Distinct PKC isozymes regulate bufalin-induced differentiation and apoptosis in human monocytic cells

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Bufalin, an Na\(^{+}\)-K\(^{+}\)-ATPase inhibitor, simultaneously induced cell differentiation and apoptosis in human monocytic leukemia THP-1 cells. In this study, we investigated the regulatory role of protein kinase C (PKC) isozymes in bufalin-induced cell differentiation and apoptosis. A PKC-specific but isoform-selective inhibitor, Ro-31–8220, and a cPKC selective inhibitor, Go–6976, caused significant attenuation of bufalin-induced interleukin-1β (IL-1β) gene expression, a mature monocytic marker, indicating that cPKC participates in the bufalin-induced cell differentiation. On the other hand, nPKCβ-subfamily and nPKCδ-defective THP-1/TPA cells displayed strong resistance to the bufalin-induced DNA ladder formation. Rottlerin, an nPKCδ-specific inhibitor, partially attenuated preapoptotic effects of bufalin, such as the limited proteolysis of nPKCζ and poly(ADP-ribose) polymerase and the cell staining by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, suggesting that nPKCδ is involved, at least in part, in bufalin-induced apoptosis. In contrast, Go–6976 and rottlerin significantly augmented bufalin-induced apoptosis and differentiation, respectively. The findings suggest that bufalin-induced cell differentiation and apoptosis are interlinked and that distinct PKC isozymes are involved in the fate of the cell.

BUFALIN, a specific inhibitor of Na\(^{+}\)-K\(^{+}\)-ATPase, has been shown to induce leukemia cell differentiation (31) and apoptosis (26) under certain experimental conditions. It has been reported that bufalin utilizes the extracellular signal-regulated kinase (ERK) cascade for signal transduction, leading not only to cell differentiation (13) but also to apoptosis (25, 26). It is thus postulated that the ERK cascade may control bufalin-induced cell differentiation and apoptosis simultaneously. It is generally accepted that an increase in ERK activity counteracts apoptotic signals, including stress-activated mitogen-activated protein kinases (MAPKs), such as c-Jun NH\(_2\)-terminal kinase (JNK) and p38, and family members of caspase-like proteases (14, 29). Nevertheless, explanations for the overlapping of responses and functions of these MAPKs between distinct stimuli (8, 10, 21) have been obscure. Therefore, bufalin induction of cell differentiation and apoptosis could be a valid experimental model to elicit precise roles of ERK and other MAPKs in cell death and survival.

We recently reported that a family of phospholipid-activated serine/threonine protein kinases, protein kinase C (PKC), plays an important role as an upper module governing the ERK protein kinase cascade in the signal transduction leading to bufalin-mediated cell differentiation (13). The findings prompted us to examine a function of PKC in bufalin-mediated apoptosis. A family of mammalian PKC is classified into three subfamilies: conventional PKC (cPKC), novel PKC (nPKC), and atypical PKC (aPKC). The cPKC subfamily is composed of α-, β-, and γ-isozymes, which are activated in a manner dependent on Ca\(^{2+}\) and diacylglycerol. The nPKC subfamily is composed of δ-, ε-, and θ-isozymes, which require diacylglycerol for the activity but are independent of Ca\(^{2+}\). The aPKC subfamily is composed of ζ- and η-isozymes, which are independent of diacylglycerol and Ca\(^{2+}\). Because of differences in tissue distribution, subcellular localization, and substrate specificity, these PKC isozymes are involved in diverse functions, including induction of cell differentiation and apoptosis (22).

We previously reported that 12-O-tetradecanoylphorbol 13-acetate (TPA)-resistant THP-1 (THP-1/TPA) cells are capable of inducing interleukin-1β (IL-1β) mRNA, a biochemical marker for mature monocytes, in response to bufalin, similar to the parent THP-1 cells (20). In the present study, we show that THP-1/TPA cells, in which cPKCβ and nPKCζ were deficient, are resistant to bufalin-induced apoptosis. Using this model system in conjunction with specific inhibitors, we demonstrate that cPKC and nPKC have divergent roles in the bufalin-mediated cell differentiation and apoptosis. Our data suggest that cPKC and nPKC direct cell differentiation and apoptosis, respec-
tively, and that these pathways are coupled in deciding the fate of the cell.

**EXPERIMENTAL PROCEDURES**

**Materials.** TPA was purchased from Sigma Chemical (St. Louis, MO). Ro-31–8220, G6-6976, and rattlerin were purchased from Calbiochem (La Jolla, CA). Monoclonal anti-PKC antibodies for immunoblot analysis and polyclonal anti-poly(ADP-ribose) polymerase (PARP) antibodies were purchased from Transduction Laboratories (Lexington, KY) and Wako Chemicals (Osaka, Japan), respectively. LY-379196 was a gift from Eli Lilly (Indianapolis, IN). Apoptosis Screening Kit was purchased from Wako Chemicals. Other chemicals were of the highest grade commercially available.

**Cell culture.** THP-1 cells (24) were obtained from Riken Cell Bank (Tsukuba, Japan). THP-1/TPA cells (20) were maintained in medium containing 100 nM TPA and were cultivated without TPA for 1 wk before use. The cells were cultivated as reported previously (20).

**Northern blot analysis.** Total RNA was isolated from cells by acid guanidinium thiocyanate-phenol-chloroform extraction as described by Chomczynski and Sacchi (1). Northern blot analysis was carried out as described previously (20). Probes used were the 1.1-kb PstI insert of a human IL-1β cDNA purified from IL-1 X-14 plasmid (17) (American Type Culture Collection) and the 0.5-kb insert of a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA purified from GD5 plasmid (16).

**DNA fragmentation.** Cells were washed with PBS and lysed in 10 mM Tris-HCl (pH 7.4) containing 10 mM EDTA and 0.5% (wt/vol) Triton X-100 for 10 min at 4°C. After centrifugation at 15,000 rpm for 5 min, the supernatant was incubated with 0.2 mg/ml RNase A at 50°C for 60 min and then with 0.2 mg/ml proteinase K at 37°C for 30 min. After precipitation with isopropanol, samples were subjected to electrophoresis on a 1% (wt/vol) agarose gel in 40 mM Tris-acetate (pH 7.5) containing 1 mM EDTA for 60 min at 100 V. DNA was visualized by ethidium bromide staining.

**Quantification of bufalin-induced apoptosis.** Apoptotic cells were quantified by a modified terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method according to the manufacturer’s instructions (Wako). Briefly, fixed cells in 96-well microplates were incubated with terminal deoxynucleotidyl transferase and fluorescein-labeled dUTP at 37°C for 30 min and then with peroxidase-conjugated anti-fluorescein antibodies at 37°C for 30 min. The peroxidase reaction was carried out with hydrogen peroxide and o-phenylenediamine as substrates and measured at 492 nm.

**Immunoblot analysis.** Cells were lysed in the boiling SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, 2% SDS, 10% glycerol, and 0.025% bromphenol blue). Denatured proteins were separated on a polyacrylamide gel (8%) and transferred to a polyvinylidene difluoride membrane (Pall Biosupport Division, Port Washington, NY) at 120 mA for 1 h with a semidry blotting apparatus. The membrane was incubated with 0.2% casein-based I-Block (Tropix, Bedford, MA) dissolved in 25 mM Tris-HCl, pH 7.4, containing 137 mM NaCl, 2.68 mM KCl, and 0.1% Tween 20 (TTBS) for 1 h at room temperature, washed with TTBS (3 times for 15 min each), and incubated for 1 h with primary antibody dissolved in the blocking solution overnight at 4°C. After it was washed with TTBS, the membrane was incubated for 1 h with the horseradish peroxidase-linked secondary antibodies. Immunoreactive proteins were detected by the enhanced chemiluminescence system (Amersham-Phar-macia Biotech, Buckinghamshire, UK).

**RESULTS**

**Responses of THP-1 and THP-1/TPA cells to bufalin.** A low concentration (30 nM) of bufalin induces differentiation of THP-1 cells characterized by continuous c-fos and egr-1 expressions (20), induction of IL-1β transcripts (Fig. 1A), and functional markers for mature monocytes, such as phagocytosis and substrate adherence (13). A higher concentration of bufalin (100 nM) also induced IL-1β mRNA, but to a lesser extent (Fig. 1A). Treatment of THP-1 cells with 100 nM bufalin induced DNA ladder formation, a typical characteristic of apoptotic cell death. Bufalin at 30 nM also induced DNA fragmentation, but to a lesser extent (Fig. 1B). We reported that inhibition of MAPK/ERK kinase (MEK), an upper kinase of ERK, abrogates IL-1β induction by bufalin (13). DNA ladder formation by bufalin was significantly suppressed by pretreatment of cells with a specific MEK inhibitor, U-0126 (3) (Fig. 1B). Bufalin induced IL-1β gene expression in THP-1/TPA cells to an extent similar to that in THP-1 cells (20) (Fig. 1A). However, apparently no ladder formation was observed when THP-1/TPA cells were exposed to 100 nM bufalin (Fig. 1B).

**PKC isozymes in THP-1 and THP-1/TPA cells.** THP-1 cells expressed α-, β-, and γ-isozymes of cPKC and the δ-isozyme of nPKC (Fig. 2). THP-1/TPA cells also expressed α- and γ-isozymes of cPKC to an extent similar to the parental cells; however, cPKCβ and nPKCδ virtually disappeared in the resistant cells (Fig. 2). Other isozymes were detected very weakly or not at all in THP-1 and THP-1/TPA cells (data not shown).

Fig. 1. Responses of THP-1 and THP-1/TPA cells to bufalin. A: THP-1 and THP-1/TPA (T/T) cells were left untreated (Cont) or treated with 30 or 100 nM bufalin (Bu) for 48 h. Top: Northern blot analysis carried out using a 32P-labeled human interleukin-1β (IL-1β)-specific probe. Bottom: 18S RNA visualized by ethidium bromide as a control of loading. B: THP-1 and THP-1/TPA cells left untreated or treated with bufalin or 10 μg/ml VP-16, which served as a positive control, for 12 h. In some cases, cells were pretreated with 3 μM U-0126 (U) for 1 h before bufalin treatment. DNA samples were analyzed by agarose gel electrophoresis and stained with ethidium bromide. Results are representative of 3 independent experiments with similar outcomes.
Effect of PKC inhibitors on bufalin-induced cell differentiation. We previously showed that pretreatment of THP-1 cells with Ro-31–8220, a specific but isozyme-nonselective PKC inhibitor (7), reduces bufalin-induced IL-1β gene expression (13). Gö-6976, which selectively inhibits cPKC isozymes (18), was also effective; bufalin-induced IL-1β gene expression was inhibited concentration dependently and apparently cancelled at 3 μM Gö-6976 (Fig. 3A). A suppressive effect of the PKC inhibitors on bufalin-induced IL-1β expression was also seen in THP-1/TPA cells to an extent similar to that observed in THP-1 cells (Fig. 3B). Interestingly, bufalin-induced IL-1β gene expression was significantly enhanced by rottlerin, an nPKCδ-specific inhibitor (6), in a concentration-dependent manner. The nPKCδ inhibitor at 2 μM augmented the bufalin-induced level of IL-1β transcripts by approximately fivefold (Fig. 3C). However, as expected, rottlerin showed no effect on bufalin-induced IL-1β gene expression in PKCδ-deficient THP-1/TPA cells (Fig. 3D).

Effect of PKC inhibitors on bufalin-induced apoptosis. THP-1 cells pretreated with PKC inhibitors followed by 100 nM bufalin were subjected to analysis of apoptotic changes. Bufalin induced limited proteolysis of 78-kDa nPKCδ and 116-kDa PARP, which is known to be catalyzed by caspase-like proteases (2, 23), resulting in the appearance of the 43- and 85-kDa fragments, respectively. Pretreatment with Gö-6976 significantly augmented limited proteolysis of PKCδ and PARP (Fig. 4A). The cPKC inhibitor also accelerated bufalin-induced DNA fragmentation (Fig. 4B) and the TUNEL-based cell staining (Fig. 4C). However, such an augmented effect was not observed when cells were pretreated with Ro-31–8220 (Fig. 4B). On the other hand, pretreatment with rottlerin suppressed the bufalin-induced level of proteolytic products of nPKCδ and PARP (Fig. 4A). In addition, rottlerin attenuated nucleosomal cleavage as determined by TUNEL staining (Fig. 4C). However, LY-379196, a specific PKCβ inhibitor (9), did not affect the preapoptotic proteolysis (data not shown).

Fig. 2. Expression of protein kinase C (PKC) isozymes in THP-1 (TH) and THP-1/TPA (T/T) cells. Cell lysates from THP-1 and THP-1/TPA cells were subjected to immunoblot analysis using monoclonal antibodies specific for α-, β-, γ-, and δ-isozymes of PKC.

Fig. 3. Effect of PKC inhibitors on bufalin-induced IL-1β gene expressions in THP-1 and THP-1/TPA cells. THP-1 and THP-1/TPA cells were pretreated with vehicle (0.1% DMSO), Gö-6976 (Go), Ro-31–8220 (Ro), or rottlerin for 1 h and then treated with 30 nM bufalin for 48 h. Northern blot analysis was carried out using a 32P-labeled IL-1β-specific probe. Ribosomal 18S RNA was visualized by ethidium bromide as a control of RNA loading (A and B). Steady-state levels of IL-1β mRNA were quantified with a Fuji BAS 3000 bioimaging analyzer and normalized with glyceraldehyde 3-phosphate dehydrogenase (C and D, bottom); values (means ± SE of 3 independent experiments) are expressed as percentage of bufalin-induced levels.
DISCUSSION

Bufalin preferentially induced THP-1 cells undergoing cell differentiation at a low concentration (30 nM) and apoptosis at a high concentration (100 nM). On the other hand, 30 nM bufalin also induced DNA fragmentation, but to a lesser degree than 100 nM bufalin. In addition, 100 nM bufalin induced IL-1β expression, but to a lesser degree than 30 nM bufalin. These results indicate that bufalin induces cell differentiation and apoptosis simultaneously. It has been suggested that the ERK cascade plays a major role in the cellular signal transduction involved in the induction of cell differentiation (13) and apoptosis (26) by bufalin, which are generally considered to be opposite responses (29). The present study also demonstrated that the specific inhibitor for the ERK cascade inhibited bufalin-induced DNA fragmentation. The query how the same molecule controls differentiation and apoptosis has arisen from these observations. One possible resolution for the issue would be that distinct upper signals utilize the ERK cascade, resulting in separate responses. It is suggested that scaffold or adaptor proteins may function in such a regulation (28, 30). On the basis of this hypothesis, we demonstrated in the present study a role of PKC isoforms in bufalin-mediated cell differentiation and apoptosis.

In addition to our previous observations that inhibition of PKC by Ro-31–8220 sequentially attenuates the bufalin-mediated ERK activation, c-fos induction, and IL-1β gene expression (13), the present study demonstrated that the cPKC-specific inhibitor Go-6976 was also effective in the expression of the monocytic marker. These results forcefully suggest that PKC, in particular cPKC, plays an important role as an upper module of the ERK cascade in bufalin-induced cell differentiation. It has been known that cPKC can directly interact with Raf-1 and, thereby, activate the ERK cascade (12). We have shown that a recently established specific inhibitor of the Na+/Ca2+ exchanger, KB-R7943 (27), suppressed ERK activation and c-fos and IL-1β gene expressions (13). Taken together, inhibition of Na+/K+ATPase by bufalin may sequentially induce an increase in Ca2+ concentration via the Na+/Ca2+ exchanger, activation of Ca2+-dependent cPKC and the ERK cascade, and the gene expressions needed for monocytic differentiation, such as c-fos and egr-1.

The present study demonstrated that cPKCβ- and nPKCδ-deficient THP-1/TPA cells displayed strong resistance to bufalin-induced apoptosis, although these cells retained the ability to differentiate in response to bufalin. Similar observations that the TPA-resistant cells, such as the HL-525 variant of HL-60 cells and the TUR variant of U-937 cells, acquired resistance to tumor necrosis factor-α- and drug-induced apoptosis have been reported (15, 19). Because these variant cells...
have been reported to be deficient in cPKCβ gene expression, it has been suggested that the PKC isozyme is involved in apoptosis mediated by certain inducers. On the other hand, nPKC is proteolytically activated by caspase-3-like proteases, along with execution of apoptosis (2). Findings that the forced expression of the catalytically active 43-kDa fragment of nPKCδ contributes to apoptosis (5). These findings prompted us to examine which PKC isozyme, cPKCβ or nPKCδ, is involved in bufalin-mediated apoptosis. The cPKC-selective and cPKCβ-specific inhibitors, G6-6976 and LY-379196, respectively, showed no inhibitory effect on bufalin-induced nPKCδ and PARP proteolysis and DNA fragmentation. However, nPKCδ inhibition by rottlerin suppressed the preapoptotic proteolysis and TUNEL staining. These results suggest that nPKCδ promotes early biochemical changes leading to the bufalin-mediated apoptosis. On the other hand, rottlerin failed to suppress the bufalin-induced DNA fragmentation (data not shown), indicating that nPKCδ is involved, at least in part, but not sufficient for bufalin-mediated apoptosis.

During the course of this study, we were surprised to find that rottlerin accelerated the bufalin-induced IL-1β gene expression. A possible interpretation of these findings is that rottlerin attenuates bufalin-induced apoptosis, resulting in more cells surviving and expressing IL-1β. Findings that rottlerin was not effective in nPKCδ-deficient THP-1/TPA cells in regard to bufalin induction of IL-1β support the idea that inhibition of apoptosis by suppression of nPKCδ activity enhances expression of the cell differentiation marker. However, cells treated with 30 nM bufalin for 48 h displayed >80% viability (20), indicating that IL-1β expressions in rottlerin-rescued cells cannot fully explain the significant effect of the nPKCδ inhibitor. Therefore, it is also possible that nPKCδ negatively regulates the bufalin-induced cell differentiation. Because rottlerin did not enhance the bufalin-induced ERK activation (not shown), nPKCδ is likely to regulate downstream signals of the ERK cascade. Although we have not identified the target molecule of nPKCδ, it may lie in upstream signals that confer the activation of c-fos and egr-1 transcription, the expressions of which are essential for cell differentiation along the monocytic lineage (11). Meanwhile, G6-6976 significantly reinforced the bufalin-induced DNA fragmentation and the proteolytic cleavage of nPKCδ and PARP. Such effects of the cPKC-specific inhibitor were abrogated in the case of the isozyme-nonspecific inhibitor Ro-31–8220, suggesting that cPKC negatively regulates other PKC signals that are involved in bufalin-mediated apoptosis. It is also possible that inhibition of cell differentiation by the cPKC inhibitor relatively enhances bufalin-mediated apoptosis.

On the basis of results presented in this study, we propose that cPKC and nPKC are involved in the signal transduction leading to bufalin-induced cell differentiation and apoptosis, respectively (Fig. 5). In addition, we suggest that bufalin-mediated cell differentiation and apoptosis are coupled to each other and that the distinct PKC isozymes direct the fate of individual cells.

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