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DEFECTIVE PHOSPHOLIPASE D ACTIVATION IN Ki-*ras*-TRANSFORMED NIH3T3 CELLS : EVIDENCE FOR DOWNSTREAM EFFECTOR OF PLC-γ1 IN PDGF-MEDIATED SIGNAL TRANSDUCTION

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SUMMARY : Platelet-derived growth factor (PDGF), a potent mitogen for fibroblasts and many other cell types, was used to examine phosphatidylcholine-speific phospholipase D (PLD), phosphoinositide-specific phospholipase C (PI-PLC) and tyrosine phosphorylation in NIH3T3 fibroblast and its Ki-*ras*-transformed derivative, DT. When cells prelabeled with [^{3}H] myristic acid were stimulated by 10 and 50 ng/ml of PDGF in presence of 0.3% butanol, formation of phosphatidylbutanol (PtdBut) was increased three to six fold in NIH3T3 fibroblasts whereas it was marginal in DT cells. Myo-[^{3}H]inositol-labeled cells showed higher inositol phosphate production in nontransformed control fibroblasts, indicating higher phospholipase C activity compared to the transformed DT cells. PDGF caused increase in tyrosine phosphorylation of a group of proteins with 110-130 kDa, PLC-γ1 and PDGF receptor in NIH3T3 cells. There was no significant increase in tyrosine phosphorylation in both PDGF receptor and PLC-γ1 in DT cells. These results suggest that PLD acts as a downstream effector molecule of PLC-γ1 in the PDGF-mediated signal transduction pathway.

Phospholipase D (PLD) has been recognized to play a key role in signal transduction (1,2) and several factors such as Ca^{2+}, protein kinase C (PKC) and GTP-binding proteins have been reported to be involved in the regulation of PLD (3,4). Tyrosine phosphorylation is also thought to play a role in the activation of PLD (5-10). The PDGF receptor possesses an intrinsic protein tyrosine kinase activity and stimulation by PDGF causes autophosphorylation of its receptor, increase in phosphoinositide hydrolysis, activation of PKC, and induction of a number of growth-related genes including c-*myc*, c-*fos* and c-*jun* (11-15). It has been shown that addition of PDGF leads to an increase in tyrosine phosphorylation of PLC-γ1, mediated by the PDGF receptor tyrosine kinase, which consequently stimulates PIP_2 hydrolysis by the activation of PLC-γ1 (13).

Several lines of evidence suggest a direct relationship between phosphoinositide-specific phospholipase C (PI-PLC) and PLD in signal transduction pathways (16,17,18). Recently, Yeo et

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Abbreviations : PDGF, platelet-derived growth factor; PI, phosphoinositide; PLC, phospholipase C; PLD, phospholipase D; PtdCho, phosphatidylcholine; PKC, protein kinase C; PI3K, phosphatidylinositol-3-kinase; PTK, protein tyrosine kinase; PtdBut, phosphatidylbutanol; GAP, GTPase-activating protein; ARF, ADP-ribosylation factor.

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al. (18) showed that activation of PLC-γ1 was necessary for the activation of PLD induced by PDGF in TRMP cells, a canine kidney epithelial cell line. Defective PLCγ binding site or kinase inactive mutants of the PDGF receptor in TRMP cells failed to activate PI-PLC and PLD induced by PDGF. Mutants defective in tyrosine phosphorylation sites of PDGF receptor that were unable to associate with PLCγ, PI3K or GAP also failed to mediate PI hydrolysis and PLD activation induced by PDGF (18). In NIH3T3 fibroblasts overexpressing PLC-γ1, PI turnover and PLD activity were enhanced by PDGF stimulation (17). However, pretreatment of cells with tyrosine kinase inhihibitors including staurosporine and genistein decreased PI-PLC as well as PLD activity in these cell lines. The PLD activity was also found to be abolished in PKC-downregulated cells (17,18), suggesting that PI-PLC serves as the upstream molecule in the PLD activation in PDGF-induced signal transduction cascade.

To study further the phosphatidylcholine (PtdCho) hydrolysis by PLD and its relationship with PI-PLC activity, we have undertaken experiments using the parental NIH3T3 fibroblasts and its *ras*-transformed DT cells (19). Lower activity of PLD has been shown earlier in transformed fibroblasts (20). Fu et al. (21) have shown a reduced PtdCho hydrolysis in *ras*-transformed fibroblasts stimulated by bradykinin. Activated *ras* gene causes the inhibition of PDGF-induced autophosphorylation of its receptor and diminishes PI-turnover and calcium mobilization (22-25). In our previous study it was shown that transformation of Ki-*ras* caused alterations in PI hydrolysis induced by PDGF or bradykinin in NIH3T3 cells (21,26). Here we have examined PLD activation and PLC-γ1 phosphorylation in DT cells in which the PDGF receptor is defective in autophosphorylation by PDGF, and proposed PLD as a downstream effector of PLC-γ1 in the PDGF-mediated signalling pathway.

MATERIALS AND METHODS

Materials : Recombinant PDGF-BB homodimer was purchased from Amersham. Monoclonal anti-phosphotyrosine antibody (PY20) was from ICN Biomedicals. Monoclonal PLC-γ1 antibody was a generous gift from Dr. S.G. Rhee (National Institute of Health, Bethesda, USA). Myo-[^{3}H]inositol (18.2 Ci/mmol) and [^{3}H]myristic acid (54 Ci/mmol) were from Amersham. Authentic phosphatidylbutanol (PtdBut) standard was synthesized from egg PtdCho using crude cabbage PLD according to the method of Yang et al. (27). Immobilon polyvinylidene difluoride (PVDF) transfer membrane filters were from Millipore. All other reagents from commercial sourses were of analytical grade.

Cell lines : Parent NIH3T3 fibroblasts and Kirsten murine sarcoma virus-transformed NIH3T3 (DT) cells (19) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Irvine) containing 100 units of penicillin and 100 μg of streptomycin (Gibco) per ml in a humidified atmosphere of 5% CO_2/95% air at 37^{0}C. Quiescent cells were prepared at subconfluent condition with DMEM containing 0.5% FBS as described (21). During maintenance of cultures, cells were changed every 6-8 passage interval.

Measurement of inositol polyphosphates : NIH3T3 and DT cells were seeded at a density of 2X10^5 cells/well in six-well culture plates and at near confluence, they were labeled with myo-[^{3}H]inositol (1.0 μCi/ml) for 24 h with inositol-free α-MEM containing 0.5% FBS. After washing three times with Hepes Buffered Salt Solution (HBSS) [145 mM NaCl, 25 mM Hepes, pH 7.35, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 0.1% bovine serum albumin (BSA)] containing 25 mM LiCl as described (26), the cells were further incubated for 20 min with the same buffer at 37^{0}C (pre-incubation). Cell stimulation was performed with the addition of PDGF-BB for the indicated periods of time. The reaction was terminated by the addition of 10% (v/v) perchloric acid (finally 3%). The mixture was neutralized by KOH. Inositol phosphates were then separated

with the stepwise gradients of ammonium formate, using Dowex AG 1X8 anion exchange resin (200-400 mesh), formate form (Bio-Rad), as described elsewhere (28) and radioactivity was counted by a scintillation counter (Beckman 6500).

Measurement of PtdBut formation : NIH3T3 and DT cells were seeded at a density of $2X10^5$ cells/35 mm dishes and the subconfluent cells were starved and labeled for 24 h with [^{3}H]myristic acid (1 μCi/ml) in 1 ml of DMEM containing 0.5% FBS. After washing three times, the labeled cells were pre-incubated for 20 min in HBSS buffer before stimulation. Cells were treated with PDGF-BB in the presence of 0.3% butanol for 10 min. The reactions were terminated by aspirating the reaction buffer and subsequent addition of 0.75 ml of ice-cold methanol and 0.75 ml of HBSS. The dish was further washed with 0.75 ml of methanol and the lipids were extracted according to the method of Bligh & Dyer (29). Lipid samples were dried under N_2 gas, re-dissolved in 25 μl of chloroform : methanol (6:1) and applied to a TLC plate (Silica Gel 60 A). The plate was then developed using the upper phase of a solvent system cosisting of ethyl acetate/iso-octane/acetic acid/H_2O (130:20:30:100, v/v) as described (18). The plate was stained with iodine vapor and [^{3}H]PtdBut produced by PLD in the cells was identified by its co-migration with a PtdBut standard prepared from cabbage (27). The scraped off spots from TLC plates were counted by a scintillation counter (Beckman 6500)

Preparation of whole cell lysate and Western blotting : Cells were seeded at a density of $5X10^5$ in 60 mm dishes and at near confluence they were starved for 16 h in DMEM containing 0.5% FBS as described (30). After stimulation the buffer was aspirated and the cells were washed two times with ice-cold PBS containing 10 mM NaF, 1 mM orthovanadate, 20 mM β-glycerol phosphate and 1 mM phenylmethyl sulfonyl fluoride (PMSF). Cells were lysed with ice-cold lysis buffer (25 mM Hepes, pH 7.35, 1% Triton X-100, 10% glycerol, 10 mM NaF, 1 mM orthovanadate, 1 mM PMSF & 10 μg/ml leupeptin). The cell suspension was then vortexed and incubated on ice for 30 min. Particulate material was removed by centrifugation at 13,000 rpm for 30 min at 4^{0}C and protein concentration of the soluble fraction was estimated by Bio-Rad protein assay kit, using bovine serum albumin (BSA) as standard. The whole cell lysates (100 μg) were separated by 6%-sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli (31). The resolved proteins were transferred to Immobilon-P membrane (millipore) at 45 mA for 1 h. Overnight blocking was performed in Tris-buffered saline consisting of 5% chicken egg albumin (sigma) and 0.1% Tween 20 (TBS-T). Monoclonal anti-phosphotyrosine antibody (PY20) was used to probe the blot (1:1000). Following incubation with anti-mouse IgG horseradish peroxidase (1:5000), the blots were developed using Amersham enhanced chemiluminescence system (ECL).

RESULTS

PLD activity was measured by the formation of [^{3}H]PtdBut which is a specific and metabolically stable product of transphosphatidylation reaction by PLD (32). We and other researchers have previously shown that transformed fibroblasts had a lower PLD activity as compared to the wild type counterparts (20,21). Fu et al. (21) showed that bradykinin, a bioactive peptide-induced PtdCho hydrolysis is much higher in parental NIH3T3 cells than DT cells. Here our experiments were designed to compare the PLD activity in intact cells of nontransformed and Ki-*ras*-transformed fibroblasts in response to PDGF. In [^{3}H] myristic acid-labeled cells, incubation for 10 min with PDGF-BB at a concentration of 10 ng/ml causes about 3.7 fold increase in PLD activity in NIH3T3 cell in presence of 0.3% butanol (Fig.1). A higher concentration of PDGF (50 ng/ml) causes 6 fold increase in PLD activity in NIH3T3 cells. In contrast, in *ras*-transformed cells, there was no significant activation of PLD induced by PDGF. Neither higher nor lower concentrations of PDGF could produce PtdBut in DT cells.

We have then examined PI hydrolysis induced by PDGF in NIH3T3 and DT cells. The maximal production of inositol phosphates was seen at 10 min after stimulation (data not shown). When myo-[^{3}H]inositol-labeled cells were stimulated by PDGF-BB (10 ng/ml) in presence of 25

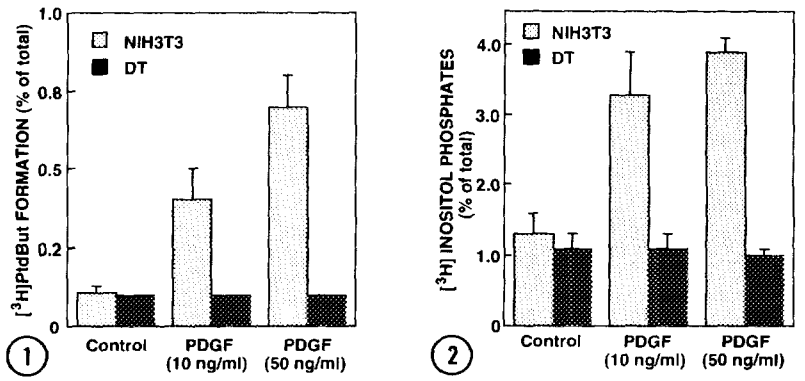

Figure 1. PDGF-induced PLD activity in NIH3T3 and DT cells : NIH3T3 [□] and DT [■] cells were labeled with [^{3}H]myristic acid (1μCi/ml) for 24 h and stimulated for 10 min with the indicated concentrations of PDGF-BB in the presence of 0.3% butanol. After lipid extraction, [^{3}H]PtdBut was analyzed by TLC as described under Materials and Methods. Results are represented as means ±SD of three separate experiments performed in duplicate.

Figure 2. PDGF-induced inositol phosphate production in NIH3T3 and DT cells : NIH3T3 [□] and DT [■] cells were labeled with myo-[^{3}H] inositol (1μCi/ml) for 24 h and stimulated with the indicated concentrations of PDGF-BB for 10 min in presence of 25 mM LiCl. Aqueous extracts were analyzed on an anion exchange column as described under Materials and Methods. The results are represented as means ±SD of three separate experiments performed in duplicate.

When myo-[^{3}H]inositol-labeled cells were stimulated by PDGF-BB (10 ng/ml) in presence of 25 mM LiCl, NIH3T3 cells showed a significant increase (about 2.5 fold) in production of inositol polyphosphates [inositol bisphosphates (IP_2) + inositol trisphosphates (IP_3)] (Fig.2). However, DT cells, under identical conditions, could not elicit any significant production of inositol phosphates. Increasing the concentrations of PDGF causes an apparent increase in PI hydrolysis in NIH3T3 fibroblasts (about 3 fold increase) but not in DT cells. The results obtained here are in accordance with the previous findings indicating the diminished PI turnover in Ki-and EJ-*ras* transformed fibroblasts (23,24,26). This reduced PI turnover was thought to be due to desensitization of the PDGF receptor in *ras*-transformed cells (23), although the exact mechanism is not clear.

To investigate the relationship between the PI hydrolysis and PLD activity in fibroblasts, we have examined tyrosine phosphorylation using monoclonal anti-phosphotyrosine antibody (PY 20) in NIH3T3 and DT cells (Fig.3A). When exposed to PDGF (10 ng/ml), NIH3T3 cells showed a marked increase in tyrosine phosphorylation of a group of proteins with 110-130 kDa, PLC-γ1 and 180-210 kDa. Autophosphorylation of the PDGF receptor (180 kDa) occurs, resulting in activation of PI-PLC (12). PDGF receptor also causes tyrosine phosphorylation of PLC-γ1 by the intrinsic tyrosine kinase activity (13). Tyrosine phosphorylation of PLC-γ1 in NIH3T3 cells exposed to PDGF-BB is correlated with production of inositol phosphates. Higher concentration of PDGF (50 ng/ml) had no significant effects on both tyrosine phosphorylation (Fig. 3A) and PI turnover (Fig. 2) in NIH3T3 fibroblasts. In contrast, DT cells stimulated by PDGF-BB did not show any tyrosine phosphorylation of either PLC-γ1 or PDGF receptor. Several earlier reports showed that *ras*

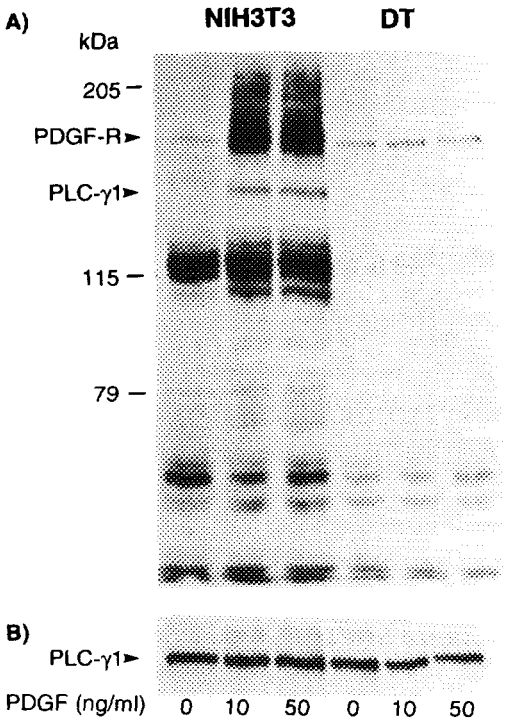

Figure 3. PDGF-induced tyrosine phosphorylation in NIH3T3 and D cells: NIH3T3 and DT cells were stimulated by PDGF-BB (10 & 50 ng/ml) for 10 min. Cell lysates resolved by 6% SDS-PAGE were immunoblotted with monoclonal anti-phosphotyrosine antibody (PY20) (A) or with anti-PLC-γ1 antibody (B) as described under Materials and Methods. Molecular weight standards used are myosin (208 kDa), β-galactosidase (115 kDa), bovine serum albumin (79 kDa).

transformation of fibroblasts causes inhibition of receptor autophosphorylation and PI hydrolysis (22,23). The content of PLC-γ1 was also analyzed in these two cell lines using monoclonal anti-PLC-γ1 antibody (Fig. 3B). Although there was marked difference in tyrosine phosphorylation of PLC-γ1, the enzyme content inferred by Western blotting was identical between these two cell lines. This was further confirmed by immunoprecipitation experiments (data not shown).

DISCUSSION

Several reports suggest the participation of agonist-induced PLD activity in signal transduction in mammalian cells (1,2,16). Although PLD has been reported to be regulated by Ca^{2+}, PKC, tyrosine kinase and GTP-binding proteins (3,4), its exact mechanisms are still not clear. Recently a small GTP-binding protein, ARF is believed to be a key factor for PLD activation (33). In the present study we have undertaken some experiments to clarify further the regulation of PLD activity. PDGF, a potent mitogen, which is a natural agonist for NIH3T3 cells was used to examine the activation of PI-PLC, PLD and tyrosine phosphorylation. After stimulation of NIH3T3 cells by PDGF, a number of events occur including autophosphorylation of its receptor, increase in PI hydrolysis, activation of PKC and expression of c-*fos*, c-*myc* and c-*jun* (11-15). In

contrast, DT cells, *ras*-transformed derivative of NIH3T3 failed to respond to PDGF to phosphorylate its own receptor and several other growth-related signals.

Previously, we have shown alterations in PtdCho and PI hydrolysis in Ki-*ras*-transformed fibroblasts (DT cells), induced by bradykinin and PDGF, resepectively (21,26). The present study demonstrated that DT cells could not produce PtdBut when stimulated by PDGF (Fig.1). PI hydrolysis was also prevented in DT cells (Fig. 2). At the optimal time of PI hydrolysis induced by PDGF (data not shown), NIH3T3 cells produce much higher amount of inositol polyphosphates compared to DT cells. Higher concentration (50 ng/ml) of PDGF also did not induce further enhancement in PI turnover (Fig.2). Recently, Lee et al. (17) using stably PLC-γ1 overexpressed NIH3T3 fibroblasts, showed that PLD activity induced by PDGF is dependent on PLC-γ1. They have shown that PLC-γ1 overexpression dramatically increases the PLD activity and that inhibition of its tyrosine phosphorylation by tyrosine kinase inhibitors (e.g. staurosporine and genistein) decreases the PLD activity in response to PDGF. Moreover, Yeo et al. (18) demonstrated in epithelial cell lines overexpressing several mutants of PDGF receptor, evidence for the direct stimulation of PLD activity by PLC-γ1. In the present experiments with DT cells which are defective in tyrosine phosphorylation of PLC-γ1 and PDGF receptor, we provide additional evidence for PLD as a downstream effector molecule of PLC-γ1 in the PDGF-mediated signal transduction in fibroblasts.

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