Anti-apoptotic role of phospholipase D in spontaneous and delayed apoptosis of human neutrophils

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Abstract Neutrophil apoptosis is a constitutive process that can be enhanced or delayed by signals induced by various stimuli. We investigated the role of phospholipase D (PLD) in neutrophil apoptosis. The apoptotic rate of neutrophils was found to be increased by 1-butanol and decreased by the exogenous addition of PLD. Moreover, the delay of apoptosis by apoptosis-delaying stimuli such as granulocyte/macrophage colony-stimulating factor or lipopolysaccharide (LPS) was also blocked by 1-butanol. Unstimulated PLD activity in cultured cells for 20 h was higher than that in freshly isolated cells and further increased in cultured cells with LPS. These results suggest that PLD is involved in the up-regulation of neutrophil survival. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Phospholipase D; Apoptosis; Neutrophil; Butanol; Propranolol

1. Introduction

The apoptosis of neutrophils has been found to be an important factor in the resolution of inflammation [1]. Neutrophil-associated functions, such as chemotaxis, phagocytosis, degranulation, and respiratory burst, are lost when the cells undergo apoptosis [2,3]. However, aged neutrophils in tissue exacerbate inflammation by releasing proteases, reactive oxygen species, and pro-inflammatory mediators [4]. Therefore, the modulation of neutrophil apoptosis would be of benefit to patients with decreased numbers of circulating neutrophils and to those with inflammatory diseases. Several factors are known to either suppress or induce neutrophil apoptosis [1,5]. However, the intracellular signaling pathways that underlie neutrophil apoptosis are still incompletely understood and controversial.

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine to generate free choline and phosphatidic acid (PA). This PA can be further metabolized by phosphatidic acid phosphohydrolase to form diacylglycerol (DAG) and by phospholipase A2 to form lysophosphatidic acid. Thus, PLD appears to amplify the signaling initiated by receptor activation. When PLD is stimulated in the presence of a primary alcohol, such as ethanol or 1-butanol, transphosphatidylation results in the formation of phosphatidylalcohol instead of PA [6]. Two types of PLD (PLD1 and PLD2) have been cloned, but they differ in terms of their activation and their subcellular localization [7,8]. Although neutrophil PLD has not been purified, it has been shown that the PLD activity of neutrophils is mainly associated with the plasma membrane, secretory vesicles, and Golgi apparatus [9], and that it is stimulated by ARF, RhoA, protein kinase C (PKC), and an unidentified 50 kDa cytosolic factor [10–12].

The physiological process of neutrophil apoptosis differs from processes identified in other cells [1,5]. It is now known that Lyn, PKC-δ, and mitogen-activated protein kinase play a role in neutrophil apoptosis [13–16]. The involvement of PLD in the determination of apoptosis has been reported in other cells, but these results remain contradictory [17]. It has also been suggested that the inhibition of PLD might be implicated in the induction of apoptosis by ceramide, since guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS)-dependent PLD activity and the levels of PLD1a and PLD1b were down-regulated in ceramide-induced apoptotic glial cells [18]. In addition, PLD activation plays an anti-apoptotic role in the hypoxia-induced cell death of PC12 cells [19]. In contrast to the above results, PLD activity was upregulated during the apoptosis of Jurkat T cells, as induced by actinomycin D, tumor necrosis factor (TNF)-α, or H_2O_2 [20]. However, the role played by PLD in neutrophil apoptosis has not been investigated. This study was designed to determine whether PLD signaling is involved in neutrophil apoptosis and whether PLD-derived PA plays a role in the modulation of neutrophil apoptosis.

2. Materials and methods

2.1. Materials

Histopaque, lipopolysaccharide (LPS), dibutyryl-cAMP (dbcAMP), phorbol 12-myristate 13-acetate (PMA), fMet-Leu-Phe (fMLP), and bacterial PLD (from *Streptomyces chromofuscus*) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), dextran and [^{3}H]1-*O*-alkyl-lysophosphatidylcholine (192 Ci/mmol) from Amersham Pharmacia Biotech (Uppsala, Sweden), granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-8 (IL-8) from R&D Systems (Minneapolis, MN, USA), and RPMI 1640 medium from Gibco-BRL (Rockville, MD, USA). All other reagents and solvents used were of the highest quality available commercially.

2.2. Cell culture

Peripheral blood neutrophils were isolated from healthy young donors using a method involving dextran sedimentation and differential

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Abbreviations: PLD, phospholipase D; LPS, lipopolysaccharide; GM-CSF, granulocyte/macrophage colony-stimulating factor; PA, phosphatidic acid; DAG, diacylglycerol; PEth, phosphatidylethanol; IL-8, interleukin-8; dbcAMP, dibutyryl-cAMP; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate)

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centrifugation through a Ficoll-Hypaque density gradient [11]. Venous blood was collected on sodium citrate solution (3.8%). The cellular part of the blood was mixed with a solution of 3% dextran in 0.9% NaCl solution and kept for 45 min at 25°C. The neutrophil-rich upper layer of the suspension was then collected and centrifuged ($250\times g$, 10 min). Residual erythrocytes were removed by hypotonic lysis and the pellet so obtained was suspended in HEPES-buffered saline (25 mM HEPES, pH 7.4, 125 mM NaCl, 0.7 mM $MgCl_2$, and 0.5 mM EDTA). The suspension was centrifuged ($250\times g$, 30 min) on Histopaque solution at 4°C. Isolated neutrophils (2×10^5/100 μl) were maintained in RPMI 1640 medium supplemented with 1% glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in 96-well flat-bottomed plates at 37°C in a humidified atmosphere containing 5% CO_2. Neutrophils were shown to be 95% pure morphologically by microscopy.

2.3. Morphological assessment of neutrophil apoptosis

Neutrophils incubated in the presence or absence of butanol or various agents were spun down on a glass slide in a cytospin (Shandon, PA, USA). Cells were fixed with methanol and stained with Giemsa staining solution. Percentages of apoptotic cells were determined by counting at least 300 cells per slide.

2.4. Measurement of phosphatidylserine (PS) exposure

PS exposure was measured by the binding of annexin V–fluorescein isothiocyanate (FITC) using protocol outlined in the TACS apoptosis detection kit (Trevigen, The Netherlands). Cells (1×10^6) were first harvested and washed with phosphate-buffered saline (PBS). They are then incubated for 15 min with annexin V–FITC and propidium iodide (PI). The cells (1×10^4) were subsequently analyzed with flow cytometer (Becton Dickinson, NJ, USA). The combination of annexin V–FITC and PI allows for the differentiation between early apoptotic cells (annexin V–FITC-positive), late apoptotic and/or necrotic cells (annexin V–FITC- and PI-positive), and viable cells (unstained).

2.5. Assay of PLD activity

Neutrophils were labeled with [^{3}H]alkyl-lysophosphatidylcholine (1.5 μCi/2×10^7 cells) for 90 min at 37°C [11], washed twice with PBS and resuspended in assay buffer (20 mM HEPES, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 1.6% ethanol, and 1 mg/ml bovine serum albumin). After being incubated for 20 min at 37°C in the presence or absence of cytochalasin B and fMLP or PMA, reactions were stopped by adding 1 ml of $CHCl_3$/CH_3OH/concentrated HCl (50:50:0.3, v/v), and 0.35 ml of 1 M HCl/5 mM EGTA. Lipids were then extracted and separated on Silica gel 60 TLC plates in a solvent system consisting of ethyl acetate/trimethyl pentane/acetic acid/H_2O (13:2:3:10, v/v). The plates were exposed to iodine vapor and [^{3}H]phosphatidylethanol (PEth) was identified by its co-migration with a PEth carrier. Radioactive [^{3}H]PEth was scraped off the plates and quantified in a liquid scintillation counter.

2.6. Statistical analysis

Results are presented as means ± S.D. Student's *t*-test for unpaired samples was used to compare means. A probability value of less than 0.05 was considered significant.

3. Results

Neutrophils rapidly undergo spontaneous apoptosis upon in vitro culture. Typical apoptotic cells displaying nuclear condensation and cytoplasmic vacuoles were readily observed (Fig. 1A). More than 50% of the cells showed apoptotic changes after 20 h of culture. Culture of neutrophils with 1-butanol for 20 h significantly increased the number of apoptotic cells (Figs. 1 and 2A). By comparison, no significant acceleration of apoptosis was detected after culturing with secondary alcohol, 2-butanol. Fig. 1B shows original recordings of the fluorescence histograms obtained using annexin V and PI. The fluorescence distribution of freshly isolated neutrophils indicates a single cell population. After 20 h of culture, two fluorescent populations were observed, one containing annexin V staining and one with normal cells. 1-Butanol increased annexin V and/or PI staining, indicating that early

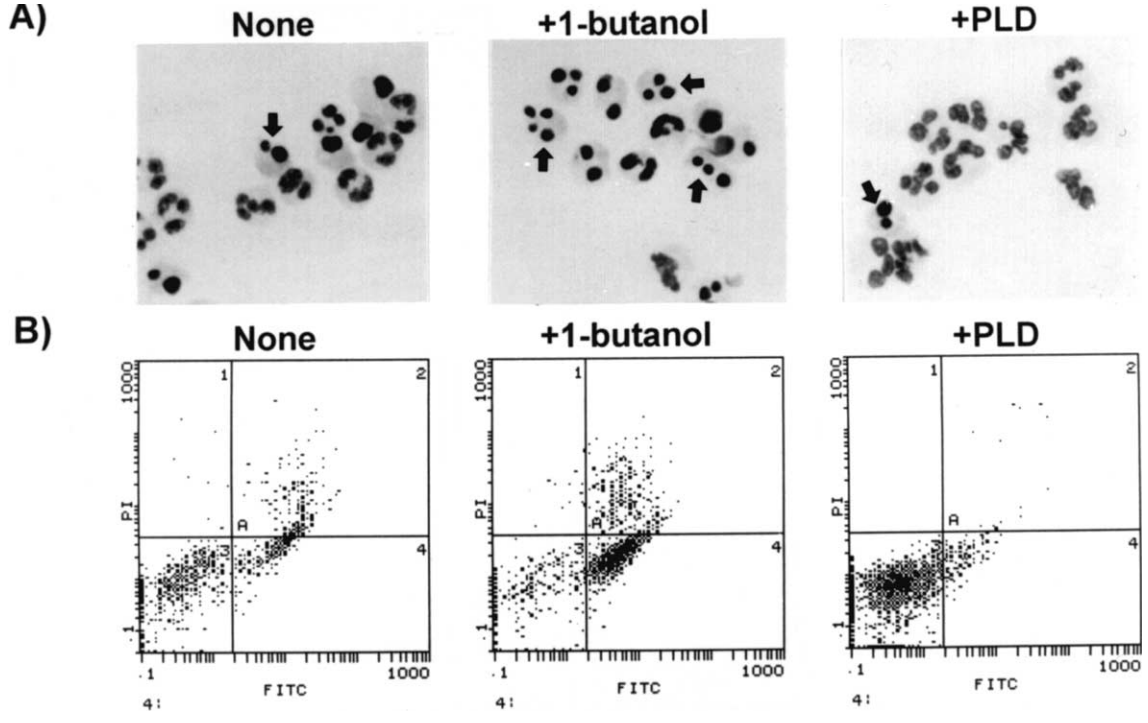

Fig. 1. Apoptotic changes of neutrophils by 1-butanol and bacterial PLD. A: Cytospin preparations of neutrophils after incubation in vitro for 20 h were stained with Giemsa solution. Solid arrows indicate apoptotic cells. Neutrophils were cultured for 20 h with medium (None), 1% 1-butanol (+1-butanol), and 100 units of bacterial PLD (+PLD). B: The cell death was assessed by double staining of cells with FITC-labeled annexin V and PI. Neutrophils were treated as in A.

and late apoptotic cells were increased by 1-butanol. When bacterial PLD (100 units) was exogenously added to the culture, the spontaneous apoptosis of neutrophils was significantly reduced. As shown in Fig. 2, the effects of 1-butanol and PLD were dose-dependent. Maximal responses of 1-butanol and bacterial PLD occurred at 1.5% and 20 units, respectively. Cell necrosis was induced at higher concentrations of 1-butanol or 2-butanol exceeding 1.5%.

Neutrophils were cultured for 20 h in the presence of GM-CSF, LPS, dexamethasone, IL-8, and dbcAMP, which are known to prolong neutrophil survival [1]. As shown in Fig. 3A, all these agents decreased both the number of apoptotic cells and the extent of DNA fragmentation. The percentage of apoptotic neutrophils was markedly augmented, by 70%, after culture with 1-butanol, even in the presence of LPS and GM-CSF. In contrast, the inhibitory effect of dbcAMP was partially suppressed by 1-butanol. To further characterize whether PLD-derived PA specifically mediates the inhibitory effect of PLD on neutrophil apoptosis, cells were pretreated with propranolol, an inhibitor of phosphatidate phosphohydrolase. The treatment of cells with propranolol increased the number of constitutive apoptotic cells from 50% to 80% (Fig. 3B). Propranolol (20 μM) also significantly partially blocked the inhibitory effect of PLD on the apoptosis of neutrophils (Fig. 3B). However, propranolol failed to block the effect of GM-CSF, LPS, and IL-8 on neutrophil apoptosis.

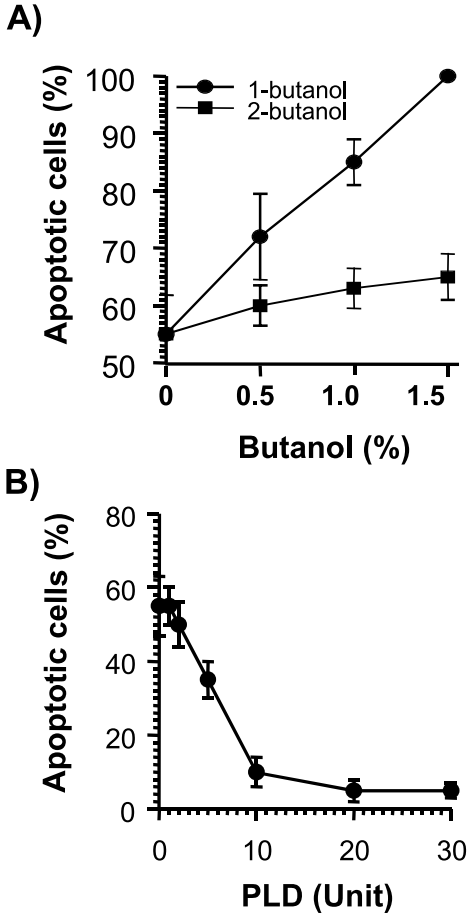

Fig. 2. Dose-dependent effects of 1-butanol and PLD on the spontaneous apoptosis of neutrophils. Neutrophils were cultured for 20 h with increasing concentrations of 1-butanol or 2-butanol (A) and bacterial PLD (B). Apoptotic cells were identified by their morphology under light microscopy and were counted. Data are reported as the percentage of cells showing the morphologic features of apoptosis. Results represent means ± S.D.

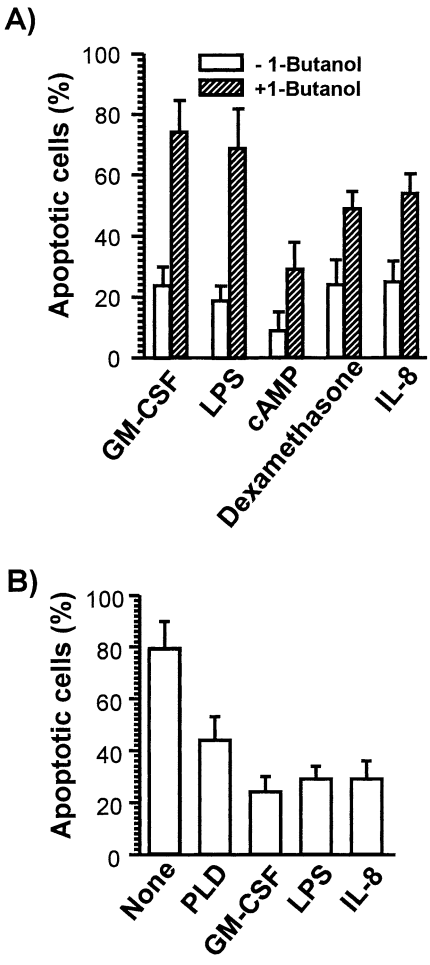

Fig. 3. Effects of 1-butanol and propranolol on the delay of neutrophil apoptosis. A: Neutrophils were pretreated with or without 1% 1-butanol for 1 h and further incubated for 20 h in the presence of 1 μg/ml GM-CSF, 1 μg/ml LPS, 300 μM dibutyryl-cAMP, 5 μM dexamethasone, and 100 ng/ml IL-8. B: Neutrophil were pretreated with 10 μM propranolol for 1 h and further incubated for 20 h in the presence or absence of bacterial PLD (20 units), GM-CSF, LPS, and IL-8. Data are reported as the percentage of cells showing morphologic features of apoptosis. Results represent means ± S.D.

Next, we measured PLD activity in cultured neutrophils with or without LPS or IL-8 for 20 h (Fig. 4). Cultured cells containing 50% apoptotic cells and 50% aged cells showed higher basal PLD activity than the freshly isolated neutrophils ($0.70 \pm 0.15\%$ vs. $1.50 \pm 0.15\%$, $P < 0.01$). Interestingly, PLD activity was further increased in cultured cells in the presence of added LPS or IL-8, and these cells contained fewer apoptotic cells than the untreated incubation, indicating that the increased PLD activity might be attributed to apoptosis-resistant aged cells. Moreover, the PLD activity of freshly isolated neutrophils was dramatically increased by 5 μM cytochalasin B and 1 μM of fMLP or 100 nM of PMA. However, these agonists failed to further stimulate PLD activity in cells cultured for 20 h with or without LPS or IL-8.

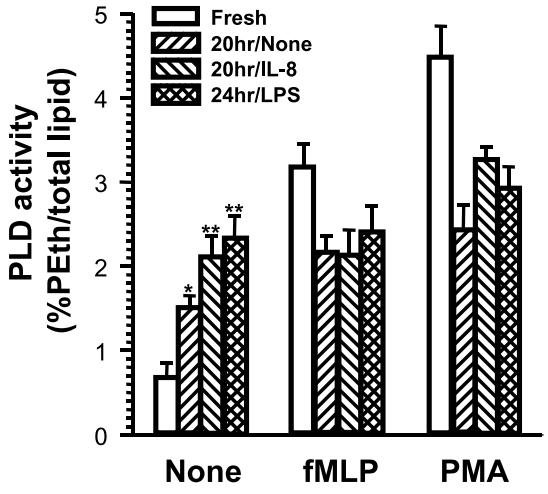

Fig. 4. Changes of PLD activity in neutrophils containing apoptotic cells. PLD activity was measured from freshly isolated neutrophils (Fresh), 20 h cultured cells in culture medium (20hr/None), 20 h cultured cells with IL-8 (20hr/IL-8), and 20 h cultured cells with LPS (20hr/LPS). Reactions were initiated by addition of mock (None), 5 μM cytochalasin B and 1 μM fMLP (fMLP), or 100 nM PMA (PMA). After 20 min, the incubations were terminated and formation of PEth was quantified as described in Section 2. Data represent means ± S.D. $*P < 0.01$, fresh cells vs. 20 h cultured cells with medium. $**P < 0.01$, 20 h cultured cells with medium vs. 20 h cultured cells with LPS or IL-8.

4. Discussion

PLD-derived PA is well known for its role in cell proliferation, but its role in non-proliferating and terminally differentiated cells like neutrophils has not been fully investigated. Cellular responses induced by exogenously added PA might differ from the responses evoked by intracellular PA, since neutrophils have specific binding sites for PA, the occupation of which leads to the mobilization of intracellular Ca^{2+} [21]. Thus, we did not consider the effect of exogenously added PA on neutrophil apoptosis. In the present study, incubation with PLD was significantly more potent than incubation with the other tested agents in terms of inhibiting neutrophil apoptosis. In neutrophils where endogenous PA and DAG formation was prevented by 1-butanol treatment, apoptosis increased. Similar apoptotic-inducing actions of 1-butanol have been observed in other cells [19]. However, there is a possibility that acute treatment of neutrophil suspension with 1% butanol can induce apoptosis by a mechanism other than inhibition of PLD and PA accumulation. It has been shown that ethanol induces the apoptosis of neutrophils by production of nitric oxide [22] and that ethanol-induced apoptosis of liver cells is initiated by the intracellular Ca^{2+} elevation in the cytoplasm [23]. However, the treatment of neutrophils with 1-butanol did not produce nitric oxide (results not shown). Furthermore, N^{w}-nitro-L-arginine methyl ester, an inhibitor of nitric oxide synthase, and EGTA, a calcium chelator, did not attenuate 1-butanol-induced apoptosis (results not shown).

One should be careful to interpret the effect of PLD on cellular responses, including apoptosis, since PA is further metabolized to DAG, which is an activator of PKC. The propranolol-mediated inhibition of DAG generation may have an influence on the apoptosis of neutrophils and may be comparatively more potent than PA in the induction or inhibition of apoptosis. The finding that the PLD-induced delay of apoptosis was partially inhibited by propranolol suggests that PA-derived DAG may be involved in the apoptotic process of neutrophils. Kanaho et al. [24] reported that ethanol significantly inhibited the chemoattractant-stimulated degranulation of neutrophils but that propranolol had no effect on degranulation. There is also a possibility that a metabolite of PA, other than DAG, might also induce apoptosis.

The apoptosis of neutrophils is modulated by various factors, including inflammatory mediators like GM-CSF, IL-8, and TNF-α [1,5]. However, it is unknown how these factors influence the lifespan of neutrophils. We can speculate that delay of apoptosis by GM-CSF, LPS, and IL-8 is mediated by PLD activation, since all these agents can modulate PLD activity. Moreover, the addition of GM-CSF primes PLD to subsequent stimulation by fMLP or PMA [25], and GM-CSF stimulated PLD activity in adherent neutrophils [26]. LPS and IL-8 stimulate PLD activity, whereas cAMP down-regulates PLD activity [27,28]. The majority of priming and activating agonists of neutrophils functions, including PLD activity, inhibit or accelerate apoptosis. On the other hand, corticosteroids and cAMP-elevating agents induce apoptosis in thymocytes and lymphocytes, but have the opposite effects in neutrophils [29,30]. However, the anti-apoptotic effects of LPS and GM-CSF were blunted by 1-butanol in our experiment, which suggests their anti-apoptotic role may be associated with the PLD pathway. In contrast, the anti-apoptotic effect of cAMP was not affected by 1-butanol as much as it was by the other agonists. Our group has shown that GTPγS-stimulated PLD activity is inhibited by protein kinase A (PKA) [31]. However, PKA is unlikely to mediate the effect of cAMP on neutrophil apoptosis because H-89, an inhibitor of PKA, did not prevent the inhibition of apoptosis by dbcAMP [32]. Although the exact target site(s) has to be identified, these finding suggests that different mechanisms are active in the modulation of neutrophil apoptosis by cAMP. Upon appropriate conditions, neutrophils are capable of producing and releasing GM-CSF or IL-8, which are potent inhibitors of apoptosis. Therefore, it is speculated that 1-butanol may affect this process. However, 1-butanol-induced neutrophil apoptosis was not blocked by preincubating with or the simultaneous addition of GM-CSF and IL-8 (results not shown).

It has been demonstrated that functional activities, including degranulation and generation of O_2^-, in response to external stimuli decline as neutrophils proceed through apoptosis [2,33,34]. PLD activity in cultured cells was higher than in freshly isolated cells, indicating that apoptotic cells appear to have a more active PLD status than aged cells. These results may be in agreement with a previous report, which showed an increased PLD activity during the H_2O_2-induced apoptosis of PC12 cells, and during the apoptosis of Jurkat T cells, as induced by TNF-α or H_2O_2 [19,20]. However, care is needed to interpret PLD activity changes in the apoptotic process since both apoptotic and aged cells are present simultaneously. Interestingly, in the present study, PLD activity was further increased in cultured cells, which contained fewer apoptotic cells, by treatment with LPS or IL-8. This result suggests that the increment of PLD activity in 20 h cultured cells may be due to the basal activity of aged cells, rather than from apoptotic cells. It was also reported that the PLD activity of HL-60 cells is not significantly changed during the ap-

optotic process [20]. It has been shown that PLD1 has low basal activity but PLD2 is constitutively active [7,8]. The high basal levels of PLD activity observed in aged cells might be due to increased expression of PLD2. Yoshimura et al. [18] showed PLD2 expression is increased in apoptotic cells, whereas the level of PLD1 mRNA is down-regulated. Unfortunately, progress in identifying the changes of PLD expression in neutrophil apoptosis has been hampered by the failure to purify the enzyme(s) to homogeneity and to detect the level of PLD isotypes exactly using commercially available anti-PLD antibodies. Thus, we performed reverse-transcriptase-mediated polymerase chain reaction to compare mRNA levels of PLD1 and PLD2 in freshly isolated cells and cells cultured for 20 h with or without LPS or IL-8. However, mRNA levels of PLD1 and PLD2 were not correlated with PLD activities (results not shown). Regardless of the changes in the PLD protein itself, PLD activity may be implicated in the process of apoptosis since butanol, propranolol, and exogenous PLD affect apoptotic rate.

On the other hand, 1-butanol caused DNA fragmentation, showing strong PI staining. This indicates that activation of endonuclease occurred. Caspase-3 activity was identified in neutrophils undergoing apoptosis [35]. In addition, actin and fodrin are substrates for caspases [36,37] and inhibit PLD activity [38,39]. Therefore, it is plausible that caspase activation leads to the degradation of actin and fodrin, and that this results in an amelioration of the inhibition of PLD activity.

In summary, this study shows that the elevation of the intracellular level of PA, formed by adding exogenous PLD, inhibits spontaneous apoptosis, which suggests that the PLD pathway is one of signaling events in the modulation of neutrophil apoptosis.

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