Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1773 (2007) 1721–1731
Biochimica et Biophysica Acta BBA
www.elsevier.com/locate/bbamcr

Up-regulation of cyclooxygenase-2 by cobalt chloride-induced hypoxia is mediated by phospholipase D isozymes in human astroglioma cells

Bong-Hyun Ahn [a,1], Mi Hee Park [b,1], Young Han Lee [c], Taeg Kyu Kwon [d], Do Sik Min [b,*]

[a] *Cardiovascular Branch National Heart, Lung and Blood Institute (NHLBI), NIH Bldg 10/CRC 5-3288 10 Center Drive, Bethesda, MD 20892, USA*
[b] *Department of Molecular Biology, College of Natural Science, Pusan National University, 30 Jangjeon dong, Geumjeong gu, Busan 609-735, Korea*
[c] *Department of Biomedical Science and Technology, Institute of Biomedical Science and Technology (IBST), Konkuk University, Seoul, Korea*
[d] *Department of Immunology, School of Medicine, Keimyung University, 194 DongSan-Dong Jung-Gu, Daegu 700-712, Korea*

Received 5 March 2007; received in revised form 2 June 2007; accepted 5 June 2007
Available online 15 June 2007

Abstract

Cyclooxygenase-2 (COX-2) is an isoform of prostaglandin H synthase induced by hypoxia and has been implicated in the growth and progression of a variety of human cancers. In the present study, we investigated the role of phospholipase D (PLD) isozymes in cobalt chloride ($CoCl_2$)-induced hypoxia-driven COX-2 expression in U87 MG human astroglioma cells. $CoCl_2$ stimulated PLD activity and synthesis of COX-2 protein in a dose and time-dependent manner. Moreover, elevated expression of PLD1 and PLD2 increased hypoxia-induced COX-2 expression and prostaglandin E2 (PGE_2) production. Pretreatment of cells with 1-butanol, but not 3-butanol, suppressed $CoCl_2$-induced COX-2 expression and PGE_2 formation. In addition, evidence that PLD activity was involved in the stimulation of COX-2 expression was provided by the observations that overexpression of wild type PLD isozymes, but not catalytically inactive PLD isozymes, stimulated $CoCl_2$-induced COX-2 expression and PGE_2 production. PLD1 enhanced COX-2 expression by $CoCl_2$ via reactive oxygen species (ROS), p38 MAPK kinase, PKC-δ, and PKA, but not ERK, whereas PLD2 enhanced $CoCl_2$-induced COX-2 expression via ROS and p38 MAPK, but not ERK, PKC-δ, and PKA. Differential regulation of COX-2 expression mediated through PLD isozymes was comparable with that of $CoCl_2$-induced PLD activity in these two PLD isozymes. Taken together, our results demonstrate for the first time that PLD1 and PLD2 isozymes enhance $CoCl_2$-induced COX-2 expression through differential signaling pathways in astroglioma cells.

Keywords: Cyclooxygenase-2; Prostaglandin E2; Cobalt chloride; Hypoxia; Phospholipase D

1. Introduction

Cyclooxygenase (COX), also referred to as prostaglandin endoperoxide synthase, is a key enzyme in the conversion of arachidonic acid to prostaglandins (PGs) and other eicosanoids. Two isoforms of COX have been identified [1]. COX-1 is expressed constitutively in many tissues and cell types, whereas COX-2 is inducible by a variety of factors, including cytokines, growth factors, and tumor promoters. COX-2 is highly expressed in a number of human cancers and cancer cell lines. COX-2 seems to be involved in the processes of malignant transformation and tumor progression by affecting cell proliferation, cell cycle progression, cell adhesion, apoptosis, and angiogenesis [2]. COX-2 overexpression has been documented in human glioma [3,4]. When the correlation between COX-2 expression and survival was examined in one study, high COX-2 expression correlated with poor survival for all malignant gliomas and demonstrated the strongest correlation with glioblastoma multiforme. An interesting observation in gliomas has been the accumulation of COX-2-expressing astrocytes during progression of oligodendrogliomas to a higher grade [5]. In light of known tumor-promoting properties of cyclooxygenase in other tumors, this observation suggests that COX-2 expression may be involved in the neoplastic progression of glioma.

* Corresponding author. Tel.: +82 51 510 3682; fax: +82 51 513 9258.
E-mail address: minds@pusan.ac.kr (D.S. Min).
[1] These authors contributed equally to this study.

0167-4889/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2007.06.001

Hypoxia is observed in nearly all solid tumors and has been associated with poor prognosis [6]. Most solid human tumors have focal hypoxic areas that cause low oxygen tension. Cobalt is an essential trace compound because it plays a critical role in the synthesis of vitamin B12. However, excessive cobalt exposure can lead to various disease such as asthma, pneumonia, or hematological abnormalities [7]. Cobalt chloride ($CoCl_2$) has been widely used as hypoxia mimic in both in vitro and in vivo studies [8]. Cobalt chloride increases the generation of oxidative stress in cells and increase the level of ROS [9–11]. Although the increase of ROS under a state of hypoxic stress occurred after exposure to both metals and hypoxia, it was not clear whether this was the stimulus for a hypoxic gene response. Cobalt chloride treatment of cells in vitro has previously been shown to induce cellular changes which are similar to these seen after hypoxia [12,13]. It has been demonstrated that COX-2 expression and activity are induced by hypoxia in prostate cancer cells and human umbilical vein endothelial cells [14,15].

Several lines of evidence have suggested a functional role for PLD in COX-2 regulation during cell activation [16–19]. PLD catalyzes the hydrolysis of phosphatidylcholine (PC) to generate a lipid mediator, phosphatidic acid (PA) and has emerged as a critical regulator of cell proliferation and survival signaling [16,20]. PA is thought to function as a second messenger in a wide range of physiological processes including mitogenesis, differentiation, and ischemia [21,22]. Although two distinct mammalian PLD isoforms, PLD1 and PLD2, have been cloned to date [22], their physiological roles are still poorly understood. Activation of PLD occurs through interactions of the ARF and Rho families as well as with protein kinase C (PKC). Recently, it has been demonstrated that PLD activity is increased by hypoxia of PC12 cells and by transient forebrain ischemia in rat hippocampus [23,24]. However, the role of PLD isozymes in hypoxia-induced COX-2 expression has not been studied in any biological system. Therefore, we investigated the role of PLD in the regulation of COX-2 expression of in astroglioma during $CoCl_2$-induced hypoxia. To the best our knowledge, this is the first study to link PLD isozymes to $CoCl_2$-induced COX-2 expression in any cell system.

2. Experimental procedures

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and LipofectAMINE plus were purchased from Invitrogen. Rabbit polyclonal COX-2 antibody was from Santa Cruz Biotechnology. Antibodies to phospho-ERK, ERK, phospho-p38, p38, pan-phospho PKC, and phospho PKA were from Cell Signaling. A polyclonal antibody that recognizes both PLD1 and PLD2 was generated as previously described [25]. Phosphatidylbutanol (PtdBut) standard was from Avanti Polar Lipid. Anti-β-tubulin antibody were from Sigma (St. Louis, MO), and NAC, PD98059, PD169316, rottelerin and H89 were from Biomol (Plymouth Meeting, PA). [9], [10-^{3}H] myristate was purchased from PerkinElmer Life Sciences. Silica gel 60 A thin layer chromatography plates were from Whatman. Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were from Kirkegaard and Perry Lab (Gaithersburg, MD). Enhanced chemiluminescence (ECL) reagents and the PGE_2 enzyme immunoassay kit were from Amersham Biosciences (Piscataway, NJ).

2.2. Methods

2.2.1. Cell culture and transfection

U87 MG human astroglioma was obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained in DMEM (Life Technologies, Inc) supplemented with 10% (v/v) heat-inactivated fetal bovine serum under 5% CO_2 For hypoxia induction, incubator conditions were either normoxic (21% O_2, 5% CO_2) or hypoxic (0.5% O_2, 5% CO_2, balance N_2) in a humidified incubator at 37 °C. U87 cells were transiently transfected for 40 h with expression plasmid encoding empty vector, using LipofectAMINE Plus (Invitrogen) according to manufacturer's instructions. U87 cells stably overexpressing wild type and catalytically inactive mutant of PLD isozyme (K898R for PLD1, K758R for PLD2) were obtained by transfection, using LipofectAMINE. Transfected cells were selected with G418 (700 μg/ml) for 21 days at 37 °C. At that time antibiotics-resistant colonies were pooled and expanded for further analysis under selective conditions.

2.2.2. In vivo PLD activity

In vivo PLD activity was determined as described previously [26]. PLD activity was assessed by measuring the formation of [^{3}H] phosphatidylbutanol (PtdBut), the product of PLD-mediated transphosphatidylation, in the presence of 1-butanol. Cells in 6-well plates were serum-starved in the presence of 2 μCi/ml [^{3}H] myristic acid. After overnight starvation, the cells were washed three times with 5 ml of phosphate-buffered saline (PBS) and pre-equilibrated in serum-free DMEM for 1 h. For the final 10 min of preincubation, 0.3% 1-butanol was included. At the end of the preincubation, cells were treated with agonists for the indicated times. The extraction and characterization of lipids by thin-layer chromatography were performed as previously described [26]. Radioactivity incorporated into total phospholipids was measured, and the results were presented as percentage of total lipid cpm incorporated into phosphatidylbutanol to normalize the results.

2.2.3. Western blot

Cells were washed twice with ice-cold phosphate-buffered saline and then lysed in the extraction buffer (20 mM HEPES, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.2% SDS, 200 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenymethylsulfonyl fluoride). The resulting cell lysates was spun at 15,000×*g* in a Eppendorf microcentrifuge for 10 min at 4 °C to pellet the unbroken cells. Protein concentrations were determined using Bradford method with bovine serum albumin as a standard. Protein samples were analyzed by SDS-polyacrylamide gel electrophoresis on 8% gels and were transferred to a nitrocellulose membrane. The blots were then blocked with 5% non-fat milk in Tris-buffered saline-Tween 20 (25 mM Tris–HCl, 150 mM NaCl, and 0.05% Tween 20) and incubated with appropriate primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were detected using enhanced chemiluminescence. resuspended in sample buffer.

2.2.4. PGE2 production assay

PGE_2 levels were determined using an enzyme immunoassay kit according to the manufacturer's instruction. Briefly, 50 μl of standard or sample was pipetted into the wells of a 96 well plate. Aliquots of mouse polyclonal PGE_2 antibody and PGE_2 conjugated to alkaline phosphatase were then added to each well and the plate was incubated at room temperature for 1 h. After incubation, the wells were washed six times with 200 μl of PBS containing 0.05% Tween 20, and the TMB substrate was added. Wells were read at 670 nm with an enzyme-linked immunosorbent assay reader 30 min after adding substrate.

2.2.5. Luciferase assay

COX-2 promoter construct was generously provided by Dr. H. Inoue (National Cardiovascular Center Research Institute, Japan). Briefly, the region from −1432 to +59 bp of COX-2 promoter was cloned into pGL2. COX-2 promoter plasmid was transfected into human U87 astroglioma cells using Lipofectamine Plus according to the manufacturer's instruction. After 24 h of transfection, the cells were treated with or without $CoCl_2$ for 12 h. Following stimulation, cells were washed with PBS and lysed in luciferase lysis buffer. The activities of *firefly* and *Renilla* luciferase in the cellular extracts were measured

using the dual-luciferase reporter assay system. Relative luciferase activity was obtained by normalizing the firefly luciferase activity against the internal control *Renilla* luciferase activity.

2.2.6. RNA isolation and reverse transcription PCR (RT-PCR)

Total RNA was isolated from cells by the Trizol method. First-strand cDNA was synthesized using 5 μg total RNA using MMLV-RTase (Promega, Mannheim, Germany). The reaction was incubated at 80 °C for 5 min, 42 °C for 90 min, and MMLV-RTase was inactivated at 95 °C for 5 min. The synthesized cDNA was amplified using COX-1, COX-2, COX-3, and GAPDH primers with Eppendorf thermocycler (Eppendorf Scientific, Westbury, NY). PCR reactions were cycled as follows: initial denaturation at 95 °C for 5 min and then 30 cycles for COX-1, COX-2, COX-3 and GAPDH at 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s. Forward primer of GAPDH was 5′-GTG GTC TCC TCT GAC TTC AAC-3′, and the reverse primer was 5′-TCT CTT CCT CTT GTG CTC TTG-3′ with 197 bp amplified fragments. The forward primer of COX-1 gene was 5′-CAATGCCACCTTCATCCG A-3′, and the reverse primer was 5′-GAGCCGCAGTTGATACTGA-3′ with 430 bp amplified fragments. The forward primer of COX-2 gene was 5′-CCTTCCTCCTGTGCCTGATG-3′, and the reverse primer was 5′-TCTAGCCAGAGTTTCACCGTA-3′ with 203 bp amplified fragments. The forward primer of COX-3 gene was 5′-CAT-GAGCCGTGAGTGCG-3′, and the reverse primer was 5′-CTGCAGAGGA-GAGAGATGAG-3′ with 102 bp amplified fragments. PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining.

2.2.7. Statistics

The results are expressed as mean±S.D. of the number of determinations indicated. Statistical significance of differences was determined by ANOVA. Significance was accepted when $p<0.05$.

3. Results

3.1. Elevated expression of PLD isozymes upregulates $CoCl_2$–induced COX-2 expression and PGE_2 production in human astroglioma cells

Cobalt has been widely used as a hypoxia mimic agent both in vitro and in vivo studies. It has been reported that $CoCl_2$ induces COX-2 protein levels in prostate cancer cells [27]. To investigate the effect of PLD on $CoCl_2$-induced COX-2 expression, we established U87 MG human astroglioma cells stably overexpressing vector, PLD1, or PLD2. In unstimulated cells, elevated expression of PLD1 or PLD2 led to somewhat higher basal expression of COX-2 protein compared with that of vector (Fig. 1A). In $CoCl_2$-stimulated cells, overexpression of PLD1 or 2 significantly increased COX-2 expression, compared with that of control cells (Fig. 1A), suggesting that elevated expression of PLD1 or PLD2 enhances $CoCl_2$-induced COX-2 expression. On the other hand, COX-2 is expressed transiently by a wide spectrum of growth factors and pro-inflammatory stimuli under certain pathophysiological conditions [28–30]. Elevated expression of PLD also increased PMA or lipopolysaccharide (LPS)-induced COX-2 expression in U87 astroglioma cells (Fig. 1B and C), COX-2 but not COX-1 and -3, was present in U87 astroglioma cells, and $CoCl_2$ increased the expression of COX-2, but not COX-2 and COX-3, using RT-PCR. (Fig. 1D), suggesting that $CoCl_2$ is responsible for induction of COX-2. Because COX-2 catalyzes biosynthesis of prostaglandin E_2 (PGE_2), we examined whether this enzyme was responsible for $CoCl_2$-induced PGE_2 production. $CoCl_2$, PMA, and LPS increased PGE_2 biosynthesis in the culture media of cells stimulated with these agents (Fig. 1E). $CoCl_2$, PMA or LPS-induced PGE_2 increase was also enhanced by elevated expression of PLD isozymes compared with that of vector cells (Fig. 1E). Both PLD1 and PLD2 contribute to $CoCl_2$-induced PGE_2 production in a similar extent in human astroglioma cells. Furthermore, $CoCl_2$-induced COX-2 protein expression was enhanced in a dose- and time-dependent manner in cells overexpressing of PLD isozymes compared with that of vector cells (Fig. 2A and B). Overexpression of PLD isozymes also increased $CoCl_2$-induced PGE_2 production in a dose-dependent manner com-

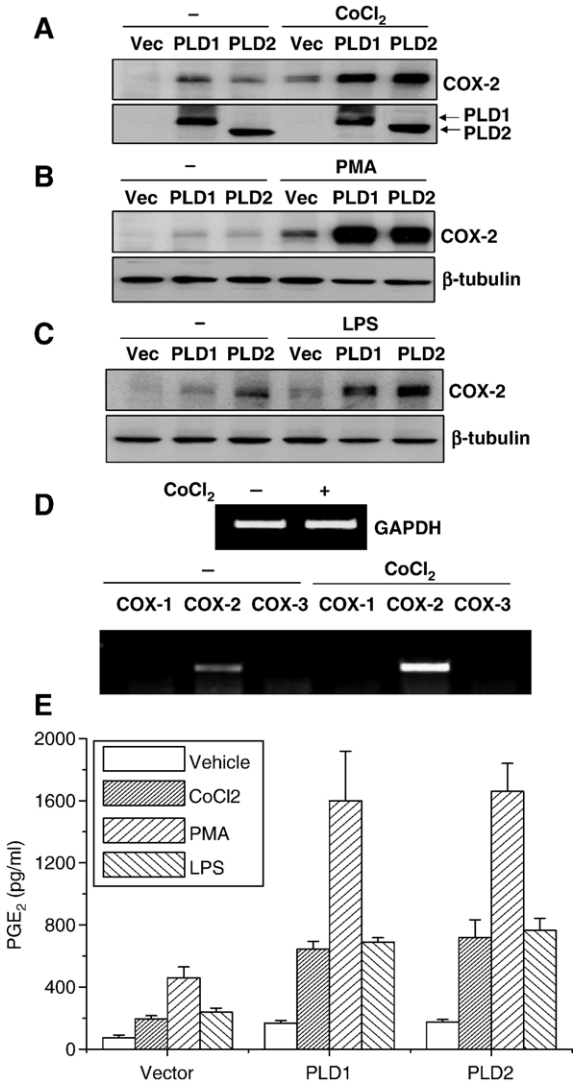

Fig. 1. Elevated expression of PLD isozymes upregulates $CoCl_2$, PMA, or LPS-induced COX-2 expression and PGE_2 production in human astroglioma cells. U87 MG human astroglioma cells overexpressing vector, PLD1 or PLD2 were stimulated without or with 200 μM of $CoCl_2$ (A), 20 nM of PMA (B) or 10 μg/ml of LPS (C) for 20 h. Equal amounts of cell lysates (40 μg) were analyzed by SDS-polyacrylamide gel electrophoresis, followed by transfer of proteins to nitrocellulose membrane and immunoblotting with antibodies to COX-2, PLD or β-tubulin antibody. (D) U87 MG cells were treated with or without $CoCl_2$ (500 μM) for 6 h. and RT-PCR analysis was performed as described in Exprimental procedures. The data shown are representative of three independent experiments. (E) The release of PGE_2 was measured from supernatants as described in Exprimental procedures. The values shown for PGE_2 production are the mean±S.D. of the three independent experiments.

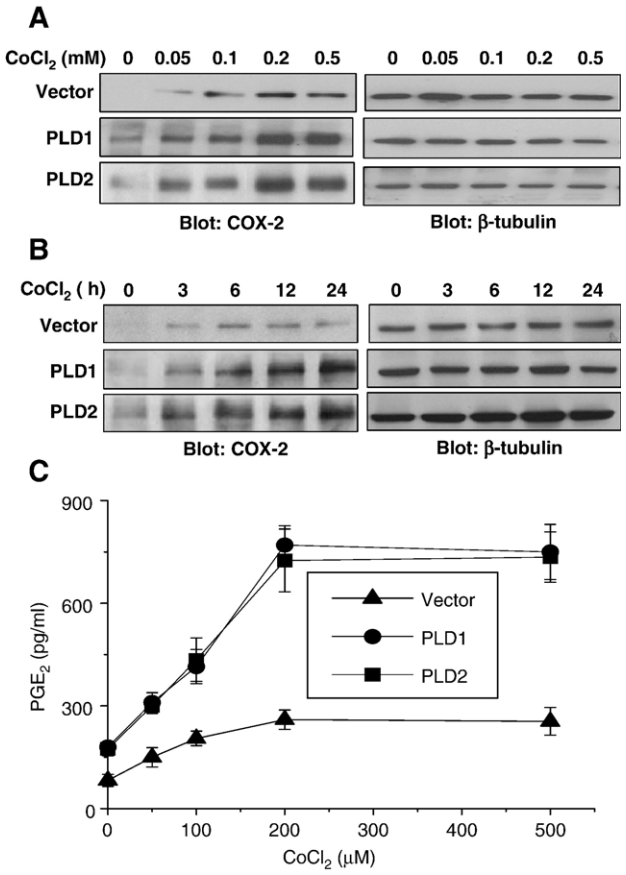

Fig. 2. Overexpression of PLD1 and PLD2 enhances $CoCl_2$-induced COX-2 expression and PGE_2 formation in a dose and time-dependent manner. (A) U87 MG human astroglioma cells overexpressing PLD1, PLD2, or vector were treated with or without various concentration of $CoCl_2$ for 20 h or 500 μM $CoCl_2$ for the times indicated. The cell lysates were prepared and analyzed for COX-2 expression by Western blot analysis. These blots are representative of results obtained from three experiments. (B) Cells were stimulated with the indicated concentrations of $CoCl_2$ for 20 h, and then the release of PGE_2 was measured from supernatants as described in Exprimental procedures. The values shown for PGE_2 production are the mean±S.D. of the three independent experiments.

pared with that of vector cells (Fig. 2C). The results indicate that elevated expression of PLD isozymes up-regulates $CoCl_2$-induced COX-2 expression and PGE_2 biosynthesis in U87 MG human astroglioma cells.

3.2. Hypoxia-induced COX-2 expression is increased by overexpression of PLD isozymes

It has been reported that hypoxia increases COX-2 protein levels in prostate cancer cells [31]. Therefore, we examined whether hypoxia-induced COX-2 expression is increased by elevated expression of PLD isozymes. The hypoxia effect was investigated under 0.5% O_2 and U87 MG astroglioma cells were incubated under hypoxic or normoxic conditions for 12 h. Hypoxia stimulated COX-2 expression and elevated expression of PLD1 or PLD2 led to higher expression of COX-2 protein in normoxic condition, compared with that of vector cells (Fig. 3A). Furthermore, overexpression of PLD1 and PLD2 up-regulated hypoxia-induced COX-2 expression compared with that of vector cells. Hypoxia-induced PGE_2 production was also increased by elevated expression of PLD isozymes and comparable with the result of hypoxia-induced COX-2 expression (Fig. 3B). These results suggest that elevated expression of PLD isozyme enhances COX-2 expression and PGE_2 formation induced by hypoxic culture.

3.3. PLD activity is involved in $CoCl_2$-induced COX-2 expression and PGE_2 production

To address the possible involvement of PLD activation in $CoCl_2$-induced COX-2 expression and PGE_2 biosynthesis, U87 human astroglioma cells prelabeled with [^{3}H] myristate were stimulated with 500 μM $CoCl_2$ for various times. And PLD activity was measured by the formation of [^{3}H] PtdBut from 1-butanol, a product specific to PLD activity. As shown in Fig. 4A and B, $CoCl_2$ induced PtdBut formation, in a time- or dose-dependent manner. $CoCl_2$-induced PLD activation was significantly increased by elevated expression of PLD1 and PLD2. Furthermore, PMA-induced PLD activation was also increased by PLD overexpression (Fig. 4C). Although the experiments showed that PLD was activated in $CoCl_2$-treated human astroglioma cells, they provided no direct evidence that PLD activity was involved in the induction of COX-2 expression. We then examined whether PLD activity affects COX-2 expression and PGE_2 production in $CoCl_2$-stimulated cells (Fig. 5). Fig. 5A

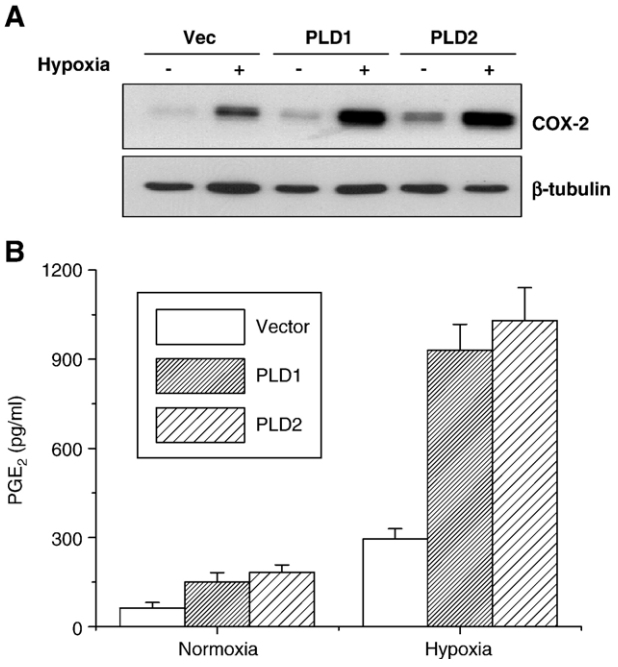

Fig. 3. Hypoxia-induced COX-2 expression is increased by overexpression of PLD isozymes. U87 MG human astroglioma cells overexpressing PLD1, PLD2, or vector were incubated for 12 h in the normoxia or in the hypoxia condition (0.5% O_2). (A) The cell lysates were immunodetected with anti-COX-2 or anti-β-tubulin antibody. The data shown are representative of three independent experiments. (B) The release of PGE_2 was measured from supernatants from normoxic and hypoxic culture. The values shown for PGE_2 production are the mean±S.D. of the three independent experiments.

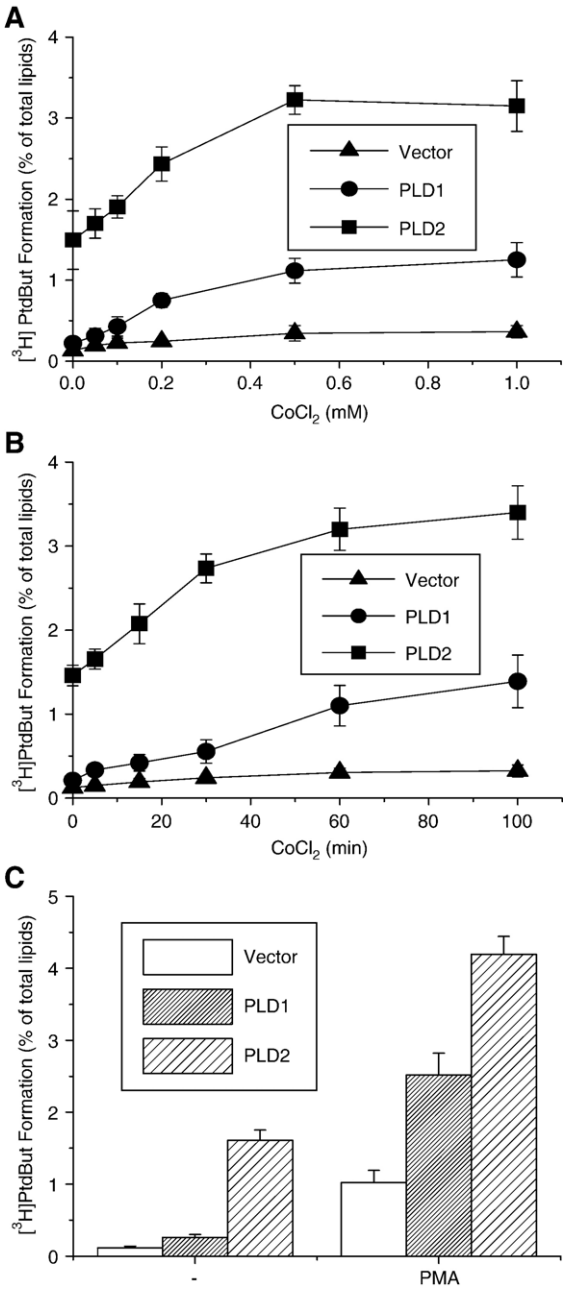

Fig. 4. Activation of PLD by $CoCl_2$ in astroglioma cells. U87 MG human astroglioma cells overexpressing PLD1, PLD2, or vector were cultured in six-well plates and labeled with 2 μCi/ml [^{3}H] myristic acid. Following washing and preincubation with DMEM, 0.1% BSA, 0.3% 1-butanol for 20 min, the cells were treated with 500 μM $CoCl_2$ for the indicated times (A), with the indicated concentration of $CoCl_2$ for 1 h (B), or 100 nM PMA for 1 h (C). The radioactivity incorporated into PtdBut was measured as described in Exprimental procedures. Data are expressed as the mean of the means±S.D. of three independent experiments.

shows the enzymatic activities and expression of PLDs in cells overexpressing wild-type PLDs and catalytically inactive mutant PLDs. $CoCl_2$-induced COX-2 expression and PGE_2 formation were significantly increased in cells expressing wild type PLDs compared with that in vector-transfected control cells, whereas there was no increase of COX-2 protein and PGE_2 formation by treatment of $CoCl_2$ in cells expressing the catalytically inactive mutants of PLD, suggesting that these effects are clearly PLD activity dependent (Fig. 5B, C). A role for PLD activity in $CoCl_2$-induced COX-2 expression received further support when 1-butanol was used to block PA production by PLD, by virtue of the formation of phosphatidylbutanol through the transphosphatidylation reaction. U87 MG cells were stimulated with $CoCl_2$ in the presence of 1% 1-butanol or 3-butanol. As shown in Fig. 5D, 1-butanol, but 3-butanol inhibited $CoCl_2$-induced COX-2 expression and PGE_2 biosynthesis. This activity dependency was further confirmed by measuring the promoter activity of COX-2 gene. A luciferase reporter plasmid containing genomic fragment corresponding to the COX-2 promoter was transfected into cells expressing vector, wild-type PLD1 or PLD2, or the catalytically inactive mutants of PLD1 and PLD2. As shown in Fig. 5E, COX-2 promoter activity was increased by treatment with $CoCl_2$. $CoCl_2$,-induced COX-2 promoter activity was significantly increased by elevated expression of wild-type PLD1 and PLD2, but not catalytically inactive mutants of PLD1 and PLD2. Taken together, these results show that $CoCl_2$-induced COX-2 expression and PGE_2 formation are increased by a PLD dependent pathway.

3.4. PLD1 and PLD2 differentially regulate $CoCl_2$-induced COX-2 expression

We tried to investigate the signaling pathway of $CoCl_2$-induced COX-2 expression via PLD isozymes. As shown in Fig. 6A, anti-oxidant (NAC) and p38 MAPK inhibitor (PD169316) suppressed $CoCl_2$-induced COX-2 expression in both PLD1 and PLD2-overexpressed astroglioma cells, suggesting that reactive oxygen species (ROS) and p38 MAPK are involved in $CoCl_2$-induced COX-2 expression via both PLD1 and PLD2 isozymes. It is known that $CoCl_2$ can mimic the hypoxia responses in many respects, including production of ROS in cultured cells [32]. The inhibitor of p42/44 MAPK, which is known to regulate COX-2 expression, did not affect the $CoCl_2$-induced COX-2 expression in PLD1 and PLD2-overexpressed astroglioma cells. Interestingly, PKC-δ specific inhibitor, rottelerin and PKA inhibitor, H89 significantly suppressed $CoCl_2$-induced COX-2 expression in PLD1-overexpressed cells, but not PLD2-overexpressed cells. In addition, we examined transcriptional activation of COX-2 using the luciferase reporter plasmid containing COX-2 promoter. $CoCl_2$-induced COX-2 promoter activity in PLD1-overexpressed cells was suppressed by pretreatment with anti-oxidant (NAC), p38 MAPK inhibitor, PLC-δ inhibitor, and PKA inhibitor (Fig. 6B), but not MEK inhibitor, whereas $CoCl_2$-induced COX-2 promoter activity in PLD2-overexpressed cells was suppressed by pretreatment with anti-oxidant and p38 MAPK inhibitor, but not PLC-δ inhibitor, PKA inhibitor, and MEK inhibitor (Fig. 6C). These results were correlated with those of $CoCl_2$-induced COX-2 protein expression, suggesting differential regulation of COX-2 by PLD isozymes. As shown in Fig. 6D, the phosphorylation of ERK by $CoCl_2$ increased gradually and peaked at 20 min and decreased thereafter. $CoCl_2$ stimulated significantly p38 MAPK, and $CoCl_2$- induced p38 MAPK peaked at 10 min and then declined. Although $CoCl_2$ stimulated phosphorylation of ERK and p38 MAPK in U87

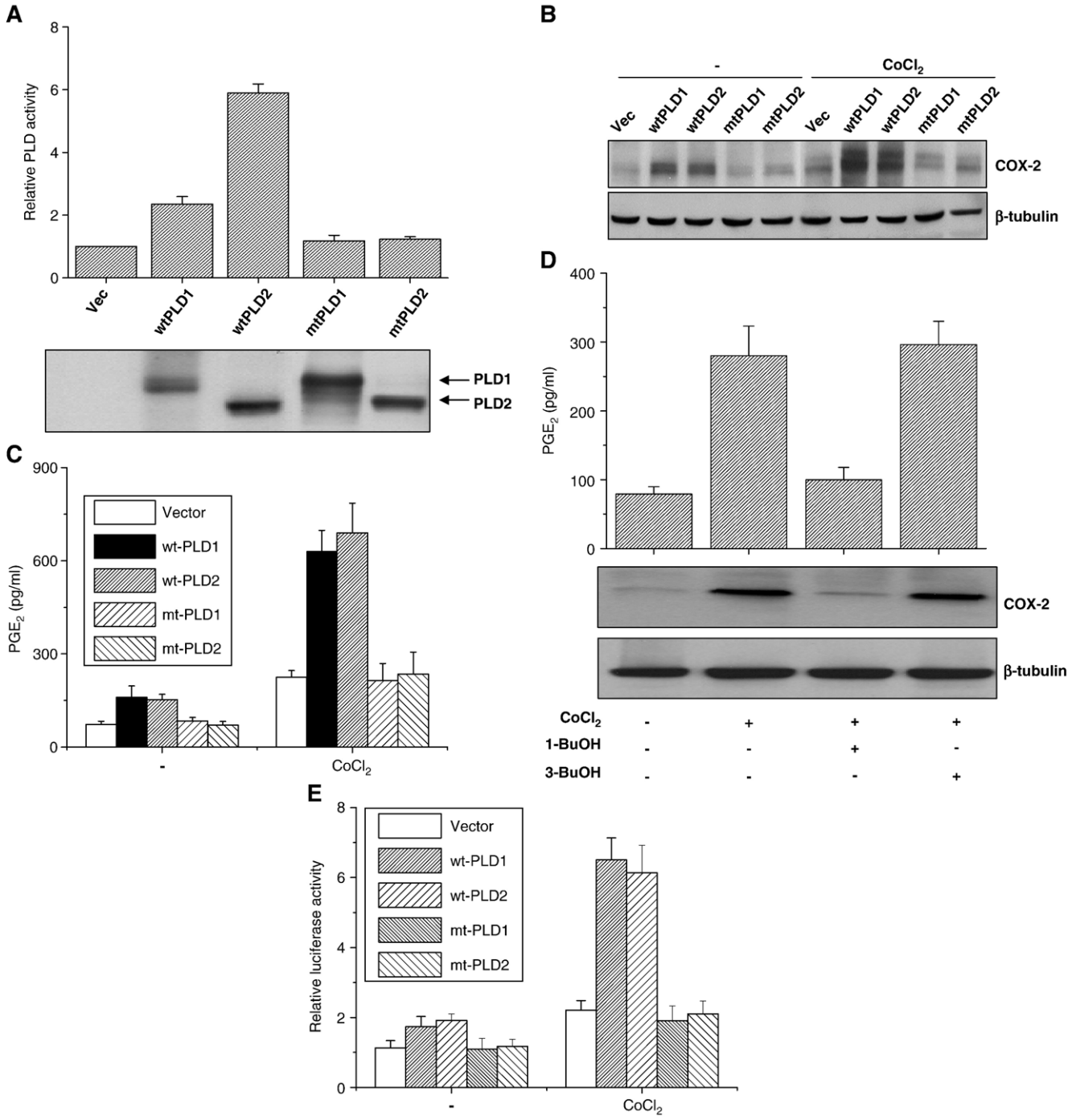

Fig. 5. PLD activity is involved in $CoCl_2$-induced COX-2 expression. (A) Upper panel: Activity of PLD in U87 MG human astroglioma cells overexpressing vector (Vec), wild-type PLD1 (wtPL1), PLD2 (wtPLD2), and their catalytically inactive mutants, PLD1 (mtPLD1), PLD2 (mtPLD2) were measured as described in Exprimental procedures. Expression of PLD isozymes were analyzed using antibody to PLD. (B) The cells were treated with 200 μM of $CoCl_2$ for 12 h and cell lysates were immunoblotted with an antibody directed against COX-2 or an anti-β-tubulin antibody. The data shown are representative of three independent experiments. (C) The release of PGE_2 was measured from supernatants as described in Exprimental procedures. The values shown for PGE_2 production are the mean±S.D. of the three independent experiments. (D) Upper panel: U87 cells were stimulated with 200 μM of $CoCl_2$ in the presence of 1% butanol or 3-butanol for 12 h. The release of PGE_2 was measured from supernatant, Lower panel: The extracted proteins were immunodetected with anti-COX-2 or anti-β tubulin antibody. (E) The cells were transfected with 25 ng of the reporter plasmid, pGL2-COX-2. After 12 h of transfection, cells were treated without or with 200 μM of $CoCl_2$ for 12 h, and luciferase activity was measured. The internal control vector (pRL-null) was used to normalize for transfection efficiency. Results show means±S.D. of three independent experiments.

astroglioma cells, both PLD1 and PLD2 isozymes may mediate $CoCl_2$ -induced COX2 expression via activation of p38 MAPK, but not ERK. We also found that $CoCl_2$ activated PKC and PKA in a time-dependent manner in U87 astroglioma cells using antibodies to pan-phospho-PKC and phospho-PKA (Fig. 6D). Furthermore, $CoCl_2$-induced PLD activation was also com-

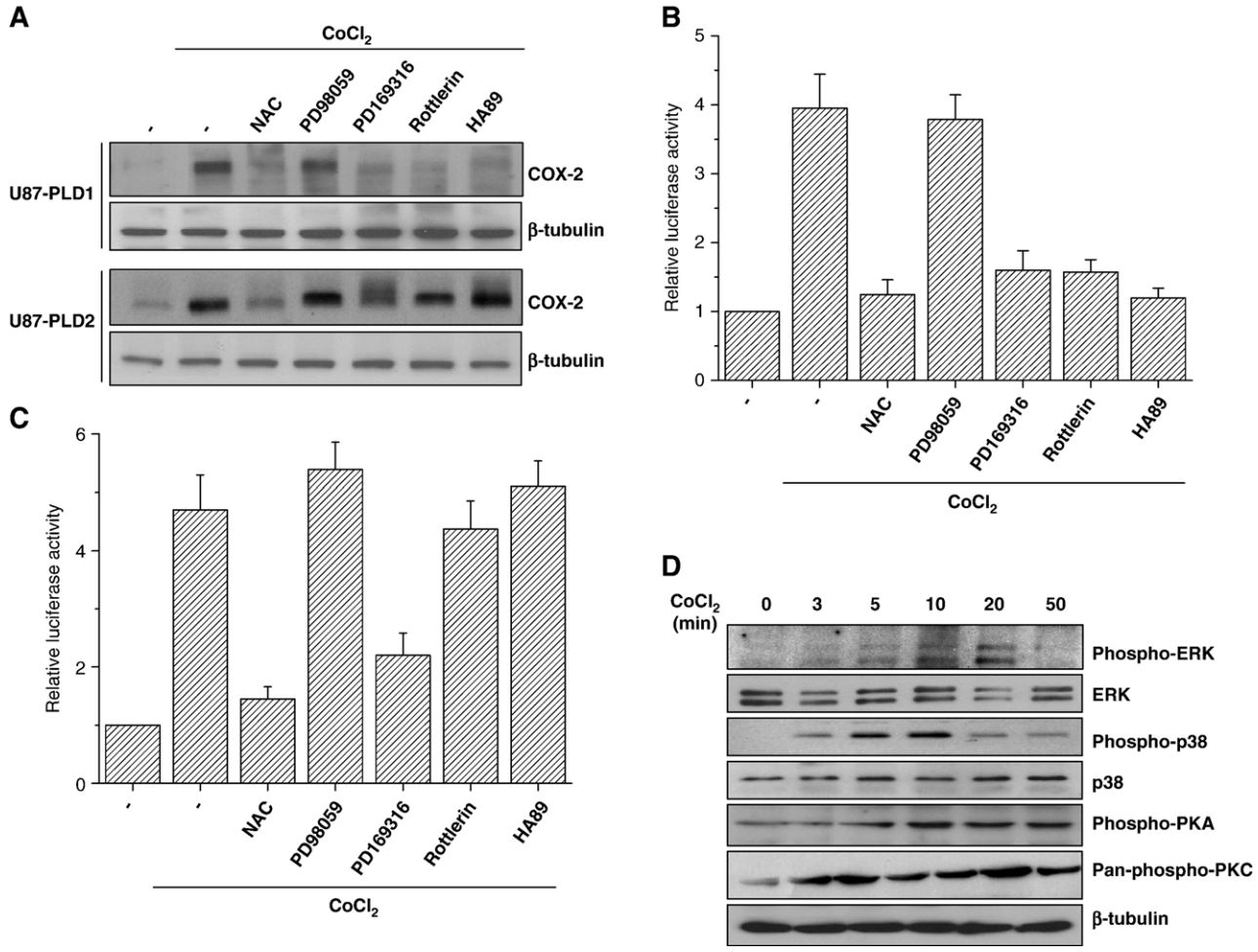

Fig. 6. Differential regulation of $CoCl_2$-induced COX-2 expression by PLD isozymes. (A) U87 cells overexpressing PLD1 or PLD2 were pretreated with NAC (10 mM), PD98059 (10 μM), PD169316 (10 μM), Rottlerin (10 μM), and HA89 (10 μM) for 30 min followed by stimulation with $CoCl_2$ (200 μM) for 12 h. Cell lysates were prepared and analyzed for COX-2 by Western blot analysis, using a specific antibody. These blots are representative of results obtained from three experiments. U87 cells overexpressing PLD1 (B) or PLD2 (C) was transfected with COX-2-Luc. After 12 h of transfection, cells were pretreated with the indicated inhibitors for 30 min and stimulated without or with $CoCl_2$ (200 μM) for 12 h, and luciferase activity was measured. The internal control vector (pRL-null) was used to normalize for transfection efficiency. Results show means ± S.D. of three independent experiments. (D) U87 cells were stimulated with $CoCl_2$ (200 μM) for the indicated times. Cell lysates were immunoblotted with the indicated antibodies. Data are representative of three experiments.

parable with that of $CoCl_2$-induced COX-2 expression (Fig. 7). PLD1 activation by $CoCl_2$ was suppressed in a dose-dependent manner by PD169316, rottlerin, and HA89, whereas $CoCl_2$-induced PLD2 activation was inhibited by PD169316, but not by rottlerin, and HA89. Both PLD 1 and PLD2 were also suppressed by antioxidant (NAC), but not by PD98059. These results suggest that PLD1 and PLD2 differentially regulate $CoCl_2$-induced COX-2 expression in astroglioma cells.

3.5. $CoCl_2$ also stimulates PGE2 production via PLA_2 and PLC signaling pathway, but pertussis toxin-insensitive manner

It has been known that hypoxic injury also involve the stimulation of phospholipase A_2 (PLA_2) and phospholipase C (PLC) in brain tissue [33]. PLA_2 and PLC liberate arachidonic acid, which is the substrate for COX, the enzyme that produces PGE_2 [34,35]. Therefore, we examined relative contribution of PLA_2, PLC and PLD in $CoCl_2$-induced PGE_2 production using its specific inhibitors (Fig. 8A). The cytosolic PLA_2 inhibitor (AACOCF3) and PLC inhibitor (U73122) suppressed $CoCl_2$-induced PGE_2 production in a similar extent, but PLD inhibitor (1-butanol, which blocks PA formation by PLD) showed dramatic decrease in $CoCl_2$-induced PGE_2 production compared with those of other two phospholipases, suggesting that PLD mainly contributes to $CoCl_2$-induced PGE_2 production and both PLA2 and PLC are also in a similar extent involved in the phenomena. Furthermore, we examined whether G protein is involved in the $CoCl_2$-induced PGE_2 production. As shown in Fig. 8B, pertussis toxin, which catalyzes ADP ribosylation of the G_i family of the α subunits, thus uncoupling them from their activating receptors, did not affect $CoCl_2$-induced PGE_2 production in PLD1 or PLD2-expressed astroglioma cells. Taken together, these results suggest that in U87 human astroglioma cells, PLD mainly contributes to $CoCl_2$-induced PGE_2 production and PLA_2 and PLC signaling pathway are also involved, but pertussis toxin-insensitive manner.

4. Discussion

The major finding of this study is that PLD is a vital component of the signal transduction pathway induced by $CoCl_2$ or hypoxia that leads to the expression of COX-2 in human astroglioma. Cellular responses to either $CoCl_2$ or hypoxia share a common mechanism for oxygen sensing, signal transduction, and transcriptional regulation [36,37]. The present study created an in vitro hypoxia-like state by treatment of astroglioma cells with $CoCl_2$ and demonstrates that astroglioma cells increase levels of COX-2 in the presence of $CoCl_2$, but not COX-1 and COX-3. It has been reported that COX-2-positive cells accumulated in human glioma tumor cells, but no COX-1 immunoreactivity was observed in the tumor cells [38]. Moreover, it has been reported that VEGF induction by $CoCl_2$-stimulated hypoxia is mediated by induction of COX-2 expression in a human metastatic prostate cancer cell line [27].

It was also suggested that significant expression of COX-2 of neoplastic astrocytes may represent induction of COX-2 by hypoxia or hypoglucosemia, as has been observed in brain infarcts [39]. COX-2 overexpression in astrocytic glioma is intimately related to features of biological aggressiveness in astrocytic gliomas, i. e. histological grade and proliferative potential and closely associated with vascular surface area and the expression of the angiogenic factor VEGF, known to drive angiogenesis in these tumors.

Little is known about how COX-2 protein levels are regulated in glioma cells, the PLDs responsible for such a regulation, and the molecular mechanisms involved. We have studied signal transduction pathways involved in $CoCl_2$-induced COX-2 expression in U87 MG astroglioma cells. $CoCl_2$ stimulated COX-2 protein expression and PGE_2 production as well as PLD activity.

Some studies have implicated a PLD-derived signaling pathway in the generation of prostaglandins in many cell types [16–19]. Kaneki et al. [40] showed that PMA-induced COX-2 expression in osteoblast-like UMR-106 cells was dependent upon PLD activity.

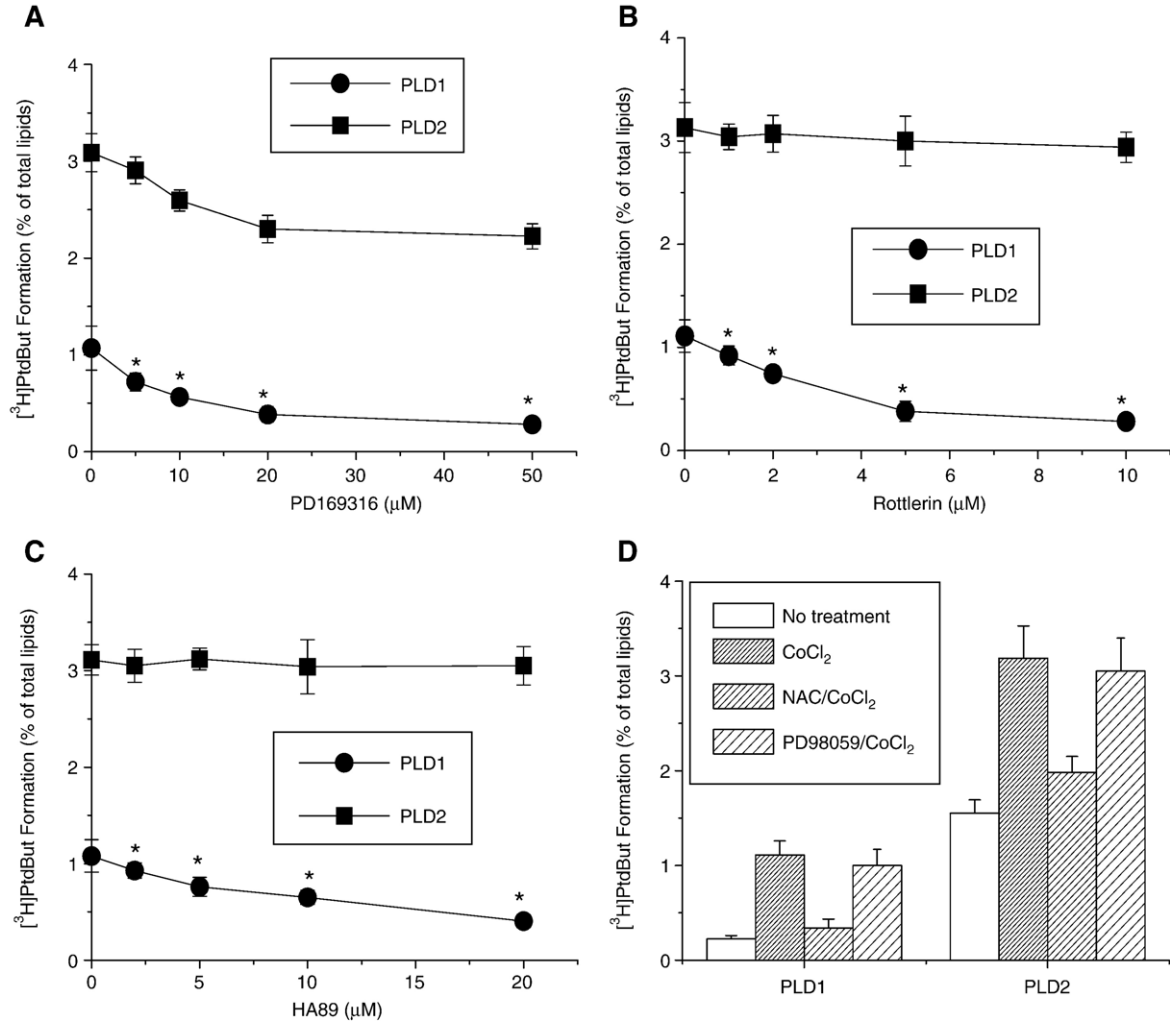

Fig. 7. Differential regulation of $CoCl_2$-induced PLD activation. U87 cells overexpressing PLD1 or PLD2 were cultured in six-well plates and labeled with 2 μCi/ml [^{3}H] myristic acid. Following washing and preincubation with DMEM, the cells were pretreated with the indicated concentration of PD98059 (A), Rottlerin (B), HA89 (C) for 30 min, and then stimulated with 500 μM $CoCl_2$ for 1 h. The labeled cells were also pretreated with NAC (20 mM) or PD98059 (10 μM) for 30 min followed by stimulation with $CoCl_2$ (500 μM) for 1 h (D). The radioactivity incorporated into PtdBut was measured as described in Exprimental procedures. Data are expressed as the mean of the means±S.D. of three independent experiments. *$p<0.05$ vs. control.

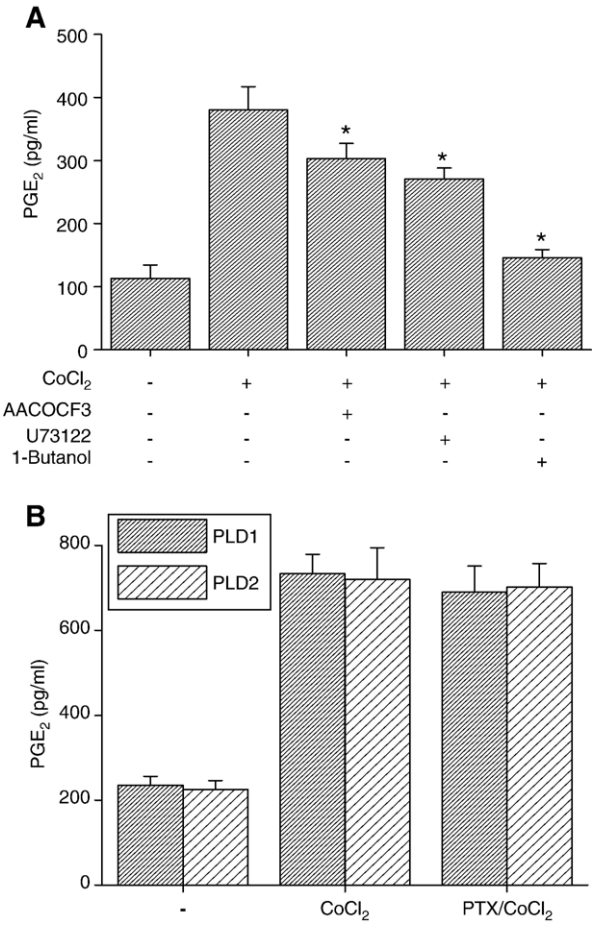

Fig. 8. PLD mainly contribute to $CoCl_2$-induced PGE_2 production and both PLA_2 and PLC, but not pertussis toxin-sensitive G protein, are involved in $CoCl_2$-induced PGE_2 production. (A) U87 cells were pretreated with AACOCF3 (10 μM), U73122 (10 μM), or 1-butanol (0.3%), and treated with $CoCl_2$ (500 μM) for 24 h. (B) U87 cells expressing vector, PLD1 or PLD2 were pretreated with pertussis toxin for 30 min and stimulated with 500 μM $CoCl_2$ for 24 h. The release of PGE_2 was measured from supernatants as described in Exprimental procedures. The values shown for PGE_2 production are the mean± S.D. of the three independent experiments. *$p<0.05$ vs. control.

It has been reported that PLD activity is up-regulated during hypoxic incubation [23] and in reactive astrocytes the hippocampus after transient forebrain ischemia [24]. $CoCl_2$ has been widely used as a hypoxia mimic in both in vitro and in vivo studies [8]. There is no direct evidence that indicates direct regulation of COX-2 expression by PLD protein levels. Our observation that elevated expression of wild types of PLD isozymes, but not catalytically inactive mutants of PLD isozymes, leads to increased expression of COX-2 by $CoCl_2$, clearly indicates a positive role for PLD isozyme in $CoCl_2$ – induced COX-2 expression in human U87 MG astroglioma cells. The evidence implicating PLD activity as an important component of these functional events relies on the use of alcohols. Importantly, inhibition of phosphatidic acid formation through the addition of the primary alcohol 1-butanol, but not tertiary butanol, suppressed $CoCl_2$ induction of COX-2 expression and PGE_2 formation. Therefore, our findings imply that PLD activity is an important factor in the suppression of $CoCl_2$-induced COX-2 expression and PGE_2 production.

PLA_2 and PLC is known to be involved in PGE_2 production. In U87 human astroglioma cells, PLD mainly contributes to $CoCl_2$-induced PGE_2 production, and both PLA_2 and PLC are also involved in the similar extent in the formation of PGE_2 by $CoCl_2$. Since PLA_2, PLC, and PLD are the part of signal transduction network, it is possible that cross-talk among these phospholipases in $CoCl_2$-induced COX2 expression and PGE_2 production. It has been reported that COX-2 expression can be regulated through different signaling pathways and that the particular signaling pathway involved is dependent on the type of stimuli [28]. Recently, we have reported that PLD isozymes mediate epigallocatechin gallate (EGCG)-induced COX-2 expression through PKC and p38 MAPK in U87 MG astroglioma cells [17]. Park et al. [18] demonstrated that in human amnion cells, IL-1β might activate PLD through an upstream protein kinase C to elicit p38 and finally induce COX-2 expression. It was reported that induction of COX-2 by interleukin-1 is mediated by both ERK and p38 kinase in murine astrocytes [30]. In the present study, we observed that elevated expression of PLD1 and PLD2 isozymes regulated differentially $CoCl_2$-induced COX-2 expression in U87 MG astroglioma cells. Although $CoCl_2$ stimulated phosphorylation of ERK and p38 MAPK in U87 astroglioma cells, both PLD1 and PLD2 isozymes may mediate $CoCl_2$ -induced COX2 expression via activation of p38 MAPK, but not ERK. $CoCl_2$ also stimulated phosphorylation of PKC and PKA. PLD1 increased $CoCl_2$ induction of COX-2 expression via ROS, p38 MAPK, PKC-δ, and PKA, but not ERK, whereas PLD2 increased $CoCl_2$-induced COX-2 expression via ROS and p38 MAPK, but not ERK, PKC-δ, and PKA. It is likely that PLD1 and PD2 act interchangeably as well as in parallel to transducer signal. Pertussis-sensitive G protein was not involved in $CoCl_2$-induced PGE_2 upregulation via PLD1 and PLD2.

It was suggested that PLD either acts on a discrete pool of substrate phosphatidylcholine (PC) separate from the bulk of cellular PC, or that the enzyme exhibits a degree of substrate specificity for selected PC molecular species [41] Therefore, it is possible that PLD1 and PLD2 act on different molecular species of PC in various cellular pools located in various subcellular fractions of human astroglioma cells.

An emerging issue in cancer research is the focus on the mechanistic link between chronic inflammation and carcinogenesis, including tumor angiogenesis [42]. COX-2 is an important inducible enzyme mediating inflammatory processes and is highly expressed in a diversity of cancer [43]. Malignant gliomas are among the most common tumors of the central nervous system. COX-2 protein expression in malignant gliomas is an interesting phenomenon, particularly in light of its better-defined role in tumorigenesis in other cancers. COX-2 expression in malignant gliomas has been associated with a higher tumor grade and worse prognosis. PLD has been also known to be involved in many aspects of cell proliferation, survival, and metastasis [44].

To the best of our knowledge, this is the first report to show that $CoCl_2$ might activate differentially PLD1 and PLD2 iso-

zymes and finally induce COX-2 expression. Further studies on physiological role of PLD in the cobalt chloride-stimulated COX-2 regulation in astroglioma cells.

Acknowledgments

This study was supported by a grant no. R01-2006-000-10521-0 (2006) from the Basic Research Program of the Korea Science & Engineering Foundation, and by a grant of 0620040 from the Nation R&D Program for Cancer Control, Korea, and by a grant 0620400-1from the National Cancer Center, Korea.

References

[1] W.L. Smith, R. Langenbach, Why there are two cyclooxygenase isozymes, J. Clin. Invest. 107 (2001) 1491–1495.
[2] C.S. Williams, M. Mann, R.N. DuBois, The role of cyclooxygenases in inflammation, cancer, and development, Oncogene 18 (1999) 7908–7916.
[3] M.H. Deininger, M. Weller, J. Streffer, et al., Patterns of cyclooxygenase-1 and -2 expression in human gliomas in vivo, Acta Neuropathol. 98 (1999) 240–244.
[4] T. Shono, P.J. Tofilon, J.M. Bruner, et al., Cyclooxygenase-2 expression in human gliomas: prognostic significance and molecular correlations, Cancer Res. 61 (2001) 4375–4381.
[5] M.H. Deininger, R. Meyermann, K. Trautmann, et al., Cyclooxygenase (COX)-1 expressing macrophages/microglial cells and COX-2 expressing astrocytes accumulate during oligodendroglioma progression, Brain Res. 885 (2000) 111–116.
[6] H. Zhong, A.M. De Marzo, E. Laughner, et al., Overexpression of hypoxia-inducible factor 1a in common human cancers and their metastasis, Cancer Res. 59 (1999) 5830–5835.
[7] R. Lauwerys, D. Lison, Health risks associated with cobalt exposure—An overview, Sci. Total Environ. 150 (1994) 1–6.
[8] G.L. Wang, G.L. Semenza, Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia, J. Biol. Chem. 268 (1993) 21513–21518.
[9] K. Kasprzak, The role of oxidative damage in metal in metal carcinogen city, Chem. Res. Toxicol. 4 (1991) 604–615.
[10] X. Wang, I. Yokoi, J. Liu, A. Nori, Cobalt(II) and nickel(II) ions as promoters of free radicals in vivo: detected directly using electron spin resonance spectrometry in circulating blood in rats, Arch. Biochem. Biophys. 306 (1993) 402–406.
[11] S. Leonard, P.M. Gannett, Y. Rojanasakul, D. Schwegler-Berry, V. Castranova, V. Vallyathan, X. Shi, Cobalt-mediated generation of reactive oxygen species and its possible mechanism, J. Inorg. Biochem. 70 (1998) 239–244.
[12] H.F. Bunn, R.P. Poyton, Oxygen sensing and molecular adaptation to hypoxia, Physiol. Rev. 76 (1996) 839–885.
[13] M.A. Goldberg, T.J. Schneider, Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin, J. Biol. Chem. 269 (1994) 4355–4359.
[14] X.H. Liu, A. Kirschenbaum, K. Yu, S. Yao, A.C. Levine, Cyclooxygenase-2 suppresses hypoxia-induced apoptosis via a combination of direct and indirect inhibition of p53 activity in a human prostate cancer cell line, J. Biol. Chem. 280 (2005) 3817–3823.
[15] J.F. Schmedtje, Y.S. Ji, W.L. Liu, R.N. DuBois, M.S. Runge, Hypoxia induces cyclooxygenase-2 via the NF-kappaB p65 transcription factor in human vascular endothelial cells, J. Biol. Chem. 272 (1997) 601–608.
[16] P.M. Steed, A.H.M. Chow, Curr. Pharm. Biotech. 2 (2001) 241–256.
[17] S.Y. Kim, B.H. Ahn, K.J. Min, Y.H. Lee, E.H. Joe, D.S. Min, Phospholipase D isozymes mediate epigallocatechin gallate-induced cyclooxygenase-2 expression in astrocyte cells, J. Biol. Chem. 279 (2004) 38125–38133.
[18] D.W. Park, Y.S. Bae, J.O. Nam, J.H. Kim, Y.G. Lee, Y.K. Park, S.H. Ryu, S.H. Baek, Regulation of cyclooxygenase-2 expression by phospholipase D in human amnion-derived WISH cells, Mol. Pharmacol. 61 (2002) 614–619.
[19] L.T. Grab, M.W. Kearns, A.J. Morris, L.W. Daniel, Differential role for and phospholipase D2 in 12-*O*-tetradecanoyl-13-phorbol acetate-stimulated MAPK activation, Cox-2 and IL-8 expression, Biochim. Biophys. Acta 1636 (2004) 29–39.
[20] G..M. Jenkins, M.A. Frohman, Phospholipase D: a lipid centric review, Cell. Mol. Life Sci. 62 (2005) 2305–2316.
[21] J.W. Phillis, M.H. O'Regan, A potentially critical role of phospholipases in central nervous system ischemic, traumatic, and neurodegenerative disorders, Brain Res. Brain Res. Rev. 44 (2004) 13–47.
[22] M.A. Frohman, T.-C. Sung, A.J. Morris, Biochim. Biophys. Acta 1439 (1999) 175–186.
[23] H. Yamakawa, Y. Banno, S. Nakashima, M. Sawada, J. Yamada, S. Yoshimura, Y. Nishimura, Y. Nozawa, N. Sakai, Increased phospholipase D2 activity during hypoxia-induced death of PC12 cells: its possible anti-apoptotic role, NeuroReport 11 (2000) 3647–3650.
[24] M.Y. Lee, S.Y. Kim, D.S. Min, Y.S. Choi, S.L. Shin, M.H. Chun, S.B. Lee, M.S. Kim, Y.H. Jo, Upregulation of phospholipase D in astrocytes in response to transient forebrain ischemia, Glia 30 (2000) 311–317.
[25] D.S. Min, B.H. Ahn, D.J. Rhie, S.H. Yoon, S.J. Hahn, M.S. Kim, et al., Expression and regulation of phospholipase D during neuronal differentiation of PC12 cells, Neuropharmacology 41 (2001) 384–391.
[26] B.H. Ahn, S.Y. Kim, E.H. Kim, K.S. Choi, T.K. Kwon, Y.H. Lee, J.S. Chang, M.S. Kim, Y.H. Jo, D.S. Min, Transmodulation between phospholipase D and c-Src enhances cell proliferation, Mol. Cell. Biol. 23 (2003) 3103–3126.
[27] X.H. Liu, A. Kirschenbaum, S. Yao, M.E. Stearns, J.E. Holland, K. Claffey, A.C. Levine, Upregulation of vascular endothelial growth factor by cobalt chloride-stimulated hypoxia is mediated by persistent induction of cyclooxygenase-2 in a metastatic human prostate cancer cell line, Clin. Exp. Metastasis 17 (2000) 687–694.
[28] B. Hinz, K. Brune, Cyclooxygenase-2–10 years later, J. Pharmacol. Exp. Ther. 300 (2002) 367–375.
[29] S.M. Prescott, F.A. Fitzpatrick, Cyclooxygenase-2 and carcinogenesis, Biochim. Biophys. Acta 1470 (2000) M69–M78.
[30] M. Caivano, P. Cohen, Role of mitogen-activated protein kinase cascades in mediating lipopolysaccharide-stimulated induction of cyclooxygenase-2 and IL-1(in RAW264 macrophages, J. Immunol. 164 (2000) 3018–3025.
[31] X.H. Liu, A. Kirschenbaum, K. Yu, S. Yao, A.C. Levine, Cyclooxygenase-2 suppresses hypoxia-induced apoptosis via a combination of direct and indirect inhibition of p53 activity in a human prostate cancer cell line, J. Biol. Chem. 280 (2005) 3817–3823.
[32] K. Guillemin, M.A. Krasnow, The hypoxic response: huffing and HIFing, Cell 89 (1997) 9–12.
[33] O.P. Mishra, M. Delivoria-Papadopoulos, Cellular mechanisms of hypoxic injury in the developing brain, Brain Res. Bull. 48 (1999) 233–238.
[34] M.A. Balboa, I. Varela-Nieto, K. Killermann Lucas, E.A. Dennis, Expression and function of phospholipase A(2) in brain, FEBS Lett. 531 (2002) 12–17.
[35] C.J. Lo, H.G. Cryer, R.V. Maier, Prostaglandin E2 production by endotoxin-stimulated alveolar macrophages is regulated by phospholipase C pathways, J. Trauma 40 (1996) 557–562.
[36] H.F. Bunt, R.O. Poyton, Oxygen sensing and molecular adaptation to hypoxia, Physiol. Rev. 76 (1996) 839–885.
[37] M.A. Goldberg, T.J. Schneider, Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin, J. Biol. Chem. 269 (1994) 4355–4359.
[38] H. Deininger, M. Weller, J. Streffer, M. Mittelbronn, R. Meyermann, Patterns of cyclooxygenase-1 and -2 expression in human gliomas in vivo, Acta. Neuropathol. (Berl.) 98 (1999) 240–244.
[39] T. Sairanen, A. Ristimaki, M.L. Karjalainen-Lindsberg, A. Paetau, M. Kaste, P.J. Lindsberg, Cyclooxygenase-2 is induced globally in infracted human brain, Ann. Neurol. 43 (1998) 738–747.

[40] H. Kaneki, J. Yokozawa, M. Fujieda, S. Mizuochi, C. Ishikawa, H. Ide, Phorbol ester-induced production of prostaglandin E2 from phosphatidylcholine through the activation of phospholipase D in UMR-106 cells, Bone 23 (1998) 213–222.
[41] Y.M.M. Heung, A.D. Postle, The molecular selectivity of phospholipase D in HL60 granulocytes, FEBS Lett. 364 (1995) 250–254.
[42] L.M. Coussens, Z. Werb, Inflammation and cancer, Nature 420 (2002) 860–867.
[43] G.A. FitzGerald, C. Patrono, The coxibs, selective inhibitors of cyclooxygenase-2, N. Engl. J. Med. 345 (2001) 433–442.
[44] D.A. Foster, L. Xu, Phospholipase D in cell proliferation and cancer, Mol. Cancer Res. 1 (2003) 789–800.