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Increased Activity of Oleate-Dependent Type Phospholipase D During Actinomycin D-Induced Apoptosis in Jurkat T Cells

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Apoptosis is an active form of cell death that can be induced by a wide variety of agents and conditions. In response to actinomycin D, hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), or TNF-\textalpha, Jurkat T cells underwent typical apoptosis. Phospholipase D (PLD) activity in intact cells determined by phosphatidylbutanol generation was up-regulated by these agents. The PLD activation was in a time-dependent manner during apoptosis. It was also shown that the PLD activity measured by using exogenous substrate in the lysate from apoptotic cells was higher than that in the lysate from control untreated cells. The PLD activity in lysate from control untreated cells was stimulated by unsaturated fatty acids (UFA), but not by guanosine 5'-\textalpha-O-(3-thiotriphosphate). However, the PLD activity in the apoptotic cell lysate was no longer enhanced by the addition of oleate, suggesting that the increased PLD activity during apoptosis was attributed to the PLD of UFA-dependent type, but not the small G protein-dependent one. In fact, the release of free oleate was increased during apoptosis. The caspase inhibitors, z-DEVD and z-VAD, effectively suppressed PLD activation and apoptosis, but UFA release was unaffected. These results suggest the possibility that UFA-dependent type PLD may be implicated in apoptotic process in Jurkat T cells. This is the first demonstration that the PLD of UFA-dependent type would be involved in cellular responses.

were from Sigma (St. Louis, MO). Authentic phosphatidylbutanol (PBut) was from Avanti Polar-Lipid (Albaster, AL). Guanosine 5'-O-(3-thiotriphosphate) (GTPγS) was obtained from Boehringer Mannheim (Mannheim, Germany). The relatively nonspecific inhibitor of caspase-1-like proteases, z-VAD.FMK, and the selective inhibitor of caspase-3, z-DEVD.FMK, were obtained from Enzyme Systems Products (Dubin, CA). Other reagents were of the highest quality available.

Cell culture

The human T cell leukemia, Jurkat T cell was obtained from RIKEN Cell Bank (Tsukuba, Japan). Cells were grown in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C.

Cell morphology

For morphologic examination, Jurkat T cells were pelleted after treatment with apoptosis inducers, and the pellets were resuspended with 1% glutaraldehyde for 30 min for fixation. Cells were then rinsed twice with PBS and stained with Hoechst 33258 (final 10 μM) for 10 min. These stained cells were observed under a nonconfocal fluorescent microscope (Olympus BX60) with excitation at 360 nm. Cells with condensed and/or fragmented nuclei were defined as apoptotic cells. To calculate the rate of apoptotic cells, more than 400 cells were counted in each sample.

Measurement of PLD activity in intact cells

PLD activity was determined by measuring the formation of [3H]PBut in the presence of butanol (0.3%, v/v), as described previously (24). For assay of PLD activity in intact cells utilizing the endogenous substrate, Jurkat cells were labeled with [3H]palmitic acid (1 μCi/ml) and HL60 cells were labeled with [3H]oleic acid (0.5 μCi/ml) for 12–15 h, and then treated with apoptosis inducers. The cells were washed twice with buffer A (25 mM HEPES, pH 7.4, 125 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM glucose, and 1 mg/ml BSA) and resuspended in buffer A. After incubation for 30 min at 37°C in the presence of butanol (0.3%, v/v), reactions were stopped by chloroform/methanol (1:2, v/v). Lipids were extracted according to the method of Bligh and Dyer (25) and separated on Silica Gel 60 TLC plates in a solvent system of the upper phase of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (13:2:3:10, v/v), as described previously (24). The plates were exposed to iodine vapor, and [3H]PBut was identified by comigration with PBut standard. The spots scraped off the plates were mixed with the scintillation mixture, and the radioactivity was counted in a liquid scintillation counter (Beckman LS-6500).

Measurement of PLD activity in cell-free lysates

Cells were washed twice with buffer B (25 mM HEPES, pH 7.4, 3 mM NaCl, 100 mM KCl, 5 mM MgCl₂, 0.5 mM PMSF, 5 mM DTT, and 10 μg/ml leupeptin) and resuspended in buffer B. Cells were then disrupted by N₂ cavitation (600 psi at 4°C for 30 min). After unbroken cells and nuclei were removed by centrifugation at 9000 × g for 5 min, the resulting supernatant was used as cell lysates for additional experiments. PLD activity in cell lysates was measured by using the exogenous substrate of phospholipid vesicles prepared according to the method of Massenburg et al. (26) and Brown et al. (27), with minor modifications. For the assay of UFA-dependent PLD activity, egg PC vesicles containing [palmitoyl-3H]DPPC (3 μCi/ml) were added to cell lysates (100 μg proteins/100 μl) in the reaction mixture containing 200 mM HEPES (pH 7), 8 mM EGTA, 2 mM KC1, 5 mM MgCl₂, and 4 mM DTT, and were stimulated with 1.8 mM sodium oleate or 1.8 mM sodium arachidonate at 37°C for 1 h in the presence of butanol (0.5%, v/v). For the assay of G protein-dependent PLD activity, phospholipid vesicles (phosphatidylethanolamine/PIP2/egg PC, 10:1:5 molar ratio) containing [palmitoyl-3H]DPPC (3 μCi/ml) were added to cell lysates (100 μg proteins/100 μl) in the reaction mixture containing 50 mM HEPES (pH 7.5), 3 mM EGTA, 80 mM KCl, 2.5 mM MgCl₂, 2 mM CaCl₂, and 1 mM DTT, and were stimulated with 10 μM GTPγS at 37°C for 1 h in the presence of 0.5% butanol. The lipid extraction and measurement of [3H]PBut radioactivity were performed as described above.

Measurement of release of UFA

Cells were labeled with [3H]oleic acid or [3H]arachidonic acid (1 μCi/ml) for 12–15 h. The labeled cells were washed twice and resuspended in RPMI 1640 medium with 10% FBS. Cells were then treated with apoptosis inducers, and reactions were terminated by the addition of chloroform/methanol (1:2, v/v). Lipids were extracted according to the method of Bligh and Dyer (25) and separated on Silica Gel 60 TLC plates in a solvent system of chloroform/acetone (96:4, v/v), as described previously (28). The plates were exposed to iodine vapor, and [3H]oleic acid or [3H]arachidonic acid was identified by comigration with each unlabeled standard. The radioactivity of spots was determined as described above.

Results

Changes in PLD activity in apoptotic Jurkat T cells

Apoptosis was reported to be induced by a wide variety of agents (29–33). After treatment of Jurkat T cells with different agents, the percentages of apoptotic cells were measured using staining dye, Hoechst 33258. Under a fluorescent microscope, nuclei of control untreated cells were observed as blue and round, characteristic of viable cells (Fig. 1A). On the other hand, treatment of cells with actinomycin D (5 μg/ml) for 9 h resulted in nuclear fragmentation and condensation, which are characteristic of apoptosis (Fig. 1B). In addition to actinomycin D, H₂O₂ (100 μM) for 6 h, and TNF-α (20 ng/ml) for 6 h, which are also known to induce apoptosis, caused characteristic nuclear fragmentation and condensation in Jurkat T cells (data not shown). Further support to indicate apoptotic change induced by these agents was provided by the characteristic pattern (laddering) of apoptotic DNA fragmentation (data not shown). Nearly 55% cells underwent apoptotic changes at 9 h after actinomycin D treatment. Similarly, 35 and 45% cells displayed apoptotic nuclei after 6-h incubation with H₂O₂ and TNF-α, respectively (Fig. 2A). We have measured PLD activity in Jurkat T cells exposed to apoptotic inducers (Fig. 2B). The PLD activity determined by [3H]PBut production in the control untreated Jurkat T cells was at the low level. However, the PLD activity was markedly elevated by each apoptotic inducer. The temporal correlation between the extent of apoptosis and PLD activity was investigated by examining changes in PLD activity in Jurkat T cells at various stages during apoptosis. Upon actinomycin D treatment, apoptotic cells showing nuclear fragmentation and condensation, as assessed by Hoechst staining, were increased in a time-dependent manner.
Activation of UFA-dependent, but not GTPγS-dependent type PLD during apoptosis in Jurkat T cells

At least two types of PLD have been described in several tissues: UFA-dependent type and small G protein-dependent type (26). To know which type is responsible for the increased PLD activity during apoptosis in Jurkat T cells, the effects of UFA and GTPγS were examined on PLD activity in the lyses. It was shown that the PLD activity in the control lyses was stimulated by either oleic acid (1.8 mM) or arachidonic acid (1.8 mM) (Figs. 5 and 6A). However, GTPγS (10 μM) failed to stimulate PLD activity in Jurkat T cells (Fig. 6B). This profile of PLD activation is characteristic of Jurkat T cells. This is not true with HL60 cells; the GTPγS-dependent PLD activity in lyses from HL60 cells was much higher than that in Jurkat T cell lyses under the same assay condition (Fig. 6B). In contrast, the PLD activity in HL60 cells was much less sensitive to UFA (Fig. 6A). These results suggested that the PLD activity in Jurkat T cells was principally activated by UFA, while not small G proteins. To confirm this, the effects of sodium olate were examined for PLD activity in lyses from control and actinomycin D-treated cells. In the lyses from control cells, the basal PLD activity was at a marginal level, but was enhanced by the addition of 1.8 mM sodium olate (Fig. 7). In contrast, the PLD activity in the lyses from actinomycin D-treated cells was already elevated to the same level as that in the oleate-added control cells. There was no further enhancement by the addition of oleate in the PLD activity in the actinomycin D-treated cell lysate, suggesting that the increased PLD activity in apoptotic cells may be due to the released UFA, probably via phospholipase A₂. Indeed, we have observed that oleic acid and arachidonic acid were released time dependently during apoptosis induced by actinomycin D in Jurkat T cells (Fig. 8). The extent of release was much greater in oleic acid than arachidonic acid. The results obtained suggest that during apoptotic process, the released UFA gave rise to activation of UFA-dependent PLD. However, biologically active metabolites of UFA by cyclooxygenase and lipoxygenase may potentially be implicated in the apoptotic events observed in this study. We have tested this possibility by using aspirin, an inhibitor for cyclooxygenase, but no effects on apoptosis induced by actinomycin D were observed (data not shown).
Inhibition of actinomycin D-induced apoptosis and PLD activation by caspase inhibitors

To further investigate the mechanisms for PLD activation during apoptosis, we have examined the effects of caspase inhibitors, z-DEVD and z-VAD. As shown in Fig. 9A, these caspase inhibitors effectively suppressed apoptosis induced by actinomycin D. Furthermore, PLD activation during actinomycin D-induced apoptosis was also prevented (Fig. 9B). To further examine the relationship between caspase activation and release of UFA, we have examined whether the release of UFA was affected by caspase inhibitors or not. However, they failed to inhibit UFA liberation during apoptotic process (data not shown).

Discussion

Accumulating evidence suggests that PLD is involved in a variety of cellular responses. Previous studies have indicated the possibility that receptor-mediated PLD activation may play essential roles in not only immediate responses such as degranulation and respiratory burst, but also long-term responses such as cell growth and differentiation (16). Furthermore, Arf or Rho family, the activating factors of PLD, are known to take great parts in vesicular trafficking (34) or cytoskeletal organization (35), respectively. Recent reports have suggested that PA generated by PLD would be implicated in membrane traffic (36) and changes of cell morphology (37). On the other hand, the Jurkat T cell line has been used as a good model for the studies of signal transduction of T lymphocytes. Previous reports have demonstrated that Jurkat T cells exhibited PLD activation in response to anti-CD3 mAb or phorbol ester (PMA) (38–40). In particular, PLD stimulation could be implicated in the regulation of nuclear events by modulating the activity of transcription factor AP-1 in Jurkat T cells (38). This report suggests that the induction of AP-1 enhancer factor activity by PA is mediated via PKC stimulation, either through a direct activating effect of PA or 1,2-diacylglycerol from PA in Jurkat T cells. The possible involvement of PLD activation in the expression of c-jun and c-fos has also been observed in several types of cells (41–43). The induction of AP-1 activity is originally thought to be implicated in cell growth or differentiation. However, recent reports have also described the role of AP-1 induction during apoptotic process (44–46). In the present study, the PLD activity was found

FIGURE 4. PLD activity measured with exogenous substrate in cell lysates from control and apoptotic Jurkat T cells. Cell lysates (100 μg of protein/assay) from control untreated Jurkat T cells or apoptotic cells treated with 5 μg/ml actinomycin D (ActD) for 9 h were incubated with substrate phospholipid vesicles containing [palmitoyl-3H]DPPC at 37°C for 60 min in the presence of 0.5% butanol. The PLD activities were determined by measuring the formation of [3H]PBut, as described in Materials and Methods. Data represent the means ± SD from two different experiments, each conducted in duplicate.

FIGURE 5. Effects of UFA on PLD activity in Jurkat T cell lysate. UFA-dependent PLD activity was measured by the method described in Materials and Methods. Cell lysates (100 μg protein/100 μl) from control Jurkat T cells were incubated with 1.8 mM sodium oleate (OLE) or 1.8 mM sodium arachidonate (ARA) and the substrate phospholipid vesicles at 37°C for 60 min in the presence of 0.5% butanol. The PLD activities were determined by measuring the formation of [3H]PBut, as described in Materials and Methods. Data represent the means ± SD from two different experiments, each conducted in duplicate.

FIGURE 6. Effects of oleate and GTPγS on PLD activities in Jurkat T cell and HL60 cell lysates. Oleate- or GTPγS-dependent PLD activity was measured by using two different assay methods, as described in Materials and Methods. A total of 1.8 mM sodium oleate (A) or 10 μM GTPγS (B) was added in the reaction mixture. The PLD activities were determined by measuring the formation of [3H]PBut. Data represent the means ± SD from two different experiments, each conducted in duplicate.

FIGURE 7. Oleate-dependent PLD activity in cell lysates from control and apoptotic Jurkat T cells. Cell lysates (100 μg protein/100 μl) from control or apoptotic cells treated with 5 μg/ml actinomycin D (ActD) for 9 h were incubated with 1.8 mM sodium oleate and the substrate phospholipid vesicles at 37°C for 60 min in the presence of 0.5% butanol. The PLD activities were determined by measuring the formation of [3H]PBut, as described in Materials and Methods. Data represent the means ± SD from two different experiments, each conducted in duplicate.
to increase concurrently with apoptosis in Jurkat T cells. Taken together, it is tempting to speculate that PLD is somehow implicated in apoptosis. Therefore, the mechanism(s) of PLD activation during apoptosis of Jurkat cells was investigated.

Several factors have been identified as activating factors for PLD activity (27, 36, 47–49). However, the regulatory mechanism in detail has remained unsolved, since PLD has not been molecularly defined until recently. Three mammalian genes encoding PLD (PLD1a, PLD1b, and PLD2) were recently identified (19–21, 50). The catalytic activities of PLD1a and PLD1b are dependent on PIP2 as an essential cofactor, and they are activated by Arf and Rho family proteins, and by PKCα (19). PLD2 also requires PIP2 for activity. On the other hand, UFA such as oleic and arachidonic acids were also known as a stimulator of PLD activity. UFA-dependent PLD has been purified from pig lung (17), but its activation mechanism(s) in detail is not known yet. In contrast, the biochemical and regulatory properties of PLD1a, PLD1b, and PLD2 were recently well characterized (19, 20, 51) and their activities are not stimulated by oleate, but rather inhibited.

It was to be noted that the increase in PLD activity was well correlated with apoptotic process in Jurkat T cells exposed to actinomycin D (Fig. 3), suggesting that activating factor(s) for PLD activity may act during apoptosis. In Jurkat T cells, the PLD activity in vitro assay systems was greatly activated by UFAs, especially oleic acid, whereas it was insensitive to GTPγS and PIP2. Interestingly, in HL60 cells that predominantly express small G protein-dependent PLD, PLD activity was not increased during actinomycin D-induced apoptosis. Thus, it was indicated that the PLD activity is principally due to the UFA-type PLD in Jurkat T cells. Furthermore, it was shown that addition of oleate did not further enhance PLD activity in the in vitro assay system in actinomycin D-treated cells. In other words, the UFA-dependent PLD was already maximally activated in the apoptotic Jurkat T cells. These results suggest that UFA-dependent PLD, but not small G protein-dependent PLD, would be responsible for the increased PLD activity during actinomycin D-induced apoptosis. This notion was supported by the increased release of UFA in apoptotic Jurkat T cells (Fig. 8). Taken together, the enhancement of PLD activity during apoptosis can be considered to be due to the increased release of UFA, for example, oleic acid. The increased release of UFA in apoptotic cells could be accounted for by stimulation of phospholipase A2 activity during apoptosis. We have observed recently that H2O2, a well-known apoptosis inducer, stimulates nonselective fatty acid release via phospholipase A2 of intracellular calcium-independent type in PC12 cells (52). However, mechanism of UFA release in detail remains to be resolved.

Caspases are a common and critical component of the cell death pathway (53, 54). In this study, we investigated the effects of caspase inhibitors on PLD activation, UFA release, and apoptosis. Apoptosis and PLD activation were effectively suppressed (Fig. 9), but UFA release was not affected. These results indicate that proteolytic process(es) by caspases is required for the activation of UFA-dependent PLD, and that PLD activation and apoptosis are closely correlated.

We attempted to block PLD activity to clarify whether PLD activation preceded or followed apoptosis. To date, however, selective inhibitors for PLD are not available. The known agents that modulate PLD activity are primary alcohols. Ethanol and butanol, substrates for transphosphatidylation activity of PLD, decrease PA production (15, 16). However, these alcohols had minimal effects on apoptotic changes: nuclear condensation and fragmentation (data not shown). These results imply that production of PA or its metabolites by PLD pathway is not prerequisite to apoptosis. However, these alcohols do not appear to largely impair breakdown of PC, a main component of membrane phospholipids. Therefore, UFA were exogenously added to Jurkat T cells, and the changes of PLD activity and nuclear staining were further examined. UFA at 0.5–1.8 mM, which stimulate PLD activity in Jurkat cell lysate, caused necrotic changes (prominent LDH release) probably due to their detergent-like effect. As evident from the experiments with caspase inhibitors, UFA is not sufficient to stimulate PLD activity. The data indicating a role for PLD in the induction/promotion of apoptosis were not obtained. Since PLD hydrolyzed PC, a main component of membrane phospholipids, unregulated PLD activation as a result of apoptosis may cause membrane damage, leading to the release of intracellular components, such as LDH. However, LDH release slightly increased in Jurkat cells treated with actinomycin D for 9 h, when nearly 55% of cells displayed characteristic

FIGURE 8. Release of UFA during apoptosis induced by actinomycin D in Jurkat T cells. Cells prelabeled with [3H]oleic acid or [3H]arachidonic acid were treated with 5 μg/ml actinomycin D for indicated times. The released [3H]oleic acid or [3H]arachidonic acid was determined as described in Materials and Methods. Data represent the means ± SD from two different experiments, each conducted in duplicate.

FIGURE 9. Effects of caspase inhibitors on apoptosis and PLD activation in Jurkat T cells. A, Jurkat T cells were preincubated for 30 min with medium containing 100 μM z-DEVD.FMK or z-VAD.FMK, and then treated with 5 μg/ml actinomycin D (ActD) for 9 h. Levels of apoptosis were quantitated by counting the typical apoptotic cells (chromatin condensation and fragmentation), as described in Materials and Methods. More than 400 cells were counted in each sample. Data shown are means ± SD from two different experiments, each performed in triplicate. B, [3H]Palmitic acid-labeled Jurkat T cells were preincubated for 30 min with medium containing 100 μM z-DEVD.FMK or z-VAD.FMK, and then were treated with 5 μg/ml actinomycin D (ActD) for 9 h. Treated cells were incubated for 30 min at 37°C in the presence of 0.3% butanol. The PLD activity was determined by measuring the formation of [3H]But, as described in Materials and Methods. Data represented the means ± SD from two different experiments, each conducted in duplicate.
apoptotic nuclei. Therefore, from the present data, it is still premature to determine the cause and effect relationship between PLD activation and induction of apoptosis. To our knowledge, this is the first demonstration of activation of UFA-PLD dependent in intact cell system.

In summary, PLD activity was elevated during apoptosis induced by actinomycin D in Jurkat T cells. Similar findings were also obtained in apoptosis induced by either H2O2 or TNF-α. Moreover, it was of great interest to note that the increased PLD activation was principally due to the oleate-dependent type PLD. Thus, these results led us to suggest that oleic acid-mediated PLD activation may be involved in the apoptotic process in Jurkat T cells. However, further studies are required to disclose the precise mechanism for the increased UFA-induced PLD activation during apoptosis in Jurkat T cells.

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