Activation of PKCα Downstream from Caspases during Apoptosis Induced by 7-Hydroxystaurosporine or the Topoisomerase Inhibitors, Camptothecin and Etoposide, in Human Myeloid Leukemia HL60 Cells*

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We previously demonstrated that the anticancer agent and protein kinase C (PKC) inhibitor 7-hydroxy-staurosporine (UCN-01) induces apoptosis independently of p53 and protein synthesis in HL60 cells. We now report the associated changes of PKC isoforms. PKCα, βI, βII, δ, and ζ activities were measured after incubation of cytosols from UCN-01-treated HL60 cells. UCN-01 had no effect on PKCζ and inhibited kinase activity of PKCβI, βII, and δ. PKCα activity was initially inhibited at 1 h, and subsequently increased as cells underwent apoptosis 3 h after the beginning of UCN-01 treatment. Camptothecin (CPT) and etoposide (VP-16) also markedly enhanced PKCα activity during apoptosis in HL60 cells. However, CPT did not affect PKCβI, βII and ζ, and activated PKCδ. PKCα activation was not due to increased protein levels or proteolytic cleavage but was associated with PKCα autophosphorylation in vitro and increased phosphorylation in vivo. We also found that not only PKC δ but also PKC β was proteolytically activated in HL60 cells during apoptosis. The PKCα activation and hyperphosphorylation were abrogated by N-benzyloxy carbonyl-Val-Ala-Asp(O-methyl)-fluoromethylketone (z-VAD-fmk) under conditions that abrogated apoptosis. z-VAD-fmk also prevented PKCδ and β proteolytic activation. Together these findings suggest that caspases regulate PKC activity during apoptosis in HL60 cells. At least two modes of activation were observed: hyperphosphorylation for PKCα and proteolytic activation for PKC δ and βI.

Protein kinase C (PKC) is a key enzyme for transduction of extracellular and phospholipid-mediated signals as well as for tumor promotion (1). The biochemical mechanisms underlying PKC action have become increasingly clear (1–3). PKC isoforms have been categorized in three subclasses: conventional PKCs (εPKCs) (α, βI, βII, and γ) which require phosphatidylserine (PS), diacylglycerol (DAG); and Ca2+; novel PKCs (nPKCs) (δ, ε, η, and θ) which require PS and DAG but not Ca2+; and the atypical PKCs (aPKCs) (ζ, τ, and λ) (3). Several observations suggest the implication of PKC in apoptosis. For example, it has been observed that the activation of PKC by exposure to phorbol 12-myristate 13-acetate (PMA) either alone or in conjunction with Ca2+ ionophore induces apoptosis in lymphoid (4–6) and myeloid (7) cells. PMA suppresses steroid-induced apoptosis in thymic lymphocytes (8) and opposes topoisomerase-induced apoptosis in HL60 cells as these cells differentiate (7). Several PKC inhibitors have been reported to induce apoptosis. Staurosporine is one of the most potent and universal inducers of apoptosis in a variety of cell lines (9). Its 7-hydroxy derivative, UCN-01, which is a more specific PKC inhibitor (10) and an effective antitumor agent (11, 12), also induces apoptosis in T lymphoblasts (13), K562, HT29 cells, and HL60 cells independently of p53 (14). UCN-01-induced apoptosis in HL60 and K562 cells is independent of protein synthesis (14). Calphostin C induces apoptosis in four human glioma cell lines (GB-1, T98G, U-373MG, and A-172) (15) and HL60 cells (16). Additional evidence for PKC activation during apoptosis is that ceramide activates PKCζ and down-regulates PKCα during apoptosis (17–19). Emoto et al. (20) reported proteolytic activation of PKCδ during apoptosis induced by DNA damaging agents (ionizing radiation, camptothecin (CPT) and 1-β-D-arabinofuranosylcytosine (ara-C)) in human myeloid leukemia U937 cells (21).

In the present study, we investigated PKC isoform activities during UCN-01-induced apoptosis in human myeloid leukemia HL60 cells. HL60 cells were chosen for these studies because they undergo rapid apoptosis to various agents including UCN-01. This apoptosis is p53-independent as HL60 cells are p53 null (22) and does not require protein synthesis (14, 23). It is preceded by transient activation of cyclin B1/Cdc2 kinase (14, 24). The changes of PKC activity were also examined in HL60 cells undergoing apoptosis after treatment with the topoisomerase inhibitors, CPT and etoposide (VP-16). We found that UCN-01 inhibited PKCβI, βII, and δ activity in drug-treated cells as well as in vitro. For PKCα, UCN-01 first inhibited the kinase activity and then activated PKCα. CPT-induced apoptosis was associated with marked increase in PKCδ activities. The mechanisms of PKC activation and their relationship to caspases (ICE-like proteases) are investigated.

MATERIALS AND METHODS

Drugs, Chemicals and Antibodies—UCN-01 was provided by Dr. H. Nakano (Kyowa Hakko Co., Japan) or the Drug Synthesis and Chemistry Branch, NCI, National Institutes of Health. CPT was provided by Dr. M. R. Wall (Research Triangle Institute, Research Triangle Park, NC) or the Drug Synthesis and Chemistry Branch, NCI, National Institutes of Health. N-benzyloxy carbonyl-Val-Ala-Asp(O-methyl)-fluoromethylketone (z-VAD-fmk) was purchased from Enzyme System Products (Dublin, CA). Other drugs and reagents, unless otherwise mentioned, were purchased from Sigma.
Anti-PKC antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit IgG horseradish peroxidase was purchased from Amersham Life Science, Inc. (Arlington Heights, IL). [γ-32P]ATP (4500 Ci/mmol), and [32P]orthophosphate were purchased from NEN Life Science Products (Boston, MA). Protein A-Sepharose 4B was from Pharmacia Biotech Inc. (Sweden). P-81 ion exchange filter paper was obtained from Whatman.

Cell Culture—Human promyelocytic leukemia HL60 cells were grown at 37 °C in the presence of 5% CO2 in RPMI 1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Isolation of Cytosol and Nuclei—Cytosols from untreated control and treated HL60 cells were extracted using nucleus buffer (150 mM NaCl, 1 mM KH2PO4, 5 mM MgCl2, 1 mM EGTA, 0.1 mM p-aminoethylbenzenesulfonylfluoride, 0.15 units/ml aprotinin, 1.0 mM Na3VO4, 5 mM HEPES (pH 7.4), 10% glycerol, and 0.3% Triton X-100) as described previously (25). Protein concentration was measured using a protein assay kit according to the manufacturer instructions (Bio-Rad).

Detection of MBP4–14 Phosphorylation—Cytosols (50 μl) from control or treated (1 × 107) HL60 cells were mixed with 50 μl of 2 × reaction buffer containing 5 mM HEPES (pH 7.4), 5 mM MgCl2, 10 μM ATP, 40 μg/ml phosphatidylserine, 3.3 μM dioleoylglycerol, 1.2 mM CaCl2, 25 mM MBP4–14, 2 μCi [γ-32P]ATP. After incubation for 7 min at 30 °C, the transfer of [32P]phosphate was quantified by the phosphocellulose paper-binding method (26).

Immunoprecipitation of PKCs—350–500 μl cytosols from control or treated (2 × 107) HL60 cells were precleared with 20 μl of protein A-Sepharose for 1 h and incubated with anti-PKC antibodies and 30 μl of protein A-Sepharose at 4 °C for 4 h. The beads were washed three times with nucleus buffer. This was followed by 2 washes with kinase buffer (50 mM Tris-HCl (pH 7.4), 10 mM NaF, 1 mM Na3VO4, 0.5 mM EGTA, 0.5 mM EDTA, 2 mM MgCl2, 5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride).

PKC Assays—Immunocomplex beads were obtained by incubating different anti-PKC antibodies with cytosolic extracts in 20 μl of reaction buffer containing 20 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 10 μM cold ATP, 0.4 mg/ml histone H1, 40 μg/ml PS, 3.3 μM dioleoylglycerol, 5 μCi of [γ-32P]ATP in the absence or presence of 1.2 mM CaCl2 depending on the PKC isoform activity measured. Incubations were carried out at 30 °C for 10 min. Samples were loaded onto 12% SDS-PAGE gels (NOVEX, San Diego, CA) and electrophoresed at 120 V for 2 h. For quantification of kinase activity in immunoprecipitates, gels were dried, and the extent of histone H1 phosphorylation was measured using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Western Blot Analysis of PKCs—Cytosolic samples were electrophoresed at 120 V on 12% SDS-PAGE gels and electrophoretically transferred to Immobilon membranes. Membranes were blocked overnight in phosphate-buffered saline-Tween 20 (PBS-T) containing 5% non-fat dried milk. Probing with selected anti-PKC antibodies (1 μg/ml in PBS-T) for 1 h was followed by incubation with horseradish peroxidase-labeled anti-rabbit IgG (1:1000 dilution). After washing in PBS-T, membranes were developed using the enhanced chemiluminescence (ECL) detection system (NEN Life Science Products).

RESULTS

UCN-01- and CPT-induced Apoptosis Are Associated with Increased PKC Activity in HL60 Cells—Our previous studies indicated that UCN-01 and CPT are potent inducers of apoptosis in HL60 cells (14, 27). After 3 h of drug treatment, 60–90% of the cells exhibit apoptosis with typical morphological changes and internucleosomal fragmentation. To investigate whether PKC was involved in apoptosis induced by these anticancer drugs, we treated HL60 cells with UCN-01 or CPT and extracted cytosol and membrane fractions at various times. The synthetic peptide, MBP4–14, was used as a specific and sensitive substrate to assay PKC activity (26). We found that phosphorylation of MBP4–14 first decreased during the first hour of UCN-01 treatment compared with control and then increased progressively above control values 3 h after the beginning of treatment. CPT increased the phosphorylation of MBP4–14 already after 1 h of treatment (Fig. 1). These measurements were performed in cytosolic fractions. In membrane fractions, there was no significant difference between basal and stimulated levels of MBP4–14 phosphorylation and no change of MBP4–14 phosphorylation after exposure to UCN-01 or CPT for 3 h (not shown). These data indicate that PKC activity is induced by UCN-01 and CPT treatment in HL60 cells undergoing apoptosis.

UCN-01- and CPT-induced Apoptosis Are Associated with Up-regulation of PKCα Activity—The above data raised the question as to what kinds of PKC isoforms were activated by UCN-01 or CPT. MBP4–14 is selective for PKCα, β, βII, and δ isoforms but has been reported to be a poor substrate for other isoforms (26). From the work by Seynaeve et al. (10), we also...
know that UCN-01 inhibits PKC\(\alpha\), \(\beta\), \(\gamma\), \(\delta\), and \(\epsilon\) and does not inhibit PKC\(\zeta\) \textit{in vitro}. Since PKC\(\gamma\) was undetectable in HL60 cells and PKC\(\epsilon\) was expressed only very weakly using Western blotting (data not shown) (1), we used anti-PKC\(\alpha\), \(\beta\)I, \(\beta\)II, \(\zeta\), and \(\delta\) antibodies and immunoprecipitation to determine the effects of UCN-01 and CPT on PKC isoform activities. Fig. 2 shows that UCN-01 inhibited all the PKC isoforms tested at 1 h of treatment, which is consistent with the known anti-PKC activity of UCN-01 \textit{in vitro} (10). However, at 3 h, PKC\(\alpha\) activity was markedly stimulated (5-fold) by UCN-01. CPT increased PKC\(\alpha\) and \(\delta\) activities but had no effects on PKC\(\beta\)I and \(\beta\)II activities. Neither UCN-01 nor CPT affected PKC\(\zeta\) activity (data not shown). These data indicate that apoptosis induced by UCN-01 and CPT treatment is associated with marked PKC\(\alpha\) activation.

**Suppression of UCN-01- and CPT-induced Apoptosis by the Caspase Inhibitor, z-VAD-fmk, Is Associated with Lack of PKC\(\alpha\) Activation**—Caspases (ICE-like proteases) are key mediators of apoptosis (28–30), and we previously showed that the caspase inhibitor z-VAD-fmk prevents UCN-01-induced apoptosis in HL60 cells (14). Fig. 3A, shows that z-VAD-fmk also prevents apoptotic DNA fragmentation induced by CPT in HL60 cells. However, z-VAD-fmk does not inhibit the DNA fragmentation induced by UCN-01 and CPT-activated cytosol in cell-free system (8, 14, 25) (Fig. 3B). Under these conditions, we examined whether z-VAD-fmk affected the PKC changes induced by UCN-01 or CPT in HL60 cells undergoing apoptosis. We found that z-VAD-fmk blocked the activation of PKC\(\alpha\) induced either by UCN-01 or CPT. However, z-VAD-fmk did not suppress the CPT-induced activation of PKC\(\delta\) (Fig. 4A) and had no effects on
PKC activity directly. CPT had no direct effect on PKC

A, B, C

Fig. 4. UCN-01- and CPT-induced apoptosis are associated with proteolytic cleavage of PKCβII and δ while PKCα and βII proteins appear unchanged. HL60 cells were treated with 10 μM UCN-01 or 1 μM CPT in the absence and presence of z-VAD-fmk. Cytosols from control and treated cells were subjected to Western blotting analysis using antibodies against PKCα, β, βII, and δ (panels A, B, C, and D, respectively).

Fig. 5. UCN-01- and CPT-induced apoptosis increase PKCα autophosphorylation in vitro. A, PKCα autophosphorylation in UCN-01- and CPT-activated cytosols was carried out essentially as described for histone H1 phosphorylation except that histone H1 was omitted. B, treatment of cells with z-VAD-fmk inhibits PKCα autophosphorylation induced by UCN-01 or CPT.

the inhibitions of PKCβII, βII, and δ activities induced by UCN-01 (Fig. 4). We next tested whether PKCα was activated in response to the topoisomerase II inhibitor, VP-16. We found that VP-16 also activated PKCα in HL60 cells undergoing apoptosis (8) (Fig. 4C). These data suggested that apoptosis induced by different pathways (topoisomerase inhibition for CPT or VP-16 or protein kinase inhibition for UCN-01) is associated with PKCα activation in HL60 cells. The observation that z-VAD-fmk blocked PKCα activation suggests that PKCα is downstream from caspases during apoptosis in HL60 cells.

Apopotic PKCα Activation Is Not Due to a Direct Effect of UCN-01 or CPT, and Suppression of PKCα Activation Is Not Due to a Direct Effect of z-VAD-fmk on PKCα Activity.—To further investigate the mechanisms by which UCN-01 or CPT regulate PKCα activity, we first tested whether UCN-01 or CPT directly affected PKCα activity using in vitro kinase assays. PKCα immunoprecipitates obtained from control cytosol were tested for kinase activity with UCN-01 or CPT. Fig. 5A shows that UCN-01 did not activate but rather inhibited PKCα activity directly. CPT had no direct effect on PKCα activity (Fig. 5A). z-VAD-fmk was also tested in this system and did not exhibit a direct effect on PKCα activation induced by UCN-01 or CPT (Fig. 5B). These results indicated that the PKC changes observed in cytosols from cells treated with UCN-01 or CPT in the absence or presence of z-VAD-fmk were indirect.

We next examined whether the activation of PKCα by UCN-01 or CPT and the effects of z-VAD-fmk might be due to alterations of the PKCα protein. Western blot analyses were carried out on cytosols from untreated, UCN-01- or CPT-treated HL60 cells using anti-PKCα antibody. PKCα protein levels remained unchanged after drug-treatments (Fig. 6A). Thus, PKCα activation by UCN-01 and CPT was not due to changes in PKCα protein levels. We also measured the other PKC isoforms. Protein levels of PKCβII, βII, and δ did not change significantly (Fig. 6, B–D). Interestingly, UCN-01 and CPT induced PKCβII and δ cleavages when HL60 cells underwent apoptosis, and z-VAD-fmk blocked these proteolytic effects. Proteolytic activation of PKCδ by caspases is consistent with results obtained in U-937 cells (21). However, our data suggest that PKCβII is also cleaved by caspases during apoptosis in HL60 cells.

Since neither proteolytic activation nor change in protein levels appear to account for PKCα activation, we next tested the effects of UCN-01 and CPT on the PKCα autophosphorylation. It is indeed known that autophosphorylation is one of the important modes of regulation of PKC activity (31). Autophosphorylation assays for PKCα were carried out essentially as described for histone H1 phosphorylation except that histone H1 was omitted from the reactions. Fig. 7A shows that treatment of HL60 cells for 3 h with either UCN-01 or CPT increased PKCα autophosphorylation (Fig. 7A) and that z-VAD-fmk blocked this increased autophosphorylation (Fig. 7B). Finally, we performed experiments to test in vivo phosphorylation of PKCα in HL60 cells treated with UCN-01. Fig. 8 shows that UCN-01 increased PKCα phosphorylation after 1 h of treatment and that z-VAD-fmk inhibited the UCN-01-induced PKCα phosphorylation. These results suggest that caspase activation during apoptosis in HL60 cells is upstream from PKCα autophosphorylation and activation.

DISCUSSION

The results of Fig. 1 show that although UCN-01 is a PKC inhibitor and CPT is a topoisomerase I inhibitor, both of them induced activation of PKC. Using immunoprecipitation assay for PKC activities, we found that UCN-01 inhibited PKCβII, βII, and δ activities in whole cells as well as in vitro but that PKCα was activated in HL60 cells undergoing apoptosis. Apoptosis induced by CPT, a DNA topoisomerase I inhibitor was also associated with activation of PKCα as well as PKCδ activation.

Caspases play a central role in the apoptosis pathways (28–30). z-VAD-fmk, a cell-permeable caspase inhibitor with broad specificity, blocks apoptosis induced by various stimuli including treatment by UCN-01 or CPT. Our results demonstrate...
that z-VAD-fmk selectively inhibited the activation of PKCa induced by UCN-01 and CPT. On the other hand, z-VAD-fmk did not affect the inhibition of PKCβII, βIII, and δ activities by UCN-01 and the activation of PKCδ by CPT. At the same time, we found that z-VAD-fmk blocked the proteolytic cleavages of PKCβ and PKCδ, suggesting that PKCδ cleavage is not the only mechanism for PKCδ activation. For PKCa, we found no evidence that PKCa activation is associated with proteolytic cleavage.

Our data (Fig. 5) rule out that UCN-01, CPT, or z-VAD-fmk directly affected PKCa. PKCa activation is not due to changes in PKCa protein levels since control and cells treated with UCN-01 or CPT in the absence and presence of z-VAD-fmk had approximately equal amounts of PKCa protein as detected by Western blot. Phosphorylation has emerged as an important mode of regulation for PKCa (3, 32). For example, mutation of Thr to Ala in PKCa (Thr-497) results in an inactive kinase (33), and PKCα autophosphorylation has been shown to regulate PKCα activity by trans-phosphorylation at the activation loop fol-

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