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Role of Phospholipase D2 in Anti-Apoptotic Signaling Through Increased Expressions of Bcl-2 and Bcl-xL

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Abstract We have previously reported that Fas-resistant A20 cells (FasR) have phospholipase D (PLD) activity upregulated by endogenous PLD2 overexpression. In the present study, we investigated how overexpressed PLD2 in FasR could generate survival signals by regulating the protein levels of anti-apoptotic Bcl-2 and Bcl-xL. To confirm the effect of PLD2 on Bcl-2 protein levels, we transfected PLD2 into wild-type murine B lymphoma A20 cells. The transfected cells showed markedly the increases in Bcl-2 and Bcl-xL protein levels, and became resistant to Fas-induced apoptosis, similar to FasR. Treatment of wild-type A20 cells with phosphatidic acid (PA), the metabolic end product of PLD2 derived from phosphatidylcholin, markedly increased levels of anti-apoptotic Bcl-2 and Bcl-xL proteins. Moreover, PA-induced expressions of Bcl-2 and Bcl-xL were enhanced by propranolol, an inhibitor of PA phospholydrolase (PAP), whereas completely blocked by mepacrine, an inhibitor of phospholipase A_2 (PLA_2), suggesting that PLA_2 metabolite of PA is responsible for the increases in Bcl-2 and Bcl-xL protein levels. We further confirmed the involvement of arachidonic acid (AA) in PA-induced survival signals by showing that 1,2-dipalmitoyl-sn-glycero-3-phosphate (DPPA), PA without AA, was unable to increase Bcl-2 and Bcl-xL proteins. Moreover, PA notably increased cyclooxygenase (COX)-2 protein expression, and PA-induced expression of both Bcl-2 and Bcl-xL was inhibited by NS-398, a specific inhibitor of COX-2. Taken together, these findings demonstrate that PA generated by PLD2 plays an important role in cell survival during Fas-mediated apoptosis through the increased Bcl-2 and Bcl-xL protein levels which resulted from PLA_2 and AA-COX2 pathway. J. Cell. Biochem. 101: 1409–1422, 2007.

Key words: FasR; phospholipase D2; phosphatidic acid; Bcl-2; Bcl-xL; phospholipase A_2; cyclooxygenase2

Fas/Apo-1/CD95 is a death domain-containing member of the tumor necrosis factor receptor (TNFR) family which can mediate apoptosis. Fas generates an apoptotic signal when activated by anti-Fas monoclonal antibody (anti-Fas mAb) or Fas ligand (Fas L) [Kang et al., 1998], and apoptosis induced by Fas activation has been shown to be involved in the regulation of lymphocytic death [Nagata and Golstein, 1995; Nagata 1997; Wang et al., 2005].

On the other hand, cancerous cells can develop their own mechanism to escape from Fas-mediated cell death [Lim et al., 2002; Kongphanich et al., 2002]. Earlier, we cloned Fas-resistant A20 cells (FasR) from wild-type murine B lymphoma A20 cells [Lim et al., 2002], and the FasR clone showed increased resistance against Fas-induced apoptosis. The isolation of FasR promoted us to elucidate the signaling pathways for anti-apoptosis and cell survival,

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and to identify crucial component involved in resistance against Fas-induced apoptotic signals; basal PLD activity of FasR was higher than that of wild-type A20 cells, which was found to be due to PLD2 overexpression [Lim et al., 2002].

Phospholipase D (PLD) is a widely distributed enzyme, which catalyzes the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA) and choline. PLD has two isoforms, PLD1 and PLD2, and they differ in the mechanism of activation and subcellular localization [Hammond et al., 1995; Colley et al., 1997]. PLD metabolism is implicated in various physiological processes, including membrane trafficking, cytoskeletal reorganization, cell proliferation [Jones et al., 1999], differentiation, survival, and apoptosis [Nakashima and Nozawa, 1999]. Furthermore, PLD plays an important anti-apoptotic role in many cells and tissues. PLD expression and activation have been observed in many cancers [Uchida et al., 1999; Zhao et al., 2000] and many breast cancer cell lines [Chen et al., 2005], and have acted as a survival factor in many other cell types [Lee et al., 2000; Yamakawa et al., 2000; Lim et al., 2002; Park et al., 2002; Zhong et al., 2003]. The role of PLD has been established through PA, catalytic product of PLD. PA can be produced via two pathways: (1) from the hydrolysis of PC by PLD [Erickson et al., 1999; Liscovitch et al., 2000], or (2) from diacylglycerol (DAG) by the action of diacylglycerol kinase (DGK) [Erickson et al., 1999].

Fas cross-linking in A20 cells increases PLD activity through activation of phosphatidylcholine-specific phospholipase C (PC-PLC) and protein kinase C (PKC) [Han et al., 1999; Shin and Han, 2000a]. Ras GTPase is also essential for the Fas-mediated PLD activation [Shin and Han, 2000b]. As mentioned above, we selected FasR clone from wild-type A20 cells by continuously treating with anti-Fas mAb, and FasR which we cloned showed increased PLD2 expression [Lim et al., 2002]. Therefore, it is quite possible that PLD2 overexpression enabled the cells to survive from Fas-induced apoptosis. However, PLD2-related survival signaling pathway during Fas-mediated apoptosis has not yet been studied.

In the present study, we observed that PA produced by PLD2 could raise the protein levels of anti-apoptotic Bcl-2 and Bcl-xL via phospholipase A_2 (PLA_2) and AA-COX2 pathway, resulting in increased cell survival from Fas-mediated cell death.

MATERIALS AND METHODS

Materials

RPMI 1640 medium, penicillin/streptomycin solution, and fetal bovine serum (FBS) were obtained from Gibco-BRL (Gaithersburg, MD). Mouse PLD2 cDNA inserted into the Xbal and Smal sites of the pCGN vector was a generous gift from Dr. Dosik Min at Dong-A University, Korea [9,10(n)-3H]. Palmitic acid was purchased from Amersham Pharmacia Biotech (Amersham Place, Little Chalfont, Buckinghamshire, England). The silica gel 60A plates for TLC were purchased from Whatman (Clifton, NJ), and phosphatidylethanol (PEt) standard, 1-palmitoyl-2-arachidonoyl-sn-Glycerol-3-phosphoate (PA) dissolved in chloroform, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (DPPA) were purchased from Avanti Polar Lipids (Alabaster, AL). Anti-Fas mAb (Jo2) was obtained from Pharmagen (San Diego, CA). Propranolol and mepacrine were purchased from Sigma-Aldrich (St. Louis, MO). NS-398 was obtained from Biomol Research Laboratories (Plymouth, PA). Used primary antibodies were as follows: Anti-PLD1 polyclonal antibody and anti-PLD2 polyclonal antibody (1:1000, Upstate Biotechnology, Lake Placid, NY). Anti-Bcl-2 monoclonal antibody, anti-Bcl-xL polyclonal antibody, anti-cleaved PARP polyclonal antibody, anti-caspase 3 polyclonal antibody, and anti-caspase 8 polyclonal antibody (1:1000, Cell Signaling Technology, Beverly, MA). Anti-cytochrome *c* monoclonal antibody (1:1000, BioSource, Camarillo, CA). Anti-COX-2 polyclonal antibody (1:1000, Chemicon International, Inc., Temecula, CA). And anti-β-actin monoclonal antibody (1:2000, Abcam, Cambridge, MA). In addition, anti-PLD polyclonal antibody, which recognize both PLD1 and PLD2, was a generous gift from Dr. Dosik Min at Dong-A University, Korea. All other chemicals were of analytical grade.

Cell Culture

A20 murine B lymphoma cell line was obtained from American Type Culture Collection, ATCC TIB-208 (Manassas, VA). A20 cells were cultured for 2 days at 37°C in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells grown at 37°C in

a humidified CO_2-controlled (5%) incubator were then washed with RPMI-1640 medium containing 0.1% (w/v) bovine serum albumin, 100 unit/ml penicillin, and 100 μg/ml streptomycin (serum-free medium) and incubated in a serum-free medium at 37°C for 18 h before treatment.

Fas-Resistance A20 Clone (FasR)

FasR, which had been generated according to our previous report [Lim et al., 2002], has been stored at −70°C, and was used for this study. Briefly, A20 cells grown in 100 mm dish (1×10^6 cells/ml) were treated with 0.2 μg/ml of anti-Fas mAb. After 24 h, the dead cells were removed by aspiration and selection with anti-Fas mAb was continued for additional 48 h. After 72 h of selection, the medium containing anti-Fas mAb was discarded. The surviving cells were resuspended in medium containing anti-Fas mAb, and were seeded on 96-well plate to 1 cells/200 μl of cell density. Each well was carefully observed under inverted microscope and wells containing more than one cell were excluded. The resulting clones were grown in the presence of anti-Fas mAb for 2 weeks, and clones showing rapid growth rate were selected and transferred onto 6-well plates. All these clones showed normal growth pattern in the presence of anti-Fas mAb. The resistant clones were then transferred to 100 mm dish and were maintained in RPMI-1640 culture medium and showed normal growth pattern in the presence of anti-Fas mAb. There was no reverse generation of Fas-sensitive phenotype in the absence of anti-Fas mAb. In present study, FasR, which showed the highest basal PLD activity, was used.

MTT Assay

The MTT assay was used to measure viability of cells treated with anti-Fas mAb. Cells (2×10^4 cells/well) were seeded in 96-well plates and allowed to adhere overnight. Subsequently, the cells were incubated with serum-free media for 18 h and were treated with 70 μM PA for 3 h after pretreatment with 10 μM propranolol or 100 μM mepacrine for 30 min. And cells were then treated with 0.2 μg/ml of anti-Fas mAb for predetermined times. 25 μl (2 mg/ml) of MTT (3-[4,5-dimethylthiazol-2y1]-2,5-diphenyltetrazilum bromide; Sigma) solution was added and incubated for 3 h at 37°C. The resulting supernatant was discarded and 100 μl of dimethyl sulfoxide (DMSO) was added to dissolve formazan crystals formed. After agitating the plates for 10 min, the optical density was immediately measured at 570 nm.

Immunoprecipitation

Anti-Fas mAb-treated cells were washed twice with ice-cold PBS and lysed in 1% Nonidet P-40 lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P40, 0.5% sodium dexoycholate, 1 mM EDTA, proteinase inhibitor mixture). The resulting cell lysates were centrifuged for 10 min at 4°C followed by incubation with 10% (w/v) protein G-Agarose in immunoprecipitation buffer (50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 0.1% Nonidet 40, 0.05% sodium deoxycholate) for 3 h at 4°C. These beads were precipitated, and supernatants were incubated with PLD antibody for 1 h at 4°C. Following the incubation with primary antibody, protein G beads were added, and the mixture was further incubated for 3 h at 4°C. The beads were subsequently washed three times each with immunoprecipitation buffer and phosphate-buffered saline PBS, and finally were resuspended in the sample buffer. The immunoprecipitated proteins were resolved by 10% SDS–polyacrylamide (PAGE) gel electrophoresis and analyzed by Western blotting with PLD antibody.

Determination of PLD Activity

PLD activity was determined by measurement of [^{3}H]PEt produced via PLD catalyzed transphosphatidylation in [^{3}H]palmitic acid labeled cells [Han et al., 1999]. Briefly, A20 cells (1.5×10^6 cells/well) cultured on 6-well plates were metabolically labeled with 1 μCi/ml of [^{3}H]palmitic acid in a serum-free medium for 18 h. And cells were then pretreated with 1% (v/v) ethanol for 15 min before stimulation with anti-Fas mAb. The cells were quickly washed with ice-cold PBS and suspended in ice-cold methanol. Lipids were extracted according to the method of Bligh and Dyer [1959]. PEt was separated by TLC on silica gel 60A plates using a solvent system of ethyl acetate/isooctane/acetic acid/water (110/50/20/100, v/v). The region corresponding to PEt band was visualized by 0.002% (v/v) primulin in 80% (v/v) acetone, scraped, and counted by a scintillation counter.

Western Blot Analysis

For analysis of expression levels of proteins such as PLD, Bcl-2, Bcl-xL, and COX-2, A20

cells were harvested and washed twice with ice-cold PBS. The cells were then lysed for 1 min by sonication in extraction buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1% Triton X-100, 1 mM PMSF, and 1 mM Na_3VO_4). The lysate was centrifuged at 13,000 rpm for 10 min at 4°C. Equal amounts of protein samples (40 μg) from cell lysates were loaded onto 10% (for PLD, cleaved PARP, and COX-2) or 12% (for Bcl-2 and Bcl-xL) SDS–PAGE and were then resolved. The proteins were transferred onto nitrocellulose membranes (Amersham Biosciences, England) using a Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell. The membranes were blocked with 7% non-fat milk in Tris-buffered saline-Tween-20 (25 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20), incubated with appropriate primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:2000, New England Biolabs, Beverly, MA), and specific protein bands were detected using enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech).

Transient Transfection of Mouse PLD2 cDNA

For overexpression of PLD2 in wild-type A20cells, 5 μg of Mouse PLD2 (mPLD2) cDNA inserted in pCGN vector was transiently introduced into A20 cells (1×10^6 cells) using Cell Line Nucleofector™ Kit R (Amaxa Biosystems) according to the manufacturer's instruction. Then, the cells were cultured at 37°C with 5% CO_2 in RPMI-1640 medium supplemented with 10% (v/v) FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. After 48 h of transfection, overexpression of PLD2 in the transfectants was confirmed by Western blot analysis, using anti-PLD polyclonal antibody.

Preparation of Cytosolic and Mitochondrial Fraction

Cells were washed twice with ice-cold PBS and resuspended in five volumes of buffer A (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 250 mM sucrose, 1 mM EGTA, pH 8.0, 1 mM EDTA, pH 8.0, 1 mM PMSF, one tablet of complete protease inhibitor (Roche Diagnostics, Indianapolis, IN)). The resuspended cells were lysed by three freeze/thaw cycles (each cycle comprised of 1 min in liquid nitrogen followed by 3 min in a 37°C water bath) and allowed to swell on ice for 15 min. The lysed cells were centrifuged for 1min, and the supernatant was further centrifuged at 15,000 rpm for 15 min at 4°C. The resulting supernatant is cytosolic protein. The mitochondrial pellets was further resuspended in 50 μl of buffer B (buffer A with 0.5% NP-40 (v/v)) and sonicated with 30 strokes, and centrifuged at 15,000 rpm for 10 min at 4°C. The concentrations of cytosolic and mitochondrial proteins were determined by Bradford method (Bio-Rad), and each of cytosolic and mitochondrial proteins (50 μg) was subjected to 12% SDS–PAGE and Western blot analysis using anti-cytochrome *c* mAb.

RESULTS

Cell Viability, Basal PLD Activity, and PLD2 Expression

As shown in Figure 1A, when the cells were treated with 0.2 μg/ml of anti-Fas mAb for 3 h, the number of viable wild-type cells was conspicuously decreased, whereas most of the Fas-resistant cells (FasR) appeared to survive. Furthermore, basal PLD activity of the FasR was about 2.5-fold higher than that of the wild-type cells, and Western blot analysis revealed that the expression level of PLD2 protein in the FasR, but not PLD1, was significantly increased (Fig. 1B). These results indicate that increased basal PLD activity due to PLD2 overexpression might be responsible for the survival of FasR after Fas activation, in good agreement with our earlier result [Lim et al., 2002].

PLD2 Protects Cells From Fas-Induced Apoptosis and Leads to Bcl-2 and Bcl-xL Protein Expressions

To examine the role of PLD2 in cell death mediated by anti-Fas mAb, wild-type A20 cells were transfected with mPLD2 cDNA. Transfection of mPLD2 led to high level of PLD2 expression and raised the PLD basal activity to almost that of FasR (Fig. 2A). When the mPLD2-transfected A20 cells were treated with 0.2 μg/ml of anti-Fas mAb for 3 h, cell death induced by anti-Fas mAb was dramatically reduced compared to wild-type A20 cells, showing similar pattern observed in FasR (Fig. 2B). Next, we explored how PLD2 could rescue the cells from cell death. Bcl-2 and Bcl-xL are known as an important anti-apoptotic protein found in many types of cells, particularly B lymphocytes. Furthermore, since A20 cells

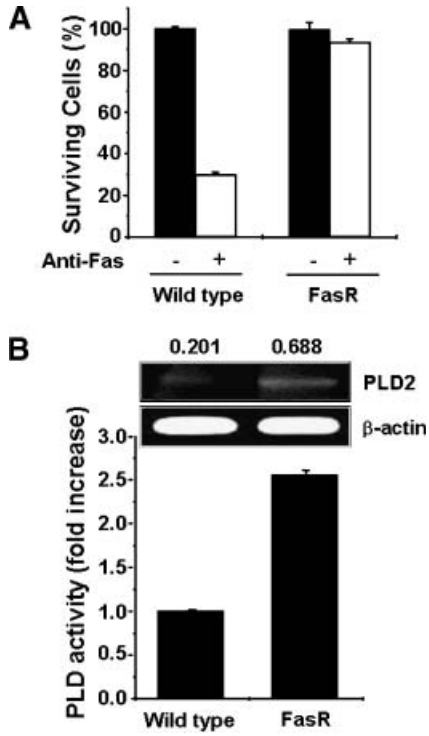

Fig. 1. Cell viabilities, basal PLD activities and PLD2 expressions of wild-type and Fas-resistant A20 cells. Fas-resistant cells were generated as described in Materials and Methods. **A**: Wild-type and Fas-resistant A20 cells were treated with 0.2 μg/ml of anti-Fas antibody for 3 h. Cell viabilities were measured using MTT assay, described in Materials and Methods. The data in this figure are expressed as a percentage relative to cells not treated with anti-Fas antibody. **B**: Wild-type and Fas-resistant A20 cells were lysed and then equal amounts of protein (40 μg) were analyzed by Western blotting using anti-PLD antibody. There was no difference in PLD1 expression levels between wild-type and Fas-resistant cells (data not shown). The relative quantities of each band, normalized to control cells, are mean ± SD from the results quantified three times using QuantityOne software (Bio-Rad). The level of basal PLD activity was determined as described in Materials and Methods. The data in this figure are expressed as fold increase relative to PEt level of wild-type cell and are mean ± SD from three independent experiments.

were derived from B lymphocytes, Bcl-2 and Bcl-xL were first suspected to be critical for the survival of A20 cells. As seen in Figure 2C, Bcl-2 and Bcl-xL protein levels in FasR were higher than that in the wild-type cells. Furthermore, mPLD2-transfectants, which introduce mPLD2 into the wild-type cells, were remarkably shown the increase in Bcl-2 and Bcl-xL protein levels. These results strongly suggest that PLD2 can act as a survival factor by increasing Bcl-2 and Bcl-xL protein levels and endow the A20 cells with resistance to Fas-induced apoptosis.

The Inhibition of Fas-Induced Apoptosis by PLD2 is Via Bcl-2 and Bcl-xL

The endogenous and exogenous PLD2 overexpression increased anti-apoptotic Bcl-2 and Bcl-xL expressions in protein level. However, it is not clear that Bcl-2 and Bcl-xL were involved in this anti-apoptotic model. Fas activation induces caspase-8 activation which can directly activate caspase-3. Herein, we observed the appearances of caspase-8, -3, and PARP cleavage products, and cytochrome *c* release in pGCN vector or PLD2-transfected cells which were treated with anti-Fas mAb for 12 h. As shown in Figure 3A, overexpression of PLD2 inhibited activation of caspase-8 and -3 as well as PARP cleavage, suggesting that PLD2 acts as a survival factor during Fas-mediated apoptosis. Next, under the same experimental condition, mitochondrial and cytosolic proteins were prepared to detect cytochrome *c* release by mitochondria activation. As shown in Figure 3B, PLD2 overexpression also prevented cytochrome *c* release from mitochondria to cytosol by Fas stimulation, suggesting that PLD2 attenuated cytochrome *c* release by increasing Bcl-2 and Bcl-xL protein expressions.

PA Increases the Expressions of Bcl-2 and Bcl-xL Proteins

In order to further elucidate whether PLD2 enhanced the expression of anti-apoptotic Bcl-2 family proteins through its product PA or any other unknown pathway, we treated the wild-type cells directly with 140 μM PA for period of times indicated (Fig. 4A) or various concentrations of PA for 3 h (Fig. 4B). The expressions of Bcl-2 and Bcl-xL proteins reached nearly maximal levels at 70 μM PA for 3 h, and the resistance of the wild-type cells to Fas-induced apoptosis was increased in a time-dependent manner (Fig. 4C). These data represent that PA produced by PLD can provoke Bcl-2 and Bcl-xL protein expressions in A20 cells.

Propranolol, a PAP Inhibitor, Potentiates PA-Induced Expressions of Bcl-2 and Bcl-xL Proteins

Next, we studied how PA upregulated anti-apoptotic proteins, such as Bcl-2 and Bcl-xL. Within cells, PA, the end product of PLD, is usually degraded to DAG by PAP [Mullmann et al., 1990] or converted into arachidonic acid (AA) and lysophosphatidic acid (LPA) by PLA_2

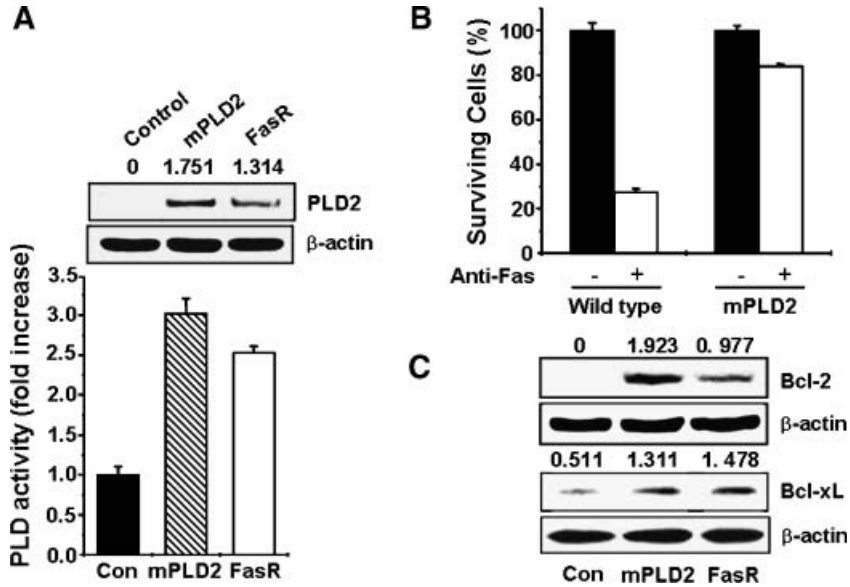

Fig. 2. Bcl-2 expression levels and cell viabilities of wild-type, Fas-resistant, and mouse PLD2 (mPLD2) cDNA-transfected A20 cells. mPLD2 gene inserted in pCGN vector was introduced into wild-type cells, using Cell Line Nucleofector™ Kit R (Amaxa Biosystems), according to the manufacturer's instruction. **A**: Wild-type, Fas-resistant, and mPLD2-transfected A20 cells were lysed and equal amounts of protein (40 μg) were subjected to Western analysis using anti-PLD antibody. The level of basal PLD activity was determined as described in Materials and Methods. The data in this figure are expressed as fold increase relative to PEt level of wild-type cell and are mean ± SD from three independent experiments. **B**: The cells were treated with or without 0.2 μg/ml of anti-Fas antibody for 3 h. Cell viabilities were measured, using MTT assay. The data in this figure are expressed as percentage relative to cells not treated with anti-Fas antibody. **C**: Cells were lysed and 40 μg of protein aliquots from each sample were subjected to SDS–PAGE. The expression levels of Bcl-2 and Bcl-xL were determined by Western blotting by using anti-Bcl-2 or Bcl-xL antibodies. The relative quantities of each band, normalized to control cells, are mean ± SD from the results quantified three times using QuantityOne software (Bio-Rad).

[Erickson et al., 1999]. Therefore, we pretreated the wild-type A20 cells with 10 μM propranolol, a well-known PAP inhibitor, for 30 min just before the treatment with 70 μM PA for 3 h. As shown in Figure 5A, PA-induced expressions of Bcl-2 and Bcl-xL proteins were slightly augmented by pretreatment of propranolol. As expected, cell viability in the propranolol-treated cells was increased in response to anti-Fas antibody treatment (Fig. 5B). These results suggest that PA induced-survival signal does not pass through DAG pathway by PAP.

Mepacrine, a PLA_2 Inhibitor, Prevents the Expressions of Bcl-2 and Bcl-xL Proteins

To investigate the role of PLA_2 in the expressions of Bcl-2 and Bcl-xL proteins, the wild-type A20 cells were pretreated with 100 μM mepacrine, a PLA_2 inhibitor, for 30 min before the treatment with 70 μM PA for 3 h. As shown in Figure 6A, pretreatment of the cells with mepacrine completely blocked PA-induced expressions of Bcl-2 and Bcl-xL proteins. As expected, PA-induced restoration of cell viability against Fas-induced cell death disappeared completely by mepacrine treatment (Fig. 6B).

PLA_2 is an Important Enzyme for PA-Induced Anti-Apoptosis

To confirm again that PA can protect cells from apoptosis induced by Fas activation, we examined the expression level of cleaved PARP, an indicator of apoptosis. First, to obtain optimal condition for observation of cleaved PARP, cells were incubated with 0.2 μg/ml or 1 μg/ml anti-Fas mAb for times indicated. As shown in Figure 7A, treatment with anti-Fas mAb increased cleaved PARP protein in a time-dependent manner at both concentrations. PA (70 μM) pretreatment for 3 h blocked PARP cleavage observed in the wild-type A20 cells treated with 0.2 μg/ml anti-Fas mAb for 3 h (Fig. 7B,C). Pretreatment of 10 μM propranolol for 30 min before treatment of 0.2 μg/ml anti-Fas mAb and/or 70 μM PA prevented PARP cleavage more effectively (Fig. 7B). In contrast, pretreatment of mepacrine (100 μM) for 30 min prior to

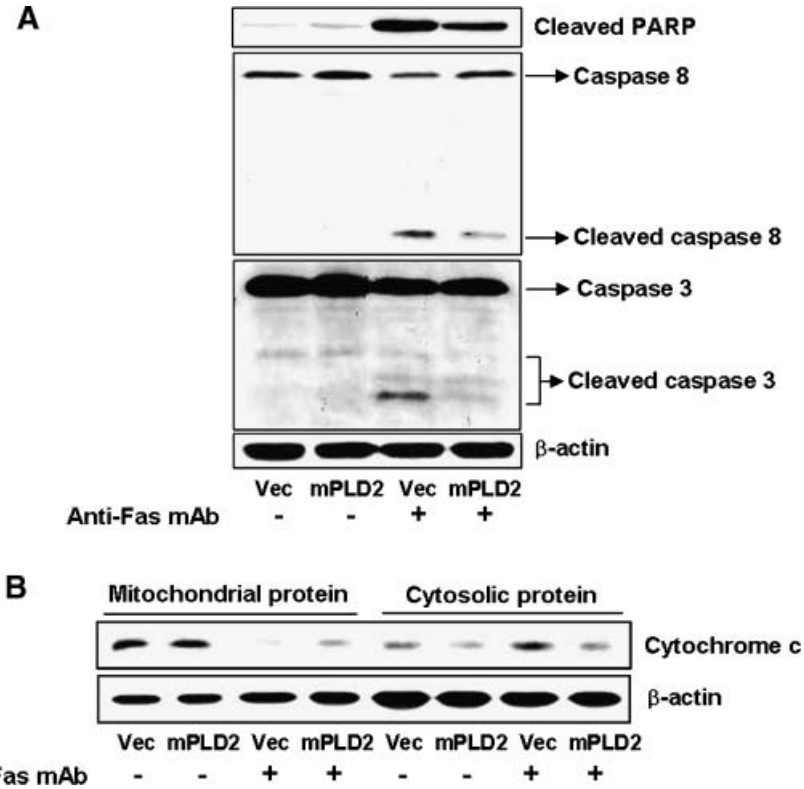

Fig. 3. Effect of PLD2 on Fas-induced cleavages of caspase-8 and -3, and cytochrome *c* release. mPLD2 gene inserted in pCGN vector was introduced into wild-type cells, using Cell Line Nucleofector™ Kit R (Amaxa Biosystems), according to the manufacturer's instruction. pCGN vector and mPLD2-transfected A20 cells were treated with anti-Fas mAb for 12 h. **A**: Equal amounts of protein (40 μg) were resolved by 10% SDS–PAGE and immunoblotted by using their specific antibodies. **B**: Equal amounts of mitochondrial and cytosolic protein (50 μg) were resolved by 12% SDS–PAGE and immunoblotted by using anti-monoclonal cytochrome *c* antibody for detection of cytochrome *c* release.

treatment of 70 μM PA failed to inhibit PARP cleavage (Fig. 7C). These results indicate that PA attenuates Fas-mediated apoptosis after hydrolysis by PLA_2, implying that one of PLA_2 products (LPA or AA) might be involved in delaying Fas-induced cell death as well as controlling the expression of anti-apoptotic proteins for the rescue of cells from Fas-induced apoptosis.

DPPA Fails to Trigger the Expressions of Anti-Apoptotic Bcl-2 and Bcl-xL Proteins

PA-specific PLA_2 converts PA into AA and LPA [Erickson et al., 1999]. To confirm which of the PA metabolites was involved in increased expressions of Bcl-2 and Bcl-xL, we treated the cells with 140 μM DPPA for indicated times. As shown in Figure 8, DPPA was not able to increase the expression of both Bcl-2 and Bcl-xL proteins, strongly indicating that AA, not LPA, acted as an important metabolite for the expression of these anti-apoptotic Bcl-2 family proteins.

AA-COX-2 Pathway is Involved in PA-Induced Expression of Bcl-2 Family Proteins

We investigated whether PA was involved in the induction of COX-2 protein, because AA is known to increase COX-2 expression [Herrmann et al., 1997]. As shown in Figure 9A, PA significantly increased COX-2 expression, and PA-induced COX-2 expression as well as the expressions of Bcl-2 and Bcl-xL proteins were blocked by pretreatment of mepacrine (Fig. 9A). To further establish the relationship between PA and AA-COX-2 pathway, cells were pretreated with 10 μM NS-398, a specific inhibitor of COX-2, before treatment of PA. Figure 9B shows that PA-induced expressions of Bcl-2 and Bcl-xL proteins were notably inhibited by NS-398, indicating that PA increased the expression of both Bcl-2 and Bcl-xL proteins through AA-COX-2 pathway.

Taken together, our findings indicate that PA generated by PLD2 upregulates the expression

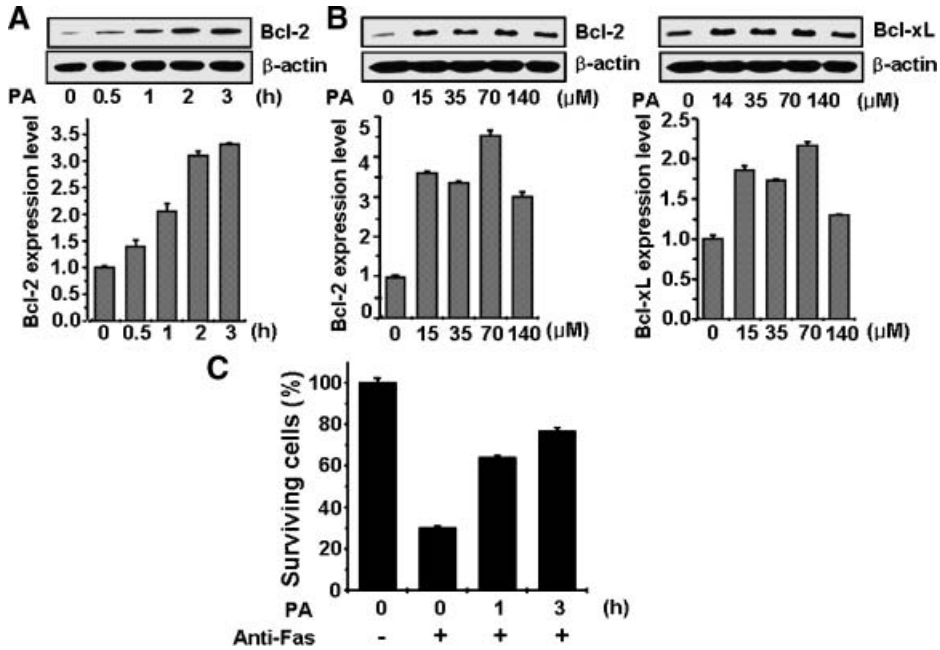

Fig. 4. Effect of PA on the expressions of Bcl-2/Bcl-xL and cell viability in wild-type A20 cells. **A**: Wild-type A20 cells on 100 mm dishes (1×10^6 cells/ml) were further cultured without FBS for 18 h, and treated with 140 μM PA for indicated times or (**B**) different concentrations of PA for 3 h. Equal amounts of protein (40 μg) were resolved by 12% SDS–PAGE and immunoblotted with anti Bcl-2 or anti-Bcl-xL monoclonal antibody. The relative quantities of each band, normalized to control cells, are mean ± SD from the results quantified three times using QuantityOne software (Bio-Rad). **C**: Wild-type A20 cells on 96-well plates were further cultured without FBS for 18 h. Cells were treated with 0.2 μg/ml of anti-Fas antibody for 3 h after treatment with PA for 1 or 3 h. Cell viabilities were measured, using MTT assay. The data in this figure are expressed as percentage relative to cells not treated with PA and anti-Fas antibody.

of both Bcl-2 and Bcl-xL proteins via PLA_2 and AA-COX-2 pathway (Fig. 10).

DISCUSSION

Bcl-2 and Bcl-xL play an important role in inhibition of apoptotic cell death [Adams and Cory, 1998; Liu et al., 2001]. They are integral membrane proteins localized mainly on the outer mitochondrial membranes and endoplasmic reticulum. All activities of the mitochondria in apoptotic signal can be blocked by Bcl-2 and Bcl-xL [Shimizu et al., 1996; Adachi et al., 1997; Kim et al., 1997; Vander Heiden et al., 1997]. Many pro-apoptotic signals converge at mitochondria and change mitochondrial membrane permeability, resulting in the release of several mitochondrial proteins into the cytoplasm which triggers the major event of mitochondria-mediated apoptosis. Pro-apoptotic proteins released from mitochondria contain cytochrome *c*, resulting in activation of caspase-3 by formation of apoptosome composed of caspase 9, apoptotic protease activating factor (Aparf-1), and dATP [Gulbins et al., 2003]. This cytosolic cytochrome *c* is necessary for the initiation of the apoptotic program. Both Bcl-2 and Bcl-xL, shown to be functionally equivalent, inhibit mitochondrial loss of cytochrome *c* [Adachi et al., 1997; Kim et al., 1997; Kluck et al., 1997; Yang et al., 1997]. Involvement of mitochondria, which serves as a gatekeeper to various pro-apoptotic proteins, also is shown for Fas-mediated apoptosis [Gulbins et al., 2003]. Upon Fas-activation, mitochondrial apoptogenic activities can also be blocked by Bcl-2 and Bcl-xL [Jaattela et al., 1995; Adachi et al., 1997; Scaffidi et al., 1998; Srinivasan et al., 1998].

PLD2, having high basal PLD activity, can be a crucial component for cell survival against apoptosis induced by various stimuli [Lee et al., 2000; Lim et al., 2002; Chen et al., 2005]. Recently, we reported that PLD2, as a survival factor against Fas-mediated apoptosis, was overexpressed in FasR cells [Lim et al., 2002]. In this study, we further investigated how PLD2 is involved in anti-apoptotic signaling pathway, and consequently found that PLD2 up-regulates the protein expressions of Bcl-2

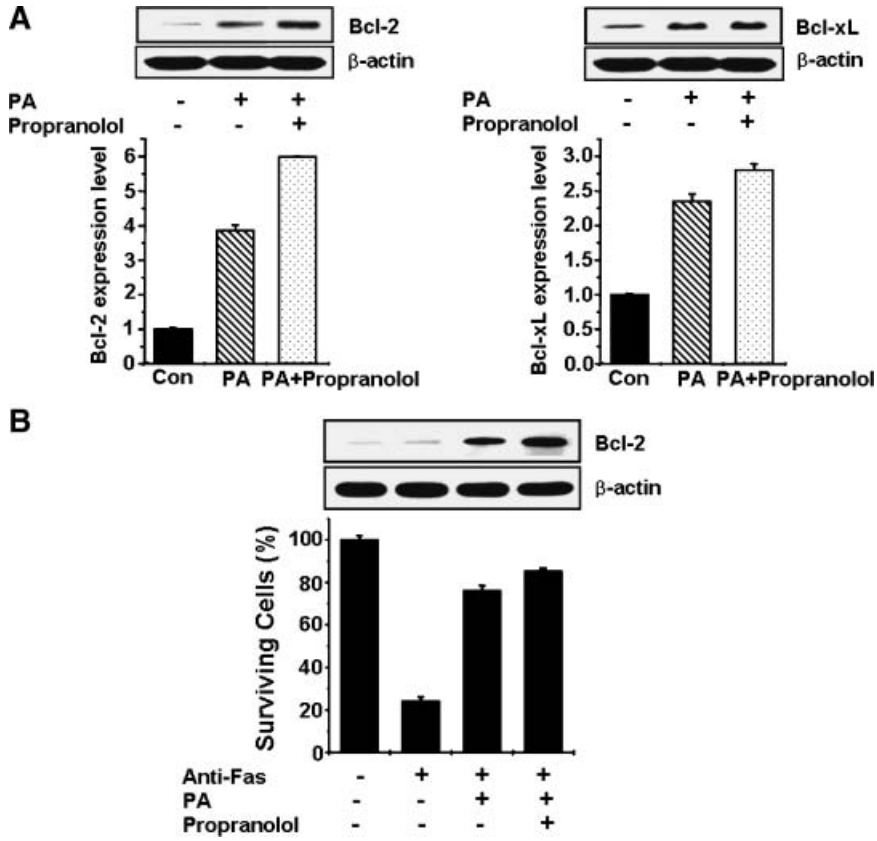

Fig. 5. Effects of propranolol on PA-induced expressions of Bcl-2 and Bcl-xL. **A**: Wild-type A20 cells were treated with 70 μM PA for 3 h after pretreatment with 10 μM propranolol for 30 min. Protein samples from cell lysates were subjected to immunoblot analysis using monoclonal Bcl-2 and polyclonal Bcl-xL antibodies. The relative quantities of each band, normalized to control cells, are mean ± SD from the results quantified three times using QuantityOne software (Bio-Rad). **B**: Wild-type A20 cells were treated with 70 μM PA for 3 h after pretreatment with 10 μM propranolol for 30 min. Forty micrograms of protein aliquots of each cell lysate were subjected to immunoblot analysis using anti-Bcl-2 monoclonal antibody, shown in upper box. PA-treated cells were washed with PBS and transferred onto 96-well plates (1×10^5 cells/ml) and treated with or without 0.2 μg/ml of anti-Fas antibody for 3 h. Cell viabilities were measured using MTT assay. The data in this figure are expressed as percentage relative to cells not treated with anti-Fas antibody.

and Bcl-xL via AA and COX-2 signaling pathway for cell survival in this model. To clarify the role of PLD2, which had been designated as a survival factor of FasR cells [Lim et al., 2002], A20 wild-type cells were treated with anti-Fas mAb after transfection with mouse PLD2 cDNA. As expected, mPLD2-transfected A20 wild-type cells showed markedly increased resistance to Fas-induced apoptosis, indicating that PLD2 is a very important enzyme for cell survival during Fas-induced apoptosis. However, the relationship between constitutive PLD2 overexpression observed in FasR and PLD2 overexpression in wild-type A20 cells is not clear. In wild-type A20 cells, to escape from Fas-mediated apoptosis, anti-Fas mAb stimulates PLD activity via Ras, PC-PLC and PKC pathway [Shin and Han, 2000a,b].

The data presented in this study indicate that PLD2 overexpression enables A20 cells to resist Fas-induced apoptosis by increasing anti-apoptotic proteins, Bcl-2 and Bcl-xL, by increasing intracellular PA level. However, it is not clear at present which event is directly responsible for the increased PLD2 expression in FasR. It is possible that there may be some mutations on PLD2 promoter, defect in the normal degradation of PLD2 or up-regulation of some transcription factors which bind to PLD2 promoter. Therefore, it is of great importance to elucidate whether FasR clone and wild-type A20 cells transfected with mouse PLD2 cDNA use the same intracellular mechanism for cell survival.

In general, PLD functions through the generation of PA, which is a functional and

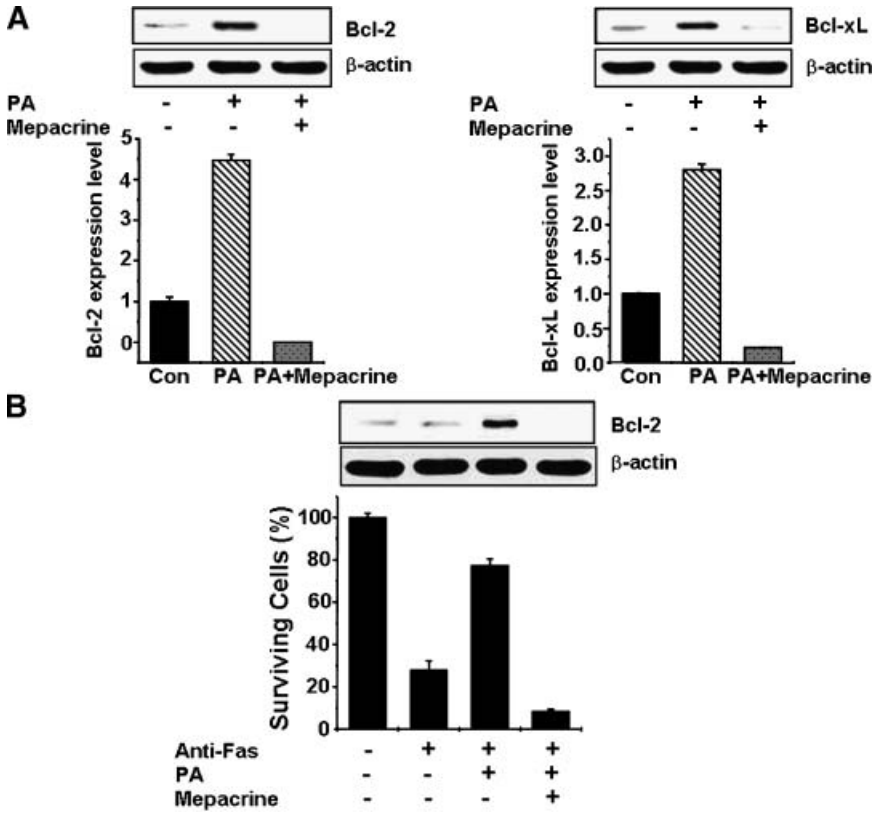

Fig. 6. Effects of mepacrine on PA-induced expressions of Bcl-2 and Bcl-xL. **A**: Wild-type A20 cells were treated with 70 μM PA for 3 h after pretreatment with 100 μM mepacrine for 30 min. The levels of Bcl-2 and Bcl-xL were determined by Western Blot using each antibody. The relative quantities of each band, normalized to control cells, were quantified using QuantityOne software (Bio-Rad). **B**: Wild-type A20 cells were treated with 70 μM PA for 3 h after pretreatment with 100 μM mepacrine for 30 min. Cells were lysed, and 40 μg protein aliquots from each cell lysate were subjected to immunoblot analysis using anti-Bcl-2 monoclonal antibody, shown in upper box. PA-treated cells were washed with PBS and transferred onto 96-well plates (1×10^5 cells/ml) and treated with or without 0.2 μg/ml of anti-Fas antibody for 3 h. Cell viabilities were measured using MTT assay. The data in this figure are expressed as percentage relative to cells not treated with anti-Fas antibody.

enzymatic product of PLD. After treatment of wild-type A20 cells with PA, the expressions of Bcl-2 and Bcl-xL proteins were increased, but PA treatment did not change the expression of pro-apoptotic Bcl-2 protein family such as Bad (data not shown). Bcl-2 family members are crucial regulators of apoptosis. The Bcl-2 family includes both anti-apoptotic molecules such as Bcl-2 and Bcl-xL, as well as pro-apoptotic molecules such as Bax, Bak, Bid, and Bad. Bcl-2 and Bcl-xL inhibit apoptosis in many types of cells including neuronal apoptotic death [Adams and Cory, 1998; Liu et al., 2001]. Those are highly activated in pancreatic cancer [Mohammad et al., 2005], and have become attractive targets for anti-cancer drug because of anti-apoptotic function [Enyedy et al., 2001; Wang et al., 2003]. By PAP, PA is converted to DAG and hydrolyzed by PLA_2 to result in AA and LPA. In our data, pretreatment of A20 cells with propranolol enhanced PA-induced expressions of Bcl-2 and Bcl-xL, indicating that inhibition of PA hydrolysis by PAP augmented PA accumulation in the cells. In contrast, pretreatment of mepacrine (PLA_2 inhibitor) completely suppressed PA-induced expressions of Bcl-2 and Bcl-xL, suggesting that PLA_2 metabolite of PA is involved in the increased expressions of anti-apoptotic Bcl-2 and Bcl-xL proteins.

PLA_2 is the specific enzyme to release AA from PA. AA, a polyunsaturated fatty acid, can be converted by COX to prostaglandin [Herrmann et al., 1997]. COX-2 expression can also inhibit Fas-mediated apoptosis [Nzeako et al., 2002]. In the present study, treatment of A20 cells with PA increased COX-2 expression. When COX-2 was blocked with NS-398, COX-2 inhibitor, PA-induced expressions of Bcl-2 and Bcl-xL proteins were suppressed. It

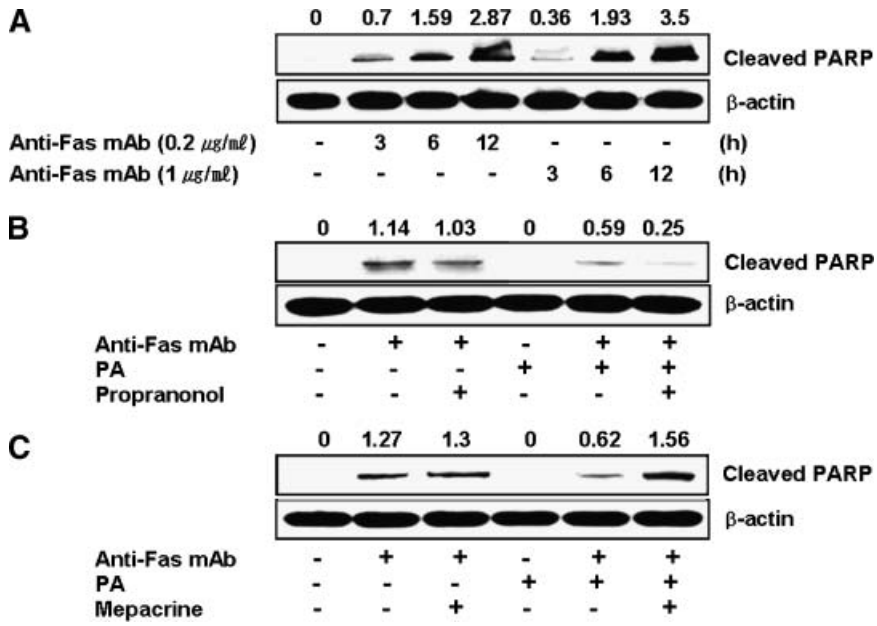

Fig. 7. Effects of pretreatment with propranolol or mepacrine on PA-induced anti-apoptosis against Fas activation. **A**: Wild-type A20 cells were treated with 0.2 μg/ml or 1 μg/ml of anti-Fas mAb for times indicated. **B**: The cells were stimulated with 70 μM PA for 3 h after pretreatment with 10 μM propranolol for 30 min, and then were incubated with 0.2 μg/ml anti-Fas mAb for 3 h. **C**: The cells were stimulated with 70 μM PA for 3 h after pretreatment with 100 μM mepacrine for 30 min, and were incubated with 0.2 μg/ml anti-Fas mAb for 3 h. Equal protein aliquots of cell lysates (40 μg) were analyzed by Western blotting, using anti-cleaved PARP antibody. The relative quantities of each band, normalized to control cells, are mean ± SD from the results quantified three times using QuantityOne software (Bio-Rad).

should be noted, however, that LPA, another PLA_2 product of PA, should also be considered. Some studies describe that PLD and PLA_2 are involved in the synthesis of LPA [Erickson et al., 1999; Moolenaar et al., 2004], and LPA increases Bcl-2 expression in lymphocytic [Hu et al., 2005] and epithelial cells [Deng et al., 2003]. To find out which PLA_2 product of PA was involved in the increased productions of Bcl-2 and Bcl-xL in A20 cells, we used DPPA, which has two palmitic acids instead of AA. However, treatment of DPPA failed to increase the expressions of Bcl-2 and Bcl-xL, indicating that AA is more important than LPA in the anti-apoptotic signaling pathway.

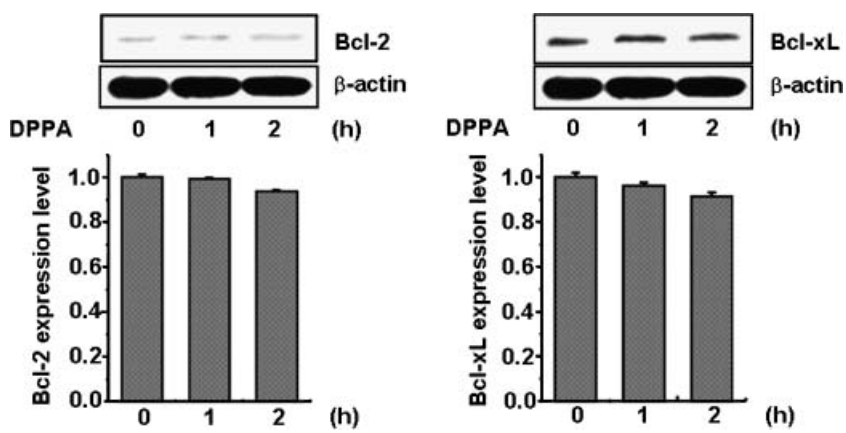

Fig. 8. Effects of DPPA on the expressions of Bcl-2 and Bcl-xL. Wild-type A20 cells were treated with or without 140 μM DPPA for times indicated. The expression levels of Bcl-2 and Bcl-xL were determined by Western blot, using their specific antibodies. The relative quantities of each band, normalized to control cells, are mean ± SD from the results quantified three times using QuantityOne software (Bio-Rad).

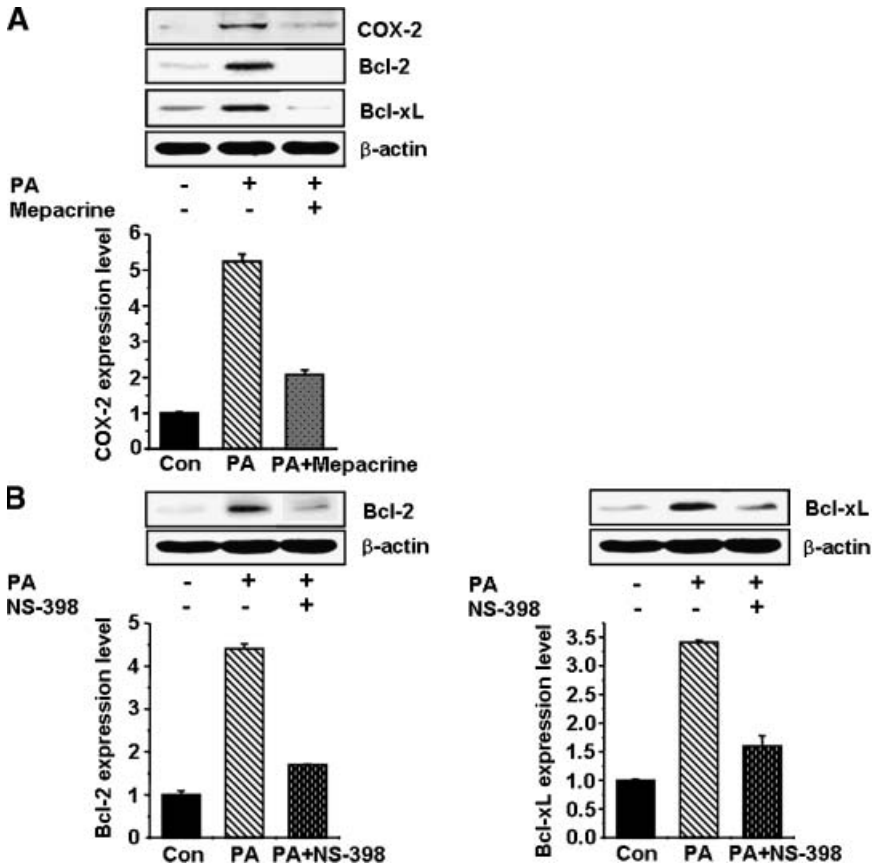

Fig. 9. Involvement of AA-COX pathway in PA-induced expressions of Bcl-2 and Bcl-xL. **A**: Wild-type A20 cells were incubated with 70 μM PA for 3 h after pretreatment with 100 μM mepacrine for 30 min. The expression of each protein was visualized by immunoblotting using specific antibodies. **B**: Wild-type A20 cells were pretreated with 10 μM NS-398 for 24 h followed by stimulation with 70 μM PA for 3 h. The expressions of anti-apoptotic proteins were detected by Western blotting, using anti-Bcl-2 and Bcl-xL antibodies. The relative quantities of each band, normalized to control cells, are mean ± SD from the results quantified three times using QuantityOne software (Bio-Rad).

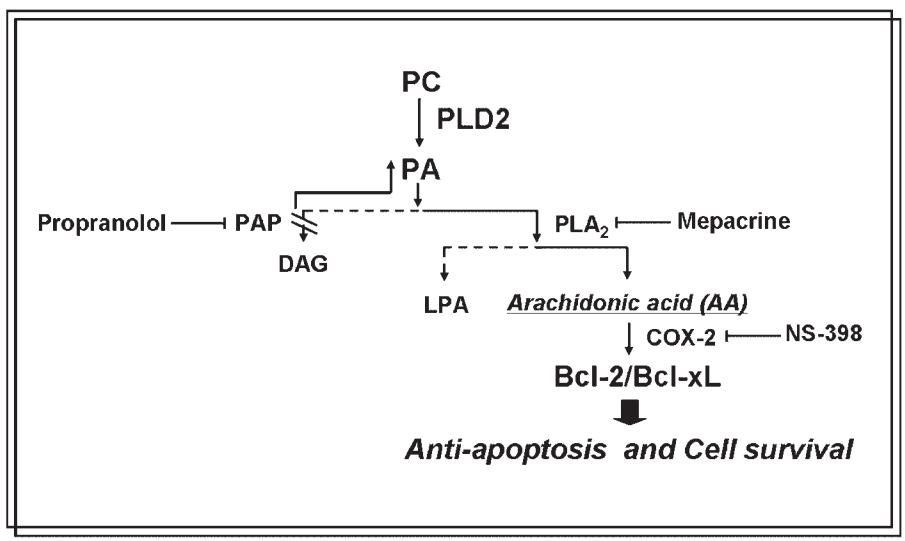

Fig. 10. The proposed model for the signaling pathway of PLD2 which is involved in cell survival during Fas-induced apoptosis. Our data suggest that PA generated by PLD2 is converted to arachidonic acid (AA) via phospholipase A_2 (PLA_2). Briefly, intracellular PA level can be increased by PLD2 overexpression and PAP inhibition. Subsequently, AA acts as an important signaling molecule to increase the expressions of Bcl-2 and Bcl-xL through COX-2. \\ means blockage of signal. And ↑ means PA accumulation by preventing PA hydrolasis induced by PAP.

To sum up, data in the present study indicate that PLD2 induces increased expressions of Bcl-2 and Bcl-xL proteins, as a survival factor during Fas-induced apoptosis. Furthermore, AA and COX-2 are the most responsible for the increased expressions of these proteins in A20 cells. To the best of our knowledge, our studies are the first report on a novel anti-apoptotic signaling pathway induced by PLD2 during Fas-mediated apoptosis, and appear to provide another anti-apoptotic pathway related to Bcl-2 and Bcl-xL.

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