Scalaradial Inhibition of Epidermal Growth Factor Receptor-Mediated Akt Phosphorylation Is Independent of Secretory Phospholipase A2

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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 about 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 indomethacin, failed to inhibit EGF-stimulated Akt phosphorylation. Furthermore, arachidonic acid, the main sPLA2-catalyzed metabolite, was not able to rescue SLD inhibition of EGF-stimulated Akt phosphorylation. Overexpression of group IIA 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) (Carpenter and Cohen, 1990; Spaargaren et al., 1991). 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 (PI3K/Akt) signaling pathway (Carpenter and Cohen, 1990; Spaargaren et al., 1991). 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 and inhibited phosphatidylinositol 3-kinase activity. This inhibition is specific for SLD because other phospholipase inhibitors, including sPLA2 inhibitor indomethacin, failed to inhibit EGF-stimulated Akt phosphorylation. Furthermore, arachidonic acid, the main sPLA2-catalyzed metabolite, was not able to rescue SLD inhibition of EGF-stimulated Akt phosphorylation. Overexpression of group IIA 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.

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ABBREVIATIONS: EGF, epidermal growth factor; EGFR, EGF receptor; PI3K, phosphatidylinositol 3-kinase; PDK, 3-phosphoinositide-dependent protein kinase; ERK, extracellular signal-regulated kinase; PLA2, phospholipase A2; sPLA2, secretory PLA2; iPLA2, Ca2+-independent PLA2; cPLA2, cytosolic phospholipase A2; SLD, 12-epi-scalaradial; AACEOCF3, arachidonyl trifluoromethyl ketone; U73122, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-triien-17-yl]amino]hexyl]-1H-pyrrrole-2,5-dione; PC, phosphatidylcholine; pEGFP, plasmid vector red-shifted green fluorescence protein; HMEC, human dermal microvascular endothelial cell; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcriptase-polymerase chain reaction; AA, arachidonic acid; MAFP, methyl arachidonyl fluorophosphonate; GFP, green fluorescence protein.
specific PL*A are now known to have multiple other effects (Cummings et al., 2000).

12-Epi-scalaradial (SLD), a natural product isolated from marine sponge (Cacospongia species), has been shown to be a selective inhibitor of sPLA2 (De Carvalho and Jacobs, 1991). SLD efficiently inhibited bee venom PL*A (IC50 = 0.07 μM) and purified human recombinant type II sPLA2 activity in vitro (IC50 = 5.4 μM) but displayed weak inhibition of cytotoxic 85-kDa PL*A 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 that 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-Aldrich (St. Louis, MO). Arachidonyl trifluoromethyl ketone (AACOCF3), U73122, and 12-epi-scalaradial were purchased from Calbiochem (San Diego, CA). Thioetheramide-PC, methyl arachidonoyl fluorophosphate, and sPLA2 assay kit were purchased from Cayman Chemical (Ann Arbor, MI). L-α-Phosphatidylisotol was purchased from Avanti Polar Lipids (Alabaster, AL). [γ-32P] ATP and protein G-Sepharose were purchased from Amersham Biosciences AB (Uppsala, Sweden). FuGENE 6 transfection reagent was purchased from Roche Diagnostics (Mannheim, Germany). pEGFP plasmid and PEGFP-PDK-1 plasmid were kind gifts from Dr. Jongkyeong Chung (Korea Advanced Institute of Science and Technology, Taejon, Korea). Human group IIA and X sPLA2 plasmids were kindly gifted from Dr. Jongkyeong Chung (Korea Advanced Institute of Science and Technology, Taejon, Korea). 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 (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 (HMECs) were from Cambrex Bio Science Walkersville (Walkersville, MD). 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 HMECs were maintained in Dulbecco’s modified Eagle's medium containing 10% FBS at 37°C with 5% CO2. All the cells were plated in six-well plates and starved in serum-free medium for 16 h before drug treatment. For transient transfection, cells grown in six-well plates at 70% confluence were transfected with pEGFP, pEGFP-PDK-1, human group IIA, 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, and 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 dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 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% nonfat milk in 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 Chemical (Rockford, IL).

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 leupeptin, 2 μM aprotinin, and 1 μM phenylmethylsulfonyl fluoride]. Insoluble material was removed by centrifugation at 12,000 rpm at 4°C for 10 min. Cell lysates (500 ng) 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-phosphatidylisotol, 50 μM ice-cold ATP, 15 μCi [γ-32P] ATP, and 20 mM MgCl2 for 30 min at 30°C in a final volume of 80 μl. Twenty microtiter of 8 M HCl was added to terminate the incubation. The lipids were extracted with 200 μl of chloroform/methanol (1:1) and centrifuged (300g) for 15 min at 4°C. The lower organic phases were resolved on a 1% potassium oxalate-pretreated silica gel 60-A thin-layer chromatography plate (Merck, Darmstadt, Germany) by chloroform/acetone/methanol/acetatic acid/water (46:17:15:14:8, v/v) and visualized by autoradiography. Identified spots containing radioactive phosphatidylinositol-3-phosphate 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 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, China) following the manufacturer’s protocols. cDNA mixtures were prepared in a 20-μl reaction using an RT-PCR kit (Promega). Briefly, 2 μg of total RNA was reverse-transcribed using 200 units of Moloney murine leukemia virus RTase H in the presence of 0.5 μg oligo(dT)18, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 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 MgCl2, 0.1% Triton X-100, 0.2 mM each of dNTP, 5 units of Taq polymerase, and 1 μM of the specific primers. The group IB sPLA2 primer sequences used were 5′-TCT TCG TGG TCT AGT CTT GTC CTT GAG TAG TCG TGC-3′ and 5′-TCA AGG CCT CAC ACT CCT TTG-3′, and 5′-TCA GAG GGT GGT GCT CCT-3′ (Degousee et al., 2002). The group IIA sPLA2 primer sequences were 5′-ATG AAC CTC CTA CTT TTG-3′ and 5′-TCA GAG GGT GGT CTC ACT-3′ (Degousee et al., 2002). The group IIA sPLA2 primer sequences were 5′-TTG GGT CTC TGG TCT TGT-3′ and 5′-TCA GAC ACG ACC TTG GGT-3′ (Degousee et al., 2002). 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, and 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 dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 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% nonfat milk in 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 Chemical (Rockford, IL).
fected with human group IIA or X sPLA2 plasmids. After transfection, the sonicated at 21 kHz and 8-EDTA (final pH 7.0) (Blom et al., 1998). The suspended cells were another 16 h, then collected in PBS (10^8 cells/ml) containing 0.34 M for 24 h, cells were starved in serum-free RPMI 1640 medium for

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 EGF-stimulated 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-regulate 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 toward 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 a dose of 30 μM (Fig. 3A). AACOCF3 (10 μM), a specific inhibitor of cPLA2 (Street et al., 1993), methyl arachidonyl fluorophosphonate (MAFP; 10 μM), an inhibitor of cyclooxygenase-2, an inhibitor of cPLA2 and iPLA2 (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–D). In addition, indomethacin (10 μM), an inhibitor of cyclooxygenases, which are downstream enzymes of PLA2 and convert AA to prostaglandins (Touhey et al., 2002), could not inhibit
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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 Translocalization.** 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

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 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 sPLA₂ inhibitor thioetheramide-PC (Thio-PC) (A), 10 μM cPLA₂ inhibitor AACOCF₃ (B), 10 μM PLA₂ 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.
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 IB, IIA, V, and X and cPLA2 IVα was determined by RT-PCR using specific primers. As shown in Fig. 7A, we detected sPLA2 IIA and cPLA2 IVα in BEL-7402 cells. We were not able to detect sPLA2 IB, V, and X even using much more cDNA templates and more PCR cycles (data not shown). These data suggest that sPLA2 IIA may be the main sPLA2 present in BEL-7402 cells. Therefore, we next transiently transfected BEL-7402 cells with sPLA2 IIA or X plasmids. The transfection efficiency was around 15 to 25% as determined by GFP transfection assay. Although the transfection efficiency was not high, the sPLA2 activity as well as mRNA level of sPLA2 IIA 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 IIA or X transfected cells but not from vector transfected cells markedly stimulated the phosphorylation of ERK1/2 (Fig. 7D), demonstrating that sPLA2 IIA and X were produced in transfected cells and secreted into medium. Importantly, overex-

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–F). Serum-starved cells were pretreated with 10 μM SLD (E) or 100 nM wortmannin (F) for 15 min and then stimulated with 10 ng/ml EGF for 3 min (B and D–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 versus 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 and 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 and 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.
Besides these well known effects on phospholipid metabolites, SLD acts as a selective inhibitor of sPLA2 (Yu et al., 1990; Beck-Speier et al., 1995) and as another structurally modified phospholipid that functions as another specific inhibitor of sPLA2 (Barnette et al., 1994; 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 nuclear factor-κB, the production of eicosanoid, and the increase in intracellular Ca2+ in response to stimuliators in different cell types (Barnette et al., 1994; Marshall et al., 1994; Thommesen et al., 1998; Baek et al., 2000). 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 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 PL2 activity (Spaargaren et al., 1992; Croxton et al., 1997; Schalkwijk et al., 1995), it is possible that SLD inhibition of the EGFR/PI3K/Akt signaling pathway is 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 mimicked by cyclooxygenase inhibitor indomethacin. Cyclooxygenases are downstream enzymes of PL2 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.

**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 nuclear factor-κB, the production of eicosanoid, and the increase in intracellular Ca2+ in response to stimuliators in different cell types (Barnette et al., 1994; Marshall et al., 1994; Thommesen et al., 1998; Baek et al., 2000). 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 the role of PI3K/Akt signaling in cellular functions.

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tion of Akt phosphorylation. ERK1/2, the other two critical protein kinase 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 sPLA2. These findings indicate that both AA and sPLA2 transfection systems 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 IB, IIA, 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 sPLA2 in BEL-7402 cells by RT-PCR. It was shown that only a 434-bp fragment of sPLA2 IIA was amplified from the total RNA of BEL-7402 cells. Therefore, it is most likely that group IIA but not other sPLA2s exerted effects in BEL-7402 cells. We transfected BEL-7402 cells with group IIA sPLA2, and an around 3-fold increase in sPLA2 activity was observed as determined by sPLA2 activity assay. The assay uses the 1,2-dithio-d analog of diheptanoyl phosphatidylcholine as a substrate that is specific for most sPLA2s (e.g., bee and cobra venoms, pancreatic, etc.) but not for cPLA2. Five micromolar of SLD is a dose that partially, but not completely, inhibited EGF-stimulated Akt phosphorylation. If SLD inhibition of EGFR-mediated Akt activation is dependent on sPLA2, overexpression of group IIA sPLA2 should reverse the inhibition of Akt phosphorylation by 5 μM SLD to some degree. However, neither group IIA 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, manoolide has previously been shown to be a potent inhibitor of venom phospholipases A2. Now it reveals that manoolide 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, AA-COCF3 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.

References

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