The Cooperative Interaction of Two Different Signaling Pathways in Response to Bufalin Induces Apoptosis in Human Leukemia U937 Cells*

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Bufalin, an active principle of Chinese medicine, chan'su, induced typical apoptosis in human leukemia U937 cells. When U937 cells were treated with $10^{-8}$ M bufalin in the absence of serum, mitogen-activated protein (MAP) kinase activity was markedly increased 6 h after the start of treatment and elevated so for 12 h. Prior to the activation of MAP kinase, increased activities of Ras, Raf-1, and MAP kinase were found, but these enzymes were transiently activated by the treatment with bufalin. These results suggest that the signal was transmitted sequentially from Ras, Raf-1, and MAP kinase to MAP kinase. In association with this signal transduction, the concentration of cAMP in the cells decreased markedly, suggesting that Raf-1 was also activated by a decrease in the extent of phosphorylation by protein kinase A. In fact, pretreatment of U937 cells with forskolin and 3-isobutyl-1-methylxanthine, which are known to increase the concentration of cAMP in the cells, and subsequent treatment with bufalin resulted in a decrease in both Raf-1 activity and DNA fragmentation. To confirm the participation of MAP kinase in the apoptotic process, antisense cDNA for MAP kinase 1 was expressed in U937 cells. The transformants were significantly resistant to both DNA fragmentation and cell death in response to bufalin. Our findings suggest that a pathway with the persistent activation of MAP kinase in U937 cells in response to bufalin is at least one of the signal transduction pathways involved in the induction of apoptosis.

We have reported a variety of differentiation-inducing agents for human leukemia cells, including camptothecin (1), VP16 (2), bufalin (3), geranylgeranylacetone (4), and daidzein (5). Recently, some differentiation-inducing agents, such as camptothecin (6–9), VP16 (6, 9, 10), cisplatin (6, 10, 11), and all-trans-retinoic acid (12) were shown to induce apoptosis in tumor and normal cells. In a previous study, we found that, at concentrations above $10^{-8}$ M, bufalin, one of the components of bufadienolides in the traditional Chinese medicine, chan'su, that is prepared from toad venom, is able to induce apoptosis in human leukemia HL-60 cells (13). Therefore, bufalin seems to act as a potent differentiation- and apoptosis-inducing agent in cancer cells.

The apoptotic process can be induced by various physical and chemical stimuli and can be modulated by inhibitors of protein kinases or of phosphatases. Okadaic acid, an inhibitor of protein phosphatases, prevents radiation-induced apoptosis in human lymphoid tumor cell lines but induces apoptosis by itself in various other lines of human and rodent cells (14). Stauroporine, a relatively nonspecific inhibitor of protein kinase C, induces apoptosis very rapidly in numerous cell lines (15). These data suggest that protein kinases and their protein phosphatases might play a crucial role in triggering the apoptotic process.

MAP kinase is known to be involved in the early events of signal transduction, and the activation of MAP kinase signal pathways is a key event in the proliferation and differentiation of cells. It has been reported that treatments with various reagents, such as EGF, NGF, platelet-derived growth factor, fibroblast growth factor, insulin, insulin-like growth factor 1, 12-O-tetradecanoylphorbol 13-acetate (TPA), and phorbol 12,13-dibutyrate, all cause the activation of MAP kinase in quiescent fibroblastic cells (16). In the present study, we investigated the signal transduction pathway of apoptosis induced by bufalin in human promonocytic leukemia U937 cells and found that treatment of U937 cells with bufalin caused the cooperative interaction of two different signal transduction pathways, leading to abnormal activation of MAP kinase.

**MATERIALS AND METHODS**

Cell Culture—Human promonocytic leukemia U937 cells were obtained from the Japanese Cancer Research Bank. The cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum at 37 °C under 5% CO₂ in air.

Chemicals and Radioisotopes—Bufalin, myelin basic protein, and alkaline phosphatase-agarose (from bovine intestinal mucosa) were purchased from Sigma. Mouse anti-MAP kinase (ERK1 + 2) and mouse anti-MAP kinase 1 (MEK1) monoclonal antibodies were obtained from Zymed Laboratories Inc. (San Francisco, CA). Rabbit anti-Raf1 polyclonal antibodies (C-12) and rat anti-v-Ha-Ras monoclonal antibodies (clone Y13-259) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and Oncogene Science (Uniondale, NY), respectively. Protein G-Sepharose F4 Fast Flow was from Pharmacia LKB Biotechnology (Uppsala, Sweden). An enzyme immunoassay kit for cAMP, the ECL Western blotting detection system, and $[^{32}P]ATP$ (3,000 Ci/mmole) were from Amersham (United Kingdom). $[^{32}P]ATP$ orthophosphoric acid in water (8,500–9,120 Ci/mmole) was purchased from Du Pont NEN. Two peptides, FLTEYVATRWWYRAPEIMLN

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+ The abbreviations used are: MAP, mitogen-activated protein; DAPI, 4',6-diamidine-2-phenylindole dihydrochloride; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; IBMX, 3-isobutyl-1-methylxanthine; MBP, myelin basic protein; MOPS, 3-(N-morpholino)propanesulfonic acid; NGF, nerve growth factor; TFA, trifluoroacetic acid; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.
and KRELVEPLTAGEAPQNALLR, were synthesized chemically (model 431A Synthesizer; Applied Biosystems, Inc., Foster City, CA) with the Fast Moc™ cycle. Cleavage from the Hmp-resin was performed with 95% (v/v) TFA. The peptides were applied to a C8 reverse-phase high performance liquid chromatography column (Prep-10 Aquapore, 10 × 250 mm). The column was washed with 0.1% (v/v) TFA and then eluted with a linear gradient of 0–20% (v/v) acetonitrile that contained 0.1% (v/v) TFA at a flow rate of 3 ml/min over the course of 60 min.

Treatment with Bufalin—Unless otherwise indicated, U937 cells (5 × 10^6 cells) were deprived of serum for 36 h before addition of bufalin and then they were treated with 10^{-8} M bufalin in serum-free RPMI 1640 supplemented with 10% FBS and leupeptin. Cell viability was determined by the trypan blue-exclusion test.

Analysis of DNA Fragmentation by Agarose Gel Electrophoresis—Cells were collected by centrifugation and washed with PBS. The washed cells were lysed in a solution of 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.5% (w/v) SDS, and 0.1% (w/v) RNase A, with incubation for 60 min at 50°C. The lysate was incubated for an additional 60 min at 30°C with 1 mg/ml protease K and then subjected to electrophoresis in a 1% (w/v) agarose gel in 40 mM Tris-acetate, pH 7.5, that contained 1 mM EDTA for 60 min at 50 V. After electrophoresis, the gel was stained with ethidium bromide.

Quantification of DNA Fragmentation—The extent of DNA fragmentation was determined by the method of Wyllie (17), with slight modifications. In brief, cells were suspended in a solution of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% (w/v) Triton X-100 and left for 20 min on ice. The suspension was centrifuged at 27,000 × g for 20 min, and the fragmented DNA was recovered from the supernatant. The pellet remaining in the centrifugation tube was sonicated for 1 min at 45 W. The amount of DNA was determined by a fluorometric method using DAPI. The fluorescence intensity was measured at 454 nm with excitation at 362 nm. The percent of fragmentation was defined as the ratio of the amount of fragmented DNA to the total amount of DNA.

Light Microscopy—Control and bufalin-treated U937 cells were collected by centrifugation at 250 × g for 5 min and washed twice with phosphate-buffered saline (PBS). The washed cells were dried on glass slides with Wright-Giemsa solution.

Preparation of Cell Lysates—U937 cells that had been treated with 10^{-8} M bufalin were collected by centrifugation and washed twice with PBS. The washed cells were lysed in 0.4 ml of lysis buffer that consisted of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% (w/v) Triton X-100 and left for 20 min on ice. The suspension was centrifuged at 27,000 × g for 20 min. The amount of DNA was determined by a fluorometric method using DAPI. The fluorescence intensity was measured at 454 nm with excitation at 362 nm. The percent of DNA fragmentation was defined as the ratio of the amount of fragmented DNA to the total amount of DNA.

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bufalin, and then it decreased slightly (Fig. 2B). It was surprising to us that the elevated activity of MAP kinase persisted for 12 h. To our knowledge, such an anomalous and continuous pattern of activation of MAP kinase has never been reported previously.

We next compared the activation of MAP kinase in U937 cells by bufalin with that by EGF. As shown in Fig. 2B, EGF induced a rapid and transient increase in the activity of MAP kinase, as demonstrated previously in other cell lines (26, 30). The extent of the activation of MAP kinase by EGF was approximately 2-fold, which was almost the same extent as that by bufalin. Immunoblot analysis of a lysate of U937 cells that had been treated with bufalin showed that the level of the phosphorylated form of MAP kinase also increased significantly 6 h after the start of treatment (Fig. 2C, P-ERK2).

Furthermore, Northern blot analysis of ERK2 revealed that its mRNA was expressed at almost a constant level for 12 h after the addition of bufalin to cultures (data not shown). These results indicated that the activation of MAP kinase was due to its phosphorylation by a protein kinase upstream of the MAP kinase signal pathway. Therefore, we examined the changes in activity of components upstream of the MAP kinase. As shown in Fig. 3, the activity of MAP kinase in U937 cells also increased during the treatment with bufalin, reaching a maximum 3 h after the start of treatment, and then it decreased gradually to the basal level. Rather similar changes in activity were observed for Raf-1 (Fig. 3B). Ras activity, measured as the ratio of Ras-bound GTP to bound (GTP + GDP), increased rapidly after the start of treatment with bufalin and then decreased rapidly 30 min after the addition of bufalin (Fig. 3C). Considering all the evidence, we can conclude that bufalin sequentially activates Ras, Raf-1, MAP kinase kinase, and MAP kinase in U937 cells.

Effects of Bufalin on the Intracellular Concentration of cAMP—Phosphorylation of Ser-43 of Raf-1 by a cAMP-dependent protein kinase (protein kinase A) is known to inhibit Raf-1 activity (27, 28). We examined whether bufalin could change the intracellular concentration of cAMP in U937 cells and, thus, affect the activity of Raf-1. The intracellular level of cAMP in U937 cells dropped sharply just after the start of the treatment with bufalin, and it decreased to approximately 50% of the control value within 6 h after the start of the treatment (Fig. 4A). By contrast, treatment of U937 cells with EGF caused a sharp and transient increase in the concentration of cAMP in the cells (Fig. 4B), as reported previously in isolated, perfused rat hearts (29). This result prompted us to examine whether agents that increase intracellular levels of cAMP, such as IBMX and forskolin, could antagonize the effect of bufalin on the activation of Raf-1. As shown in Fig. 5, the activation of Raf-1 in U937 cells by bufalin was significantly
inhibited by pretreatment of cells with IBMX or forskolin. The results suggest that a marked decrease in the concentration of cAMP in U937 cells upon treatment with bufalin results in inactivation of protein kinase A, with subsequent activation of Raf-1. The above results raise the possibility that IBMX and forskolin might inhibit the fragmentation of DNA in U937 cells that is induced by bufalin by inhibiting Raf-1 via its phosphorylation. Indeed, the induction of fragmentation of DNA by bufalin was inhibited by pretreatment of cells with either IBMX or forskolin (Fig. 6). The inhibitory effect on bufalin-induced fragmentation of DNA in U937 cells became evident 6 to 12 h after the start of treatment with bufalin. Thus, Raf-1 was activated by two mechanisms, namely, as a consequence of the increased activity of Ras, as described above, and as a result of a decrease in levels of cAMP. It seems likely that together these factors caused the persistent activation of MAP kinase.

Effect of Expression of Antisense cDNA for MAP Kinase Kinase on the Induction of Apoptosis—To verify the contribution of the MAP kinase signaling pathway to apoptosis caused by bufalin, sense and antisense cDNAs for MAP kinase kinase were inserted into the cloning site of the mammalian expression vector pMAM-neo. Transcription of the insert in this vector is under the control of the Rous sarcoma virus promoter and a dexamethasone-inducible long terminal repeat of a mouse mammary tumor virus (30). The linked selectable marker (neo), which confers resistance to G418, is controlled by the SV40 promoter. The expression plasmids were used to transfect U937 cells, and G418-resistant colonies were subjected to assays of protein kinase activity (Fig. 7). When cells transfected with pMAM-neo (no insert), with pMAM-sense MAP kinase kinase-neo, and with pMAM-antisense MAP kinase kinase-neo were treated with bufalin without pretreatment with dexamethasone, approximately 1.5-fold activation of MAP kinase kinase was observed in each case (Fig. 7A), indicating that the three lines of transfected cells retained the responsiveness to bufalin. The activities of MAP kinase kinase in cells that had been transfected with pMAM-neo or pMAM-sense MAP kinase kinase-neo and pretreated with 1 μM dexamethasone for 20 h were slightly increased by treatment with 10^{-8} M bufalin for 3 h. By comparison, the activation of MAP kinase kinase by bufalin in cells that had been transfected with pMAM-antisense MAP kinase kinase-neo was markedly decreased by the pretreatment with dexamethasone. The activities of MAP kinase kinase in the transfected cells responded similarly to dexamethasone and bufalin, respectively. As shown in Fig. 7B, activation of MAP kinase by bufalin in U937 cells that had been transfected with pMAM-antisense MAP kinase kinase-neo was also significantly suppressed by the pretreatment with dexamethasone (p < 0.001).

We also examined the effect of bufalin on the induction of apoptosis in these transformants. As can be seen from Fig. 8, the fragmentation of DNA induced by bufalin in cells that had been transfected with pMAM-antisense MAP kinase kinase-neo was significantly inhibited by the pretreatment with dexamethasone. The results together suggest that the prolonged activation of MAP kinase by bufalin, a result of the cooperative interaction of two signaling pathways, induces apoptosis in U937 cells.

**DISCUSSION**

In the absence of serum, 10^{-8} M bufalin induces apoptosis in human leukemia U937 cells. With the rapid increase in the concentration of cAMP in U937 cells upon treatment with bufalin, the activity of MAP kinase also increased markedly with time. MAP kinase activity has been reported to be stimulated by exposure of fibroblastic or PC12 cells to growth factors, such as EGF (26, 31) or NGF (26). Upon treatment of these cells with EGF, maximal activation of MAP kinase occurs within 1 min after the addition of EGF, and the activity then returns rapidly to the basal level (26). Upon treatment of PC12 cells with NGF, maximal activation of MAP kinase is observed 5 min after the addition of NGF, and the activity then diminishes gradually (26). In contrast to the effects of EGF or NGF on MAP kinase activity, bufalin induced a gradual but anomalous increase in MAP kinase activity in leukemia U937 cells. The maximal activity of MAP kinase, which was about 2.4 times the basal level, was attained 6 h after the addition of bufalin to the U937 cells, and near-maximal activity was still retained 12 h after...
the start of treatment with bufalin. Simultaneous with the activation of MAP kinase, transient increases in the activities of enzymes in the MAP kinase cascade, such as Ras, Raf-1, and MAP kinase kinase, were found. These results indicate that the bufalin signal was transduced through MAP kinase signaling pathways.

It is well known that intracellular concentrations of cAMP are increased by certain hormones. EGF increases the intracellular concentration of cAMP in rat heart (29), mammary gland (32), and in several lines of epithelial cells when they are treated with IBMX and forskolin (33). The present study demonstrated that levels of cAMP in U937 cells were markedly decreased by bufalin. Recently, Wu et al. (27) demonstrated that an increased concentration of cAMP inhibited the activity of MAP kinase by phosphorylating Ser-43 in the regulatory domain of Raf-1. It was, therefore, expected that the extent of phosphorylation of Raf-1 would be decreased by bufalin, with subsequent activation of Raf-1. Indeed, we observed that MAP kinase was activated both by the activation of Ras and by the decrease in the concentration of cAMP. Moreover, expression of antisense DNA for MAP kinase kinase in U937 cells inhibited both the activation of MAP kinase kinase, as well as that of MAP kinase, and the induction of apoptosis by bufalin. These findings suggest that the abnormal and continuous activation of MAP kinase is at least one of the signal transduction pathways involved in the induction of apoptosis in U937 cells by bufalin.

Recently, Cowley et al. (34) and Mansour et al. (35) demonstrated that constitutive activation of MAP kinase kinase is necessary and sufficient for differentiation of PC12 cells (34) and for transformation of NIH3T3 cells (34, 35). Our findings suggest that the activation of MAP kinase kinase, at least in some cases, in human leukemia cells induces cell death through apoptosis.

The activity of MAP kinase is also regulated by phosphatases. Therefore, there is a possibility that the persistent and anomalous activation of MAP kinase by bufalin might be due to the inhibition of phosphatases such as PAC1 (36) and CL100 (37). We cannot exclude this possibility at the present stage of our investigations.

A possible candidate for the receptor for bufalin in leukemia cells is Na\(^+\),K\(^+\)\(-\)ATPase (3) since the activity of this enzyme in various tumor cells is strongly inhibited by bufalin. Although the signal transduction pathway from Na\(^+\),K\(^+\)\(-\)ATPase to Ras is unknown at present, we have provided evidence in this report for the interaction of two different signaling pathways in U937 cells. The signal transduction pathway from Na\(^+\),K\(^+\)\(-\)ATPase on the surface of U937 cells to Ras and the possible participation of MAP kinase phosphatase in the signal pathway induced by bufalin merit further study.

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