FEBS Letters 580 (2006) 6224–6232

Phospholipase D protects ECV304 cells against TNFα-induced apoptosis

Helene Birbes[1,2], Caroline Zeiller[2], Hiba Komati, Georges Némoz, Michel Lagarde, Annie-France Prigent*

INSERM U585 and INSA-Lyon and IMBL, Bldg Louis Pasteur,11 avenue Jean Capelle, F-69621 Villeurbanne Cedex, France

Received 28 July 2006; revised 4 October 2006; accepted 9 October 2006

Available online 19 October 2006

Edited by Vladimir Skulachev

Abstract **Tumor necrosis factor α (TNFα), a pleiotropic cytokine, activates both apoptotic and pro-survival signals depending on the cell model. Using ECV304 cells, which can be made TNFα-sensitive by cycloheximide (CHX) co-treatment, we evaluated the potential roles of ceramide and phospholipase D (PLD) in TNFα-induced apoptosis. TNFα/CHX induced a robust increase in ceramide levels after 16 h of treatment when cell death was maximal. PLD activity was increased at early time point (1 h) whereas both PLD activity and PLD1 protein were strongly decreased after 24 h. TNFα/CHX-induced cell death was significantly lowered by exogenous bacterial PLD and phoshatidic acid, and in cells overexpressing PLD1. Conversely, cells depleted in PLD proteins by small interference RNA (siRNA) treatment exhibited higher susceptibility to apoptosis. These results show that PLD exerts a protective role against TNFα-induced cell death.**

Keywords: Phospholipase D; siRNA; Tumor necrosis factor α; Ceramide; Apoptosis; Survival; ECV304 cell

1. Introduction

Tumor necrosis factor α (TNFα) is a pleiotropic cytokine that exerts various biological effects in different cell types. In numerous tumor cells or virally infected cells, TNFα can initiate an apoptotic cascade that involves a variety of mediators and regulators including proteases, the sphingolipid ceramide, and members of the Bcl-2 family. However, most normal cells including endothelial cells are resistant to TNFα [1–3], but can be rendered sensitive when protein or RNA synthesis inhibitors such as cycloheximide (CHX) or actinomycin D are associated to TNFα treatment [2,4]. The sensitizing effect of CHX to TNFα treatment is believed to result from the inhibition of synthesis of some NF-κB-dependent survival factors [5,6]. The death induced by TNFα in the presence of CHX shows typical morphological and biochemical features of apoptosis and is blocked by anti-apoptotic proteins such as E1B, a viral homologue of Bcl-2 protein [7] or the cowpox virus CrmA protein [8]. Although the role of ceramide as a second messenger in apoptosis signaling has been a matter of debate, its involvement in the transmission of the death signal initiated by TNF receptor activation is now well recognized. In different cell types, TNFα has been shown to increase intracellular ceramide levels either through the activation of sphingomyelinases (SMases) or by a mechanism involving de novo ceramide synthesis [9–11].

On the other hand, phospholipase D (PLD) through its reaction product, phosphatidic acid (PA), has been shown to play a key role in the proliferation of various cell models, and there is a growing body of evidence linking PLD activity with mitogenic signaling. Thus, PLD activity has been shown to increase in response to various growth factors and in cells transformed by a variety of oncogenes [12]. However, the role of PLD in apoptosis remains controversial, both pro- and anti-apoptotic effects being reported depending on the cell type and the apoptotic stimulus considered [13,14]. Indeed, some reports indicated that PLD activity is upregulated during apoptosis of hematopoietic cells such as Jurkat T-cells [15] and murine B lymphoma A20 cells [16] or normal rat fibroblasts [17]. Conversely, a downregulation of PLD has been reported to occur during the ceramide-induced apoptosis of rat C6 glial cells [18] and the human keratinocyte cell line HaCaT [19]. Interestingly, recent studies are beginning to point out a protective role of PLD against apoptosis of either normal [20,21] or transformed cells [22,23] induced by different stimuli.

In the present study, we set out to investigate whether ceramide and PLD were involved in TNFα-induced apoptosis of ECV304 cells, a model of TNFα-resistant cells, which can be made TNFα-sensitive by CHX co-treatment. We first investigated the involvement of ceramide in the apoptotic response to TNFα/CHX. Then, we examined whether the apoptotic response of ECV304 cells to TNFα could be modified by changes in their PLD activity or PA content. PLD activity increases were mimicked either by addition of exogenous bacterial PLD or PA, or through PLD1 and PLD2 overexpression, whereas 1-butanol which prevents normal PLD functioning and PLDI- and PLD2-siRNA were used to lower endogenous PLD activity. Herein, our data provide evidence that PLD has a survival role counteracting TNFα-induced cell death in ECV304 cells.

*Corresponding author. Fax: +33 4 72 43 85 24.
E-mail address: annie-france.prigent@insa-lyon.fr (A.-F. Prigent).

[1]Present address: Université Pierre et Marie Curie-Paris 6, INSERM UMR 706, Institut du Fer-à-Moulin, 17 rue du Fer-à-Moulin, Paris, F-75005.
[2] These authors contributed equally to this work.

Abbreviations: Cer, ceramide; TNFα, Tumor necrosis factor α; PLD, phospholipase D; PA, phosphatidic acid; DGK, diacylglycerol kinase; DAG, diacylglycerol; SMase, sphingomyelinase; PBS, phosphate-buffered saline

0014-5793/$32.00
doi:10.1016/j.febslet.2006.10.026

2. Materials and methods

2.1. Materials

TNFα was from Peprotech (AbCys, Paris, France). Triton X-100 was from Pierce. Silica Gel G60 and LK6D Silica Gel G60 (Whatman) TLC plates were from VWR International (Fontenay-sous-Bois, France). [^{33}P]-ATP, [^{3}H]-palmitic acid, MP Hyperfilm, ECL were from Amersham Biosciences (Orsay, France). HRP-conjugated anti-mouse IgG antibody was from Jackson Immunoresearch Laboratories (Soham, UK). Bradford protein assay and HRP-conjugated anti-rabbit IgG antibody were from Bio Rad (Marnes-La-Coquette, France). Immobilon P membranes were from Millipore (St Quentin Yvelines, France). Annexin V-conjugated Alexa Fluor 594 was from Molecular Probes. Phospholipase D from *Streptomyces chromofuscus*, L-α dipalmitoyl and L-α dioctanoyl phosphatidic acids (sodium salts), anti α-tubulin monoclonal antibody were from Sigma–Aldrich (L'Isle d'Abeau, France). *Escherichia coli* DAG Kinase and cycloheximide were from Calbiochem (Merck Biosciences, France). TransPEI transfection reagent and negative control siRNA were from Eurogentec (Anger, France). C_2-ceramide and dihydroceramide were purchased from Avanti Polar Lipids (Biovalley, France).

2.2. Cell culture

ECV-304, an immortalized human vascular endothelial cell line, was obtained from American Type Culture Collection (Rockville, MD). Cells were grown in M199 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, in 5% CO_2 at 37 °C in a humidified incubator. Treatments were carried out on 80–90% confluent cells.

2.3. Cell viability assay

Viability of cultured cells was measured at various time points using the trypan blue exclusion test or the MTT colorimetric assay (Roche Diagnostics, Meylan, France) as described previously by Mosmann [24]. In this latter case, cells were cultured in 96-well culture plates at a cell density of 10^5 cells/ml. At the end of the treatment period in absence or presence of TNFα or TNFα/CHX, 10 μl of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) labeling reagent (0.5 mg/ml final concentration) was added to each well. The cells were incubated for further 4 h. 100 μl solubilization solution was then added and plates were allowed to stand overnight at 37 °C in a humidified atmosphere. The optical density (OD) was measured the next day at 550 and 690 nm wavelength using an ELISA plate reader (PowerWave X, Biotek Instruments, Winooski, USA). The number of viable cells was directly correlated to the difference of absorbance measured at 550 and 690 nm. Results were normalized relative to their respective controls (without TNFα or TNFα/CHX) taken as 100. In some experiments, apoptosis was ascertained by annexin-V binding. After treatment, cells were washed once with PBS then incubated with 1 μg/ml annexin-V conjugated to Alexa Fluor 594 (Molecular Probes) in annexin-binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$) at 37 °C. After 30 min of incubation, cells were examined by fluorescence microscopy and counted for annexin-V binding. Different fields (at least 300 cells) were counted for each experiment.

2.4. Ceramide measurement

ECV 304 cells cultured in 10 cm Petri dishes were treated for the indicated times with TNFα in the presence or absence of CHX. Culture medium was removed and centrifuged to collect floated dead cells. Attached cells were washed twice with ice-cold PBS, scraped in 1 ml methanol, and pooled with pelleted floating cells. Total lipids were then extracted by the method of Bligh and Dyer [25]. Lipids were then dried and an aliquot was taken from each sample for total lipid phosphate determination as described [26]. Measurement of ceramide levels was performed using the DAG kinase assay as previously described [26,27]. Briefly, lipid samples and standards were sonicated in 20 μl of mixed micelles (7.5% β-*n*-octyl-D-glucopyranoside/25 mM dioleyl phosphatidylglycerol) and incubated for 30 min at 37°C. Then, 70 μl of enzyme reaction buffer (75 mM imidazole pH 6.6, 71 mM LiCl, 17.8 mM $MgCl_2$, 1.5 mM EGTA, 0.25 mM DTPA, 2.8 mM DTT and 3 μg *E. coli* DAG kinase) and 10 μl of ATP mixture (10 mM ATP and 0.2 μCi/μl [γ-^{33}P]ATP in 5 mM imidazole) was added to samples, and the mixture incubated for 1 h at room temperature. Phosphorylated ceramide and DAG were extracted with chloroform/methanol. Lipid extracts, DAG and ceramide standards (0–600 pmol) were then spotted onto TLC plates and developed in chloroform/acetone/methanol/acetic acid/ water (50:20:15:10:5, by volume). Radioactive PA and ceramide-1-P were visualized and quantified by autoradiography.

2.5. PLD assay in intact cells

PLD was determined on the basis of its transphosphatidylation activity. As it is known that serum starvation increases ceramide levels, ECV304 cells were labeled in complete medium containing 2 μCi/ml [^{3}H]-palmitic acid for 24 h at 37 °C. Cells were then washed twice with PBS, shifted to complete culture medium and treated for the indicated times with CHX or with TNFα plus CHX; 1-butanol (1% final concentration) was added during the last 30 min of treatment. Cells were then collected and lipids extracted by the method of Bligh and Dyer in the presence of 50 μM butylhydroxylated toluene. Phosphatidylbutanol was separated by bidimensional TLC using chloroform/methanol/28% ammonia (65:35:5.5, by volume) for the first migration, and ethyl acetate/isooctane/acetic acid (9:5:2, by volume) for migration in the second dimension. TLC plates were then stained with Coomassie Brilliant Blue R and phosphatidylbutanol spots were scraped off and the radioactivity determined by liquid scintillation counting. Radioactivity associated with phosphatidylbutanol was expressed as percentage of total phospholipid radioactivity.

2.6. Transient transfections

pEGFP-PLD1b and -PLD2 constructs were prepared as previously described [28]. Transient transfections using transPEI reagent were performed according to the manufacturer's recommendations. Briefly, transPEI reagent diluted in 150 mM NaCl was mixed with 4.5 μg pEGFP-PLDI or pEGFP-PLD2 DNA and left in contact for 30 min. The mix was then added dropwise to 3×10^5 cells suspended in 4.5 ml of complete culture medium. The cells were plated (10^5 cells/well) and cultured for 12 h in complete culture medium before TNFα or TNFα/CHX treatment for an additional 9 h period. Control cells were transfected with 4.5 μg of pEGFP DNA (empty vector) in the same experimental conditions. SiRNA for PLD1 (hPLD1 target sequence: AAGTTAAGAGGAAATTCAAGC) and siRNA for PLD2 (hPLD2 target sequence: GACACAAAGTCTTGATGAG) were designed according to Fang et al. [29] and Powner et al. [30], respectively. A siRNA without sequence similarity with any known mammalian gene was used as a negative control. Transfection of siRNA was performed using Xtreme Gene reagent (Roche) with 25 nM siRNA in antibiotic-free medium. The cells were kept for 12 h in this medium and then shifted to complete culture medium for 24 h before treatment with TNFα/CHX.

2.7. Western blotting experiments

Cells were homogenized in 20 mM Tris/HCl, pH 7.6, buffer containing 100 mM NaCl, 1% Triton, and protease inhibitors cocktail. Cell lysates were mixed with Laemmli buffer supplemented with 2 M urea, boiled for precisely 1 min, and separated on 8% SDS–polyacrylamide gel including 4 M urea. The blots were probed with PLD1- and PLD2-specific polyclonal antibodies kindly provided by Dr S. Bourgoin (Laval University, Canada), diluted 1:2000. Immunoblots were revealed with the ECL detection system. After stripping, the membranes were reprobed with an anti-α-tubulin monoclonal antibody for normalization. Proteins were assayed by Bradford method.

2.8. Statistical analysis

Data were compared by ANOVA (Statview II for Macintosh) followed by protected *t* test for multiple comparison. Paired sample means were compared using the *t* test. *P* values of 0.05 or less were considered statistically significant.

3. Results and discussion

3.1. Cytotoxic effects of TNFα/CHX in ECV304 cells

TNFα has been shown to induce apoptotic signals together with the activation of survival pathways [31,32]. The survival pathways induced by TNFα are known to require de novo

protein synthesis, and blocking protein synthesis render the cells sensitive to TNFα-induced cell death [11]. Treatment of ECV304 cells for up to 24 h with TNFα (200 ng/ml) alone did not induce any cell death (Fig. 1A and B) showing that although ECV304 are immortalized cells, they are spontaneously resistant to the cytotoxic effects of TNFα. We thus considered to evaluate TNFα cytotoxicity in the absence of protein synthesis, and as a preliminary step, we addressed the effects of cycloheximide on protein synthesis in ECV304 cells. [^{3}H]-leucine incorporation was inhibited in a dose-dependent manner when ECV304 cells were treated with CHX. At a concentration of 10 μg/ml, CHX markedly inhibited [^{3}H]-leucine incorporation by around 97.5% (data not shown) but did not induce any cell death (Fig. 1B). In contrast, when cells were treated with increasing concentrations of TNFα in the presence of 10 μg/ml CHX, a dose-dependent decrease of cell survival was observed (Fig. 1A). The percentage of live cells decreased to approximately 25% of control after 24 h of treatment with the combination of TNFα (200 ng/ml) and CHX (10 μg/ml) as measured by MTT assay. Time-course studies showed that cell death induced by the combination of TNFα and CHX started from 6 h (around 15%) and reached a maximum at 16 h (around 75%), as evaluated by trypan blue exclusion test (Fig. 1B). Cells undergoing apoptosis present characteristic features, such as phosphatidylserine (PS) externalization, an early event in apoptosis. The presence of PS in the outer leaflet of ECV304 cell membrane was thus evaluated by means of specific PS labeling with annexin-V conjugated to a fluorescent dye. After 6 h of treatment with the combination of TNFα and CHX, the proportion of annexin-V labeled cells was markedly higher (around 30%) than the proportion of cells excluding the blue dye (Fig. 1C). This result indicates that, at this early time point, around half of cells with externalized PS were still alive. When cells were treated with TNFα or CHX alone the percentage of annexin-V positive cells remained lower than 7%, whatever the duration of treatment, and similar to that measured in untreated cells (Fig. 1C).

3.2. Effect of TNFα/CHX on ceramide levels

TNFα is known to increase the intracellular levels of ceramide during apoptosis in a number of cell types [9,10,27]. Treatment of ECV304 cells with TNFα alone did not induce

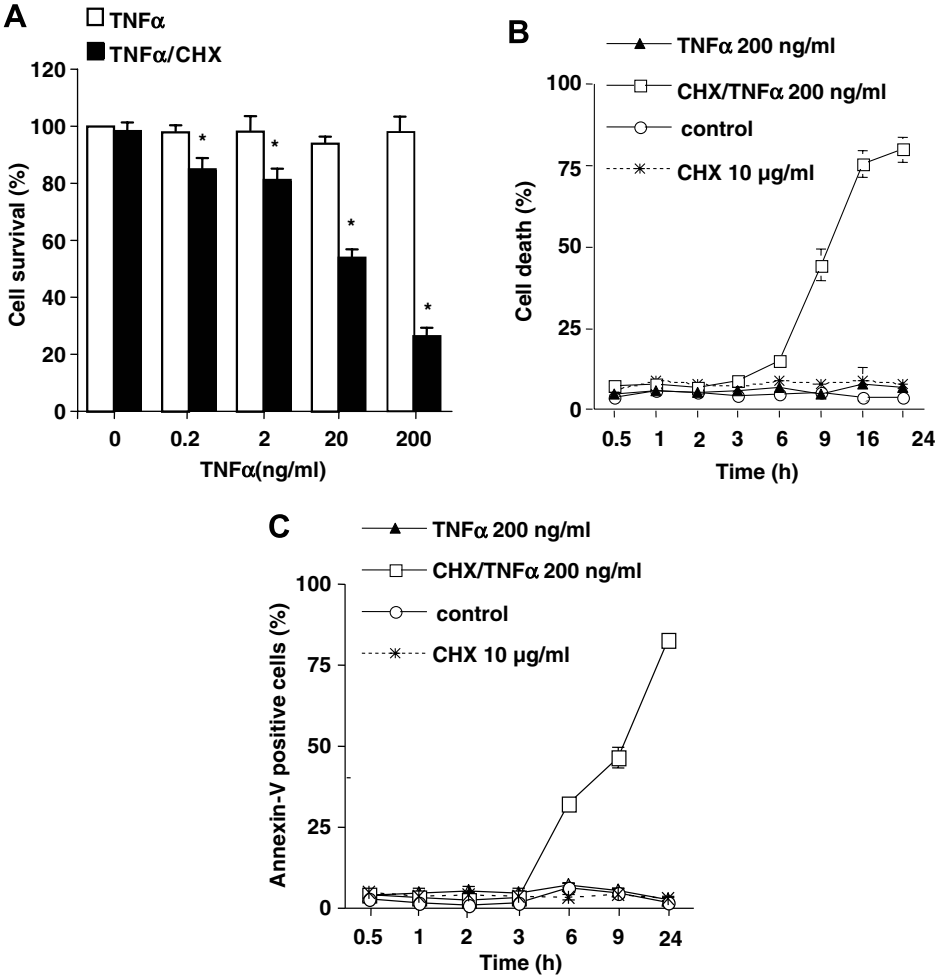

Fig. 1. Effect of TNFα/CHX on ECV304 cell death. (A). ECV304 cells were exposed to increasing concentrations of TNFα in the absence or presence of CHX (10 μg/ml) for 24 h. Cell survival was determined by MTT assay. Results are means ± S.E. of three separate experiments. ∗ indicates a significant difference ($P < 0.05$) as compared with TNFα alone. (B) EVC-304 cells were treated with 200 ng/ml TNFα in absence or presence of CHX (10 μg/ml) for the indicated periods of times. Cell death was determined by trypan blue exclusion assay. Data shown are means ± S.E. of at least three separate experiments. (C) ECV304 cells were treated as in B and cell death was estimated by annexin V-binding as described in Section 2. For each condition, the percentage of annexin-V labeled cells was determined in at least 10 different fields. Data shown are from two separate experiments.

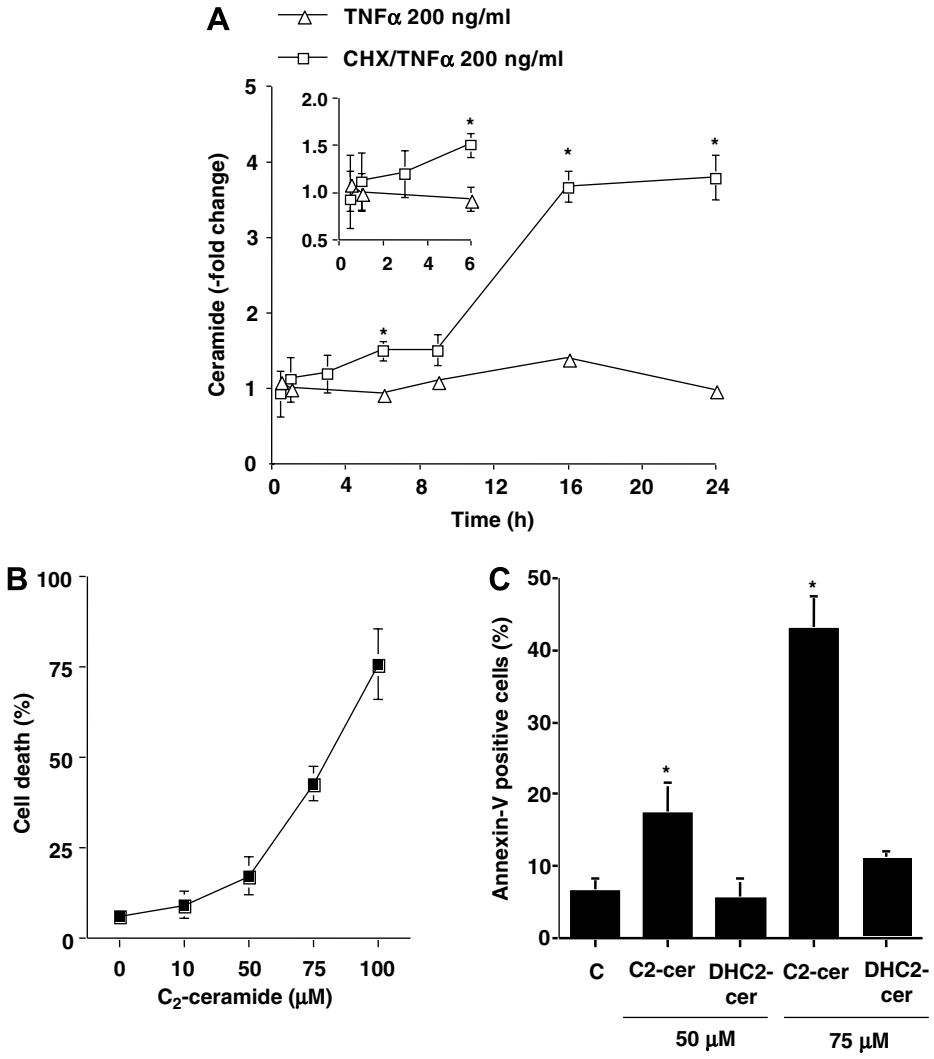

Fig. 2. Time-course of ceramide production and pro-apoptotic effects of C_2-ceramide. (A) ECV304 cells were treated with TNFα (200 ng/ml) in the absence or presence of CHX (10 μg/ml) for the indicated periods of times. At the end of treatment, lipids were extracted and ceramide levels were measured using the DGK assay as described in Section 2. Results are means ± S.E. of five to six independent experiments. The inset shows the early time points of ceramide production. ∗ indicates a significant difference ($P < 0.05$) as compared with TNFα alone. (B) ECV304 cells were treated with increasing concentrations of C_2-ceramide (0–100 μM) for 24 h and cell death was measured by trypan blue exclusion assay. (C) ECV304 cells were treated with 50 or 75 μM of C_2-ceramide (C_2-cer) or dihydroceramide (DHC_2-cer) for 24 h, and cell death was measured by annexin V-binding assay. ∗ indicates a significant difference ($P < 0.05$) as compared with the control.

significant changes in ceramide levels as compared to untreated control cells (Fig. 2A). In contrast, TNFα plus CHX treatment caused a biphasic increase of ceramide levels. A first and modest increase of ceramide level (1.5-fold) was observed at 6 h (Fig. 2A, inset). At later time points, a robust increase of ceramide levels was observed, which reached a maximum at 16 h.

3.3. Effect of exogenous ceramide on ECV304 cell death

The involvement of ceramide in ECV304 cell apoptosis induced by TNFα/CHX was further explored using a cell permeable ceramide. As shown in Fig. 2B, treatment of ECV304 cells with C_2-ceramide for 24 h, at concentrations ranging from 10 to 100 μM caused a dose-dependent cell death, with around 75% of the cells undergoing death at 100 μM. Apoptosis was also evaluated by assessment of annexin-V binding (Fig. 2C). In these experiments, 40% of cells treated with 75 μM C_2-ceramide underwent apoptosis. As a control, the effect of the biologically inactive ceramide precursor, C_2-dihydroceramide, was also evaluated. No significant pro-apoptotic effect was found at concentrations up to 75 μM (Fig. 2C). These results indicate that C_2-ceramide was able to induce apoptosis of ECV304, suggesting that cell death induced by TNFα/CHX could be mediated, at least partly, through the observed ceramide generation.

3.4. Effect of TNFα/CHX and exogenous ceramide on PLD activity and expression

We next investigated whether PLD activity and expression were modified after 1, 6, and 24 h of treatment with 200 ng/ml TNFα in the presence of 10 μg/ml CHX. After 1 h of treatment, a time point when neither ceramide level nor cell survival was affected, PLD activity was significantly increased (+40%, $P < 0.02$) as compared to cells incubated with CHX alone (Fig. 3B). After 6 h, which corresponds to the first modest

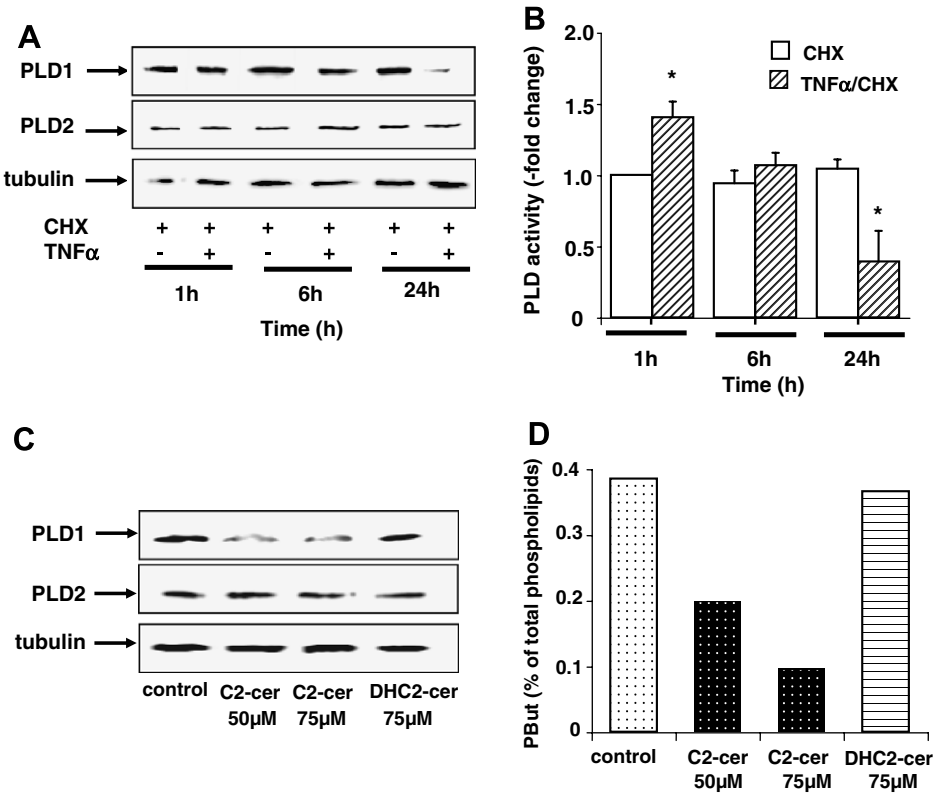

Fig. 3. Effect of TNFα/CHX and exogenous ceramide on PLD expression and activity. (A) ECV304 cells were treated with CHX (10 μg/ml) in the absence or presence of TNFα (200 ng/ml) for the indicated periods of time. At the end of treatment, cells were harvested and PLD expression was measured by Western blotting as described in Section 2. The blots showing PLD1, PLD2 and α-tubulin are representative of 2 (PLD2) to 3 (PLD1, tubulin). (B) ECV304 cells were prelabeled with [^{3}H]-palmitate for 24 h and then treated with CHX (10 μg/ml) in absence or presence of TNFα (200 ng/ml) for the indicated periods of time. 30 min before the end of the treatment, cells were incubated with 1% 1-butanol. Results expressed relative to the radioactivity incorporated in total phospholipids are normalized with respect to control cells incubated with CHX (10 μg/ml) for 1 h taken as 1. They are means ± S.E. of three independent experiments. Data were analyzed by ANOVA and means were compared by a protected *t* test. ∗ significantly different from CHX alone, $P < 0.05$. (C) ECV304 cells were treated for 24 h with C2-ceramide (50 or 75 μM) or dihydro C2-ceramide (75 μM). At the end of treatment, cells were harvested and PLD expression was measured by Western blotting as described in Section 2. Blots shown are representative of two. (D) ECV304 cells were prelabeled with [^{3}H]-palmitate for 24 h and then treated with C_2- or dihydro C_2-ceramide, as indicated, for 24 h. 30 min before the end of the treatment, cells were incubated with 1% 1-butanol. Results are from one representative experiment.

increase of ceramide level, PLD activity returned close to control level, whereas a marked fall (−61%, $P < 0.002$) was observed at 24 h when both ceramide increase and cell death were maximal (Fig. 3B). No significant variations of PLD activity were observed either in untreated cells or cells treated with TNFα alone at any time point (not shown). As illustrated on the representative blots shown in Fig. 3A, the large decrease in PLD activity observed after 24 h of treatment with TNFα/CHX could be attributed to a marked reduction of PLD1 expression (−57%, $P < 0.02$) as compared to levels observed in CHX treated cells, as indicated by ANOVA analysis of densitometric evaluation from three separate experiments (not shown). No significant variation of PLD1 expression could be detected in untreated cells or cells treated by TNFα alone. PLD2 expression remained unchanged whatever the nature and duration of cell treatment (Fig. 3A, quantification not shown). In addition, a direct inhibition of PLD activity by increased ceramide levels could also be involved at this later time point. Indeed, ceramide has been reported to inhibit PLD activity both in cell-free systems [33] and in intact cells [34]. In the present study, we observed that PLD activity of ECV304 cells was highly sensitive to C_2-ceramide inhibition (50% and 75% inhibition at 50 and 75 μM, respectively, Fig. 3D) whereas dihydro C_2-ceramide was inactive. Furthermore, as shown in Fig. 3C, C_2-ceramide also reduced PLD1 expression of ECV304 cells after 24 h of treatment (−43 and −49% at 50 and 75μM, respectively, $P < 0.01$, quantification not shown). This suggests that ceramide could act both by inhibiting PLD activity and decreasing the expression of the PLD1 isoform. On the whole, these results show that as long as PLD activity was maintained elevated, apoptosis did not occur and suggest that PLD activity exerted a protective effect against apoptosis in ECV304 cells.

3.5. Effect of PLD on TNFα/CHX-induced cell death

To explore further the potential protective role of PLD in ECV304 cells, we investigated the effect of exogenous addition of a bacterial PLD from *Streptomyces chromofuscus* (scPLD) on TNFα/CHX-induced cell death. Exogenous scPLD has been shown to efficiently protect neutrophils from spontaneous as well as FAS-induced apoptosis [21] and to inhibit hypoxia-induced apoptotic cell death in PC12 cells [35]. Addition of scPLD to ECV304 cells prior to treatment with 20 ng/ml TNFα in the presence of 10 μg/ml CHX significantly decreased TNFα/CHX death promoting effect, as shown by the dose-dependent increase of cell survival measured by the MTT assay

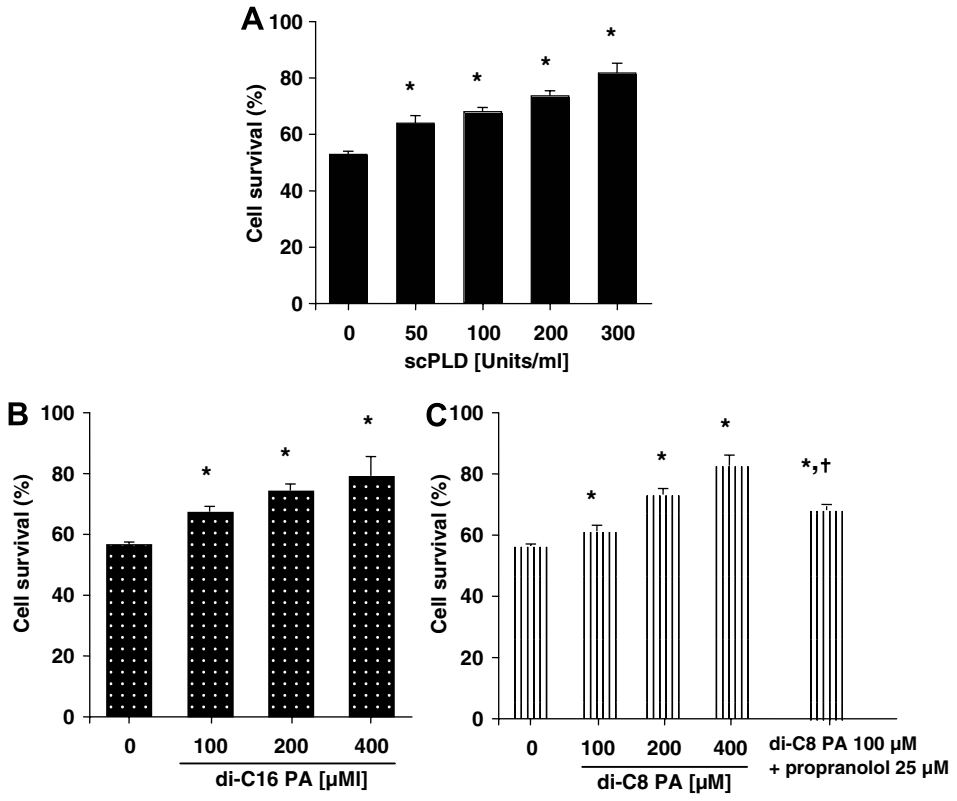

Fig. 4. Effect of exogenous bacterial PLD and phosphatidic acid on TNFα/CHX-induced cell death. ECV304 cells were treated with increasing concentrations of (A) bacterial scPLD, (B) di-C16-PA (C) di-C8-PA or 100 μM di-C8-PA plus 25 μM propranolol, for 30 min prior to treatment with or without 20 ng/ml TNFα plus 10 μg/ml CHX, for 24 h. At the end of the incubation period, cell viability was measured by the MTT assay. Results are expressed relative to cell survival measured in the absence of TNFα/CHX taken as 100. They are means ± S.E. of three separate experiments performed with six replicates. ∗ significantly different from cell survival without scPLD or PA, $P < 0.05$. † significantly different from 100 μM di-C8 PA alone, $P < 0.05$.

(Fig. 4A). These results suggest that the newly synthesized PA has a protective effect against apoptosis although we cannot exclude a contributing effect of other bioactive lipids, due to the limited specificity of scPLD toward phosphatidylcholine. The protective effect of PA was supported further by results of experiments showing that addition of dipalmitoyl PA (di-C16-PA) or the cell-permeant dioctanoyl PA (di-C8-PA) significantly increased cell survival to TNFα/CHX treatment (Fig. 4B and C). Moreover, the protective effect of 100 μM di-C8-PA was further increased by propranolol, which prevents PA dephosphorylation by PA phosphohydrolase and then enhances PA accumulation. These results together with the lack of effect of the DAG kinase inhibitor R59022 (data not shown) suggest that PA itself, rather than PA derived metabolites, protects ECV 304 cells against TNFα/CHX-induced cell death.

To confirm that PLD has an anti-apoptotic effect in ECV304 cells, we transiently overexpressed GFP-PLD1 and -PLD2 fusion proteins prior to TNFα/CHX treatment of the cells. The efficiency of transfection estimated by observing the bright fluorescence of transfected cells was similar for both vectors (around 20%). Transient overexpression of both fusion proteins significantly increased PLD activity as compared to cells transfected with the empty vector harboring only GFP (Fig. 5A). ECV304 cells overexpressing PLD1 (Fig. 5B) exhibited a higher percentage of live cells than cells transfected with the empty vector ($P < 0.05$). Cells overexpressing PLD2 exhibited a similar trend although difference with the empty vector did not reach significance ($P = 0.1$). The lower protective effect of overexpressed PLD2 can hardly be explained by a lower transfection efficiency because the percentages of PLD1- and PLD2-transfected cells were very similar. It may be speculated that overexpressed PLD2, but not PLD1, would be partially mislocated with respect to the endogenous protein. Although fully catalytically active, mislocated PLD2 might have impaired protective effects or might have trapped endogenous activators, thus preventing the correctly located PLD2 to be functional. We next examined whether PLD inhibition by 1-butanol could potentiate the apoptotic effect of TNFα/CHX, using a suboptimal concentration of TNFα (0.2 ng/ml), which only gave minimal cell death in the presence of 10 μg/ml CHX. Given the transphosphatidylation property of PLD, the use of a primary alcohol is a convenient way to divert phosphatidic acid synthesis toward that of the biologically inactive phosphatidylalcohol, and thus to mimic PLD inhibition. As shown in Fig. 5C, addition of 0.5% 1-butanol to ECV304 cells prior to treatment with TNFα/CHX for 24 h, markedly decreased cell survival. Control treatment by 2-butanol, an isomer not recognized by PLD, had no effect on TNFα/CHX-induced cell death, which rules out non specific effects of alcohol treatment. Finally, in a more straightforward approach, we examined the effect of PLD1 and PLD2 knockdown by means of specific siRNA on TNFα/CHX-induced cell death. As shown in Fig. 5E, both PLD1- and PLD-2 siRNA reduced the level of

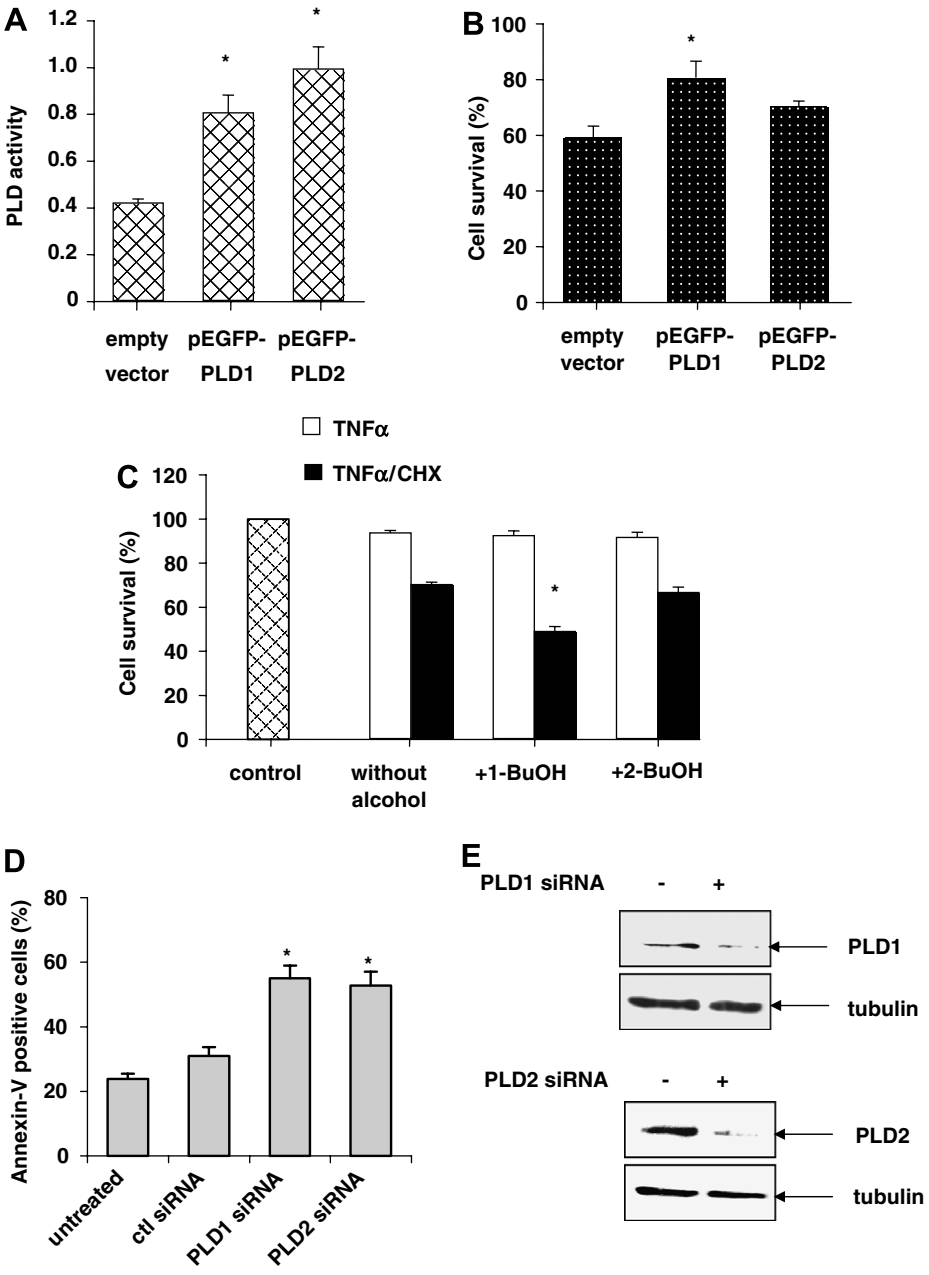

Fig. 5. Effect of changes in PLD activity on TNFα/CHX-induced cell death. (A and B) ECV304 cells were transiently transfected with pEGFP-PLD1, -PLD2 and empty vector as described in Section 2. In (A), PLD activity was measured as described in the legend of Fig. 4. The radioactivity of phosphatidylbutanol was expressed in percent of radioactivity of total phospholipids. Results are means ± S.E. of three separate experiments. ∗ significantly different from empty vector, $P < 0.05$. In (B), transfected cells were treated with 20 ng/ml TNFα in the presence of 10 μg/ml CHX for 16 h, or left untreated, and cell viability was measured by the MTT assay. Results are expressed relative to cell survival measured in the absence of TNFα/CHX taken as 100. They are means ± S.E. of three separate experiments performed with six replicates. ∗ significantly different from empty vector, $P < 0.05$. (C) ECV304 cells were incubated in the absence or presence of either 1-butanol or 2-butanol (0.5% final concentration) for 10 min before addition of 0.2 ng/ml TNFα in the presence or absence of 10 μg/ml CHX for 24 h. Cell viability was determined by MTT assay. Results are expressed relative to cell survival measured in the absence of TNFα or TNFα/CHX taken as 100. They are means ± S.E. of two separate experiments performed with six replicates. ∗ significantly different from TNFα/CHX without butanol, $P < 0.05$. (D) ECV304 cells were transfected with negative control siRNA (ctl siRNA), with PLD1- or PLD2-siRNA or left untreated and then stimulated with 20 ng/ml TNFα in the presence of 10 μg/ml CHX for 16 h. Cell death was measured by annexin-V binding assay. For each condition, the percentage of annexin-V labeled cells was determined in at least 10 different fields. ∗ significantly different from control siRNA, $P < 0.05$. (E) ECV304 cells were transfected with PLD1- or PLD2-siRNA or left untreated, then lysed and subjected to Western blot analyses with anti-PLD1 or anti-PLD2 antibodies. Blots were reprobed with anti α-tubulin antibody.

the corresponding PLD proteins by around 80%. PLD1 as well as PLD2 silencing significantly increased cell death measured by annexin-V labeling as compared to untreated cells or to cells transfected with the negative control siRNA (Fig. 5D).

In conclusion, an increase of ceramide level and a concomitant inhibition of PLD activity/expression were observed in the course of ECV304 cell death induced by TNFα/CHX. The present results showing that both exogenous bacterial PLD

and PLD overexpression counteract TNFα-induced apoptosis, and that depleting PLD proteins strongly potentiates cell death, bring strong support to a role of PLD activity as a survival pathway in TNFα/CHX-treated cells. Although the functional connexion between PLD1 and cell survival remains to be determined, several hypotheses can be envisaged to explain the pro-survival effect of the PLD product, PA. Among the various PA targets identified to date, sphingosine kinase-1 (SK1) is of special interest due to its key role in regulating the balance between pro- and anti-apoptotic sphingolipids [36]. SK1 has been shown to translocate to PA-enriched membranes upon cell stimulation, the translocation being accompanied by a marked activation [37]. Thus, increasing PA level in ECV304 cells might result in an increased level of the pro-survival S1P at the expense of ceramide/sphingosine. The mammalian target of rapamycin mTOR is another PA-activated protein which plays a crucial role in the commitment of cells to life or death [38]. Thus, PLD-dependent mTOR activation is considered to provide survival signals in most cancer cells [39]. The fact that TNFα/CHX-induced cell death was markedly increased by rapamycin in ECV304 cells (our unpublished results) suggests that the latter pathway may be involved in the protection of ECV304 cells against apoptosis.

Acknowledgments: This work was supported by INSERM and by the Groupe de Réflexion sur la Recherche Cardiovasculaire (GRRC). H.B. was a recipient of a fellowship from GRRC.

References

[1] Wallach, D. (1997) Cell death induction by TNF: a matter of self control. Trends Biochem. Sci. 22, 107–109.
[2] Muppidi, J.R., Tschopp, J. and Siegel, R.M. (2004) Life and death decisions: secondary complexes and lipid rafts in TNF receptor family signal transduction. Immunity 21, 461–465.
[3] Varfolomeev, E.E. and Ashkenazi, A. (2004) Tumor necrosis factor: an apoptosis JuNKie? Cell 116, 491–497.
[4] Nolop, K.B. and Ryan, U.S. (1990) Enhancement of tumor necrosis factor-induced endothelial cell injury by cycloheximide. Am. J. Physiol. 259, L123–L129.
[5] Hannun, Y.A. (1996) Function of ceramide in coordinating cellular responses to stress. Science 274, 1805–1976.
[6] Baichwal, V.R. and Baeuerle, P.A. (1997) Activate NF-κB or die? Curr. Biol. 7, R94–R96.
[7] White, E., Sabbatini, P., Debbas, M., Wold, W.S.M., Kusher, D.I. and Gooding, L.R. (1992) The 19-kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by tumor necrosis factor α. Cell. Biol. 12, 2570–2580.
[8] Miura, M., Friedlander, R.M. and Yuan, J. (1995) Tumor necrosis factor-induced apoptosis is mediated by a CrmA-sensitive cell death pathway. Proc. Natl. Acad. Sci. USA 92, 8318–8322.
[9] Dbaibo, G.S., El-Assaad, W., Krikorian, A., Liu, B., Diab, K., Idriss, N.Z., El-Sabban, M., Driscoll, T.A., Perry, D.K. and Hannun, Y.A. (2001) Ceramide generation by two distinct pathways in tumor necrosis factor alpha-induced cell death. FEBS Lett. 503, 7–12.
[10] Luberto, C., Hassler, D.F., Signorelli, P., Okamoto, Y., Sawai, H., Boros, E., Hazen-Martin, D.J., Obeid, L.M., Hannun, Y.A. and Smith, G.K. (2002) Inhibition of tumor necrosis factor-induced cell death in MCF7 by a novel inhibitor of neutral sphingomyelinase. J. Biol. Chem. 277, 41128–41139.
[11] Xu, J., Yeh, C.-H., Chen, S., He, L., Sensi, S.L., Canzoniero, L.M., Choi, D.W. and Hsu, C.Y. (1998) Involvement of de novo ceramide biosynthesis in tumor necrosis factor-alpha/cycloheximide-induced cerebral endothelial cell death. J. Biol. Chem. 273, 16521–16526.
[12] Foster, D.A. and Xu, L. (2003) Phospholipase D in cell proliferation and cancer. Mol. Cancer Res. 1, 789–800.
[13] Nozawa, Y. (2002) Roles of phospholipase D in apoptosis and pro-survival. Biochim. Biophys. Acta 1585, 77–86.
[14] McDermott, M., Wakelam, M.J.O. and Morris, A.J. (2004) Phospholipase D. Biochem. Cell. Biol. 82, 225–253.
[15] Kasai, T., Ohguchi, K., Nakashima, S., Ito, Y., Naganawa, T., Kondo, N. and Nozawa, Y. (1998) Increased activity of oleate-dependent type phospholipase D during actinomycin D-induced apoptosis in Jurkat T cells. J. Immunol. 161, 6469–6474.
[16] Kang, J.H., Shin, I. and Han, J.S. (1998) Changes of phospholipase D activity in TNF-alpha and anti-Fas/Apo1 monoclonal antibody induced apoptosis in HL-60 and A20 cells. Exp. Mol. Med. 30, 21–27.
[17] Zhong, M., Joseph, T., Jackson, D., Beychenok, S. and Foster, D.A. (2002) Elevated phospholipase D activity induces apoptosis in normal rat fibroblasts. Biochem. Biophys. Res. Commun. 298, 474–477.
[18] Yoshimura, S., Sakai, H., Ohguchi, K., Nakashima, S., Banno, Y., Nishimura, Y., Sakai, N. and Nozawa, Y. (1997) Changes in the activity and mRNA levels of phospholipase D during ceramide-induced apoptosis in rat C6 glial cells. J. Neurochem. 69, 713–720.
[19] Iwasaki-Bessho, Y., Banno, Y., Yoshimura, S., Ito, Y., Kitajima, Y. and Nozawa, Y. (1998) Decreased phospholipase D (PLD) activity in ceramide-induced apoptosis of human keratinocyte cell line HaCaT. J. Invest. Dermatol. 10, 376–382.
[20] Zheng, X.L., Gui, Y., Du, G., Frohman, M.A. and Peng, D.Q. (2004) Calphostin-C induction of vascular smooth muscle cell apoptosis proceeds through phospholipase D and microtubule inhibition. J. Biol. Chem. 279, 7112–7118.
[21] Lee, S.-Y., Oh, J.-Y., Lee, M.-J., Jang, M.-J., Park, H.-Y., Kim, J.-W., Min, D.S., Park, Y.-M., Chang, Y.-C., Bae, Y.-S. and Kwak, J.-Y. (2004) Anti-apoptotic mechanism and reduced expression of phospholipase D in spontaneous and Fas-stimulated apoptosis of human neutrophils. Eur. J. Immunol. 34, 2760–2770.
[22] Zhong, M., Shen, Y., Zheng, Y., Joseph, T., Jackson, D. and Foster, D.A. (2003) Phospholipase D prevents apoptosis in v-Src-transformed rat fibroblasts and MDA-MB-231 breast cancer cells. Biochem. Biophys. Res. Commun. 302, 615–619.
[23] Yamada, M., Banno, Y., Takuwa, Y., Koda, M., Hara, A. and Nozawa, Y. (2004) Overexpression of phospholipase D prevents actinomycin D-induced apoptosis through potentiation of phosphoinositide 3-kinase signalling pathways in Chinese-hamster ovary cells. Biochem. J. 378, 649–656.
[24] Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.
[25] Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. Can. J. Med. Sci. 37, 911–917.
[26] Dbaibo, G.S., Pushkareva, M.Y., Rachid, R.A., Alter, N., Smyth, M.J., Obeid, L.M. and Hannun, Y.A. (1998) p53-dependent ceramide response to genotoxic stress. J. Clin. Invest. 102, 329–339.
[27] Birbes, H., El Bawab, S., Hannun, Y.A. and Obeid, L.M. (2001) Selective hydrolysis of a mitochondrial pool of sphingomyelin induces apoptosis. FASEB J. 14, 2669–2679.
[28] Diaz, O., Mebarek-Azzam, S., Benzaria, A., Dubois, M., Lagarde, M., Némoz, G. and Prigent, A.F. (2005) Disruption of lipid rafts stimulates phospholipase D activity in human lymphocytes: implication in the regulation of immune function. J. Immunol. 175, 8077–8086.
[29] Fang, Y., Park, I.H., Wu, A.L., Du, G., Huang, P., Frohman, M.A., Walker, S.J., Brown, H.A. and Chen, J. (2003) PLD1 regulates mTOR signaling and mediates Cdc42 activation of S6K1. Curr. Biol. 13, 2037–2044.
[30] Powner, D.J., Payne, R.M., Pettitt, T.R., Giudici, M.L., Irvine, R.F. and Wakelam, M.J. (2005) Phospholipase D2 stimulates integrin-mediated adhesion via phosphatidylinositol 4-phosphate 5-kinase Igamma b. J. Cell Sci. 118, 2975–2986.
[31] Karsan, A., Yee, E. and Harlan, J.M. (1996) Endothelial cell death induced by tumor necrosis factor-alpha is inhibited by the Bcl-2 family member, A1. J. Biol. Chem. 271, 27201–27204.
[32] Cooper, J.T., Stroka, D.M., Brostjan, C., Palmetshofer, A., Bach, F.H. and Ferran, C. (1996) A20 blocks endothelial cell activation through a NF-kappaB-dependent mechanism. J. Biol. Chem. 271, 18068–18073.

[33] Venable, M.E., Bielawska, A. and Obeid, L.M. (1996) Ceramide inhibits phospholipase D in a cell-free system. J. Biol. Chem. 271, 24800–24805.
[34] Gomez-Munoz, A., Martin, A., O'Brien, L. and Brindley, D.N. (1994) Cell-permeable ceramides inhibit the stimulation of DNA synthesis and phospholipase D activity by phosphatidate and lysophosphatidate in rat fibroblasts. J. Biol. Chem. 269, 8937–8943.
[35] Yamakawa, H., Banno, Y., Nakashima, S., Sawada, M., Yamada, J., Yoshimura, S., Nishimura, Y., Nozawa, Y. and Sakai, N. (2000) Increased phospholipase D2 activity during hypoxia-induced death of PC12 cells: its possible anti-apoptotic role. Neuroreport 11, 3647–3650.
[36] Taha, T.A., Hannun, Y.A. and Obeid, L.M. (2006) Sphingosine kinase: biochemical and cellular regulation and role in disease. J. Biochem. Mol. Biol. 39, 113–131.
[37] Delon, C., Manifava, M., Wood, E., Thompson, D., Krugmann, S., Pyne, S. and Ktistakis, N.T. (2004) Sphingosine kinase 1 is an intracellular effector of phosphatidic acid. J. Biol. Chem. 279, 44763–44774.
[38] Fang, Y., Vilella-Bach, M., Bachmann, R., Flanigan, A. and Chen, J. (2001) Phosphatidic acid-mediated mitogenic activation of mTOR signaling. Science 294, 1942–1945.
[39] Chen, Y., Rodrik, V. and Foster, D.A. (2005) Alternative phospholipase D/mTOR survival signal in human breast cancer cells. Oncogene 24, 672–679.