LY294002 Enhances Boswellic Acid-induced Apoptosis in Colon Cancer Cells

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Abstract. *Background: Boswellic acids, a type of pentacyclic triterpenoids, have been shown to induce apoptosis in colon cancer cells. The phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) pathway is crucial for cell proliferation and survival. Whether the apoptotic effects of boswellic acid could be affected by inhibition of PI3K/Akt pathway was examined. Materials and Methods: Colon cancer HT29 cells were treated with 3-acetyl-11-keto-β boswellic acid AKBA in the absence and presence of LY294002 or Wortmanin, inhibitors of PI3K. Apoptosis was determined by flow cytometry and caspase assay. The activation of Akt was examined by Western blot. Results: AKBA at 30 μM only slightly induced apoptosis. Preincubation of the cells with LY294002 or wortmannin significantly enhanced the AKBA-induced apoptosis up to 20-fold. Further study showed that at the doses used, AKBA induced phosphorylation of Akt at both Ser473 and Thr308 positions, indicating an activation of the PI3K/Akt pathway. Conclusion: AKBA may activate the PI3K/Akt pathway and inhibition of the PI3K pathway significantly enhances AKBA-induced apoptosis.*

The interest seeking naturally occurring components with anti-inflammatory and anticancer properties is increasing. One group of such compound is triterpenoids (1). Among thousands of triterpenoids found in plants, boswellic acids are very promising due to their effectiveness and stability (2, 3). Boswellic acids are extracted from the gum resin of *Boswellia serrata,* a kind of deciduous tree growing in the dry part of India, China, and other countries. The gum resin of *Boswellia serrata* has been used as an antiinflammatory agent for hundreds of years (4). Recent studies at cell and molecular levels found that boswellic acids inhibit several important signalling molecules such as 5-lipoxygenase (5), leukocyte elastase (6), nuclear factor κB (NF-κB) (7, 8), and cyclooxygenase (Cox) 1 (9). For diseases of the intestinal tract, boswellic acids were reported to inhibit the carcinogen-induced formation of colonic aberrant crypt foci (10) and alleviate inflammation (3, 11, 12).

We previously reported that boswellic acids induced apoptosis in both colon and liver cancer cells by a pathway dependent on caspase-8 and caspase-3 activation but independent of Fas/Fas ligand interaction (13, 14). We also showed that boswellic acids were able to induce up-regulation of p21 and down-regulation of cyclin D and cyclin-dependent kinase-4 (CDK4), leading to suppression of pRb phosphorylation (15). Among different types of boswellic acids, 3-acetyl-11-keto-beta boswellic acid AKBA is most effective (13, 14).

The phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt) pathway is important for cell survival and tissue repair. Activation of PI3K pathway affects several proliferative and inflammatory molecules such as β-catenin, CDK and NF-κB, resulting in cell proliferation, inflammation and migration (16). In the present investigation, we examined whether the apoptotic effects of AKBA in colon cancer cells can be modified by inhibition of the PI3K pathway.

Materials and Methods

Materials. HT-29, HCT-116, SW480 and LS174T colon cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). PI3K inhibitor LY294002 (LY29) was purchased from Biomol International Incorporation (Plymouth Meeting, PA, USA) and wortmannin was from Sigma Aldrich (St Louis, MO, USA). AKBA was from ChromaDax Incorporation purity >90% (Santa Ana, CA, USA). Polyclonal antibodies against total Akt1 and phosphorylated Akt1 at Ser473 and Thr308 sites were obtained from Cell Signalling Technology (Danvers, MA, USA). Substrates for caspase-3, -8 and -9 were from Biomol International Incorporation.

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Cell culture. HT-29 and HCT-116 cells were cultured in modified McCoy 5A medium supplemented with 10% fetal bovine serum (FBS v/v). LS174T cells were cultured in RPMI-1640 medium supplemented with 5% FBS, and SW480 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% FBS. The cells were maintained at 37˚C in an incubator containing 5% CO_2. AKBA was dissolved in ethanol as a stock (40 mM). The cells at about 80% confluence were treated with AKBA, LY29, or wortmannin alone for 16 h or pre-treated with LY29 or wortmannin for 1 h followed by the addition of AKBA and incubation for a further 16 h. After stimulation, the cells were scripted, centrifuged and lysed as described (13). The cell-free extract was used for caspase analysis.

Apoptosis assay. Apoptosis was evaluated by flow cytometry and caspase assay as described elsewhere (15, 17). Flow cytometric analysis was performed in a BD Coulter flow Cytometer (Fullerton, CA, USA). The cell cycle phase distribution was analyzed by WINMDI software. The percentage of the sub-G_1 population was used as a surrogate of apoptosis. For caspase activity determination, 20 µl of cell-free extract were added to 100 µl of assay buffer containing 0.2 mM specific substrates as described elsewhere (13). The kinetic cleavage of the substrate was measured by a microplate reader at 405 nm and the activities were calculated according to the manufacturer's instructions.

Western blot. After treatment, the cells were lysed in a buffer containing 20 mM Tris, pH7.5, 150 mM NaCl, 0.5% Triton® X100 w/v, 1 mM EDTA, 1 mM Na pyrophosphate, 1 mM glycerol-2-phosphate, 1 mM Na_3VO_4, 0.25% NP40, and 1 mM phenylmethylsulphonyl fluoride (PMSF). Fifty micrograms of proteins were subjected to 12% SDS PAGE, followed by transferring the proteins to nitrocellulose membrane by electroblotting. The membranes were incubated with the primary and then with secondary antibodies conjugated with horseradish peroxidase. The levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as a loading control.

Statistical analysis. All studies were performed in triplicate in at least two separate experiments. Non-paired Student's *t*-test was applied for comparison of the mean values of two groups and $p<0.05$ was considered as significant.

Results

LY29 significantly enhanced AKBA-induced apoptosis in HT29 colon cancer cells. The concentration of AKBA used in this part of the study was 30 µM, which was based on our preliminary studies showing that at this concentration AKBA did not induce mass apoptosis by itself. As shown in Figure 1, when HT29 cells were treated with 30 µM AKBA or 30 µM LY29 alone for 16 h, only slight apoptosis was identified in flow cytometry. However, pre-treating the cells with 30 µM LY29 for 1 h significantly enhanced the apoptotic responses to AKBA, as shown by a marked increase of the sub-G_1 fraction. Quantification showed that co-treatment of LY29 and AKBA induced 5- and 20-fold increases of the sub-G_1 fraction compared with single LY29 and AKBA treatment, respectively (Figure 2 A). Similar results were also obtained when the cells were treated with wortmannin, another inhibitor of PI3K (Figure 2 B). To further confirm the apoptosis, the activities of caspase-3, -8 and -9, the key enzymes involved in apoptosis, were determined. As shown in Figure 3, treating the cells with AKBA or LY29 alone only increased the activity of caspase-3 slightly, but not those of caspase-8 and -9. However, pre-treating the cells with LY29 sharply increased the AKBA-induced activities of all three caspases.

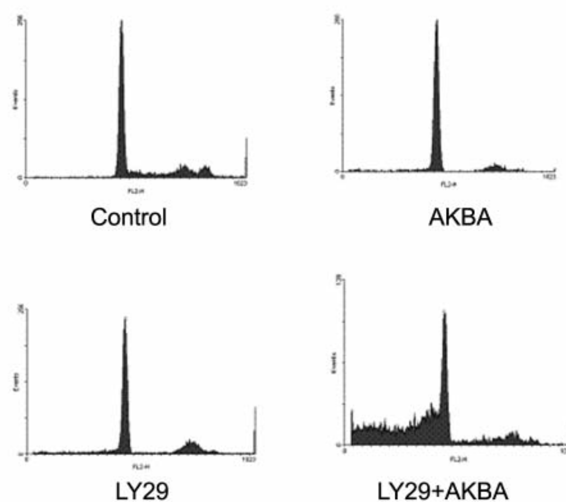

Figure 1. *LY9 enhanced AKBA-induced apoptosis as assayed by flow cytometry. HT29 cells were treated with 30 µM AKBA, or 30 µM LY29 alone for 16 h, or were pre-treated with 30 µM LY29 for 1 h followed by addition of AKBA 30 µM and incubation for 16 h LY29+AKBA. DNA was stained with propidium iodide.*

AKBA-induced phosphorylation of Akt1 in colon cancer cells. Since LY29 and wortmannin are inhibitors of PI3K and the main target of PI3K is Akt, we further examined the phosphorylation of Akt in the presence of AKBA. We found that at these concentrations, AKBA induced phosphorylation of Akt1 at Ser 473 in a time- and dose-dependent manner (Figure 4 A and B). Further studies showed that AKBA also induced phosphorylation of Akt at Thr308, another phosphorylation site for Akt activation. In all cases, the levels of total Akt1 were not affected by AKBA, indicating AKBA did not affect the expression of Akt itself.

We also examined the effect of AKBA on Akt phosphorylation in other colon cancer cells and found that AKBA at 30 µM also induced the phosphorylation of Akt1 in HCT-116, SW480 and LS174T cell lines (Figure 5).

Discussion

The anticancer and apoptotic effects of AKBA have been intensively studied and well documented in the last decade in both cell culture studies and animal models (2, 3). In the present work, we found that two inhibitors of PI3K were able to increase AKBA-induced apoptosis significantly.

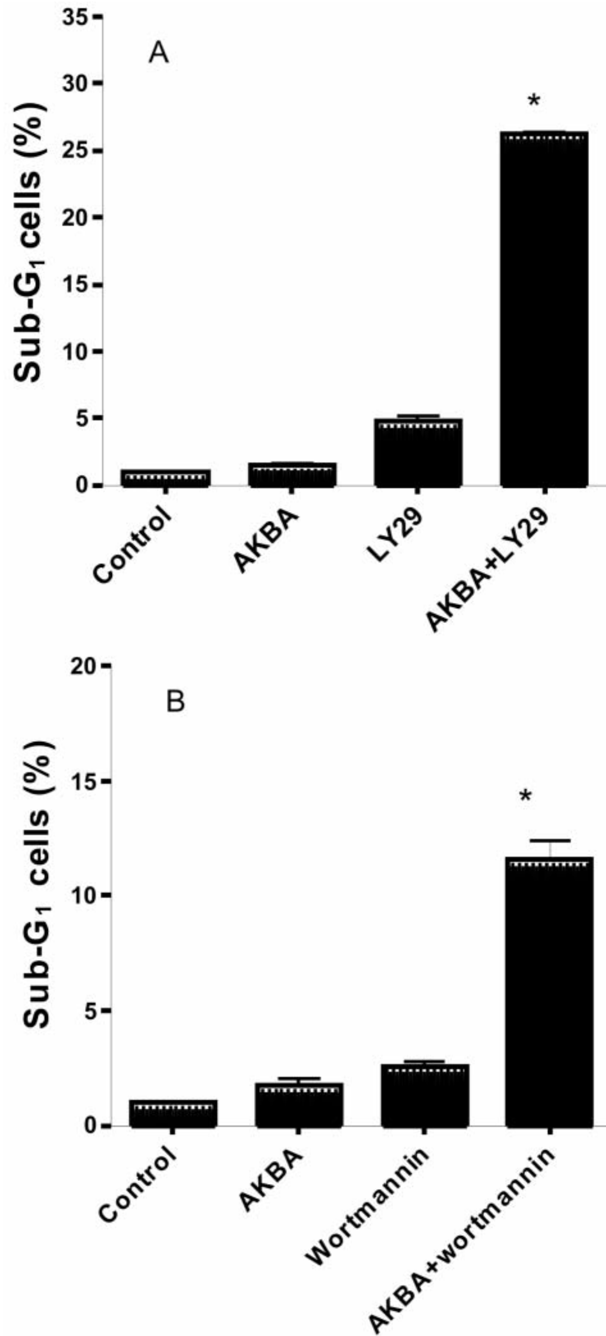

Figure 2. *Quantification of the effects of LY29 on AKBA-induced apoptosis in colon cancer cells. In A, HT29 cells were treated as described in Materials and Methods. In B, the cells were treated similarly as in A except LY29 was replaced by wortmannin. After treatment, the cell cycle distribution was analyzed by flow cytometry and the percentage of sub-G_1 cells was calculated. *p<0.05 compared to the cells treated with AKBA or PI3K/Akt inhibitor alone.*

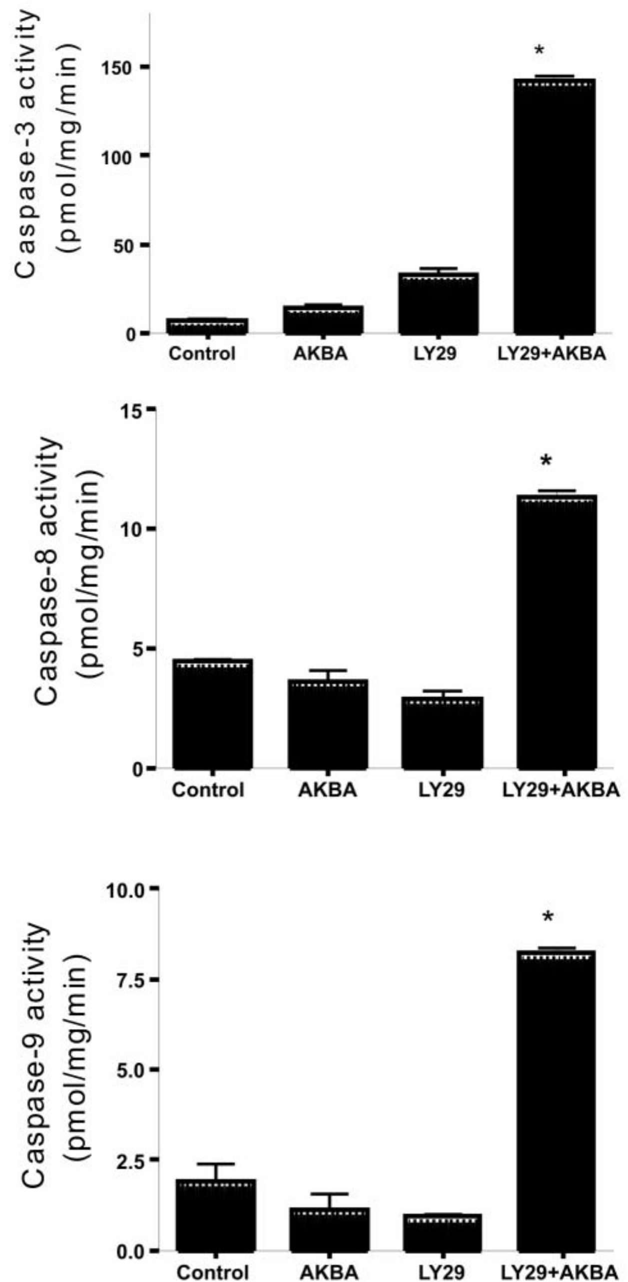

Figure 3. *LY29 enhanced caspase activities in responses to AKBA. The cells were treated as described in Materials and Methods. Activities of caspase-3, 8 and 9 were assayed after AKBA treatment. *p<0.05 compared to the cells treated with AKBA or LY29 alone.*

PI3K is one of the key molecules involved in promoting cell growth, cell survival, tissue repair and renewal (16). Activated PI3K induces phosphorylation and activation of Akt through PIP3-dependent kinase (PDK). Akt is a key molecule that phosphorylates many downstream targets such as mammalian target of rapamycin (mTOR), murine double minute 2 (MDM2), Raf, inhibitory κB kinase (IKK), p21, p27 and glycogen synthase kinase-3B (GSK-3B). leading to initiation of cell division and inhibition of apoptosis (18). Over activation

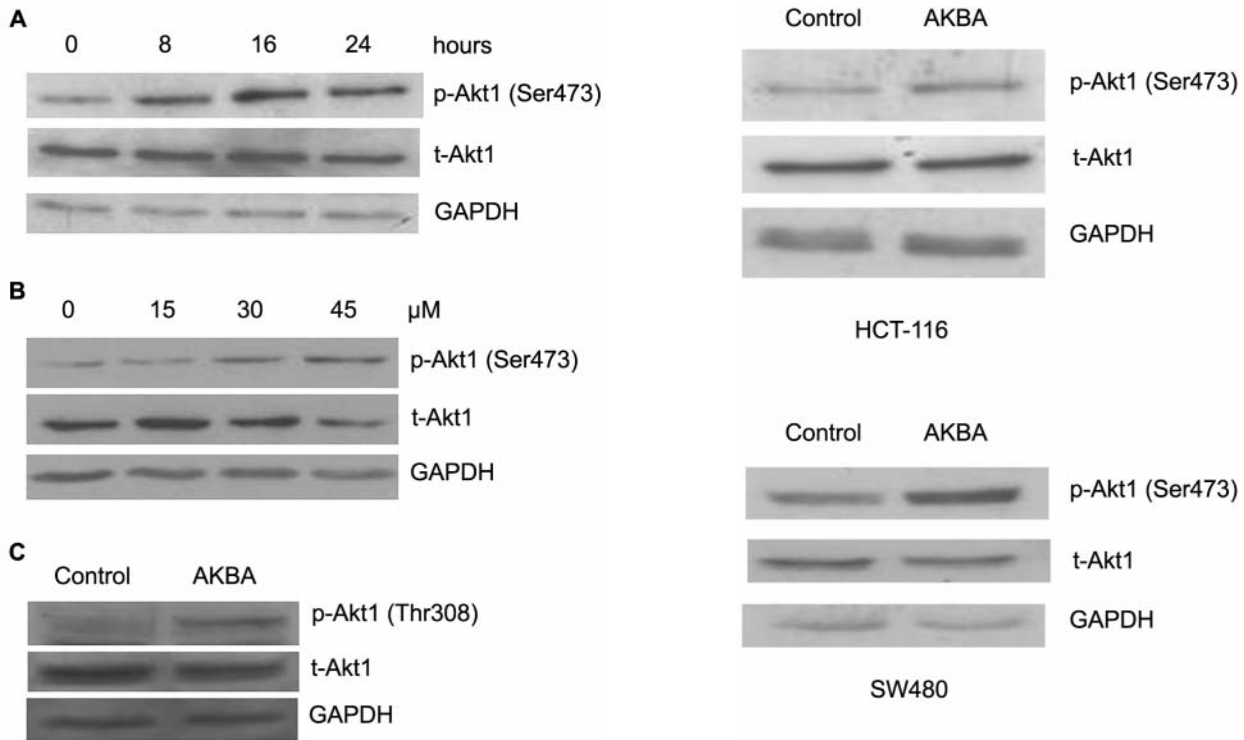

Figure 4. *AKBA induced phosphorylation of Akt1 in HT-29 cells. HT-29 cells were treated with 30 μM AKBA for different times A, or with AKBA at different concentrations for 16 h B. Phosphorylated Akt1 at Ser473 p-Akt1-Ser473 and total Akt1 t-Akt1 were assayed by Western blot. GAPDH was used as a control. C, HT29 cells were treated with 30 μM AKBA for 16 h and the phosphorylation of Akt1 at Thr308 was assayed by Western blot.*

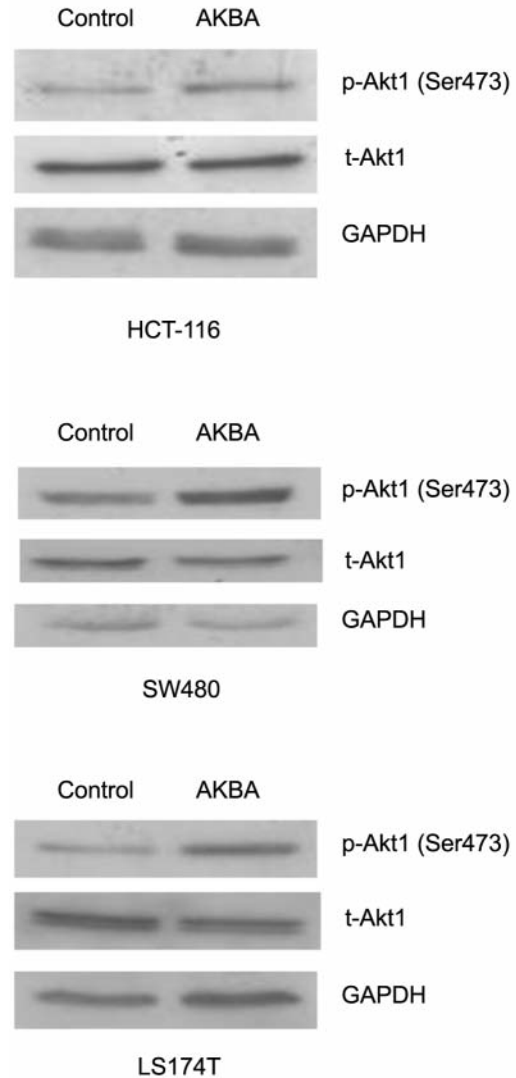

Figure 5. *Phosphorylation of Akt1 in other colon cancer cell lines. HCT116, SW480 and LS174T cells were treated with 30 μM AKBA for 16 h. The changes of phosphorylated Akt (p-Akt1 at Thr473) and total Akt1 (t-Akt1) were assayed by Western blot. GAPDH was used as a housekeeping control.*

of PI3K/Akt is a major event underlying tumorigenesis in many organs. In the intestinal tract, activating mutation or overexpression of PI3K/Akt has been identified in 57% of colorectal cancer cases (19). In the present study, we clearly showed that inhibition of PI3K pathway sharply enhanced AKBA-induced apoptosis. Such an enhancement is not simply due to a suppression of the basal PI3K activity, because we showed that AKBA actually stimulated phosphorylation of Akt at both Ser473 and Thr308 sites. Phosphorylation of both Ser473 and Thr 308 sites has been shown to be necessary for full activation of Akt (20). The activation induced by AKBA was relatively rapid and was demonstrated not only in HT29 cells but also in three other colon cancer cell lines, indicating it is a common phenomenon for colonic cells.

AKBA is a compound that is commonly considered as inducing apoptosis. The finding of the activation of PI3K/Akt by AKBA is novel. In fact, evidence is accumulating that some anticancer drugs can stimulate both the survival and apoptotic pathways in cancer cells (21). The fate of cancer cells in response to these anticancer drugs are determined by interplay of the two competing networks. In the present study, the apoptotic effect AKBA at the concentration used was relatively weak, whereas the phosphorylation of Akt induced by it was clear. The AKBA-induced activation of PI3K/Akt might counteract its apoptotic effect, which may explain why inhibition of PI3K/Akt significantly increased apoptosis induced by AKBA. We reported previously that AKBA could stimulate the expression of $p21^{waf1/cip1}$, which may also provide survival advantages in colon cancer cells (15). Whether there is a link between the PI3K/Akt activation and $p21^{waf1/cip1}$ expression in colon cancer cells is unknown. However, in other cell lines such as ovarian cells and vascular smooth muscle cells, activation of PI3K/Akt has been shown to be crucial for expression of $p21^{waf1/cip1}$ (22, 23).

Due to the importance of the PI3K/Akt pathway, PI3K has become a target for novel drug development (24). Our finding that the apoptotic effects of AKBA can be significantly enhanced by inhibition of PI3K may be of pharmacological significance. Although boswellic acids are generally considered to be safe, a toxic effect of AKBA at 50 μM which induced intestinal inflammation has been reported (25). Combination of PI3K inhibitor with AKBA might be a useful strategy to lower the dose of boswellic acid used and meanwhile enhance its apoptotic effect.

Acknowledgements

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