 1993). However, many of the PLA₂ inhibitors originally thought to be selective for a specific PLA₂ are now known to have multiple other effects (Cummings et al., 2000).

12-epi scalaradial (SLD), a natural product isolated from marine sponge (*Cacospongia sp*), has been shown to be a selective inhibitor of sPLA₂ (De Carvalho and Jacobs, 1991). SLD efficiently inhibited bee venom PLA₂ (IC₅₀=0.07 μM) and purified human recombinant type II sPLA₂ activity in vitro (IC₅₀=5.4 μM), but displayed weak inhibition of cytosol 85 kDa-PLA₂ from U937 cells (IC₅₀ = 28 μM) (De Carvalho and Jacobs, 1991; Hope et al., 1993). However, whether this compound has other effects besides inhibiting sPLA₂ activity is still unknown. Previous studies demonstrated that SLD exerted inhibitory effect on HL-60 cell proliferation (Liu and Levy, 1997). Recent unpublished work in our laboratory indicated that SLD also inhibited the proliferation of many other cell lines. Given EGFR/PI3K/Akt signaling pathway plays important roles in cell proliferation, we investigated the effect of SLD on EGFR-mediated signaling. Our results revealed that SLD inhibited EGFR-mediated Akt phosphorylation and this novel effect was independent of sPLA₂.

Materials and Methods

Materials. Epidermal growth factor was purchased from Promega (Madison, WI). Insulin, wortmannin and arachidonic acid were purchased from Sigma (St. Louis, MO). Arachidonyl trifluoromethyl ketone (AACOCF₃), U73122 and 12-epi-scalaradial were purchased from Calbiochem (San Diego, CA). Thioetheramide-PC, methyl arachidonyl fluorophosphonate and sPLA₂ assay kit were purchased from Cayman Chemical Co. (Ann Arbor, MI). L- α -phosphatidylinositol was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). [γ -³²P] ATP and protein G-Sepharose were purchased from Amersham Biosciences (Uppsala, Sweden). FuGENE 6 transfection reagent was purchased from Roche Molecular Biochemicals (Mannheim, Germany). pEGFP plasmid and pEGFP-PDK-1 plasmid were kind gifts from Dr. Jongkyeong Chung (Korea Advanced Institute of Science and Technology, Korea). Human group II A and X sPLA₂ plasmids were kind gifts from Dr. Gerard Lambeau (the Institut de Pharmacologie Moleculaire et Cellulaire, France) (Cupillard et al., 1997). Antibodies to Akt, ERK1/2, phospho-Akt (Ser473) and phospho-ERK1/2 were purchased from Cell signaling (Beverly, MA). Antibody to PI3 kinase p85 was purchased from Upstate Biotechnology Inc (Lake placid, NY).

Cell Culture and Transfection. Hepatocellular carcinoma BEL-7402 cells were from Cell Bank of Shanghai Institute for Biological Sciences, Chinese Academy of Science (Shanghai, China). Breast cancer MDA-MB-435 cells were from American

Type Culture Collection (Manassas, VA). Human dermal microvascular endothelial cells (HMEC) were from Clonetics (San Diego, CA). BEL-7402 cells were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS) at 37 °C in an atmosphere of 5 % CO₂. MDA-MB-435 cells and HMEC were maintained in DMEM containing 10 % FBS at 37 °C with 5 % CO₂. All the cells were plated in 6-well plates and starved in serum-free medium for 16 h before drug treatment. For transient transfection, cells grown in 6-well plates at 70 % confluence were transfected with pEGFP, pEGFP-PDK-1, human group II A or human group X sPLA₂ plasmids using the FuGENE 6 transfection reagent according to the manufacturer's instructions. After transfection for 24 h, cells were starved in serum-free RPMI 1640 medium for 16 h before drug treatment.

Preparation of Cell Lysates and Western Blot. After drug treatment, cells were washed twice with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) and total cell lysates were collected in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2 % SDS, 0.1 % Bromophenol Blue, 10 % glycerol). Cell lysates containing equal amount of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. After being blocked in 5 % non-fat milk in TBST (Tris-buffered saline with 0.1% Tween 20, pH 7.6), membranes were incubated with the appropriate primary antibodies at 4 °C overnight and exposed to secondary antibodies for 2 h at room temperature. Immunoreactive proteins were visualized using the enhanced chemiluminescence

system from Pierce (Rockford, IL).

PDK-1 Translocation Assay. BEL-7402 cells were grown on glass coverslips in 6-well plates and transfected with pEGFP or pEGFP-PDK-1 plasmids. After transfection for 24 h, cells were starved in serum-free RPMI 1640 medium for 16 h, then stimulated with EGF in the presence or absence of SLD or wortmannin. Stimulation was terminated by washing cells with ice-cold PBS and fixing cells with immediate addition of 4 % paraformaldehyde in PBS for 10 min at 4 °C. Fixed cells were mounted on slide glasses with 50 % glycerol in carbonate buffer (pH 9.0) and observed with a laser-scanning confocal microscope (Leica).

PI3K Activity Assay. The assay was performed as described (Whitman et al., 1985). Briefly, cells were washed twice with ice-cold PBS and lysed on ice in lysis buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 % (v/v) glycerol, 1 % Nonidet P-40, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 2 μM leupeptinin, 2 μM aprotinin and 1 mM phenylmethylsulfonyl fluoride). Insoluble material was removed by centrifugation at 12,000 rpm at 4 °C for 10 min. Cell lysates (500 μg) were subjected to immunoprecipitation with anti-PI3K p85 antibody for 2 h followed by the addition of 20 μl of 1:1 slurry of protein G-Sepharose beads for an additional hour at 4 °C. The immunocomplexes were incubated with 0.1 mg/ml L-α-phosphatidylinositol, 50 μM ice-cold ATP, 15 μCi of [γ-³²P] ATP, and 20 mM MgCl₂ for 30 min at 30 °C

in a final volume of 80 μ l. 20 μ l of 8 M HCl was added to terminate the incubation. The lipids were extracted with 200 μ l of chloroform/methanol (1:1) and centrifuged (300 \times g) for 15 min at 4 $^{\circ}$ C. The lower organic phases were resolved on a 1 % potassium oxalate pretreated silica gel 60- \AA TLC plate (Merck) by chloroform/acetone/methanol/acetic acid/water (46:17:15:14:8, v/v) and visualized by autoradiography. Identified spots containing radioactive PI[3]P were scraped off the silica gel plates and quantitated by scintillation counting.

RT-PCR. BEL-7402 cells were transfected with human group II A or X sPLA₂ plasmids. After transfection for 24 h, cells were starved in serum-free RPMI 1640 medium for 16 h. Total RNA was isolated using TRIzol reagent (Sangon, Shanghai) following the manufacturer's protocols. cDNA mixtures were prepared in a 20- μ l reaction using a RT-PCR kit (Promega). Briefly, 2 μ g of total RNA was reverse-transcribed using 200 units of M-MLV RTase H in the presence of 0.5 μ g oligo (dT)₁₈, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.5 mM of each dNTP and 20 units of RNase Inhibitor. The reaction mixture was incubated for 60 min at 42 $^{\circ}$ C (transcription) followed by 15 min at 70 $^{\circ}$ C (inactivation of reverse-transcription). PCR was performed in 50- μ l reactions containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.1 % Triton X-100, 0.2 mM of each dNTP, 5 units of Taq polymerase, and 1 μ M of the specific primers. The group I B sPLA₂ primer sequences used were: 5'-TCC TTG TGC TAG CTG TGC TG-3' and 5'-TGA AGG CCT CAC ACT CTT TG-3' (Degousee et al.,

2002). The group II A sPLA₂ primer sequences were: 5'-ATG AAG ACC CTC CTA CTG TT-3' and 5'-TCA GCA ACG AGG GGT GCT CC-3' (Degousee et al., 2002). The group V sPLA₂ primer sequences were: 5'-TTG GTT CCT GGC TTG TAG TGT G-3' and 5'-TGG GTT GTA GCT CCG TAG GTT T-3' (Degousee et al., 2002). The group X sPLA₂ primer sequences were: 5'-GAT CCT GGA ACT GGC AGG AA-3' and 5'-TCA GTC ACA CTT GGG CGA GT-3' (Degousee et al., 2002) and the group IV α cPLA₂ primer sequences were: 5'-CTC TAG TCC TCC GTT CAA GGA AC-3' and 5'-TTG CAA ACT GCC TCA GCA TCA-3' (Wu et al., 1998). The PCR reaction for group I B, II A, V and X sPLA₂ was conducted for 4 min at 95 °C, 35 cycles of amplification using a step program (95 °C, 30 s; 56 °C, 45 s; 72 °C, 50 s), followed by 10 min at 72 °C to ensure a complete extension of the amplified DNA. The PCR reaction for group IV α cPLA₂ was conducted for 4 min at 95 °C, 30 cycles of amplification using a step program (94 °C, 45 s; 56 °C, 45 s; 72 °C, 1.5 min), followed by a 10-min final extension at 72 °C. The PCR products were electrophoresed on a 1.5 % agarose gel and stained with ethidium bromide.

Measurement of sPLA₂ Activity. BEL-7402 cells were transfected with human group II A or X sPLA₂ plasmids. After transfection for 24 h, cells were starved in serum-free RPMI 1640 medium for another 16 h, then collected in PBS (10⁸ cells/ml) containing 0.34 M sucrose, 10 mM HEPES, 100 μ g/ml phenylmethylsulfonyl fluoride, 1 mM tosyl-leucyl chloromethylketone, 0.1 mM leupeptin, and 5 mM EDTA (final PH 7.0) (Blom et al., 1998). The suspended cells were sonicated at 21

kHz and 8 μ m peak-to-peak amplitude for 3×15 -second intervals at 4 °C. sPLA₂ activity was measured using sPLA₂ Activity Assay Kits from Cayman Chemical Co. (Ann Arbor, MI).

Results

SLD dose- and time-dependently inhibited EGF-stimulated Akt phosphorylation.

First, we examined if there existed classical PI3K-dependent signaling pathway in EGF-stimulated Akt activation in BEL-7402 cells. Serum-starved BEL-7402 cells were incubated with EGF for different periods of time. The addition of EGF rapidly stimulated Akt phosphorylation with the maximal stimulation at 5 min (data not shown). Pretreatment of cells with PI3K inhibitor wortmannin resulted in a total inhibition of EGF-stimulated Akt phosphorylation (data not shown). Our data indicated that EGF stimulated Akt phosphorylation, which is required for Akt activation, and this stimulation was through classical PI3K-dependent signaling pathway in BEL-7402 cells.

Next, we investigated the role of SLD in EGFR-mediated Akt activation. We incubated BEL-7402 cells with SLD for 15 min before EGF stimulation and observed SLD dose-dependently inhibited EGF-stimulated Akt phosphorylation with an IC_{50} of $2.9 \pm 0.44 \mu\text{M}$ (Fig. 1A). In addition, SLD inhibited EGF-stimulated Akt phosphorylation in a treatment-schedule-dependent manner: only pretreatment of cells with SLD before EGF stimulation, but not treatment of cells with SLD plus EGF simultaneously, or treatment of cells with SLD after EGF stimulation, could SLD markedly inhibit Akt phosphorylation (Fig. 1B), suggesting that SLD acts on steps upstream of Akt.

To test whether SLD also down-regulated EGFR/Akt signaling transduction in other cell types, we pretreated breast cancer MDA-MB-435 cells and human

endothelial cell HMEC with 10 μ M SLD before EGF stimulation using the same protocol as BEL-7402 cells'. Our results revealed that EGF-induced Akt phosphorylation in these cells was also sensitive to SLD pretreatment (Fig. 2). More importantly, insulin, another important agonist for tyrosine kinase receptor, stimulated Akt phosphorylation in BEL-7402 cells and this stimulation was also inhibited by SLD pretreatment (Fig. 2). Taken together, these data indicate that the effect of SLD on EGF-stimulated Akt phosphorylation is not cell type-specific and SLD may also down-regulated other tyrosine kinase receptor-mediated signaling transduction.

Other phospholipase inhibitors did not inhibit EGF-stimulated Akt phosphorylation. Since SLD was a well-known selective inhibitor of sPLA₂, we next investigated whether other phospholipase inhibitors have the same inhibitory effect on Akt phosphorylation. Among the tested inhibitors, the substrate analog thioetheramide-PC showed selectivity towards sPLA₂ and has been shown to markedly reduce the sulfite-induced generation of arachidonic acid (AA) metabolites in alveolar macrophages as well as rosiglitazone-induced prostaglandin E₂ release in rat aortic vascular smooth muscle cells (Yu et al., 1990; Beck-Speier et al., 2003; Bishop-Bailey and Warner, 2003). We incubated BEL-7402 cells with different doses of thioetheramide-PC before EGF stimulation. To our surprise, thioetheramide-PC failed to inhibit Akt phosphorylation even at dose of 30 μ M (Fig. 3A). Arachidonyl trifluoromethyl ketone (AACOCF₃, 10 μ M), a specific inhibitor of cPLA₂ (Street et al., 1993), methyl arachidomyl fluorophosphonate (MAFP, 10 μ M), an inhibitor of cPLA₂ and iPLA₂ (Lio et al., 1996; Cummings et al., 2000), and U73122 (10 μ M), an

inhibitor of phospholipase C (Zheng et al., 1995), had no effect on EGF-stimulated Akt phosphorylation, either (Fig. 3, B, C and D). In addition, indomethacin (10 μ M), an inhibitor of cyclooxygenases, which are downstream enzymes of PLA₂ and convert AA to prostaglandins (Touhey et al., 2002), could not inhibit EGF-stimulated Akt phosphorylation (Fig. 3E). These results indicate the inhibitory effect on EGFR-mediated Akt phosphorylation is specific for SLD.

SLD blocked EGF-induced PDK-1 membrane translocation. Akt activation requires phosphorylations at two critical residues: Thr308 at the activation loop of the catalytic domain and Ser473 at the hydrophobic C terminal domain (Alessi et al., 1996). Upon EGF stimulation, PI3K increases the levels of phosphatidylinositol 3, 4, 5-trisphosphate (PIP₃), which recruits PDK-1 as well as Akt to the plasma membrane where PDK-1 phosphorylates Thr308 of Akt (Scheid et al., 2002; Stephens et al., 1998). Previous results showed that only using pretreatment protocol was SLD able to inhibit EGF-stimulated Akt phosphorylation. So we hypothesize that SLD inhibits the upstream regulators of Akt. First we examined the effect of SLD on PDK-1 membrane translocation. Green fluorescence protein (GFP)-fused PDK-1 plasmid was transfected into BEL-7402 cells and the effect of SLD on translocation of GFP-PDK-1 protein to plasma membrane was visualized by laser-scanning confocal microscope. As seen from Fig. 4, EGF rapidly induced GFP-PDK-1 membrane translocation within 3 min and this translocation was totally inhibited by pretreatment with SLD (10 μ M) for 15 min. As a positive control, wortmannin also effectively abrogated the PDK-1 membrane translocation. However, localization of

GFP was not affected by EGF stimulation (Fig. 4B), indicating that this assay is specific for PDK-1.

SLD inhibited PI3K activity. As the subcellular localization of PDK-1 is regulated mainly by the level of PI3K product PIP₃, we next examined the activity of PI3K in the presence or absence of SLD in BEL-7402 cells by measuring the [³²P]-labeled phosphatidylinositol-3-phosphate (PI[3]P) produced by PI3K. Our results showed that EGF treatment markedly increased PI3K activity, while pretreatment with SLD (10 μM) for 15 min strongly inhibited PI3K activity (Fig. 5). These results suggest that SLD inhibits PI3K activity to block the coupling between PDK-1 and Akt in the plasma membrane, which then inhibits Akt phosphorylation.

sPLA₂'s metabolite AA could not reverse SLD inhibition of Akt phosphorylation.

sPLA₂ exhibits its biological functions partially through enzymatically hydrolyzing membrane phospholipids to release AA and other lipid metabolites (Kudo and Murakami, 2002; Hanasaki and Arita, 2002). However, our results showed AA (30 μM) could not rescue SLD inhibition of EGF-stimulated Akt phosphorylation (Fig. 6A), suggesting that this inhibitory effect of SLD may be independent of sPLA₂. To confirm this hypothesis, we first used ERK1/2 as a control to determine whether AA is active or not in our system because AA has been reported to stimulate ERK1/2 phosphorylation (Dulin et al., 1998; Silfani and Freeman, 2002). As seen from Fig. 6B, direct addition of 30 μM AA to BEL-7402 cells rapidly stimulated ERK1/2 phosphorylation (Fig. 6B). Second, we tested the effect of SLD on ERK1/2 phosphorylation. Although we did not see the inhibitory effect of SLD on

EGF-stimulated ERK1/2 phosphorylation (data not shown), we clearly observed treatment of BEL-7402 cells with 10 μ M SLD for 5 min significantly inhibited ERK1/2 phosphorylation under basal conditions (complete medium without FBS) (Fig. 6C). More importantly, another sPLA₂ inhibitor thioetheramide-PC also significantly reduced basal level of ERK1/2 phosphorylation (data not shown), indicating that sPLA₂s are involved in the maintenance of basal ERK1/2 phosphorylation. Third, we tested whether AA could reverse SLD inhibition of basal ERK1/2 phosphorylation. AA pretreatment totally abrogated the inhibitory effect of SLD on ERK1/2 phosphorylation (the percentage inhibition of ERK1/2 phosphorylation by SLD was 46.4 ± 3.8 % in control cells, 1.7 ± 1.2 % in 15 min-AA- pretreated cells, and 4.5 ± 1.0 % in 60 min-AA-pretreated cells) (Fig. 6C). These data indicate that SLD inhibits ERK1/2 phosphorylation through sPLA₂ and AA is capable of reversing the inhibitory effect of SLD on sPLA₂-mediated biological effects, at least ERK1/2 activation. The failure of AA to reverse the inhibitory effect of SLD on Akt implies that SLD inhibition of EGF-stimulated Akt phosphorylation may be independent of sPLA₂.

Overexpression of sPLA₂s did not reverse SLD inhibition of Akt phosphorylation. To further determine whether the inhibitory effect of SLD on EGFR-mediated Akt phosphorylation was independent of sPLA₂, we tested the effect of overexpression of sPLA₂s in BEL-7402 cells. Endogenous expression of sPLA₂ I B, II A, V and X, and cPLA₂ IV α was determined by RT-PCR using specific primers. As shown in Fig.7A, we detected sPLA₂ II A and cPLA₂ IV α in BEL-7402

cells. We were not able to detect sPLA₂ I B, V and X even using much more cDNA templates and more PCR cycles (data not shown). These data suggest that sPLA₂ II A may be the main sPLA₂ present in BEL-7402 cells. So, we next transiently transfected BEL-7402 cells with sPLA₂ II A or X plasmids. The transfection efficiency was around 15-25 % as determined by GFP transfection assay. Although the transfection efficiency was not high, the sPLA₂ activity as well as mRNA level of sPLA₂ II A and X in transfected cells was markedly increased and the increased sPLA₂ activity in transfected cells was significantly inhibited by SLD (Fig. 7, B and C). In addition, medium collected from sPLA₂ II A or X transfected cells but not from vector transfected cells markedly stimulated the phosphorylation of ERK1/2 (Fig. 7D), demonstrating that sPLA₂ II A and X were produced in transfected cells and secreted into medium. Importantly, overexpression of these sPLA₂s reversed SLD inhibition of ERK1/2 phosphorylation (the percentage inhibition of ERK1/2 phosphorylation by SLD in vector, sPLA₂ II A or X transfected cells was 46.7 ± 6.5 %, 14.4 ± 6.0 % and 6.2 ± 3.8 %, respectively) (Fig. 7E). These results demonstrated that SLD inhibited ERK1/2 phosphorylation through sPLA₂s, which was consistent with the conclusion from AA experiments. However, overexpression of neither sPLA₂ II A nor sPLA₂ X reversed SLD (5 μ M) inhibition of Akt phosphorylation (Fig. 7F). Of note, the SLD concentration (5 μ M) used here was relative lower as compared with the concentrations used in previous experiments and 5 μ M SLD only partially inhibited EGF-stimulated Akt phosphorylation (Fig. 7F). The percentage inhibition of EGF-stimulated Akt

phosphorylation by SLD (5 μ M) in vector, sPLA₂ II A or sPLA₂ X transfected cells were 60.9 ± 2.0 %, 59.9 ± 4.1 % and 60.5 ± 3.7 %, respectively) (Fig. 7F). Taken together, these data indicate that SLD inhibition of EGFR-mediated Akt phosphorylation is independent of sPLA₂.

Discussion

Previous studies show that SLD is a selective inhibitor of sPLA₂ (De Carvalho and Jacobs, 1991; Hope et al., 1993; Marshall et al., 1994). In the present study, we also showed that SLD effectively inhibited sPLA₂ activity (Fig. 7B). The selective inhibition of sPLA₂ by SLD blocked the release of AA, the activation of inducible NO synthase and transcription factor NF- κ B, the production of eicosanoid, and the increase in intracellular Ca²⁺ in response to stimulators in different cell types (Marshall et al., 1994; Baek et al., 2000; Thommesen et al., 1998; Barnette et al., 1994). Besides these well known effects on phospholipid metabolism, we demonstrated here that SLD dose- and time-dependently inhibited EGF-stimulated Akt phosphorylation in hepatocellular carcinoma BEL-7402 cells as well as in breast cancer MDA-MB-435 cells and human endothelial cells HMEC, and more importantly, SLD inhibited PDK-1 membrane translocation and PI3K activity. Our results clearly show that inhibition of PI3K/Akt signaling is novel and may be a common pharmacological effect of SLD. This is of importance, given that the role of PI3K/Akt signaling in cellular functions. In addition, SLD also inhibited insulin-stimulated Akt phosphorylation, suggesting SLD acts on a target which is a common event in receptor tyrosine kinase-mediated signaling. However, whether the target is PI3K or the upstream kinases of PI3K remains to be further identified.

Since SLD is an effective inhibitor of sPLA₂ and EGF has been shown to stimulate PLA₂ activity (Spaargaren et al., 1992; Schalkwijk et al., 1995; Croxtall et al., 1995), it is possible that SLD inhibition of EGFR/PI3K/Akt signaling pathway

through sPLA₂. However, thioetheramide-PC, a structurally modified phospholipid that functions as another specific inhibitor of sPLA₂ (Yu et al., 1990; Beck-Speier et al., 2003; Bishop-Bailey and Warner, 2003), did not affect EGFR-mediated Akt phosphorylation, indicating this inhibition is a specific effect of SLD and may be independent of sPLA₂. This hypothesis is supported by the following facts. First, SLD inhibition of EGFR-mediated Akt phosphorylation was not mimicked by cyclooxygenase inhibitor indomethacin. Cyclooxygenases are downstream enzymes of PLA₂ and convert AA to prostaglandins (Touhey et al., 2002). Second, sPLA₂-catalyzed metabolite AA was not able to reverse the inhibitory effect of SLD on EGF-stimulated Akt phosphorylation. Third, overexpression of sPLA₂ did not reverse SLD inhibition of Akt phosphorylation. ERK1/2, the other two critical protein kinases in tyrosine kinase receptor-mediated signaling, serve as controls in present study. Although we did not see the inhibitory effect of SLD on EGF-stimulated ERK1/2 phosphorylation, it seems to us that the maintenance of basal activity of ERK1/2 is dependent of sPLA₂ since SLD inhibits basal ERK phosphorylation and this effect is reversed by AA and overexpression of sPLA₂s. These findings indicate that both AA and sPLA₂ transfection system work well in our systems and that SLD has both sPLA₂-dependent and -independent pharmacological effects in BEL-7402 cells.

Among 10 sPLA₂ enzymes that have been described in mammals, group I B, II A and V sPLA₂ are relatively widespread and abundant in tissues (Kudo and Murakami, 2002). We used primers for these three sPLA₂ enzymes as well as group

X sPLA₂ to detect the expression of sPLA₂s in BEL-7402 cells by RT-PCR. It was shown that only a 434-bp fragment of sPLA₂ II A was amplified from the total RNA of BEL-7402 cells. Therefore, it was most likely group II A but not other sPLA₂s exerted effects in BEL-7402 cells. We transfected BEL-7402 cells with group II A sPLA₂ and around 3-fold increase in sPLA₂ activity was observed as determined by sPLA₂ activity assay. The assay uses the 1, 2-dithio analog of diheptanoyl phosphatidylcholine as a substrate which is specific for most PLA₂s (e.g., bee and cobra venoms, pancreatic, etc) but not for cPLA₂. Five μM of SLD is a dose which partially, but not completely, inhibited EGF-stimulated Akt phosphorylation. If SLD inhibition of EGFR-mediated Akt activation is dependent on sPLA₂, over-expression of group II A sPLA₂ should reverse the inhibition of Akt phosphorylation by 5 μM SLD to some degree. However, neither group II A nor group X sPLA₂ was observed to abrogate the effect of SLD on Akt. Based on the facts mentioned above, we believe that SLD inhibition of EGFR-mediated Akt phosphorylation is independent of sPLA₂.

Many PLA₂ inhibitors originally thought to be selective for a specific PLA₂ are now known to inhibit other isoforms. For example, manoalide has previously been shown to be a potent inhibitor of venom phospholipases A₂. Now it reveals that manoalide inhibits both general PLA₂ and phosphoinositide-specific phospholipase C (Bennett et al., 1987; Cummings et al., 2000). MAFP, the inhibitor of cPLA₂ with an IC₅₀ of ~0.5 μM for purified cPLA₂, also inhibits iPLA₂ purified from murine macrophage-like P388D1 cells with IC₅₀ of 0.5 μM (Lio et al., 1996). The failure of

other PLA₂ inhibitors AACOCF3 and MAFP, and phospholipase C inhibitor U73122, to inhibit EGFR-mediated Akt phosphorylation suggests it is not likely that SLD inhibited Akt phosphorylation through inhibiting other isoforms of phospholipases, at least three isoforms, cPLA₂, iPLA₂ and phospholipase C.

In summary, this study revealed that SLD possessed a novel effect besides its inactivating sPLA₂, i.e., SLD inhibits EGFR-mediated Akt activation. This novel effect of SLD is independent of sPLA₂. Provided that Akt is a critical mediator in cellular biology, our findings provide important evidence for further exploring the pharmacological effects of SLD.

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Footnotes

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Legends for Figures

Fig. 1: The effect of SLD on EGF-stimulated Akt phosphorylation. A, Serum-starved BEL-7402 cells were pretreated with different doses of SLD as indicated for 15 min, then stimulated with 10 ng/ml EGF for 5 min. B, Serum-starved BEL-7402 cells were stimulated with 10 ng/ml EGF for 5 min, with 10 μ M SLD added into medium 15 min before (t_{+15}), simultaneously with (t_0) or 2 min after (t_{-2}) EGF stimulation. Samples were resolved by SDS-PAGE and immunoblotted with antibodies specific for phospho-Akt or Akt. Data shown were representative of at least three independent experiments. p-Akt/Akt was quantitated by densitometric analysis, where control value represents 1. Values represented are mean \pm S.D. (bars), n = 3. * p < 0.05, ** p < 0.01 and ^{ns} p > 0.05 vs EGF stimulation alone.

Fig. 2: EGF- and insulin-stimulated Akt phosphorylation was inhibited by SLD in different cell lines. Endothelial cell HMEC, breast cancer cell MDA-MB-435 and hepatocellular carcinoma cell BEL-7402 were serum-starved overnight, then treated with 10 μ M 12-epi-scalaradial (SLD) for 15 min followed by 10 ng/ml EGF or 1 μ g/ml insulin stimulation for 5 min. Cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies specific for phospho-Akt and Akt. Data shown were representative of three independent experiments.

Fig. 3: Effects of other phospholipase inhibitors on EGF-stimulated Akt phosphorylation. Serum-starved BEL-7402 cells were pretreated with different doses of sPLA₂ inhibitor thioetheramide-PC (Thio-PC) (A), 10 μM cPLA₂ inhibitor AACOCF₃ (B), 10 μM cPLA₂ and iPLA₂ inhibitor MAFP (C), 10 μM phospholipase C inhibitor U73122 (D) or 10 μM cyclooxygenase inhibitor indomethacin (Indo) (E) for 15 min, then stimulated with 10 ng/ml EGF for 5 min. Cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies specific for phospho-Akt and Akt. Data shown were representative of at least three independent experiments.

Fig. 4: EGF-induced membrane localization of PDK-1 was blocked by SLD. BEL-7402 cells grown on coverslips were transfected with pEGFP plasmid (A and B) or pEGFP-PDK-1 plasmids (C, D, E and F). Serum-starved cells were pretreated with 10 μM SLD (E) or 100 nM wortmannin (F) for 15 min, then stimulated with 10 ng/ml EGF for 3 min (B, D, E and F). Cells were observed with a laser-scanning confocal microscope. Data shown were representative of three independent experiments.

Fig. 5: SLD inhibited EGF-stimulated PI3K activity. Serum-starved BEL-7402 cells were pretreated with 10 μM SLD or 100 nM wortmannin (Wort) for 15 min, then stimulated with 10 ng/ml EGF for 3 min. PI3K was immunoprecipitated and the kinase activity in the immunocomplexes was measured as described under "Materials and Methods." Data shown were representative of three independent

experiments. Values represented are mean \pm S.D. n = 3. $**p < 0.01$ vs EGF stimulation alone.

Fig. 6: EGF-stimulated Akt phosphorylation was not reversed by AA. A, Serum-starved BEL-7402 cells were pretreated with 10 μ M SLD in the presence or absence of 30 μ M AA for 15 min, then stimulated with 10 ng/ml EGF for 5 min. B, Serum-starved BEL-7402 cells were treated with 30 μ M AA for indicated times. C, Serum-starved BEL-7402 cells were pretreated with 30 μ M AA for 15 min or 1 h, then treated with 10 μ M SLD for 5 min. After treatment, cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies specific for phospho-Akt, phospho-ERK1/2, Akt and ERK1/2. Data shown were representative of three independent experiments.

Fig. 7: SLD-inhibited Akt phosphorylation was not reversed by overexpression of sPLA₂s. A, Expression of sPLA₂ I B (341 bp), II A (434 bp), V (358 bp), X (370 bp) and cPLA₂ IV α (554 bp) in BEL-7402 cells was assessed by RT-PCR. B, BEL-7402 cells transfected with human sPLA₂ II A or X plasmids were serum-starved and subjected to sPLA₂ activity assay as described under “Material and Methods”. Values represented are mean \pm S.E.M. (bars), n = 3. $**p < 0.01$ vs control. C, Expression of sPLA₂ II A (434 bp) or X (370 bp) in serum-starved BEL-7402 cells transfected with human group II A (lane 2) or X (lane 4) sPLA₂ plasmids was assessed by RT-PCR. D, BEL-7402 cells were serum-starved for 16

h, then stimulated with medium collected from vector, sPLA₂ II A or X transfected cells for 5 min. E, Serum-starved BEL-7402 cells transfected with vector, group II A or X sPLA₂ plasmids were treated with 10 μM SLD for 5 min. F, Serum-starved BEL-7402 cells transfected with group II A or X sPLA₂ plasmids were pretreated with 5 μM SLD for 15 min, then stimulated with 10 ng/ml EGF for 5 min. Cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies specific for phospho-Akt, phospho-ERK1/2, ERK1/2 and Akt (D, E and F). Data shown were representative of three independent experiments.

A

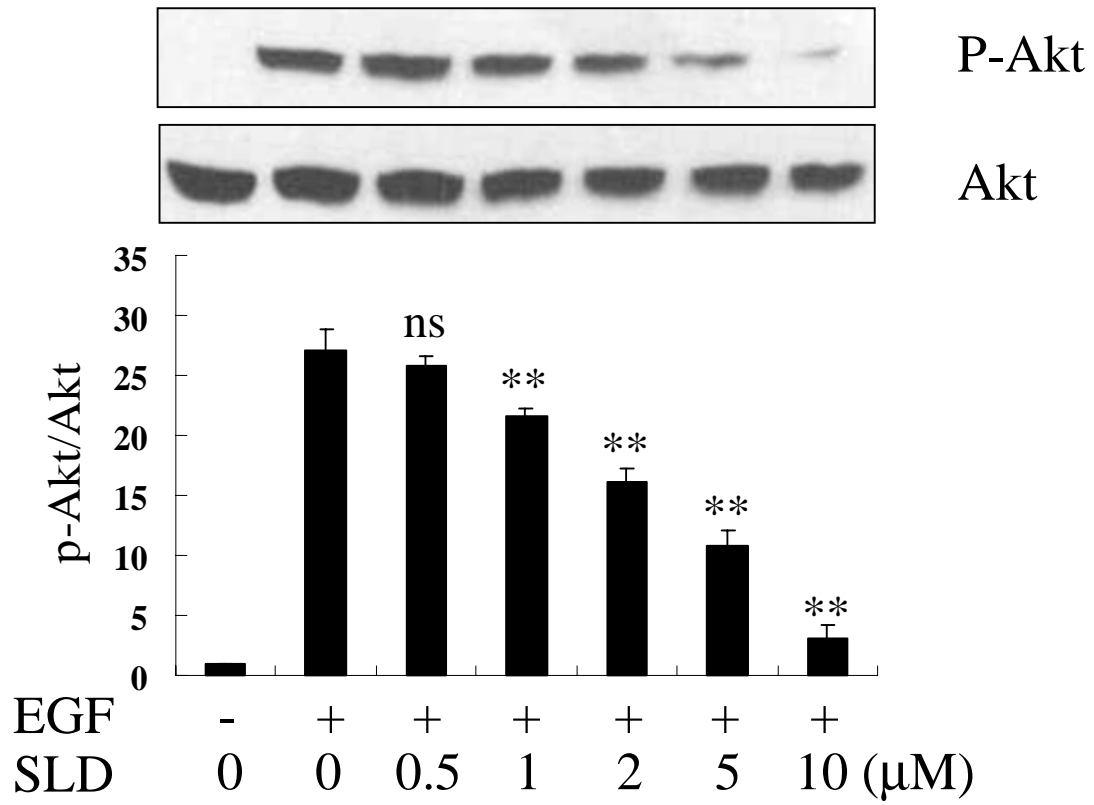


Fig. 1A

B

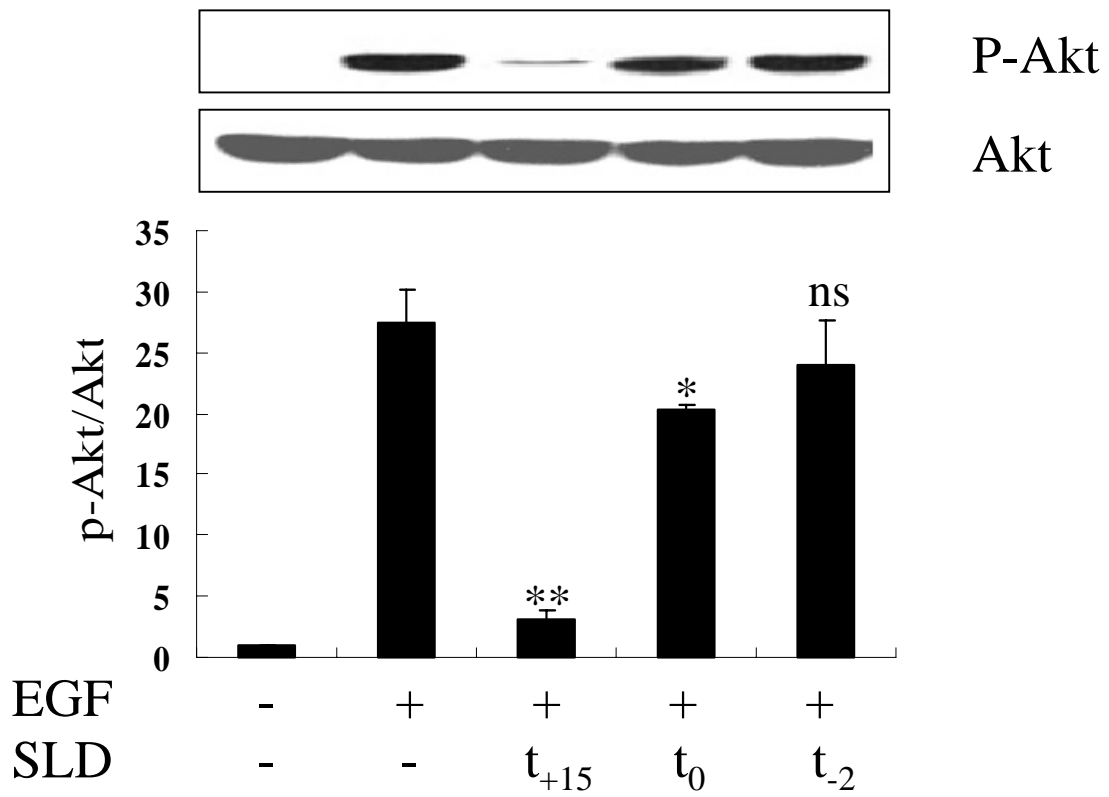
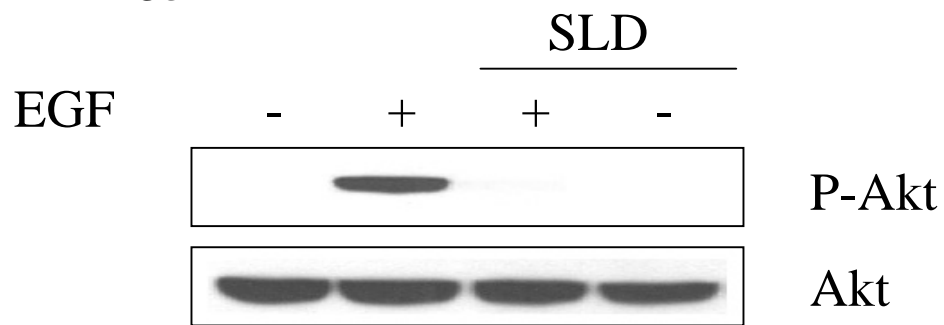
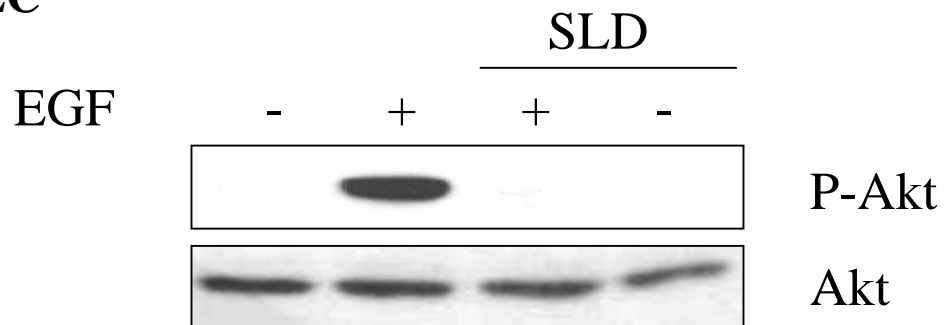


Fig. 1B

MDA-MB-435



HMEC



BEL-7402

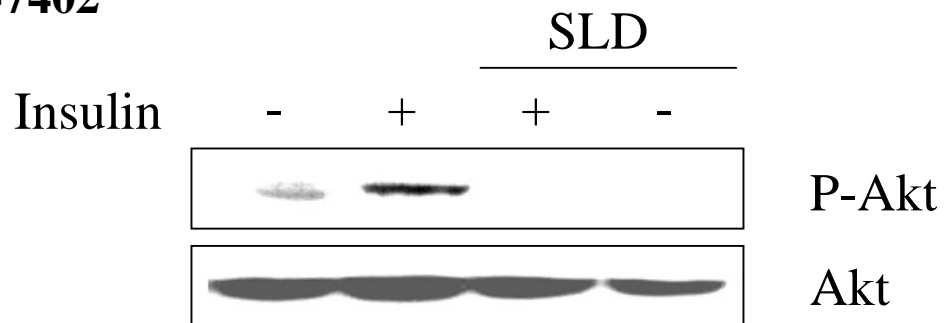


Fig. 2

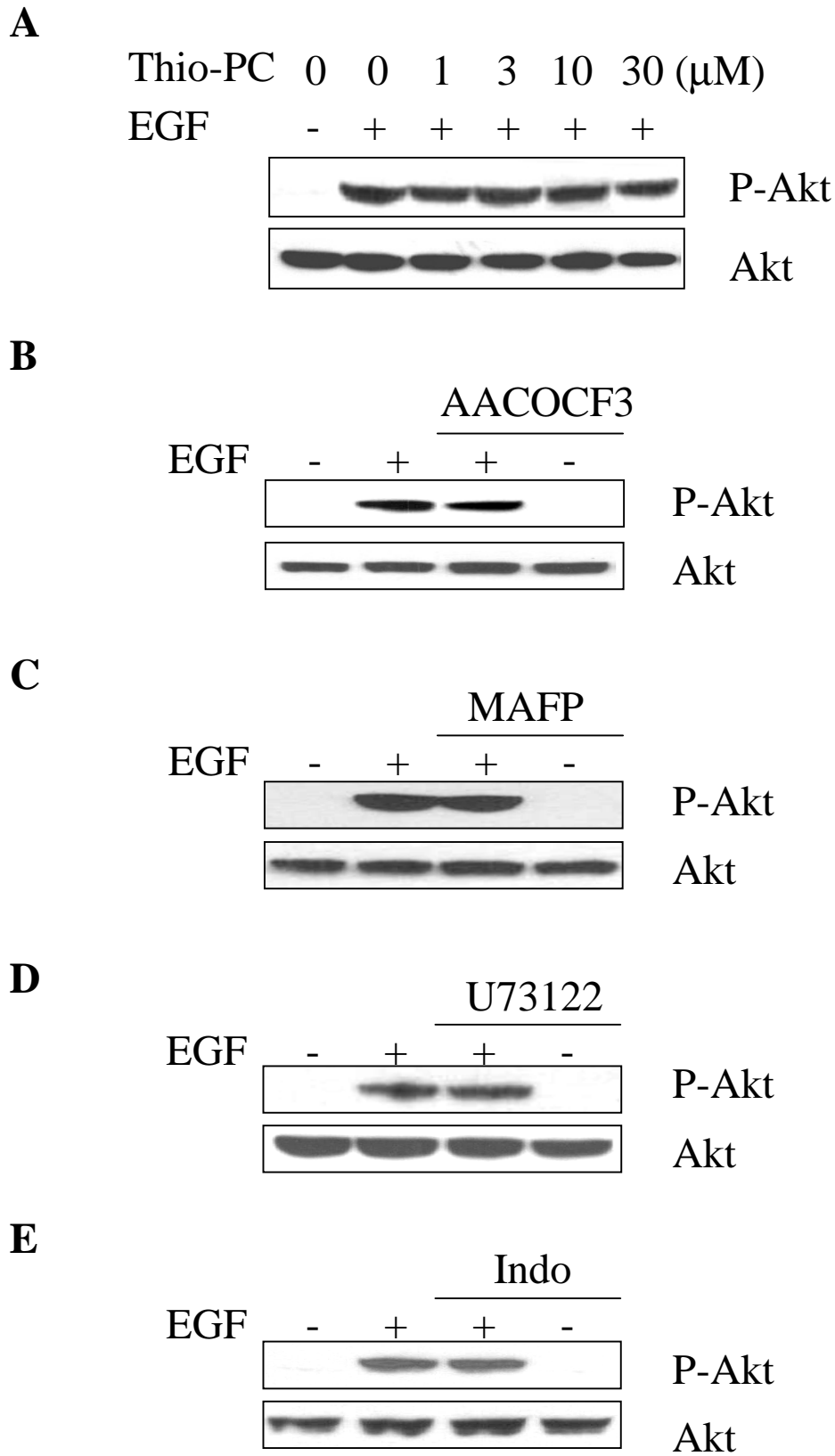


Fig. 3

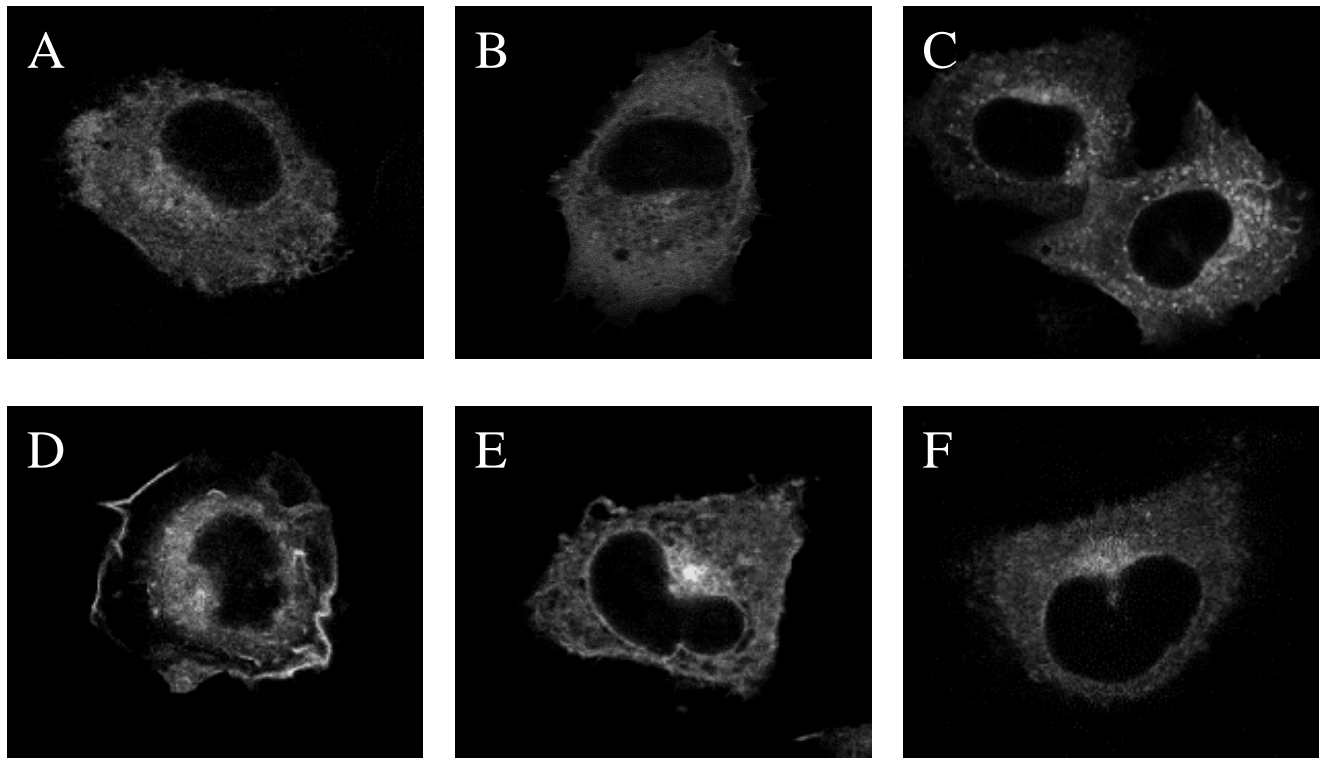


Fig. 4

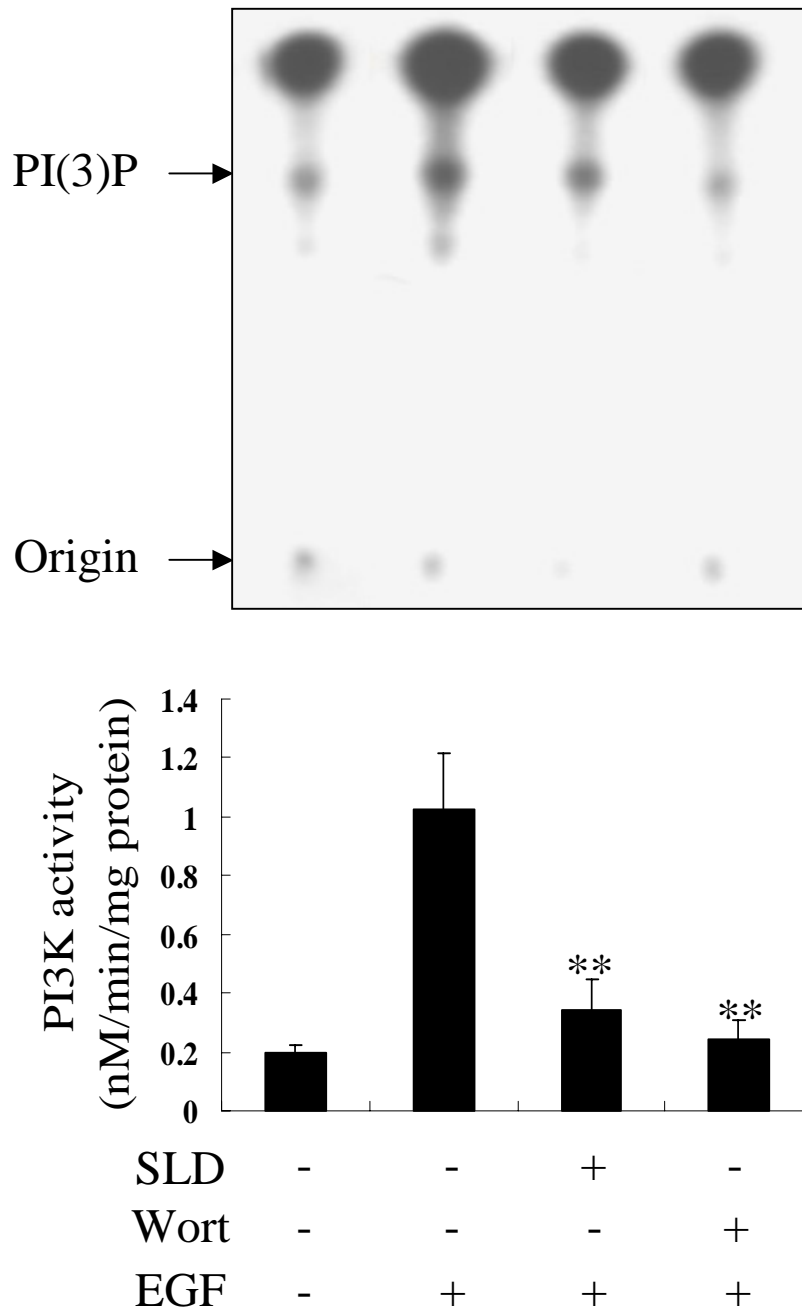
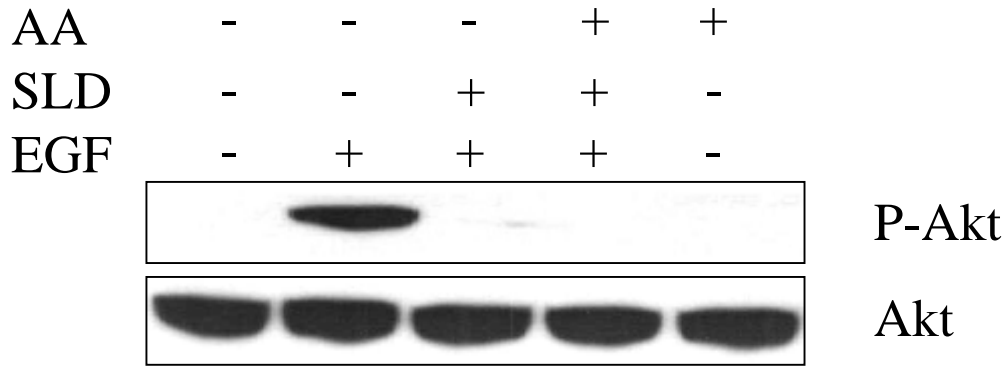
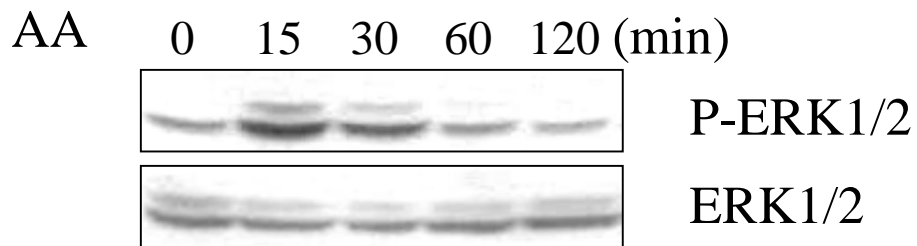


Fig. 5

A



B



C

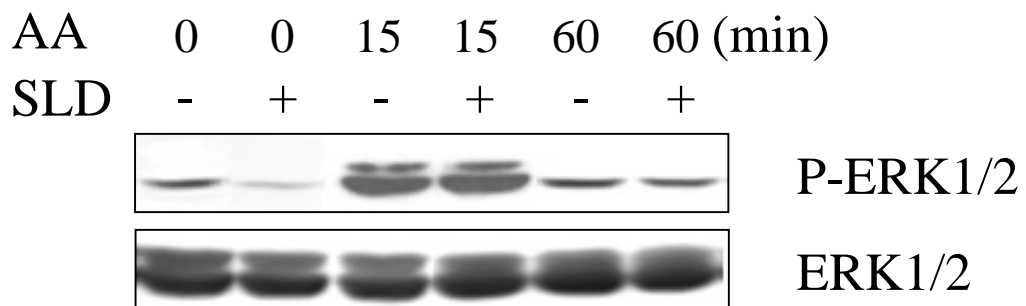
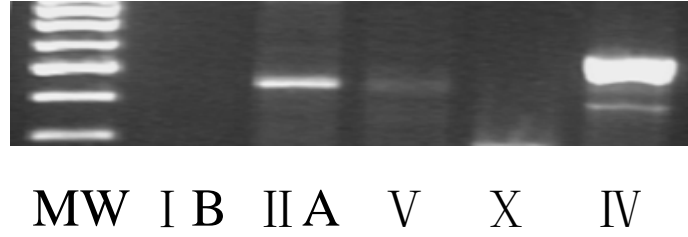
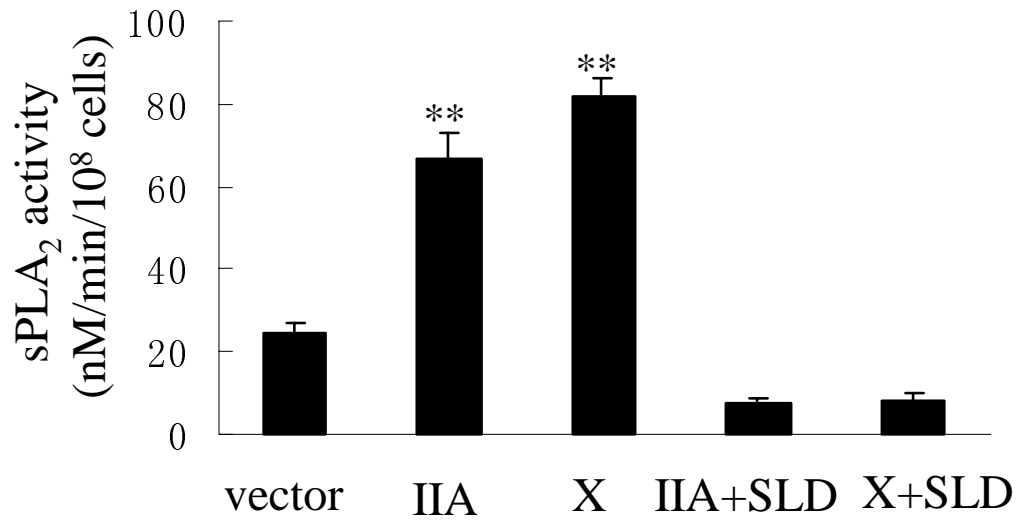


Fig. 6

A



B



C

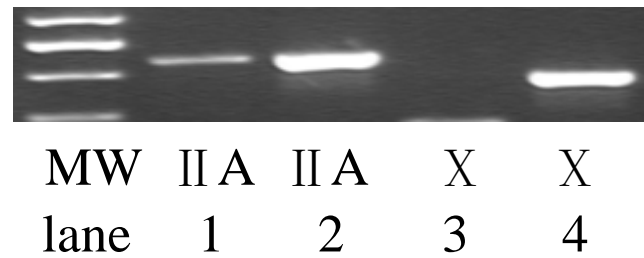


Fig. 7A, B and C

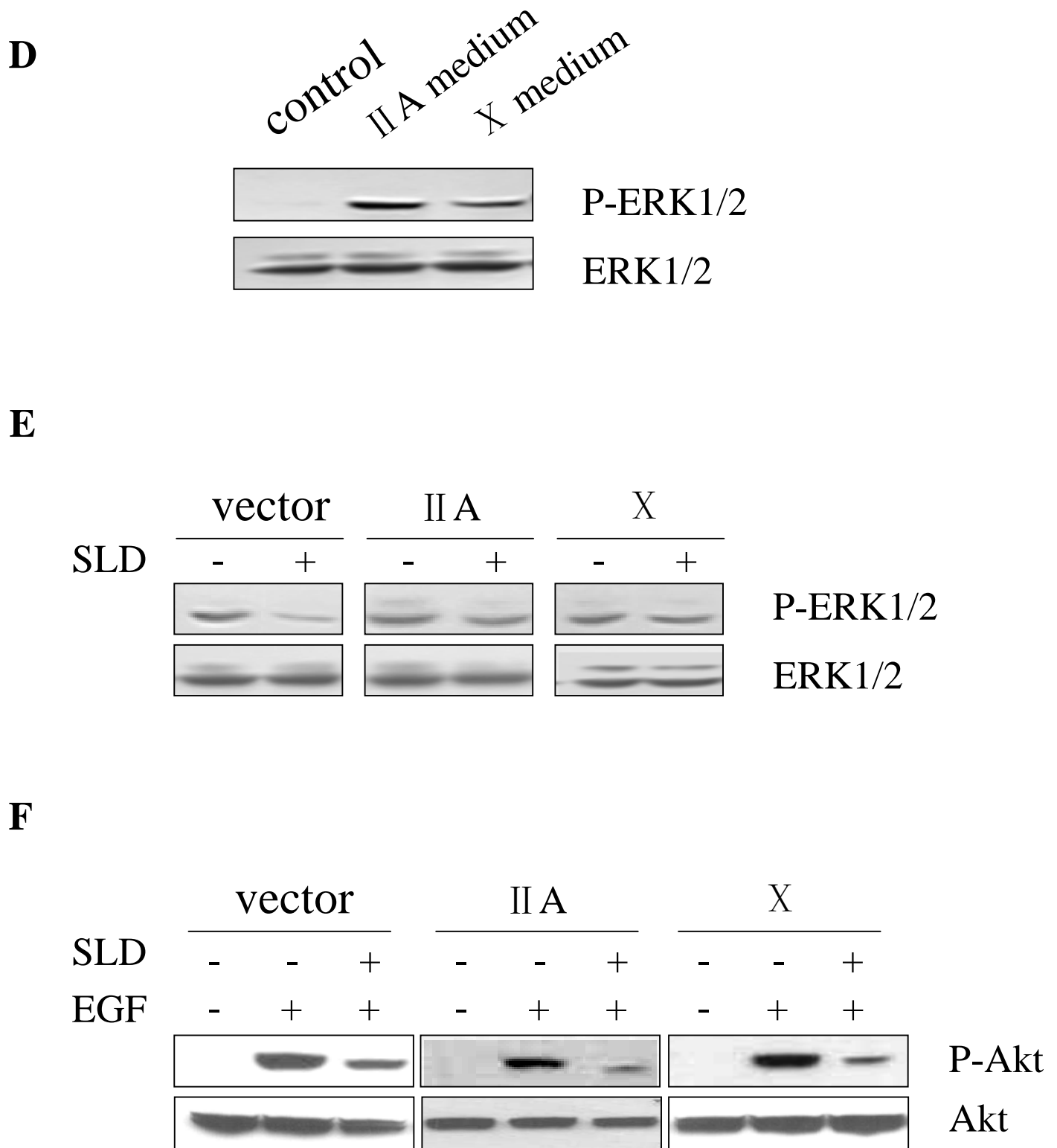


Fig. 7D, E and F