Implication of Phospholipase D2 in Oxidant-induced Phosphoinositide 3-Kinase Signaling via Pyk2 Activation in PC12 Cells*

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The role of phospholipase D (PLD) activation in hydrogen peroxide (H₂O₂)-induced signal transduction and cellular responses is not completely understood. Here we present evidence that Ca²⁺-dependent tyrosine kinase, Pyk2, requires PLD activation to mediate survival pathways in rat pheochromocytoma PC12 cells under oxidative stress. The H₂O₂-induced phosphorylation of two Pyk2 sites (Tyr²⁸⁶⁰, and Tyr⁸⁸¹) was suppressed by 1-butanol, an inhibitor of transphosphatidylation by PLD, and also by transfection of catalytically negative mouse PLD2K758R (PLD2KR). Furthermore, we found that PLD2 was associated with Pyk2 and Src, and that activation of PLD2 was required for H₂O₂-enhanced association of Src with Pyk2 leading to full activation of Pyk2. H₂O₂-induced phosphorylation of Akt and p70S6K was dependent on phosphatidylinositol 3-kinase (PI3K) activity and was abolished by 1-butanol but not t-butanol. Furthermore, the PI3K/Akt activation in response to H₂O₂ was reduced by transfection of either PLD2KR or the dominant negative Pyk2DN. This study is the first demonstration that PLD2 activation is implicated in Src-dependent phosphorylation of Pyk2 (Tyr²⁸⁶⁰ and Tyr⁸⁸¹) by promoting the complex formation between Pyk2 and activated Src in PC12 cells exposed to H₂O₂, thereby resulting in activation of the survival signaling pathway PI3K/Akt/p70S6K.

It is known that the cellular redox state is an important mediator of various signaling systems (1–3). The major signaling pathways and/or key mediators to influence survival of cells subjected to oxidant injury are the phosphorylation cascades leading to activation of tyrosine phosphorylation of several growth factor receptors and their subsequent downstream signaling events, including Akt and mitogen-activated protein kinases (4–9). A number of studies have demonstrated that hydrogen peroxide (H₂O₂) stimulates phospholipase D (PLD), which generates phosphatidic acid (PA), a second messenger in various types of cell, such as endothelial cells (10, 11), fibroblasts (12, 13), PC12 cells (14–17), vascular smooth muscle cells (18), and leukemia Li210 cells (19). As the mechanisms underlying the oxidant-induced PLD activation, various factors including protein-tyrosine kinase (10, 11, 13, 14), protein kinase C (PKC) (13, 16, 19), and mitogen-activated protein kinase (15, 17) have been proposed. Direct or indirect tyrosine phosphorylation has been thought to be involved in PLD activation in response to H₂O₂ exposure. In HL60 cells, PLD1 is directly phosphorylated by peroxovanadate (20). Min et al. (13) also demonstrate that in the presence of vanadate, H₂O₂ stimulates PLD1 activity and its tyrosine phosphorylation in Swiss-3T3 cells. Our previous studies have suggested that Pyk2 and/or p38 mitogen-activated protein kinase and ERK1/2 were involved in H₂O₂-induced PLD activation by using their inhibitors in PC12 cells (14–15, 17). However, despite many investigations, the biological roles and the exact mechanisms of the oxidant-induced PLD activation remains to be elucidated.

Several reports show that the survival signaling via the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is activated by stresses such as heat, hypoxiatoric stress, and H₂O₂ (21–23). However, little is known regarding the mechanisms for activation of PI3K and Akt caused by stresses. There are reports suggesting that the PLD stimulation participates in the pro-survival signaling at the early stage of the apoptotic process (16, 24, 25). We have previously shown that the PLD activation induced by sphingosine 1-phosphate (S1P), but not insulin-like growth factor-I, was implicated in the S1P3-mediated PI3K and Akt activation in Chinese hamster ovary cells (26, 27). Furthermore, it has been demonstrated that actinomycin D-induced apoptosis was prevented by PLD1 or PLD2 overexpression through activation of the PI3K/Akt signaling pathway, suggesting the notion that PLD plays a role in stimulation of cell survival pathway (28). However, the exact mechanism underlying the activation of the PI3K/Akt pathway via PLD has not been defined. In the present study, we have attempted to gain further insight into the role of PLD activation in response to oxidative stress in PC12 cells and have shown that PLD activation was involved in the PI3K/Akt activation through protein-tyrosine kinases Pyk2 and Src. Thus, these results provided evidence indicating a novel role for PLD2 in the Pyk2/PI3K/Akt pathway that acts as a survival signaling in response to H₂O₂.

EXPERIMENTAL PROCEDURES

Materials—[9,10⁻³H]Palmitic acid (54.0 Ci/mmol) and [γ⁻³²P]ATP (3000 Ci/mmol) were from PerkinElmer Life Sciences. Rabbit polyclonal
antibodies against Ser473-phosphorylated Akt, Tyr204-phosphorylated ERK1/2, Thr32/Ser34-phosphorylated p70S6K, Tyr402-phosphorylated S6K1 and S6K2, Tyr881-phosphorylated Src, and Akt. Rabbit polyclonal anti-PLD1 and -PLD2 antibodies were prepared as described previously (28). Anti-rabbit IgG and anti-mouse IgG antibodies conjugated with horse-radish peroxidase and the chemiluminescence kit (ECL system) were obtained from Amersham Biosciences. Expression plasmids containing wild-type and catalytically negative mouse PLD2 (K758R) in pCGN were kindly supplied by Dr. Michael A. Frohman (Center for Developmental Genetics and Department of Pharmacology, State University of New York at Stony Brook, Stony Brook, NY). All other reagents were obtained from standard commercial sources.

Cell Culture and Transfections—The PC12 cell line was a generous gift from Dr. Y. Sugimoto (Shirakawa Institute of Animal Genetics, Fukushima, Japan). PC12 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 5% horse serum, 100 units of penicillin G/ml, and 100 μg of streptomycin/ml as described previously (14). The cells were grown to the subconfluent stage at 37 °C in a humidified atmosphere containing 5% CO2.

For transient transfection PC12 cells were plated at 8 × 105 in 100-mm dishes and transfected with plasmid DNA (pCGN-catalytically inactive mouse PLD2KR in pcDNA) or PRK5-dominant negative Pyk2 from BioSource International, Inc. (Camarillo, CA). Mouse monoclonal anti-Pyk2 and Src were from BD Transduction Laboratories (Lexington, KY). Rabbit polyclonal antibodies against Tyr881- and Tyr580-phosphorylated Pyk2 were from BD Transduction Laboratories, Inc. (Santa Cruz, CA). Mouse monoclonal anti-Pyk2 and Src were from Cell Signaling Inc. (Beverly, MA). Rabbit polyclonal antibodies against p70S6K, β-actin, mouse monoclonal antibody against Src, and protein A/G Plus-agarose were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-rabbit IgG and anti-mouse IgG antibodies conjugated with horse-radish peroxidase and the chemiluminescence kit (ECL system) were obtained from Amersham Biosciences. Expression plasmids containing wild-type and catalytically negative mouse PLD2 (K758R) in pCGN were kindly supplied by Dr. Michael A. Frohman (Center for Developmental Genetics and Department of Pharmacology, State University of New York at Stony Brook, Stony Brook, NY). All other reagents were obtained from standard commercial sources.

Measurement of PLD Activity—PLD activity was assayed by measuring the formation of [3H]phosphatidylbutanol by transphosphatidylation. PC12 cells were cultured in 35-mm-diameter tissue culture dishes at 5 × 105 cells/dish and grown for 3 days. The subconfluent cells were labeled for 3 h with [3H]palmitic acid (3 μCi/dish) and then stimulated with 0.5 mM H2O2 for 10 min. The cell lysates were subjected to Western blot analysis with antibodies to phosphorylated Akt, Tyr204-phosphorylated S6K1 and S6K2, Tyr881-phosphorylated Src, and Akt. The data represent the mean ± S.E. of three different experiments.
Phosphoinositide 3-Kinase Assay—PI3K activity was assayed as described previously (27). The immunoprecipitation was performed using anti-phosphotyrosine (PY-20) and p110α PI3K antibodies, and PI3K activity in the immunoprecipitates was assayed using PI as substrate.

Western Blot Analysis—After growing to subconfluence in growth medium in 100-mm dishes, PC12 cells were incubated for 6 h in serum-free Dulbecco’s modified Eagle’s medium and with 100 μM Na3VO4 or other agents for 30 min before treatment with 0.5 mM H2O2 or EGF, harvested in ice-cold lysis buffer (1% Nonidet P-40, 0.5% sodium cholate, 1% SDS, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 20 mM HEPES, 3 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 20 μg/ml β-glycerophosphate, 1 mM sodium fluoride, and 1 mM sodium orthovanadate, pH 7.4), and sonicated. Protein concentrations were determined using the Bradford protein assay reagent (Bio-Rad) with bovine serum albumin as standard. The total cell lysate (50–100 μg protein) was subjected to electrophoresis on 10% SDS-polyacrylamide gels, then transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked using 5% bovine serum albumin. Phosphorylation of Akt, ERKs, p70S6K, Pyk2, and Src was measured by immunoblotting with rabbit polyclonal antibodies specific for Ser473-phosphorylated Akt, Tyr204-phosphorylated ERK1/2, Thr389-phosphorylated p70S6K, Tyr402-phosphorylated Pyk2, and Tyr416-phosphorylated Src, respectively. Total amounts of Akt, ERK1/2, p70S6K, Pyk2, and Src were determined by immunoblotting with rabbit polyclonal antibodies against Akt, ERKs, p70S6K, Pyk2, or Src, respectively. After repeated washes, bound antibodies were detected using the ECL Western-blotting detection system. The density of protein bands was determined with a densitometer (Atto densitograph, Version 2.5; Atto Ltd., Tokyo, Japan).

Immunoprecipitation Analysis—For immunoprecipitation, 300 μg of protein of cell lysate was diluted with 300 μl of the buffer containing 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 20 mM HEPES, 3 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 20 μg/ml β-glycerophosphate, 1 mM sodium orthovanadate, and 1 mM sodium fluoride, pH 7.4, and 2 μg of the appropriate antibody (anti-PLD2, anti-Pyk2, or anti-Src) at 4 °C for 3 h followed by incubation with protein A/G-agarose 4B overnight. Agarose beads were collected by centrifugation, washed 3 times with the same buffer, resuspended in SDS sample buffer, and then boiled for 10 min. Proteins were separated as described for Western blot analysis.

RESULTS AND DISCUSSION

Phosphorylation of Src and Pyk2 in H₂O₂—EGF-stimulated PC12 Cells—Our previous studies have demonstrated that H₂O₂-induced PLD activation in PC12 cells was inhibited by protein-tyrosine kinase inhibitors, such as PP2, genistein, and herbimycin, but enhanced in the presence of vanadate, a protein-tyrosine phosphatase inhibitor (14, 15, 17). These observations lead us to assume that tyrosine phosphorylation of PLD2 expressed in this cell may regulate its activity. However, PLD2 tyrosine phosphorylation by H₂O₂ was marginal, and direct phosphorylation of the enzyme was unlikely to stimulate the activity. Other studies also report that tyrosine phosphorylation of PLD enzyme is not involved in the regulation of its activity (29, 30). The depletion of exogenous Ca²⁺ by EGTA resulted in a complete abolishment of H₂O₂-mediated PLD activation in PC12 cells (14, 15). It was then considered that Src-type protein-tyrosine kinase and a Ca²⁺-dependent proline-rich cytoplasmic protein-tyrosine kinase (Pyk2), which is highly active in PC12 cells, should be involved in H₂O₂-mediated up-regulation of PLD activity in PC12 cells.

It has been reported that autophosphorylation of Pyk2 (Tyr⁴⁰⁵) leads to Src activation, which causes further phosphorylation of two other tyrosine residues (Tyr⁵⁸⁰, Tyr⁸⁸¹) in the C terminus of Pyk2, resulting in the full activation of Pyk2 (31–33). We examined phosphorylation of Src and Pyk2 (Tyr⁴⁰⁵, Tyr⁵⁸⁰, and Tyr⁸⁸¹) after H₂O₂ treatment of PC12 cells. For site-specific phosphorylation of Pyk2, the antibodies were used that specifically recognize phospho-Pyk2 (Tyr⁴⁰⁵, autophosphorylation and Src binding site; Tyr⁵⁸⁰, regulatory kinase activation loop, Tyr⁸⁸¹, Grb2-SH2 binding site) in PC12 cells. As shown in Fig. 1, in the presence of vanadate, H₂O₂ induced a rapid, remarkable tyrosine phosphorylation of Src and three Pyk2 sites (Tyr⁴⁰⁵, Tyr⁵⁸⁰, and Tyr⁸⁸¹). Similar levels of Pyk2 phosphorylation were observed in three tyrosine phosphorylation sites. On the other hand, upon stimulation with EGF, tyrosine phosphorylation of Src and these three Pyk2 sites was much weaker. The PLD activation in response to H₂O₂ was much higher than EGF stimulation in PC12 cells (data not shown), suggesting involvement of PLD activation in phosphorylation of Pyk2 and Src.

Involvement of PLD Activation in Tyrosine Phosphorylation of Src and Pyk2 in PC12 Cells Exposed to H₂O₂—We examined involvement of Src and PLD in site-specific tyrosine phosphorylation of Pyk2. As depicted in Fig. 2A, the blockade of Src kinase by PP2 prevented phosphorylation of Pyk2 (Tyr⁴⁰⁵ and Tyr⁵⁸⁰) but not autophosphorylation at Tyr⁴⁰⁵, which is the binding site for the SH2 domain of Src (31). In fact, the dominant negative Pyk2 (Y402F,Y881F; Pyk2DN) prevented H₂O₂-induced Src phosphorylation in PC12 cells (data not shown). Therefore, activation of Src via Pyk2-dependent mechanism was likely to occur in PC12 cells exposed to H₂O₂. To examine the involvement of PLD in the Pyk2 and Src tyrosine phosphorylation, we used 1-butanol, which inhibits PA generation by PLD activation and t-butanol, its inactive analogue. As shown in Fig. 2, A and C, 1-butanol, but not t-butanol, caused reduction of phosphorylation.
of Pyk2 (Tyr580 and Tyr881) but not Pyk2 (Tyr402) and Src tyrosine phosphorylation. In contrast, there were no inhibitory effects of 1-butanol on EGF-induced phosphorylation of Pyk2 and Src (Fig. 2B). These results suggest that PLD activation is involved in phosphorylation at Tyr580 and Tyr881 but not Tyr402 of Pyk2 and Src phosphorylation.

To ascertain the involvement of PLD activation in Pyk2 (Tyr580 and Tyr881) phosphorylation induced by H2O2, we have transfected catalytically inactive PLD2-cDNA (K758R; PLD2KR) into PC12 cells and examined phosphorylation of Pyk2 (Tyr580 and Tyr881). Overexpression of PLD2KR significantly reduced the H2O2-induced phosphatidylinositol formation as compared with that of the vector control (Fig. 3A). In PLD2KR-overexpressed PC12 cells, H2O2-induced Pyk2 (Tyr580 and Tyr881) phosphorylation was much reduced (Fig. 3, B and C), whereas phosphorylation of Pyk2 (Tyr402) and Src was not affected. These results provided confirmative support for data from the experiments with 1-butanol (Fig. 2, A and C), indicating that PLD2 was implicated in phosphorylation of Pyk2 (Tyr580 and Tyr881) via Src activation in PC12 cells exposed to H2O2.

Association of PLD2 with Pyk2 and Src in Control and PLD2KR-overexpressed PC12 Cells—It has been known that Pyk2 has both kinase and scaffolding functions and that in many cell systems, Pyk2 is associated with Src and other SH2-containing proteins including PI3K (32, 33). In PC12 cells the requirement for both Pyk2 and Src in H2O2-induced PLD activation suggests that these kinases would be associated with PLD2. Using a co-immunoprecipitation approach, we found that PLD2 was implicated in phosphorylation of Pyk2 (Tyr580 and Tyr881) via Src activation in PC12 cells exposed to H2O2.
with antibodies to PLD2, Pyk2, phosphorylated Akt, or total Akt.

**Densitometric analysis of 32P-labeled inositol trisphosphate in**

[59x478]after transfection the cells pretreated with vanadate were stimulated

**negative PLD2R-cDNA or dominant negative Pyk2DN-cDNA, and at 48 h**

Lower panel

precipitates were subjected to Western blot analysis with the antibody ([P-Tyr] as described under “Experimental Procedures.” IP3P, inositol trisphosphate. B and C, the cells were transfected with catalytically negative PLD2R-cDNA or dominant negative Pyk2DN-cDNA, and at 48 h after transfection the cells pretreated with vanadate were stimulated with 0.5 mM H2O2 for 10 min. PLK activity was assayed in immunoprecipitates ([P-Tyr] with antibody against phosphotyrosine ([P-Tyr]) as described under “Experimental Procedures.”)

**The Western blot (upper panel) is representative of three different experiments.**

**Involvement of PLD2 Activation in Phosphorylation of Akt and ERK1/2 in H2O2-treated PC12 Cells—**

Previous studies have suggested that the H2O2-induced PLD2 activation in PC12 cells is involved in the anti-apoptotic process (16, 24, 25). However, the precise mechanism of the role of PLD2 activation remains to be elucidated. It has been reported that oxidative stress by reactive oxygen species (ROS) induces the activation of mitogenic or survival signaling, including serine/threonine protein kinase Akt and ERK1/2 (6, 8, 9). In PC12 cells, both Akt and Erk1/2 were phosphorylated by H2O2 in a time-dependent manner, although their temporal profiles were distinct from those observed in EGF stimulation (Fig. 5A). Phosphorylation of Akt and ERK1/2 by H2O2 showed slow increases peaking at 10 and 20 min, respectively, whereas EGF induced rapid increases in their phosphorylation.

In PC12 cells, bradykinin, and carbacbol have been shown to transactivate EGF receptor leading to ERK1/2 activation (35, 36). Furthermore, Pyk2 is implicated in calcium ionophore- and phorbol ester-stimulated ERK1/2 phosphorylation via a direct interaction with Src (32, 37) or via EGF receptor transactivation (38). To know which pathway mediates H2O2-induced Akt and ERK activation, we examined the effect of a specific inhibitor of the EGF receptor-tyrosine kinase, AG1478, on phosphorylation of these kinases in H2O2-treated PC12 cells. As shown in Fig. 5B, treatment of PC12 cells with AG1478 reduced phosphorylation of ERK1/2 in response to H2O2 by ~80%, whereas it completely blocked EGF-induced ERK1/2 phosphorylation. On the other hand AG1478 had no significant effect on the H2O2-induced Akt phosphorylation under the condition where EGF-induced Akt phosphorylation was completely abolished. These results suggest that H2O2-induced ERK1/2 activation was largely dependent on EGF receptor transactivation, but Akt activation was not.

To examine the upstream events of H2O2-induced Akt activation, PC12 cells were pretreated with various inhibitors, including genistein, PI3K inhibitor (LY294002), and calcium chelator (EGTA) and then exposed to H2O2. H2O2-induced Akt phosphorylation was reduced by LY294002 and genistein in a dose-dependent manner and was diminished by EGTA (Fig. 6, A and B), suggesting that Akt phosphorylation in response to H2O2 was mediated by PI3K and calcium-dependent tyrosine phosphorylation.
PI3K that overexpression of PLD2KR attenuated activation of Pyk2 through Pyk2/Src in PC12 cells exposed to H2O2. By overexpression of either PLDKR or Pyk2DN (Fig. 7), the survival signaling is mediated by the Pyk2(Tyr402)/Src/PLD2(PA)/p70S6K pathway. H2O2-induced ERK activation is mainly mediated by transactivation of EGFR receptor (EGFR).

As described above, H2O2-induced Akt activation was likely to be dependent on PI3K by using LY294002 (Fig. 6, A and B). To ascertain this notion, we examined PI3K activation by H2O2 exposure. H2O2 caused marked stimulation of PI3K activity, but this activation was suppressed by 1-butanol (Fig. 7A), suggesting involvement of PLD in PI3K activation. However, PI3K activation by EGF stimulation was not prevented by 1-butanol (data not shown). It was also observed that p110 β-type, but not p110α-type, was attributed to the H2O2-induced PI3K activation (Fig. 7B). Moreover, to assess whether PLD activity is involved in H2O2-induced PI3K activation, PC12 cells were transfected with PLD2KR-cDNA. Fig. 7B showed that overexpression of PLD2KR attenuated activation of PI3Kβ. The activation of PI3Kβ by H2O2 was also repressed in Pyk2DN-overexpressed cells (Fig. 7, B and D). Accordingly, as expected, Akt phosphorylation induced by H2O2 was reduced by overexpression of either PLDKR or Pyk2DN (Fig. 7, C and D). Taken together, it was indicated that PLD2 activity was implicated in the PI3K/Akt signaling pathway mediated through Pyk2/Src in PC12 cells exposed to H2O2.

It has recently been reported that PLD2 activation is involved in the proliferation signaling pathway through mTOR/p70S6 kinase (p70S6K), where mTOR is activated by direct interaction with PA generated by PLD activity (39). In PC12 cells p70S6K was markedly phosphorylated with stimulation by H2O2 (Fig. 8). However, the H2O2-induced phosphorylation of p70S6K was completely abolished by either LY294002 or 1-butanol. In contrast, p70S6K phosphorylation induced by EGF was not affected by these inhibitors. These results suggested that PI3K-dependent p70S6K activation induced by H2O2 was regulated by the upstream PLD2 activation, which is required for full activation of Pyk2.

In conclusion, we have shown for the first time that PLD2 is associated with Pyk2 and Src in PC12 cells where the Pyk2 acts as a scaffold protein. As indicated in Fig. 9, we could demonstrate a possible implication of PA generated by H2O2-induced PLD2 activation in Src-dependent phosphorylation of Pyk2 (Tyr580 and Tyr881). Furthermore, we showed that PLD2 activation by H2O2 was most likely to participate in stimulation of the PI3K/Akt/p70S6K pathway by Pyk2/Src activation involved in regulation of cell survival of PC12 cells. On the other hand, the major pathway of H2O2-induced ERK activation, which is mediated by transactivation of EGFR receptor, was independent of PLD2 activation in PC12 cells.

REFERENCES