JPET Fast Forward. Published on May 27, 2005 as DOI: 10.1124/jpet.105.086520
This article has not been copyedited and formatted. The final version may differ from this version.

Scalaradial Inhibition of Epidermal Growth Factor Receptor-mediated Akt Phosphorylation Is Independent of Secretory Phospholipase A_2

Yili Xie, Lunhua Liu, Xiaochun Huang, Yuewei Guo and Liguang Lou*

Shanghai Institute of Materia Medica, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai 201203, China

Running title: Inhibition of EGF-stimulated Akt activation by scalaradial

*To whom correspondence should be addressed:
Dr. Liguang Lou, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zuchongzhi Road, Shanghai 201203, China. Tel: 86-21-5080 6056; Fax: 86-21-5080 7088; E-mail: lglou@mail.shcnc.ac.cn

Text pages: 19

Table: 0

Figures: 7

Reference: 39

Words in the Abstract: 178

Words in the Introduction: 435

Words in the Discussion: 884

The abbreviations used are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; PI3K, phosphatidylinositol 3-kinase; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PDK-1, 3-phosphoinositide-dependent protein kinase 1; ERK, extracellular signal-regulated kinases; $sPLA_2$, secretory phospholipase A_2; $cPLA_2$, cytosolic phospholipase A_2; HMEC, human dermal microvascular endothelial cells.

Recommended Section: Cellular & Molecular

JPET Fast Forward. Published on May 27, 2005 as DOI: 10.1124/jpet.105.086520
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

The marine natural product 12-epi scalaradial (SLD) is a specific secretory phospholipase A_2 ($sPLA_2$) 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 $sPLA_2$ inhibitor thioetheramide-PC, cytosolic PLA_2 inhibitor arachidonyl trifluoromethyl ketone, cytosolic PLA_2 and Ca^{2+}-independent PLA_2 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 $sPLA_2$-catalyzed metabolite, was not able to rescue SLD inhibition of EGF-stimulated Akt phosphorylation. Overexpression of group ⅡA or group Ⅹ $sPLA_2$ 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 $sPLA_2$.

JPET Fast Forward. Published on May 27, 2005 as DOI: 10.1124/jpet.105.086520
This article has not been copyedited and formatted. The final version may differ from this version.

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 A_2 (PLA_2) 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 PLA_2s: the 14-kDa secretory PLA_2 ($sPLA_2$), the Ca^{2+} -independent PLA_2 ($iPLA_2$), the 85-kDa cytosolic PLA_2 ($cPLA_2$), and platelet-activating factor acetylhydrolases (Kudo and Murakami, 2002; Murakami et al., 2000). To study the role of different PLA_2s in eicosanoid production as well as other pathophysiological functions such as inflammation, considerable effort has been put into developing specific inhibitors for different PLA_2s. These inhibitors included marine natural

products and PLA_2 substrate and product analogs (Hope et al., 1993). However, many of the PLA_2 inhibitors originally thought to be selective for a specific PLA_2 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 $sPLA_2$ (De Carvalho and Jacobs, 1991). SLD efficiently inhibited bee venom PLA_2 (IC_{50}=0.07 μM) and purified human recombinant type II $sPLA_2$ activity in vitro (IC_{50}=5.4 μM), but displayed weak inhibition of cytosol 85 kDa-PLA_2 from U937 cells (IC_{50} = 28 μM) (De Carvalho and Jacobs, 1991; Hope et al., 1993). However, whether this compound has other effects besides inhibiting $sPLA_2$ 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 $sPLA_2$.

JPET Fast Forward. Published on May 27, 2005 as DOI: 10.1124/jpet.105.086520
This article has not been copyedited and formatted. The final version may differ from this version.

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 ($AACOCF_3$), U73122 and 12-epi-scalaradial were purchased from Calbiochem (San Diego, CA). Thioetheramide-PC, methyl arachidonyl fluorophosphonate and $sPLA_2$ assay kit were purchased from Cayman Chemical Co. (Ann Arbor, MI). L-α-phosphatidylinositol was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). [γ-^{32}P] 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 ⅡA and Ⅹ $sPLA_2$ 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 % CO_2. MDA-MB-435 cells and HMEC were maintained in DMEM containing 10 % FBS at 37 °C with 5 % CO_2. 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 ⅡA or human group Ⅹ $sPLA_2$ 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 Na_2HPO_4, 1.8 mM KH_2PO_4, 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 polyvinylidine 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 $MgCl_2$, 1 mM $CaCl_2$, 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-^{32}P] ATP, and 20 mM $MgCl_2$ for 30 min at 30 °C

in a final volume of 80 µl. 20 µl of 8 M HCl was added to terminate the incubation. The lipids were extracted with 200 µl of chloroform/methanol (1:1) and centrifuged (300 × *g*) for 15 min at 4 °C. The lower organic phases were resolved on a 1 % potassium oxalate pretreated silica gel 60-Å 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 ⅡA or Ⅹ $sPLA_2$ 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-µl reaction using a RT-PCR kit (Promega). Briefly, 2 µg of total RNA was reverse-transcribed using 200 units of M-MLV RTase H in the presence of 0.5 µg oligo $(dT)_{18}$, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 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 °C (transcription) followed by 15 min at 70 °C (inactivation of reverse-transcription). PCR was performed in 50-µl reactions containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X-100, 0.2 mM of each dNTP, 5 units of Taq polymerase, and 1 µM of the specific primers. The group Ⅰ B $sPLA_2$ 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 ⅡA $sPLA_2$ 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 $sPLA_2$ 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 $sPLA_2$ 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 Ⅳα $cPLA_2$ 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 ⅠB, ⅡA, Ⅴ and Ⅹ $sPLA_2$ 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 Ⅳα $cPLA_2$ 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 $sPLA_2$ Activity. BEL-7402 cells were transfected with human group ⅡA or Ⅹ $sPLA_2$ plasmids. After transfection for 24 h, cells were starved in serum-free RPMI 1640 medium for another 16 h, then collected in PBS (10^8 cells/ml) containing 0.34 M sucrose, 10 mM HEPES, 100 μg/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 μm peak-to-peak amplitude for 3×15-second intervals at 4 ℃. $sPLA_2$ activity was measured using $sPLA_2$ 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 ± 0.44 μM (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 μM 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 $sPLA_2$, 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 $sPLA_2$ 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 E_2 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 μM (Fig. 3A). Arachidonyl trifluoromethyl ketone ($AACOCF_3$, 10 μM), a specific inhibitor of $cPLA_2$ (Street et al., 1993), methyl arachidomyl fluorophosphonate (MAFP, 10 μM), an inhibitor of $cPLA_2$ and $iPLA_2$ (Lio et al., 1996; Cummings et al., 2000), and U73122 (10 μM), 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 μM), an inhibitor of cyclooxygenases, which are downstream enzymes of PLA_2 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 (PIP_3), 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 μM) 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 PIP_3, we next examined the activity of PI3K in the presence or absence of SLD in BEL-7402 cells by measuring the [^{32}P]-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.

$sPLA_2$'s metabolite AA could not reverse SLD inhibition of Akt phosphorylation. $sPLA_2$ 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 $sPLA_2$. 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 μM SLD for 5 min significantly inhibited ERK1/2 phosphorylation under basal conditions (complete medium without FBS) (Fig. 6C). More importantly, another $sPLA_2$ inhibitor thioetheramide-PC also significantly reduced basal level of ERK1/2 phosphorylation (data not shown), indicating that $sPLA_2$s 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 $sPLA_2$ and AA is capable of reversing the inhibitory effect of SLD on $sPLA_2$-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 $sPLA_2$.

Overexpression of $sPLA_2$s 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 $sPLA_2$, we tested the effect of overexpression of $sPLA_2$s in BEL-7402 cells. Endogenous expression of $sPLA_2$ ⅠB, ⅡA, Ⅴ and Ⅹ, and $cPLA_2$ Ⅳα was determined by RT-PCR using specific primers. As shown in Fig.7A, we detected $sPLA_2$ ⅡA and $cPLA_2$ Ⅳα in BEL-7402

cells. We were not able to detect $sPLA_2$ ⅠB, Ⅴ and Ⅹ even using much more cDNA templates and more PCR cycles (data not shown). These data suggest that $sPLA_2$ II A may be the main $sPLA_2$ present in BEL-7402 cells. So, we next transiently transfected BEL-7402 cells with $sPLA_2$ ⅡA or Ⅹ plasmids. The transfection efficiency was around 15-25 % as determined by GFP transfection assay. Although the transfection efficiency was not high, the $sPLA_2$ activity as well as mRNA level of $sPLA_2$ ⅡA and Ⅹ in transfected cells was markedly increased and the increased $sPLA_2$ activity in transfected cells was significantly inhibited by SLD (Fig. 7, B and C). In addition, medium collected from $sPLA_2$ ⅡA or Ⅹ transfected cells but not from vector transfected cells markedly stimulated the phosphorylation of ERK1/2 (Fig. 7D), demonstrating that $sPLA_2$ ⅡA and Ⅹ were produced in transfected cells and secreted into medium. Importantly, overexpression of these $sPLA_2$s reversed SLD inhibition of ERK1/2 phosphorylation (the percentage inhibition of ERK1/2 phosphorylation by SLD in vector, $sPLA_2$ ⅡA 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 $sPLA_2$s, which was consistent with the conclusion from AA experiments. However, overexpression of neither $sPLA_2$ ⅡA nor $sPLA_2$ Ⅹ reversed SLD (5 μM) inhibition of Akt phosphorylation (Fig. 7F). Of note, the SLD concentration (5 μM) used here was relative lower as compared with the concentrations used in previous experiments and 5 μM SLD only partially inhibited EGF-stimulated Akt phosphorylation (Fig. 7F). The percentage inhibition of EGF-stimulated Akt

phosphorylation by SLD (5 μM) in vector, $sPLA_2$ ⅡA or $sPLA_2$ 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 $sPLA_2$.

Discussion

Previous studies show that SLD is a selective inhibitor of $sPLA_2$ (De Carvalho and Jacobs, 1991; Hope et al., 1993; Marshall et al., 1994). In the present study, we also showed that SLD effectively inhibited $sPLA_2$ activity (Fig. 7B). The selective inhibition of $sPLA_2$ by SLD blocked the release of AA, the activation of inducible NO synthase and transcription factor NF-κB, the production of eicosanoid, and the increase in intracellular Ca^{2+} 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 $sPLA_2$ and EGF has been shown to stimulate PLA_2 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 $sPLA_2$. However, thioetheramide-PC, a structurally modified phospholipid that functions as another specific inhibitor of $sPLA_2$ (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 $sPLA_2$. 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 PLA_2 and convert AA to prostaglandins (Touhey et al., 2002). Second, $sPLA_2$-catalyzed metabolite AA was not able to reverse the inhibitory effect of SLD on EGF-stimulated Akt phosphorylation. Third, overexpression of $sPLA_2$ 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 $sPLA_2$ since SLD inhibits basal ERK phosphorylation and this effect is reversed by AA and overexpression of $sPLA_2$s. These findings indicate that both AA and $sPLA_2$ transfection system work well in our systems and that SLD has both $sPLA_2$-dependent and –independent pharmacological effects in BEL-7402 cells.

Among 10 $sPLA_2$ enzymes that have been described in mammals, group ⅠB, ⅡA and Ⅴ $sPLA_2$ are relatively widespread and abundant in tissues (Kudo and Murakami, 2002). We used primers for these three $sPLA_2$ enzymes as well as group

Ⅹ sPLA$_2$ to detect the expression of sPLA$_2$s in BEL-7402 cells by RT-PCR. It was shown that only a 434-bp fragment of sPLA$_2$ ⅡA was amplified from the total RNA of BEL-7402 cells. Therefore, it was most likely group ⅡA but not other sPLA$_2$s exerted effects in BEL-7402 cells. We transfected BEL-7402 cells with group ⅡA sPLA$_2$ and around 3-fold increase in sPLA$_2$ activity was observed as determined by sPLA$_2$ activity assay. The assay uses the 1, 2-dithio analog of diheptanoyl phosphatidylcholine as a substrate which is specific for most PLA$_2$s (e.g., bee and cobra venoms, pancreatic, etc) but not for cPLA$_2$. 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 sPLA$_2$, over-expression of group ⅡA sPLA$_2$ should reverse the inhibition of Akt phosphorylation by 5 μM SLD to some degree. However, neither group ⅡA nor group Ⅹ sPLA$_2$ 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 sPLA$_2$.

Many PLA$_2$ inhibitors originally thought to be selective for a specific PLA$_2$ are now known to inhibit other isoforms. For example, manoalide has previously been shown to be a potent inhibitor of venom phospholipases A$_2$. Now it reveals that manoalide inhibits both general PLA$_2$ and phosphoinositide-specific phospholipase C (Bennett et al., 1987; Cummings et al., 2000). MAFP, the inhibitor of cPLA$_2$ with an IC$_{50}$ of ~0.5 μM for purified cPLA$_2$, also inhibits iPLA$_2$ purified from murine macrophage-like P388D1 cells with IC$_{50}$ of 0.5 μM (Lio et al., 1996). The failure of

other PLA_2 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, $cPLA_2$, $iPLA_2$ and phospholipase C.

In summary, this study revealed that SLD possessed a novel effect besides its inactivating $sPLA_2$, i.e., SLD inhibits EGFR-mediated Akt activation. This novel effect of SLD is independent of $sPLA_2$. Provided that Akt is a critical mediator in cellular biology, our findings provide important evidence for further exploring the pharmacological effects of SLD.

References

Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P and Hemmings BA (1996) Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J* 15:6541-6551.

Baek SH, Kwon TK, Lim JH, Lee YJ, Chang HW, Lee SJ, Kim JH and Kwun KB (2000) Secretory phospholipase A_2-potentiated inducible nitric oxide synthase expression by macrophages requires NF-κB activation. *J Immunol* 164:6359-6365.

Barnette MS, Rush J, Marshall LA, Foley JJ, Schmidt DB and Sarau HM (1994) Effects of scalaradial, a novel inhibitor of 14 kDa phospholipase A_2, on human neutrophil function. *Biochem Pharmacol* 47:1661-1667.

Beck-Speier I, Dayal N, Denzlinger C, Haberl C, Maier KL, Ziesenis A and Heyder J (2003) Sulfur-related air pollutants induce the generation of platelet-activating factor, 5-lipoxygenase- and cyclooxygenase-products in canine alveolar macrophages via activation of phospholipases A_2. *Prostaglandins Other Lipid Mediat* 71:217-234.

Bennett CF, Mong S, Wu HL, Clark MA, Wheeler L and Crooke ST (1987) Inhibition of phosphoinositide-specific phospholipase C by manoalide. *Mol Pharmacol* 32:587-593.

Bishop-Bailey D and Warner TD (2003) PPARγ ligands induce prostaglandin production in vascular smooth muscle cells: indomethacin acts as a peroxisome proliferator-activated receptor-γ antagonist. *FASEB J* 17:1925-1927.

Blom M, Tool ATJ, Wever PC, Wolbink GJ, Brouwer MC, Calafat J, Egesten A,

Knol EF, Hack CE, Roos D and Verhoeven AJ (1998) Human eosinophils express, relative to other circulating leukocytes, large amounts of secretory 14-kD phospholipase A_2. *Blood* 91:3037-3043.

Carpenter G and Cohen S (1990) Epidermal growth factor. *J Biol Chem* 265:7709-7712.

Croxtall JD, Choudhury Q, Tokumotos H and Flower RJ (1995) Lipocortin-1 and the control of arachidonic acid release in cell signalling. Glucocorticoids (changed from glucorticoids) inhibit G protein-dependent activation of $cPLA_2$ activity. *Biochem Pharmacol* 50:465-474.

Cummings BS, McHowat J and Schnellmann RG (2000) Phospholipase A_2s in cell injury and death. *J Pharmacol Exp Ther* 294:793-799.

Cupillard L, Koumanov K, Mattéi MG, Lazdunski M and Lambeau G (1997) Cloning, chromosomal mapping, and expression of a novel human secretory phospholipase A_2. *J Biol Chem* 272:15745-15752.

De Carvalho MS and Jacobs RS (1991) Two-step inactivation of bee venom phospholipase A_2 by scalaradial. *Biochem Pharmacol* 42:1621-1626.

Degousee N, Ghomashchi F, Stefanski E, Singer A, Smart BP, Borregaard N, Reithmeier R, Lindsay TF, Lichtenberger C, Reinisch W, Lambeau G, Arm J, Tischfield J, Gelb MH and Rubin BB (2002) Groups IV, V, and X phospholipases A_2s in human neutrophils. Role in eicosanoid production and gram-negative bacterial phospholipid hydrolysis. *J Biol Chem* 277:5061-5073.

Dulin NO, Alexander LD, Harwalkar S, Falck JR and Douglas JG (1998)

Phospholipase A_2-mediated activation of mitogen-activated protein kinase by angiotensin II. *PNAS* 95:8098-8102.

Dumont JE, Jauniaux JC and Roger PP (1989) The cyclic AMP-mediated stimulation of cell proliferation. *Trends Biochem Sci* 14:67-71.

Emlet DR, Moscatello DK, Ludlow LB and Wong AJ (1997) Subsets of epidermal growth factor receptors during activation and endocytosis. *J Biol Chem* 272:4079-4086.

Hanasaki K and Arita H (2002) Phospholipase A_2 receptor: a regulator of biological functions of secretory phospholipase A_2. *Prostaglandins Other Lipid Mediat* 68–69:71-82.

Hope WC, Chen T and Morgan DW (1993) Secretory phospholipase A_2 inhibitors and calmodulin antagonists as inhibitors of cytosolic phospholipase A_2. *Agents Actions* 39:C39-C42.

Kudo I and Murakami M (2002) Phospholipase A_2 enzymes. *Prostaglandins Other Lipid Media* 68–69:3-58.

Lio YC, Reynolds LJ, Balsinde J and Dennis EA (1996) Irreversible inhibition of Ca^{2+}-independent phospholipase A_2 by methyl arachidonyl fluorophosphonate. *Biochim Biophys Acta* 1302:55-60.

Liu Y and Levy R (1997) Phospholipase A_2 has a role in proliferation but not in differentiation of HL-60 cells. *Biochim Biophys Acta* 1355:270-280.

Lou LG, Urbani J, Ribeiro-Neto F and Altschuler DL (2002) cAMP inhibition of Akt is mediated by activated and phosphorylated Rap1b. *J Biol Chem* 277:32799-32806.

Luo J, Manning BD and Cantley LC (2003) Targeting the PI3K-Akt pathway in human cancer: rationale and promise. *Cancer Cell* 4:257-262.

Marshall LA, Winkler JD, Griswold DE, Bolognese B, Roshak A, Sung CM, Webb EF and Jacobs R (1994) Effects of scalaradial, a type II phospholipase A_2 inhibitor, on human neutrophil arachidonic acid mobilization and lipid mediator formation. *J Pharmacol Exp Ther* 268:709-717.

Murakami M, Nakatani Y, Kuwata H and Kudo I (2000) Cellular components that functionally interact with signaling phospholipase A_2s. *Biochem Biophys Arch* 1488:159-166.

Okano JI, Gaslightwala I, Birnbaum MJ, Rustgi AK and Nakagawa H (2000) Akt/protein kinase B isoforms are differentially regulated by epidermal growth factor stimulation. *J Biol Chem* 275:30934-30942.

Schalkwijk CG, Spaargaren M, Defize LH, Verkleij AJ, van den Bosch H and Boonstra J (1995) Epidermal growth factor (EGF) induces serine phosphorylation-dependent activation and calcium-dependent translocation of the cytosolic phospholipase A_2. *Eur J Biochem* 231:593-601.

Scheid MP, Marignani PA and Woodgett JR (2002) Multiple phosphoinositide 3-kinase-dependent steps in activation of protein kinase B. *Mol Cell Biol* 22:6247-6260.

Silfani TN and Freeman EJ (2002) Phosphatidylinositide 3-kinase regulates angiotensin II-induced cytosolic phospholipase A_2 activity and growth in vascular smooth muscle cells. *Arch Biochem Biophys* 402:84-93.

Spaargaren M, Defize LH, Boonstra J and de Laat SW (1991) Antibody-induced dimerization activates the epidermal growth factor receptor tyrosine kinase. *J Biol Chem* 266:1733-1739.

Spaargaren M, Wissink S, Defize LH, de Laat SW and Boonstra J (1992) Characterization and identification of an epidermal-growth-factor-activated phospholipase A_2. *Biochem J* 287:37-43.

Stephens L, Anderson K, Stokoe D, Erdjument-Bromage H, Painter GF, Holmes AB, Gaffney PR, Reese CB, McCormick F, Tempst P, Coadwell J and Hawkins PT (1998) Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. *Science* 279:710-714.

Street IP, Lin HK, Laliberte F, Ghomashchi F, Wang Z, Perrier H, Tremblay NM, Huang Z, Weech PK and Gelb MH (1993) Slow- and tight-binding inhibitors of the 85-kDa human phospholipase A_2. *Biochemistry* 32:5935-5940.

Thommesen L, Sjursen W, Gåsvik K, Hanssen W, Brekke OL, Skattebøl L, Holmeide AK, Espevik T, Johansen B and Lægreid A (1998) Selective inhibitors of cytosolic or secretory phospholipase A_2 block TNF-induced activation of transcription factor nuclear factor-κB and expression of ICAM-1. *J Immunol* 161:3421-3430.

Touhey S, O'Connor R, Plunkett S, Maguire A and Clynes M (2002) Structure-activity relationship of indomethacin analogues for MRP-1, COX-1 and COX-2 inhibition. Identification of novel chemotherapeutic drug resistance modulators. *Eur J Cancer* 38:1661-1670.

Whitman M, Kaplan DR, Schaffhausen B, Cantley L and Roberts TM (1985) Association of phosphatidylinositol kinase activity with polyoma middle-T competent for transformation. *Nature* 315:239-242.

Wu YL, Jiang XR, Newland AC and Kelsey SM (1998) Failure to activate cytosolic phospholipase A_2 causes TNF resistance in human leukemic cells. *J Immunol* 160:5929-5935.

Yu L, Deems RA, Hajdu J and Dennis EA (1990) The interaction of phospholipase A_2 with phospholipid analogues and inhibitors. *J Biol Chem* 265:2657-2664.

Zheng L, Paik WY, Cesnjaj M, Balla T, Tomic M, Catt KJ and Stojilkovic SS (1995) Effects of the phospholipase-C inhibitor, U73122, on signaling and secretion in pituitary gonadotrophs. *Endocrinology* 136:1079-1088.

Footnotes

This work was supported by grant from National Natural Science Foundation of China № 30370729 (to L.L.).

Address correspondence to: Dr. Liguang Lou, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zuchongzhi Road, Shanghai 201203, CHINA.

JPET Fast Forward. Published on May 27, 2005 as DOI: 10.1124/jpet.105.086520
This article has not been copyedited and formatted. The final version may differ from this version.

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 μM 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 ± 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 μM 12-epi-scalaradial (SLD) for 15 min followed by 10 ng/ml EGF or 1 μg/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 $sPLA_2$ inhibitor thioetheramide-PC (Thio-PC) (A), 10 μM $cPLA_2$ inhibitor $AACOCF_3$ (B), 10 μM $cPLA_2$ and $iPLA_2$ 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 ± 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 μM SLD in the presence or absence of 30 μM AA for 15 min, then stimulated with 10 ng/ml EGF for 5 min. B, Serum-starved BEL-7402 cells were treated with 30 μM AA for indicated times. C, Serum-starved BEL-7402 cells were pretreated with 30 μM AA for 15 min or 1 h, then treated with 10 μM 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 sPLA$_2$s. A, Expression of sPLA$_2$ ⅠB (341 bp), ⅡA (434 bp), Ⅴ (358 bp), Ⅹ (370 bp) and cPLA$_2$ Ⅳα (554 bp) in BEL-7402 cells was assessed by RT-PCR. B, BEL-7402 cells transfected with human sPLA$_2$ ⅡA or Ⅹ plasmids were serum-starved and subjected to sPLA$_2$ activity assay as described under "Material and Methods". Values represented are mean ± S.E.M. (bars), n = 3. $^{**}p < 0.01$ *vs* control. C, Expression of sPLA$_2$ⅡA (434 bp) or Ⅹ (370 bp) in serum-starved BEL-7402 cells transfected with human group ⅡA (lane 2) or Ⅹ (lane 4) sPLA$_2$ plasmids was assessed by RT-PCR. D, BEL-7402 cells were serum-starved for 16

h, then stimulated with medium collected from vector, $sPLA_2$ ⅡA or Ⅹ transfected cells for 5 min. E, Serum-starved BEL-7402 cells transfected with vector, group Ⅱ A or Ⅹ $sPLA_2$ plasmids were treated with 10 μM SLD for 5 min. F, Serum-starved BEL-7402 cells transfected with group ⅡA or Ⅹ $sPLA_2$ 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.

JPET Fast Forward. Published on May 27, 2005 as DOI: 10.1124/jpet.105.086520
This article has not been copyedited and formatted. The final version may differ from this version.

A

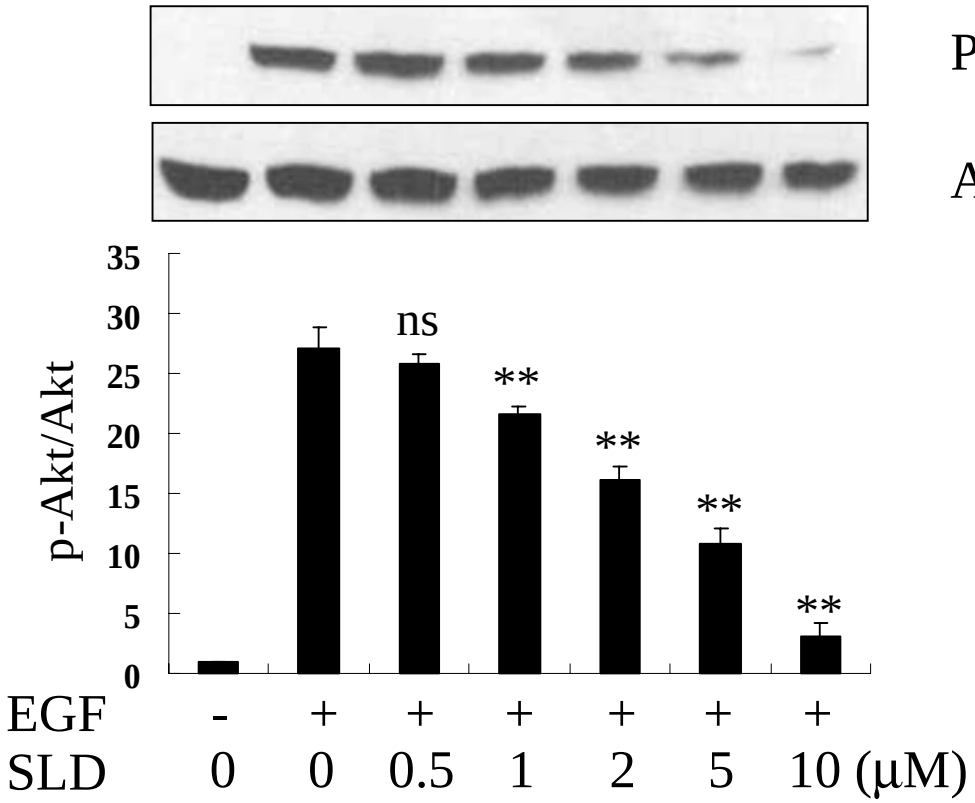


Fig. 1A

JPET Fast Forward. Published on May 27, 2005 as DOI: 10.1124/jpet.105.086520
This article has not been copyedited and formatted. The final version may differ from this version.

B

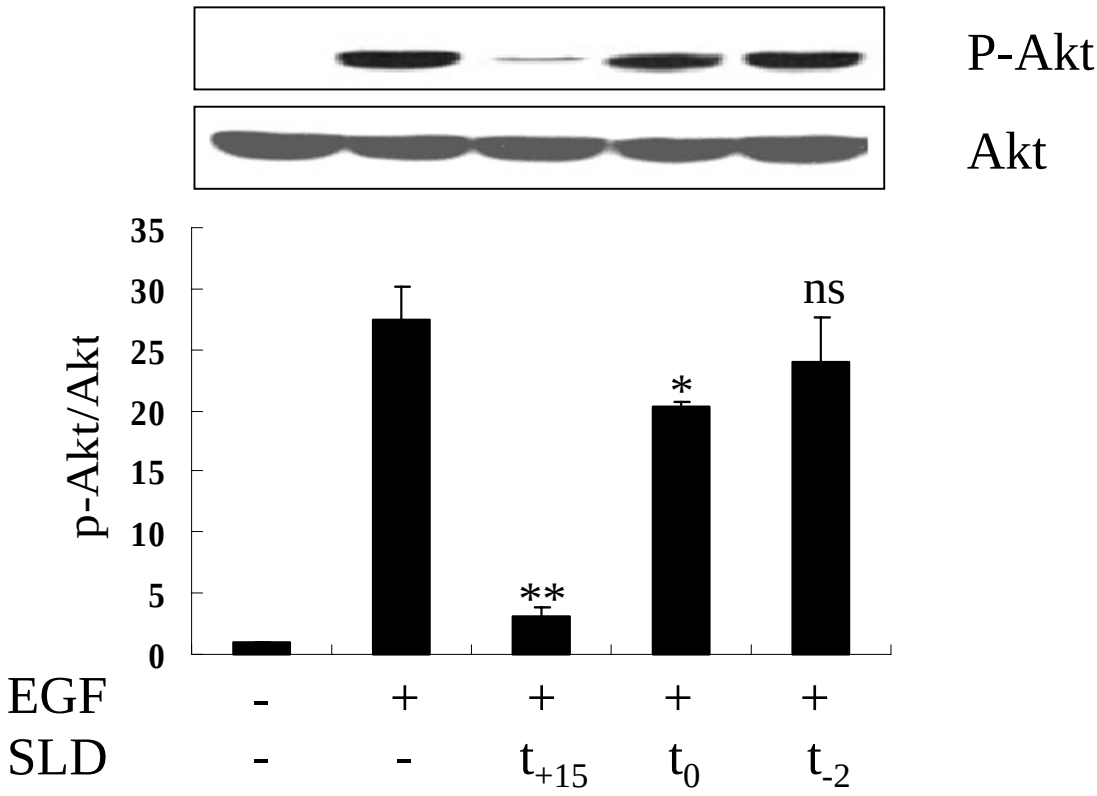
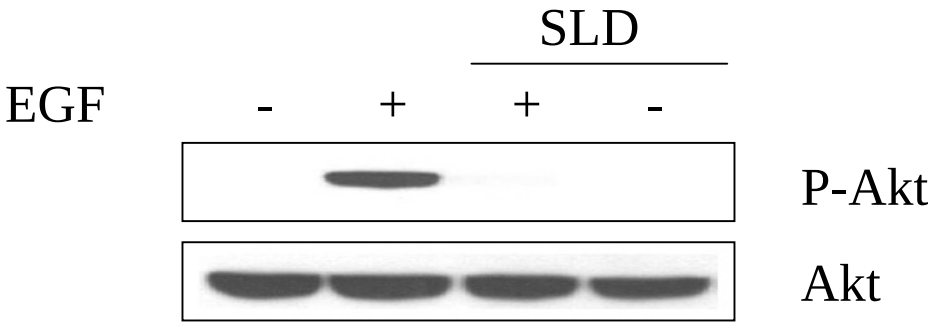


Fig. 1B

JPET Fast Forward. Published on May 27, 2005 as DOI: 10.1124/jpet.105.086520
This article has not been copyedited and formatted. The final version may differ from this version.

MDA-MB-435



HMEC

BEL-7402

Fig. 2

JPET Fast Forward. Published on May 27, 2005 as DOI: 10.1124/jpet.105.086520
This article has not been copyedited and formatted. The final version may differ from this version.

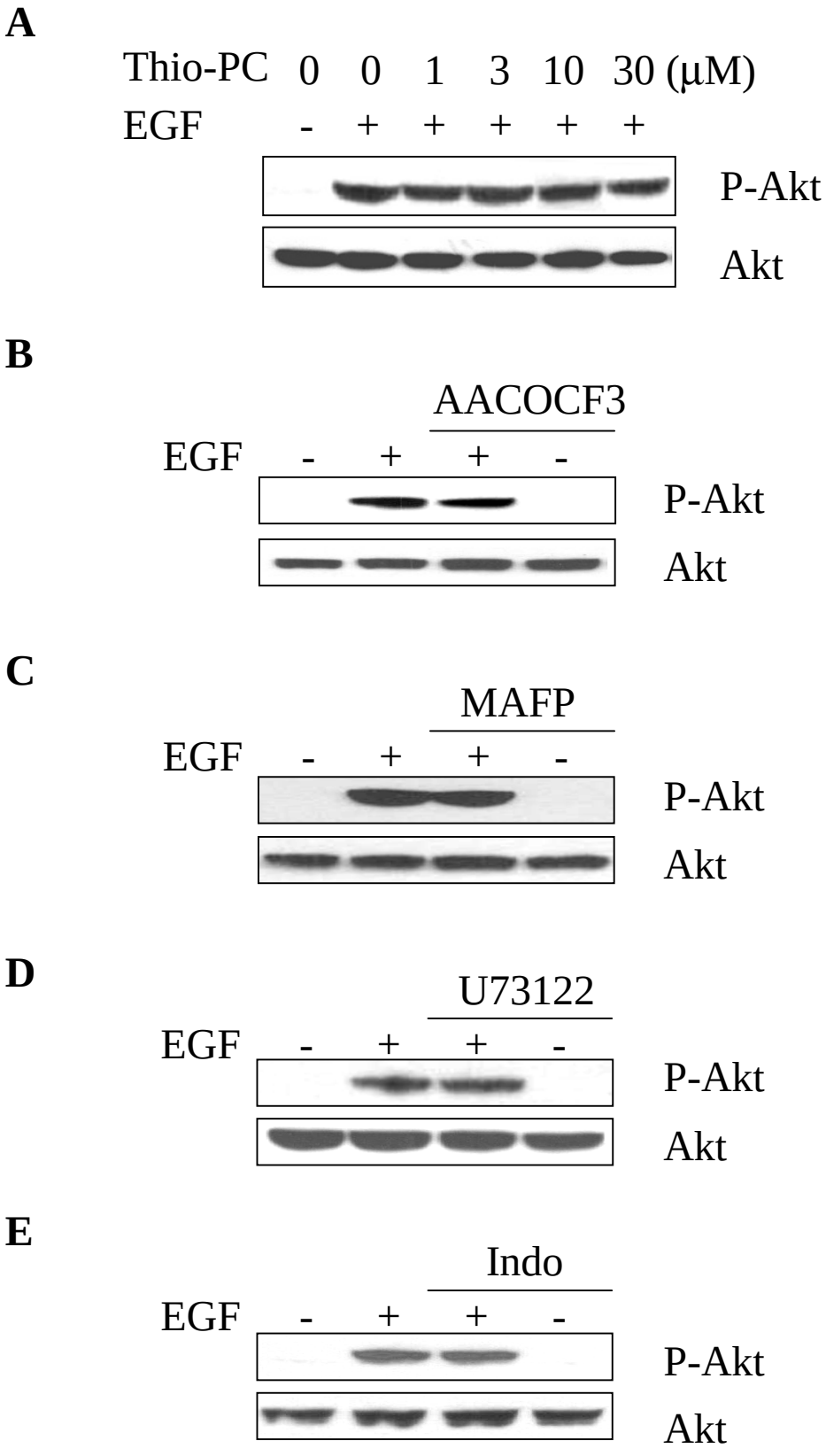


Fig. 3

JPET Fast Forward. Published on May 27, 2005 as DOI: 10.1124/jpet.105.086520
This article has not been copyedited and formatted. The final version may differ from this version.

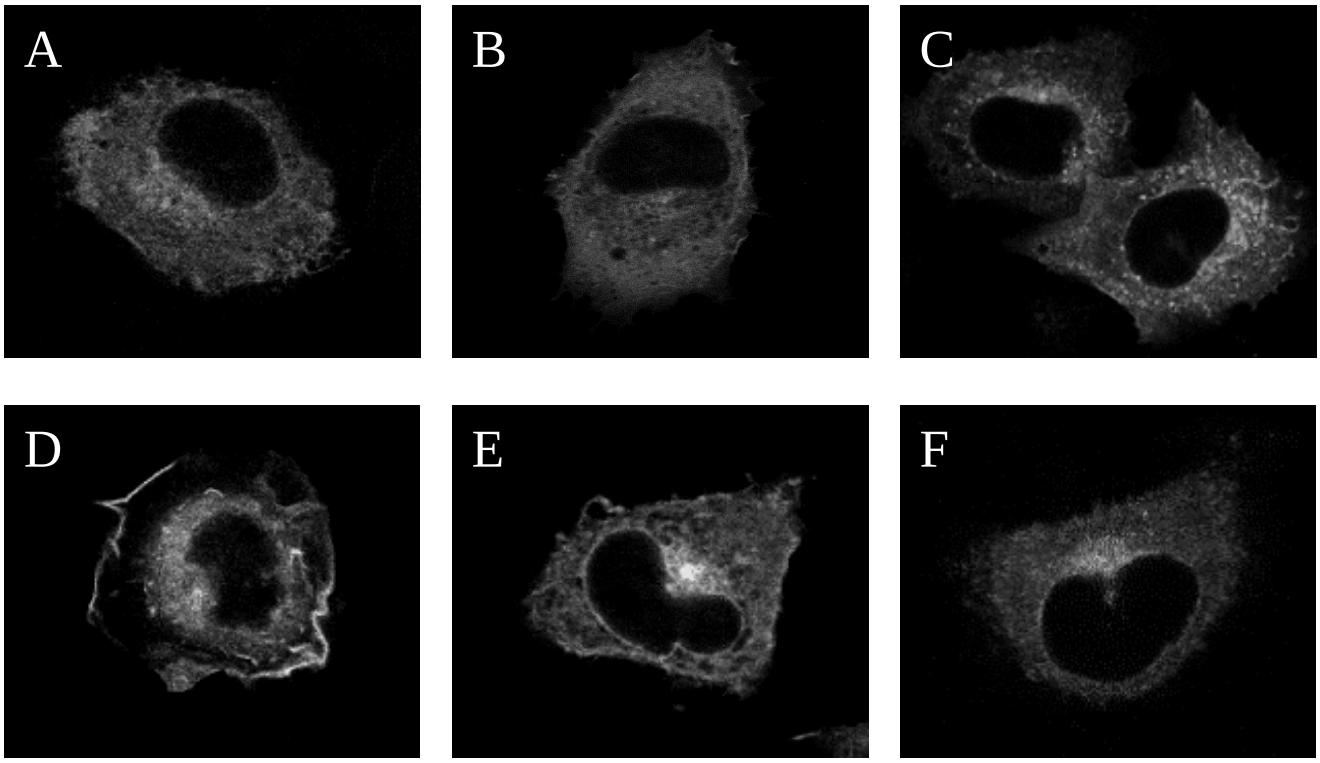


Fig. 4

JPET Fast Forward. Published on May 27, 2005 as DOI: 10.1124/jpet.105.086520
This article has not been copyedited and formatted. The final version may differ from this version.

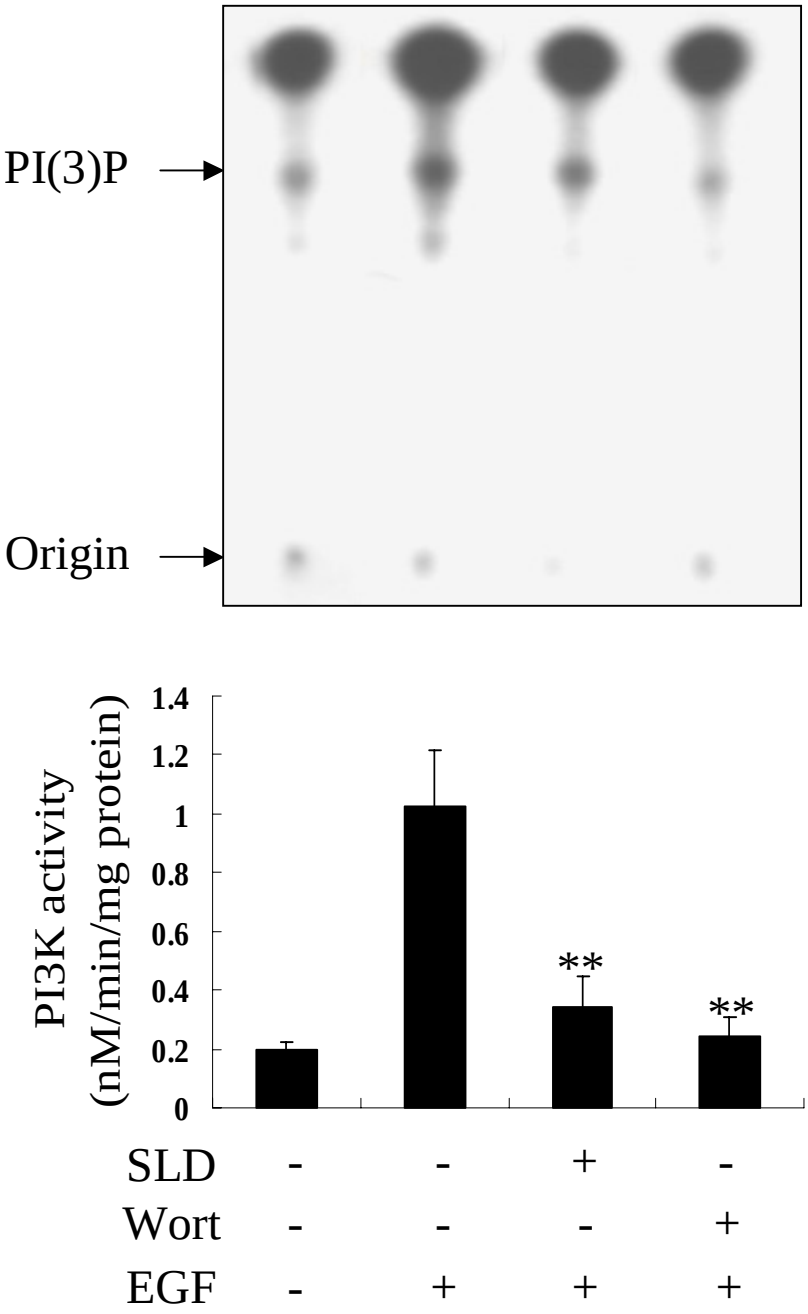
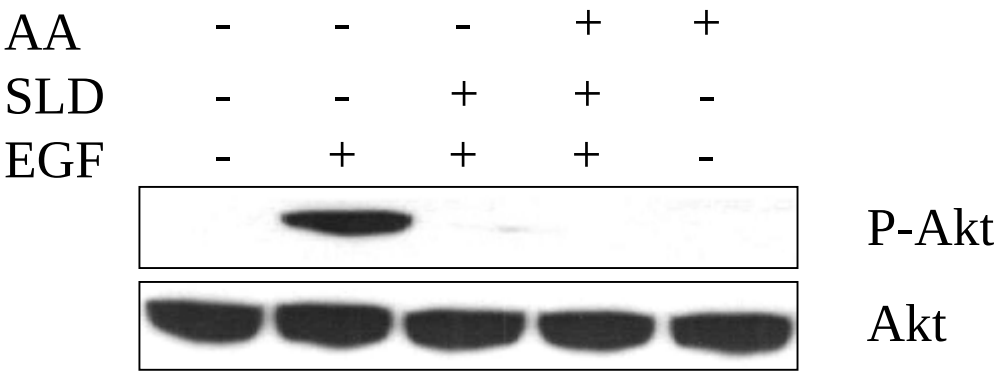


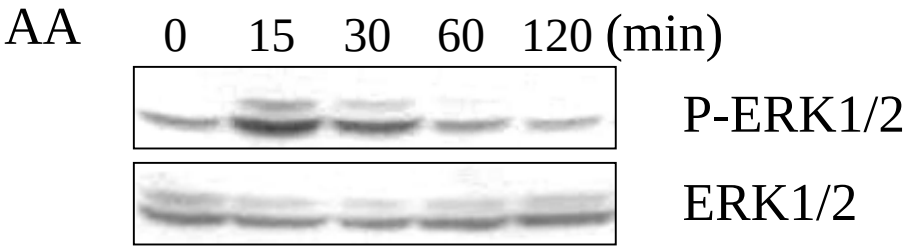
Fig. 5

JPET Fast Forward. Published on May 27, 2005 as DOI: 10.1124/jpet.105.086520
This article has not been copyedited and formatted. The final version may differ from this version.

A



B



C

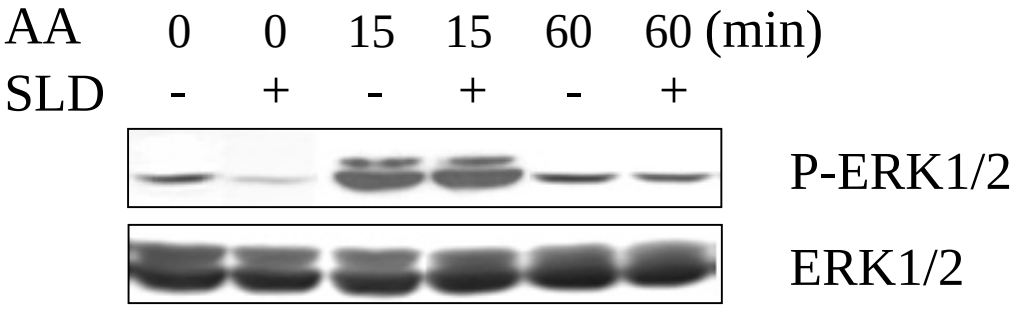
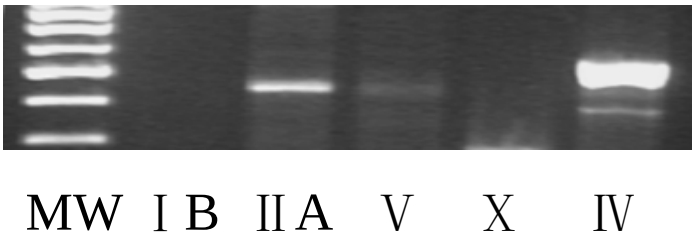


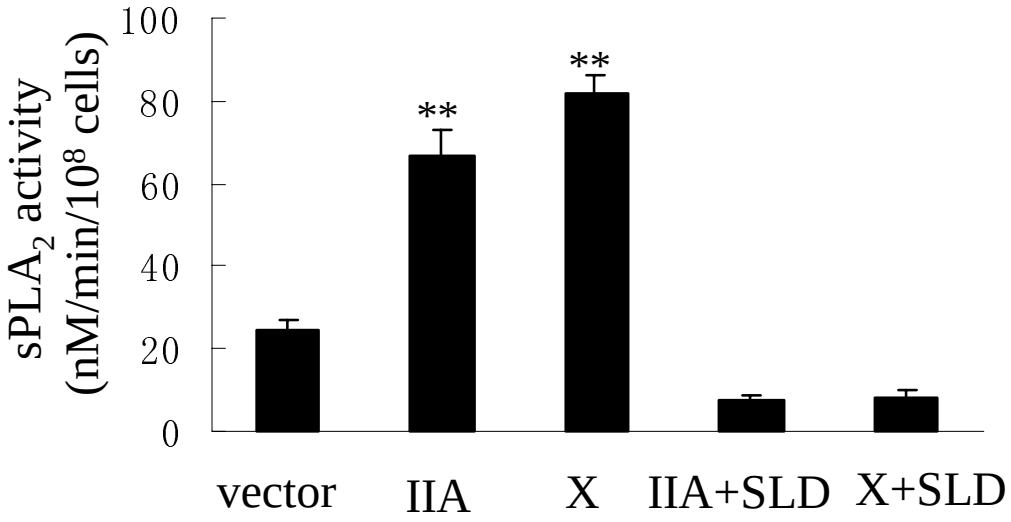
Fig. 6

JPET Fast Forward. Published on May 27, 2005 as DOI: 10.1124/jpet.105.086520
This article has not been copyedited and formatted. The final version may differ from this version.

A



B



C

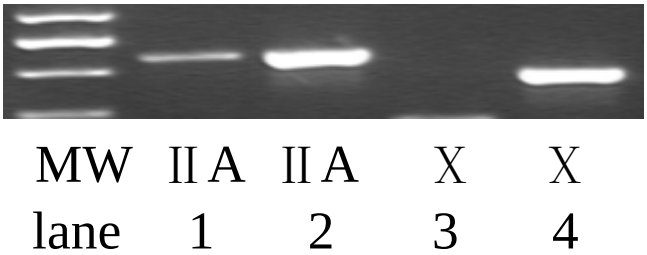
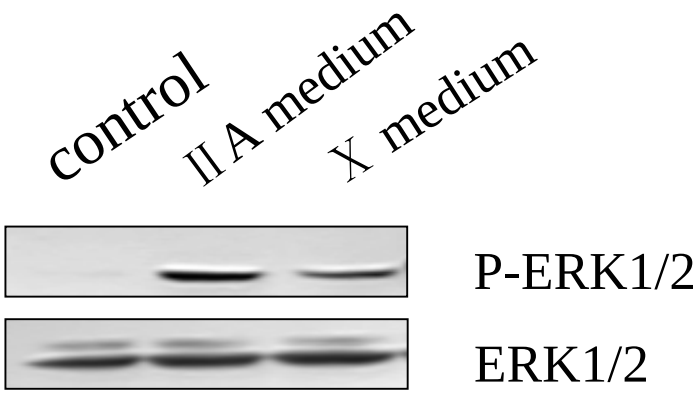


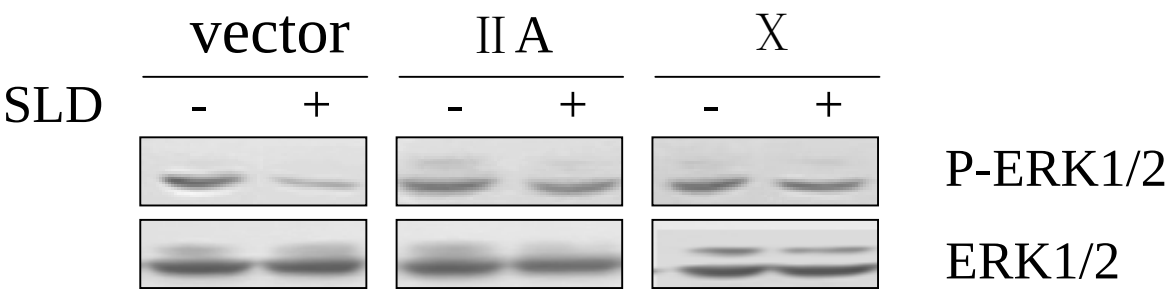
Fig. 7A, B and C

JPET Fast Forward. Published on May 27, 2005 as DOI: 10.1124/jpet.105.086520
This article has not been copyedited and formatted. The final version may differ from this version.

D



E



F

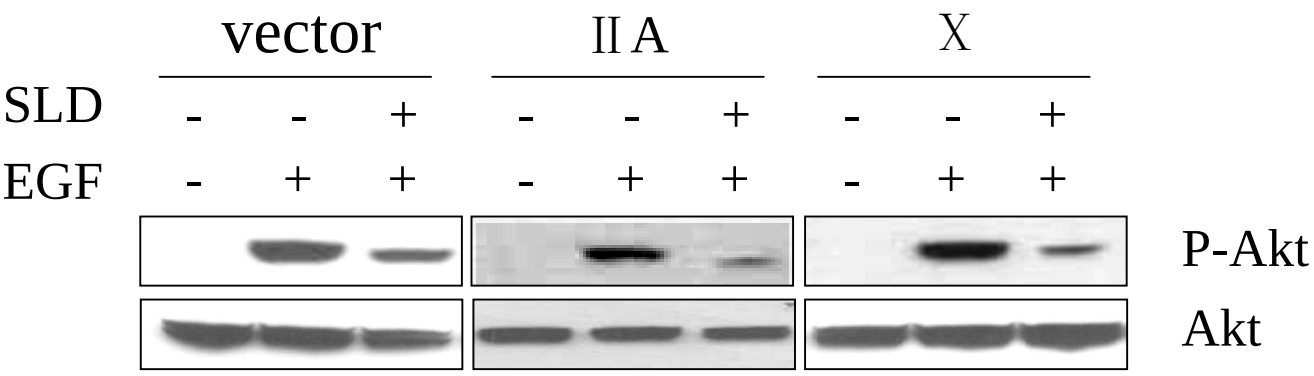


Fig. 7D, E and F