Elevated L-PGDS activity contributes to PMA-induced apoptosis concomitant with downregulation of PI3-K

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Submitted 29 May 2002; accepted in final form 28 August 2002


First published September 11, 2002; 10.1152/ajpcell.00247.2002.—Recently we demonstrated the induction of apoptosis by the addition of recombinant lipocalin-type prostaglandin D2 synthase (L-PGDS) to the culture medium of LLC-PK1 cells. Because protein kinase C (PKC) has been shown to be involved in the apoptotic process of various cell types, we examined the potential role of L-PGDS in phorbol 12-myristate 13-acetate (PMA)-induced apoptosis. We report here the enzymatic activation and phosphorylation of L-PGDS in response to phorbol ester in cell culture and the direct phosphorylation of recombinant L-PGDS by PKC in vitro. Treatment of cells with PMA or L-PGDS decreased phosphatidylinositol 3-kinase (PI3-K) activity and concomitantly inhibited protein kinase B (PKB/Akt) phosphorylation, which led to the hypophosphorylation and activation of Bad. In addition, hypophosphorylation of retinoblastoma protein was also observed in response to L-PGDS-induced apoptosis. Cellular depletion of L-PGDS levels by using an antisense RNA strategy prevented PI3-K inactivation by phorbol ester and inhibited caspase-3 activation and apoptosis. We conclude that phorbol ester-induced apoptosis is mediated by L-PGDS phosphorylation and activation by PKC and is accompanied by inhibition of the PI3-K/PKB anti-apoptotic signaling pathways.

lipocalin-type prostaglandin D2 synthase; phorbol 12-myristate 13-acetate; phosphatidylinositol 3-kinase; apoptosis; LLC-PK1; protein kinase C; 3-β-trace protein

LIPOCALIN-TYPE PROSTAGLANDIN D2 SYNTHASE (L-PGDS), an isomerase that converts prostaglandin (PG)H2 into PGD2, is a unique enzyme in that it also functions as a transporter of small, lipophilic molecules such as bile salts and retinol (26, 27). Recently we added the novel role of apoptotic inducer to the list of L-PGDS protein functions (13, 18). L-PGDS, which is overexpressed in Escherichia coli, was added exogenously to LLC-PK1 cells, where it caused a fivefold increase in apoptosis. The apoptosis observed appeared to involve activation of the caspase-3 pathway and presumably the L-PGDS enzymatic end product, 15-deoxy-D12,14PGJ2 (15-dPGJ2).

15-dPGJ2 is a natural peroxisome proliferator-activated receptor-γ (PPAR-γ) ligand that is known to induce apoptosis. Because the process was dependent on the presence of calcium in the medium, we decided to investigate the role of calcium-dependent protein kinase C (PKC) in L-PGDS-mediated apoptosis.

PKC refers to a family of serine and threonine kinases that have traditionally been associated with the regulation of cell growth and differentiation in response to a variety of stimuli (16). More recently, members of the PKC family have been linked to cell death and apoptosis of various cells (3, 17). For example, phorbol ester induces apoptosis in the renal epithelial cell line LLC-PK1 (11), PKCδ downregulation suppresses apoptotic signals in 3Y1 rat fibroblasts (31), tyrosine phosphorylation of PKCε is essential for its apoptotic effects (2), PKCζ modulates thromboxane A2-mediated apoptosis in myocytes (22), and PKCδ knock-out mice show a marked decrease in cellular apoptosis and increased cell proliferation (12). In one study of phorbol 12-myristate 13-acetate (PMA)-induced apoptosis (11), the process seemed to be the result of a conflict between growth-retarding signals elicited by PMA and growth-promoting signals stimulated by serum. The conflict apparently led to DNA damage, which in turn led to apoptosis. When the conflict was prevented by serum starvation, apoptosis was suppressed.

The activation or inhibition of several other downstream signal transduction proteins by a variety of cytokines within a cell has been shown to alter the balance between cell proliferation and apoptosis. Classically, stimulation of the phosphatidylinositol 3-kinase (PI3-K) pathway and subsequent protein kinase B (PKB/Akt) phosphorylation have been shown to be anti-apoptotic. Inhibition of PI3-K phosphorylation by the stimulation of phosphoinositide-specific inositol polyphosphate 5-phosphatase IV inhibits PKB phosphorylation and leads to apoptotic cell death in human embryonic kidney cells (9). Similarly, phosphorylation of the retinoblastoma protein (pRb) is known to promote cellular proliferation and inhibit cellular apoptosis (29, 30). Conversely, proapoptotic events such as...
the activation of PKC but not extracellular signal-related kinase (ERK) have been demonstrated to play a role in vitamin E succinate-induced apoptosis of HL-60 cells (1).

In the present study, we investigated the molecular mechanism of L-PGDS-mediated apoptosis and the involvement of PKC. We report on the phosphorylation and enzymatic activation of L-PGDS by PKC in response to phorbol ester. L-PGDS phosphorylation, induced by PMA, was accompanied by decreases in PI3-K and enzymatic activation of L-PGDS by PKC in response to phorbol ester. L-PGDS phosphorylation, induced by PMA, was accompanied by decreases in PI3-K and enzymatic activation of L-PGDS by PKC in response to phorbol ester.

**EXPERIMENTAL PROCEDURES**

**Materials.** Cell culture reagents, antibiotics, antimycotics, fetal bovine serum, Lipofectamine, genetin (G418), and all media were purchased from Life Technologies (Grand Island, NY). The γ-[32P]ATP (sp. act., 3,000 Ci/mmol) and [35S]-orthophosphate were purchased from Dupont/New England Nuclear (Boston, MA). Electrophoresis reagents were obtained from Bio-Rad (Richmond, CA). Biocinchoninic acid protein assay reagent was purchased from Pierce (Rockford, IL). The PKC inhibitor Gö-6976 was purchased from Calbiochem (San Diego, CA). Restriction endonucleases, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay reagents, and PKC enzyme (a combination of α-, β-, and γ-isozymes) were obtained from Roche Molecular Biochemicals (Indianapolis, IN). The caspase-3 activity apoptotic detection kit was purchased from R&D (Minneapolis, MN). Antibodies against actin, Akt, Bad, p55, p110, pRb, and ERK were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All phospho-antibodies were purchased from Cell Signaling (Beverly, MA). Antibody against L-PGDS and the PGD2-methoxime (MOX) immunoassay kit were from Cayman Chemical (Ann Arbor, MI). The PKC activator PMA was purchased from Biomol (Plymouth Meeting, PA). Enhanced chemiluminescence reagent was from Amersham Pharmacia Biotech (Piscataway, NJ). The mammalian expression vector pcDNA3. L-PGDS depletion was accomplished by infection of LLC-PK1 cells with a retro-viral construct expressing an L-PGDS antisense sequence.

**Cell culture.** LLC-PK1 cells were the parent strain of LLC-PK1 cells. Each cell line was maintained in DMEM/F-12 medium supplemented with 10% fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin (Life Technologies, Gaithersburg, MD) at 37°C. Next, the cells were labeled with [32P]orthophosphate (0.1 mCi/ml) overnight and exposed to various agents. The cells were rinsed three times with PBS that contained sodium vanadate (10 mM) and were immunoprecipitated.

**Immunoprecipitation.** Cells were lysed in a buffer that contained 50 mM HEPES, pH 7.5, 2 mM EDTA, 1% Triton X-100, 100 mM NaCl, 50 mM β-glycerophosphate, 100 mM NaF, 100 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 2 μM microcystin, and a cocktail of protease inhibitors. Equal amounts of precleared protein lysate (usually between 500 and 1,000 μg) were immunoprecipitated with the appropriate rabbit polyclonal antibody for 2 h at 4°C and were collected with protein A-Sepharose via incubation overnight. The immunoprecipitated protein was either assayed for kinase activity or it received immunoblot analysis.

**Immunoblot analysis.** Culture plates were washed four times with ice-cold PBS, which was followed by the addition of cell lysis buffer that contained 50 mM Tris·HCl, pH 7.5, 2.0 mM EDTA, 2.0 mM EGTA, 1.0% SDS, 1.0 mM benzo- dino, 2.0 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/ml each of leupeptin, aprotinin, antipain, soybean trypsin inhibitor, and pepstatin A. When phosphorylation was detected, PBS and cell lysis buffer contained 2 mM sodium orthovanadate and 1 μm microcystin at 4°C. Plates were scraped, and the cell lysate was sonicated and centrifuged at 2,000 g for 5 min. Typically, 50 μg of protein was mixed with Laemml sample buffer (that contained 0.1% bromophenol blue, 1.0 M NaH2PO4, pH 7.0, 50% glycerol, and 10% SDS) and boiled for 5 min before it was loaded onto an SDS-PAGE gel. The separated proteins were transferred to polyvinylidene difluoride (PVDF) membrane and probed with the appropriate antibody before detection via enhanced chemiluminescence reagent and subsequent autoradiography were performed. The intensity of the signal was quantitated by densitometric analysis of the autoradiograms.

**PKC activity assay.** In vitro PKC activity was measured as described by Kitano et al. (10). Briefly, the 50-μl reaction mixture contained 20 mM Tris·HCl, pH 7.5, 10 mM MgCl2, 0.5 mM CaCl2, 5 mM dithiothreitol, 1.5 μg L-PGDS substrate where indicated, mixed micelles (0.31 mg/ml phosphatidylserine, 60 μg/ml dolein, 0.03% Triton X-100 in 20 mM Tris·HCl, pH 7.5, sonicated separately), and 0.25 μM/mL PKC enzyme diluted in 20 mM Tris·HCl, pH 7.5, 0.5 mM EDTA, with 0.5 mM EGTA. The reaction was initiated by the addition of 5 μM [32P]ATP (10 μCi/μl) and was incubated at 30°C for 30 min. After termination of the reaction with 1.5 μl of 10 mM ATP, 25 μl of the reaction mixture was removed and 5× Laemmli gel buffer was added. The sample was heated to 95°C for 5 min, separated on a 12% SDS-PAGE gel, and transferred to PVDF membrane; the dried membrane was exposed to film overnight.

**Assay of L-PGDS enzymatic activity.** L-PGDS enzymatic activity was determined using a PGD2-MOX enzyme immunoassay kit. Cells were treated as described and lysed by freeze-thawing in 0.1 M Tris·HCl (pH 7.5) and 1 mM PMSF. Equal amounts of whole-cell lysates were used as the enzyme source. The reaction mixture contained 0.1 M Tris·HCl (pH 7.5), 1.0 mM β-mercaptoethanol, 40 μM PGH2 as the substrate, and cell extracts in a final volume of 50 μl. The reaction was initiated by addition of the substrate to the reaction mixture, incubated for 1 min at 25°C, and terminated by heating the mixture to 100°C for 10 min. The resulting PGD2 product in the reaction mixture was quantitated according to the manufacturer’s instructions.

**PI3-K activity assay.** Anti-p110 and anti-p85 antibodies were used to immunoprecipitate PI3-K activity from 500 μg of precleared lysate. Assays were performed as previously described (20).

**Apoptotic activity.** Apoptosis was quantitated by either TUNEL staining as previously described (13, 18) or by measuring the activation of caspase-3 activity as per the manufacturer’s procedure.

**Construction, transfection, and selection of stable L-PGDS antisense cell lines.** LLC-PK1 cells were the parent strain used for transfection with the mammalian expression system vector pcDNA3. L-PGDS depletion was accomplished by in-
Chronic PMA treatment induces apoptosis via PKC. Previously, we demonstrated the induction of apoptosis by chronic exposure of LLC-PK₁ cells to L-PGDS (13). Similarly, Lee and Rosson (11) had demonstrated phorbol ester-induced apoptosis in this cell line when serum was present. We decided to study whether the L-PGDS-induced apoptosis that we had observed previously was linked to PKC. Consequently, we treated cells with PMA in the presence and absence of the PKC inhibitor compound Go6-976, which selectively inhibits the PKCo and PKCβ isoforms, to test whether PKC activation induced apoptosis. Figure 1 demonstrates that chronic PMA exposure results in a twofold induction of apoptosis, which is significantly inhibited by the PKC inhibitor compound Go6-976. Similar data using the TUNEL assay for apoptotic quantification were generated (data not shown).

PMA increases L-PGDS phosphorylation and enzymatic activity. Because it appeared clear that the PKC pathway had a role in L-PGDS-induced apoptosis in LLC-PK₁ cells, and subsequent amino acid-sequence analysis of the L-PGDS protein (using the Prosite program) revealed three potential PKC phosphorylation sites, we decided to investigate whether PKC could phosphorylate L-PGDS and possibly modulate its enzymatic activity. When 32P-labeled cells were stimulated with 100 nM PMA, immunoprecipitation of L-PGDS revealed a threefold induction of L-PGDS phosphorylation after chronic exposure to PMA for 15 h in the presence of serum (Fig. 2A, lane 3). Figure 2B shows no change in the relative amount of L-PGDS protein from all treatments.

To help confirm the cell culture data, the phosphorylation of L-PGDS by PKC was studied in vitro. Figure 3A demonstrates the direct phosphorylation of L-PGDS by PKC after 30 min of incubation in the presence of γ-[32P]ATP. When either the substrate (L-PGDS) or the enzyme (PKC) were left out of the reaction mixture (Fig. 3A, lanes 1 and 2, respectively), no phosphorylation of L-PGDS was observed. A low level of PKC autophosphorylation can also be observed in lanes 1 and 3. Figure 3B demonstrates the presence and location of the L-PGDS protein in lanes 2 and 3.

We next examined whether PKC could activate endogenous L-PGDS enzymatic activity. LLC-PK₁ cells were treated with PMA for 10 min or 15 h, and the L-PGDS enzymatic activity of cell extracts was assayed and compared with untreated cells. Exposure of cells to PMA for 10 min caused a threefold increase, and a 15-h PMA exposure caused a sevenfold increase in L-PGDS enzymatic activity (Fig. 4). These observations suggest that sustained L-PGDS activation may be required for PMA-induced apoptosis.
Cells that express L-PGDS antisense mRNA resist PMA-induced caspase-3 activation and apoptosis. Because we observed that PMA-induced apoptosis was accompanied by L-PGDS phosphorylation and enzymatic activation, we decided to confirm the role of L-PGDS in PMA/PKC-induced apoptosis by creating a cell line that overexpresses antisense L-PGDS mRNA to decrease L-PGDS protein expression. More than 200 clones were screened and assayed for the reduction in L-PGDS protein synthesis. Figure 5A illustrates a Western blot analysis of two stable clones with L-PGDS protein expression inhibited by fivefold (lanes 2 and 3). Lane 1 represents wild-type LLC-PK1 cells, and lane 4 is a transfected cell line harboring the empty expression vector. The reductions in L-PGDS levels were not due to loading errors as is demonstrated by the equal expression of actin among all cell lines (Fig. 5B). When the antisense cells were exposed to 100 nM PMA for 15 h, there was an almost complete loss of the ability of PMA to induce apoptosis as measured by TUNEL assay (Fig. 5C) and caspase-3 activity (Fig. 5D). These data lend further support to the role of L-PGDS in phorbol ester-induced apoptosis.

Chronic treatment with PMA or L-PGDS inhibits PI3-K signaling. The PI3-K pathway is traditionally perceived as anti-apoptotic. For example, several...
growth factors that inhibit apoptosis also stimulate the activation of PI3-K. Zhou et al. (32) demonstrated that activation of PI3-K by the inhibition of Na,K-ATPase is cytoprotective in LLC-PK1 cells. More importantly, inhibitors of PI3-K such as wortmannin and LY-294002 inhibit PKB/Akt phosphorylation and have been shown to enhance apoptosis when added to LLC-PK1 cells as measured by the activation of caspase-3 and -9 (8, 25). We found similar results in our system, where 100 nM wortmannin caused a twofold increase in the apoptotic index (apoptotic indexes: control, 5%; wortmannin, 12%). Because PI3-K is considered to be a classic anti-apoptotic pathway that is activated for cell survival, the effects of chronic treatment with either PMA or recombinant L-PGDS on PI3-K activity were investigated. Cells incubated with 100 nM PMA or 50 μg/ml L-PGDS for 15 h were immunoprecipitated with anti-p85 antibody and then assayed for PI3-K enzymatic activity in p85 immunoprecipitates. As shown in Fig. 6A (lane 3), exposure of LLC-PK1 cells to PMA resulted in a marked decrease in the amount of phosphatidylinositol 3,4,5-trisphosphate (PIP3) formed. Treatment with PKC inhibitor prevented the PMA-induced decrease in PI3-K activity and restored PIP3 formation (Fig. 6A, lane 4). More importantly, PMA failed to inhibit PI3-K activity in L-PGDS-depleted cells (Fig. 6A, lane 7). Treatment of LLC-PK1 cells with recombinant L-PGDS also inhibited PI3-K enzymatic activity (Fig. 6B). The reduction in PI3-K activity in L-PGDS-treated cells was due to a marked reduction in the association of the p110 catalytic subunit with the p85 regulatory subunit (Fig. 6C). The levels of the p85 PI3-K subunit were not altered due to L-PGDS treatment (Fig. 6D).

PMA- or L-PGDS-induced inhibition of PI3-K is accompanied by Akt inactivation and Bad activation. Because PI3-K enzymatic activities were decreased in PMA- and L-PGDS-treated cells, we decided to study whether substrates downstream of PI3-K and related to apoptosis were also affected. Therefore, the effects of PMA and L-PGDS on PKB/Akt and Bad phosphorylation were determined. Chronic incubation with PMA or L-PGDS for 15 h resulted in a three- or sixfold decrease in Akt phosphorylation, respectively (Fig. 7A, compare lanes 1 and 3 or 5 and 6), whereas Akt protein levels remained constant. The level of Bad phosphorylation similarly decreased by 50% with exposure to either PMA or L-PGDS (Fig. 7B, compare lanes 1 and 3 or 5 and 6).
and 6). The effects of PMA on Akt dephosphorylation as well as Bad dephosphorylation could be prevented by pretreatment with Go6976 (Fig. 7, A and B, lane 4).

L-PGDS inhibits pRb phosphorylation but has no effect on ERK phosphorylation. Phorbol ester-induced apoptosis of LLC-PK1 cells has been associated with the hypophosphorylation of pRb (11). We decided to investigate the effect of L-PGDS on pRb levels and phosphorylation. When 50 μg/ml L-PGDS was added to cells for 15 h, there was a 2.5-fold decrease in pRb phosphorylation (Fig. 8A). The level of ERK phosphorylation, however, was not affected (Fig. 8B).

**DISCUSSION**

In the present study, we demonstrate that chronic exposure of LLC-PK1 cells to phorbol ester leads to the phosphorylation and enzymatic activation of L-PGDS and appears to be mediated via PKC. Although not conclusive, this interpretation is supported by the in vitro data, the 32P-labeled L-PGDS immunoprecipitation rates in response to chronic PMA exposure, and the potential PKC phosphorylation sites found within the L-PGDS amino acid sequence. In addition, mutations within several of the PKC phosphorylation site consensus sequences resulted in an L-PGDS protein with limited ability to induce apoptosis when added to cells exogenously (data not shown). Enzymatic activation of L-PGDS in response to chronic PMA treatment is consistent with the work of Mahmud et al. (14), who have demonstrated the activation of hematopoietic PGDS activity in response to phorbol esters in human megakaryoblastic cells.

L-PGDS, when added to cells at high levels, is only able to efficiently induce apoptosis when serum is present in the culture medium. We found similar results with PMA, where the full enzymatic activation of L-PGDS and the induction of apoptosis were found only after chronic exposure in the presence of serum. It is quite possible that only under these conditions are so-called “growth conflicts” in place, which allow for the induction of apoptosis. The effect of serum on PMA-induced apoptosis has been studied previously (29, 30), and it was concluded that the conflict between pRb-dependent growth signals and caspase activity is required for PKC-signaled apoptosis. Similarly, Lee and Rosson (11) demonstrated that only in the presence of serum was apoptosis induced in LLC-PK1 renal cells after chronic exposure to PMA. A requirement for serum to facilitate apoptosis seems logical, because selective programmed cell death, apoptosis, is usually observed under conditions where serum is plentiful.

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**Fig. 7. Effects of PMA and L-PGDS on Akt and Bad phosphorylation.** LLC-PK1 cells at 90% confluence were exposed to 100 nM PMA for 15 h either alone or in combination with 500 nM Go6976 or 50 μg/ml L-PGDS, and protein (50 μg) was separated by SDS-PAGE. Proteins were transferred to a PVDF membrane and probed for expression of phospho-Akt and Akt (A) and phospho-Bad and Bad (B).

**Fig. 8. Effect of L-PGDS on retinoblastoma protein (pRb) and extracellular signal-related kinase (ERK) phosphorylation.** LLC-PK1 cells at 90% confluence were exposed to 50 μg/ml L-PGDS (lane 2) for 15 h, and protein (50 μg) was separated by SDS-PAGE. Proteins were transferred to a PVDF membrane and probed for expression of phospho-pRb and pRb (A) and phospho-ERK and ERK (B).

**Fig. 9. Schematic of PKC-induced apoptosis using L-PGDS.** Under conditions of PKC stimulation such as inflammation, there is an increased PKC activation, which leads to increased L-PGDS serine phosphorylation (ser-P). Result is an inhibition of the antiapoptotic PI3-K/Akt pathways and a concomitant hypophosphorylation of pRb and Bad, which increase apoptosis.
and some cells need to continue to proliferate. An example is embryogenesis, where cells are both proliferating and undergoing apoptosis. Alternatively, there may be a threshold of PGD₂ products that is needed for the apoptotic signal to proceed, and these products could be limited by the PGH₂ substrate. This phenomenon requires further examination.

It appears that PKC₅₆ and PKC₅₁ are the most relevant isoforms involved with L-PGDS phosphorylation. This notion is supported by the data involving the indolocarbazole inhibitor Gö-6976, which selectively inhibits these PKC isoforms (15). The fact that these are the classic calcium-dependent isoforms is consistent with the calcium dependence we have previously observed with L-PGDS-induced apoptosis. Other forms, i.e., PKCθ and PKCe, which have been shown to induce apoptosis in certain cell lines, appear to involve p38 mitogen-activated protein kinase (MAPK) and operate independently of the PI3-K pathway (5, 15, 21–23). We also observed no change in ERK phosphorylation in response to L-PGDS. It is interesting to note that selenocompounds, which are known to inhibit L-PGDS enzymatic activity (6) and L-PGDS-induced apoptosis (15), have been shown to preferentially inhibit the calcium-dependent isoforms of PKC (4). In addition, bile salts, which are transported by L-PGDS in vivo, have been shown to activate PKC and induce apoptosis in hepatocytes (7). The possibility of other members of the MAPK family such as c-Jun NH₂-terminal kinase and p38 MAPK participating in L-PGDS-mediated apoptosis needs to be examined so that we can more clearly define the role of the MAPK pathway in this process. We are currently investigating these isoforms.

The molecular mechanism of L-PGDS-induced apoptosis appears to be at least in part via the inhibition of the PI3-K pathway and the subsequent inhibition of the phosphorylation of Bad and pRb. Usui et al. (28) have demonstrated a link between PKC and PI3-K activity and subsequent hypophosphorylation of the downstream targets Bad and pRb. Figure 9 is a schematic that summarizes the effects of L-PGDS activation. These results indicate that phorbol ester-induced apoptosis in LLC-PK₁ cells is mediated at least in part by L-PGDS phosphorylation by PKC, and the mechanism may involve the inhibition of the PI3-K/PKB anti-apoptotic signaling pathways.

We thank Dr. Najma Begum for assistance with the manuscript revisions. This work was supported in part by a Grant-in-Aid Award from the American Heart Association Heritage Affiliate, a Career Development Award from the American Diabetes Association, and Winthrop-University Hospital.

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