Modulation of Apoptosis by Berberine through Inhibition of Cyclooxygenase-2 and Mcl-1 Expression in Oral Cancer Cells

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Abstract. *Background: We have previously shown that berberine exerts its anti-inflammatory effects through inhibition of cyclooxygenase-2 (COX-2) expression. In this study, we explored the biochemical influence of berberine-induced COX-2 reduction and apoptosis. Materials and Methods: KB cells were treated with berberine, and the apoptosis was measured by morphology and caspase-3 activity. The effects of prostaglandin E_2 (PGE_2) on berberine-mediated cell growth were also determined. The expression of COX-2, Bcl-2, Mcl-1, Akt and phosphorylated Akt in berberine-treated KB cells, with or without PGE_2, were assessed by Western blots. Results: Berberine induced apoptosis in KB cells, and was partially reversed by incorporation of PGE_2. Berberine treatment inhibited COX-2 and Mcl-1 expression dose-dependently, but not Bcl-2. PGE_2 induced COX-2 and Mcl-1 expression and reversed the repressive effect of berberine on Mcl-1. In addition, PGE_2 had no effect on total Akt, but slightly reversed the phosphorylated Akt, which was decreased by berberine. Conclusion: These results suggest that berberine-induced apoptosis might be COX-2-dependent and is related to decreased Akt phosphorylation and Mcl-1 expression.*

Berberine, an isoquinoline alkaloid, is the major ingredient of many medicinal herbs, such as *Hydrastis canadensis* (goldenseal), *Cortex phellodendri* (Huangbai) and *Rhizoma coptidis* (Huanglian) (1). Berberine has many pharmacological effects including inhibition of DNA and protein synthesis, arrest of cell cycle progression and anti-inflammatory effects (2-4). Recently, we have shown that berberine exerts its anti-inflammatory effects through

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inhibition of cyclooxygenase (COX)-2 expression and prostaglandin (PG) E_2 production (5).

COXs plays an important role in the synthesis of prostaglandins from arachidonic acid, and two closely related forms of COXs have been identified, COX-1 and COX-2. COX-1 is considered as a "housekeeping" protein and is involved in physiological functions, whereas COX-2, an inducible protein, is related to pathological conditions (6). Epidemiological studies indicated that long-term consumption of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) reduces the incidence of numerous cancers (7-9). These observations on COXs inhibitors implicated that COXs, especially COX-2 expression, may be involved in the development of malignant tumors (10). In tumorigenesis, COX-2 activation results in increased production of PGE_2, which can promote tumor growth by stimulating angiogenesis (11) and invasiveness (12), as well as inhibiting immune surveillance (13) and apoptosis (14).

Many studies have explored the anti-tumor potential of NSAIDs through the inhibition of COXs and prostaglandins. NSAIDs can induce various cancer cells to undergo apoptosis (15,16). Li *et al.* showed that NS-398, a specific COX-2 inhibitor, induced apoptosis in two COX-2-positive esophageal cancer cell lines but not in a COX-2-negative cell line, and the apoptosis was inhibited by PGE_2 (17). Sheng *et al.* also indicated that PGE_2 induced Bcl-2 protein 4 to 5-fold in a human colon carcinoma cell line HCA-7 (18). These results suggest that COX-2 expression and production of PGE_2 regulate the growth of cancer cells and prevent apoptosis.

The Bcl-2 proteins are a family of proteins involved in the regulation of apoptosis. Some of these proteins (such as Bcl-2 and Bcl-XL) are anti-apoptotic, while others (such as Bad or Bax) are pro-apoptotic, the sensitivity of cells to apoptotic stimuli depending on the balance of pro- and anti-apoptotic bcl-2 proteins (19,20). Lin *et al.* indicated that Mcl-1, but not other Bcl-2 members, was significantly up-regulated by COX-2 transfection or PGE_2 treatment in

human lung adenocarcinoma CL1.0 cells. This suggested that the expression of Mcl-1 is regulated by COX-2 (21).

In our previous study (5), berberine reduced COX-2 protein expression and decreased PGE_2 production in oral cancer cells. In this report, we further demonstrated that berberine inhibited COX-2 expression concomitantly with inducing apoptosis in KB cells, and examined the regulation of anti-apoptotic proteins by berberine *via* signal transduction pathway.

Materials and Methods

Cell line. Human oral epidermal carcinoma KB cells, obtained from the American Type Culture Collection (ATCC), were cultured in RPMI 1640 (Gibco BRL, Gaithersburg, MD, USA) and supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA) and antibiotics (100 μg/ml streptomycin and 100 units /ml penicillin) (Gibco BRL). The cells were maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO_2.

Cell viability. Trypan blue exclusion and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay were employed for cell proliferation evaluation. Briefly, cells were seeded in 10-cm petri dishes, incubated for 24 h, and treated with different concentrations (1, 10, and 100 μM) of berberine (Sigma, St. Louis, MO, USA). At designated times, floating and trypsinized cells were counted under microscope by the trypan blue exclusion method.

KB cells were cultured in 96-well plates for 24 h, the medium containing different concentrations of berberine with or without 4 μg/ml PGE_2 (Cayman Chemical, Ann Arbor, MI, USA), being refreshed every 24 h. At the indicated treatment period, MTT (0.5 mg/ml, Sigma) was added to the culture medium 3 h prior to harvest. The supernatant was then removed and 100 μl dimethylsulphoxide (DMSO, Sigma) was added. A Max 250 microplate reader (Molecular Devices, Menlo Park, CA, USA) was used for colorimetric reading at 570 nm. Cell viability is expressed as percentage of the 24 h control value. All experiments were performed three times in triplicate.

Measurement of caspase-3-like activity. The colorimetric CaspACE Assay system (Promega, Madison, WI, USA) was used to measure the caspase-3-like (DEVDase) activity. Cells were treated with berberine for 3 or 12 h, then the cellular extract was mixed with colorimetric substrate which was cleaved by DEVDase to generate chromophore p-nitroaniline and monitored at 405 nm.

Morphological characterization of cell death. KB cells were plated on eight-well glass chamber slide (Nunc Inc., Naperville, IL, USA), and treated with various concentrations of berberine for 12 h. The morphology of hematoxylin-eosin-stained cells was examined under microscope.

Western blotting. Cells were exposed to berberine with or without 4 μg/ml PGE_2 (Cayman Chemical) for 1, 3 or 6 h. Cell lysates (20 μg) were separated by 10% SDS polyacrylamide gel electrophoresis, electroeluted to polyvinylidene fluoride membrane, and probed for specific proteins using primary antibodies against COX-2 (Cayman Chemical), Bcl-2, Mcl-1, total-Akt or phospho-Akt (Ser 473)

(Santa Cruz Biotechnology, Santa Cruz, CA, USA). Actin was probed by an actin antibody (Chemicon, Temecula, CA, USA) and used as a protein loading control. The specific protein and primary antibody complex were detected by a peroxidase-coupled goat anti-mouse or goat anti-rabbit antibody (Pierce, Rockford, IL, USA). The reaction product was visualized by the "Enhanced Chemiluminescence" detection kit (Pierce). The intensity of protein bands was measured by a densitometer (Personal Densitometer SI, Molecular Dynamics, San Jose, CA, USA). The expressions of specific proteins were normalized by actin band intensity.

Statistics. Comparison of the means of various treatment groups was made by one-way analysis of variance (ANOVA) using the SigmaStat (Jandel Scientific, IRC, USA) software. A $p < 0.05$ is considered as statistically significant.

Results

Berberine inhibited proliferation of KB cells. To evaluate the effects of berberine on proliferation of KB cells, we collected cells treated with various concentrations of berberine for 12-72 h, and then counted the attached and floating cells by trypan blue exclusion assay. Cells treated with 1 μM berberine had a similar growth pattern to the control; however, higher concentrations of berberine inhibited cell growth (10 μM, 100 μM) at 24, 48 and 72 h. The number of viable cells showed a gradual decrease after 100 μM berberine treatment at 48 and 72 h (Figure 1A). Concurrently, berberine induced death of KB cells in a time- and dose-dependent manner (Figure 1B). These results indicated that berberine-induced inhibition of KB cells is related to cell death.

In order to investigate the effect of PGE_2 on the growth of KB cells in the presence or absence of berberine, the MTT assay was utilized for evaluation of cell proliferation (Figure 1C). In comparison with that of the 24 h control group, 4 μg/ml PGE_2 treatments alone or in combination with 1 μM berberine increased KB cells proliferation about 20%. Nevertheless, at 48 and 72 h after treatment, PGE_2 had no significant effect on the berberine-induced growth inhibition of KB cells.

Berberine induced apoptosis in KB cells. To determine whether berberine induced apoptosis in KB cells, we monitored changes in the caspase-3 activity and cell morphology after berberine treatment. The activity of caspase-3 did not show any alteration in KB cells after a 3-h berberine treatment. However, cells treated with 10 or 100 μM berberine for 12 h showed 1.6-fold and 3-fold increase of caspase-3 activity, respectively, as compared to the control group (Figure 2A). In addition, KB cells treated with 100 μM berberine for 12 h had cellular membrane blebbing indicating apoptosis (Figure 2B).

Berberine inhibited the expression of Mcl-1 in KB cells. Since we had previously found that berberine inhibited COX-2 expression and reduced the secretion of PGE_2 in cancer cells
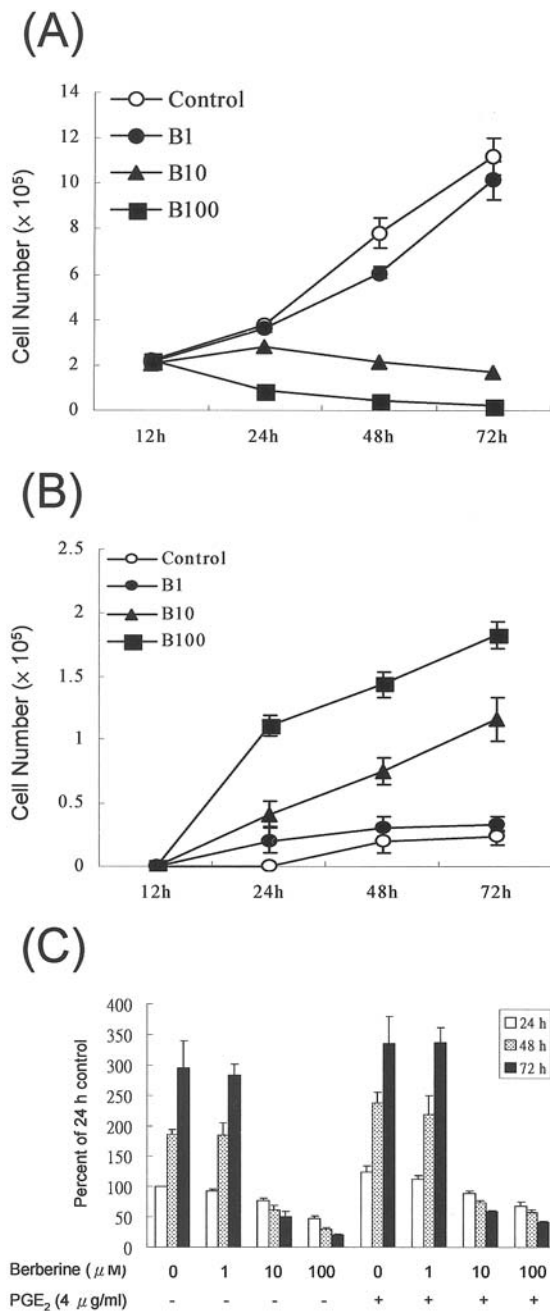
Figure 1. *The effect of berberine and PGE$_2$ on proliferation of KB cells. Viable cells (A) and dead cells (B) were determined by trypan blue dye exclusion. The effects of PGE$_2$ on berberine-treated KB cells were evaluated by MTT assay at indicated time points and expressed as a percentage of that of the 24 h control group (C). Data are expressed as mean±SD from 3 independent experiments.*
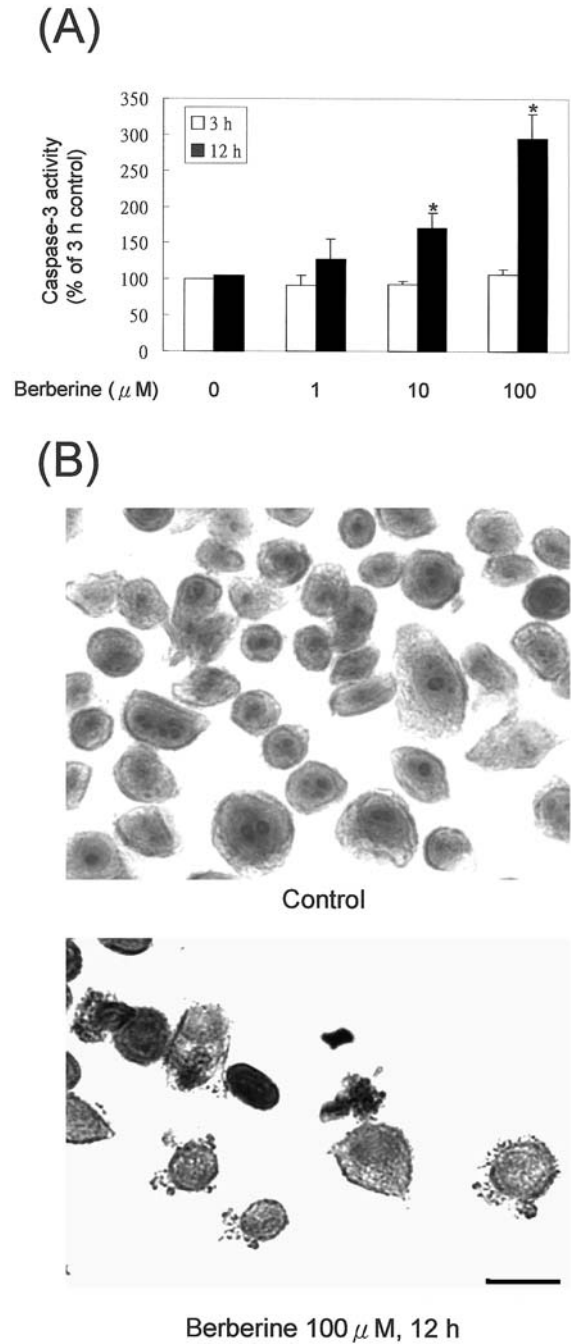
Figure 2. *(A) Caspase-3-like protease activity in KB cells treated with berberine. Data are expressed as the percentages of the 3 h control. Values represent mean±SD from 3 separate experiments. * p<0.05 as compared with 3 h control group. (B) Membrane of KB cells showed blebbing under 100 μM berberine treatment (hematoxylin and eosin, original magnification X 200. Scale bar, 20 μm).*

(5), we further investigated the expression of apoptotic proteins in KB cells after treatment with berberine. In addition, we also evaluated the effect of supplemental PGE$_2$ on berberine-induced apoptotic proteins in KB cells. KB cells treated with 10 and 100 μM berberine for 3 h had significantly reduced COX-2 expression (0.68-fold and 0.01-
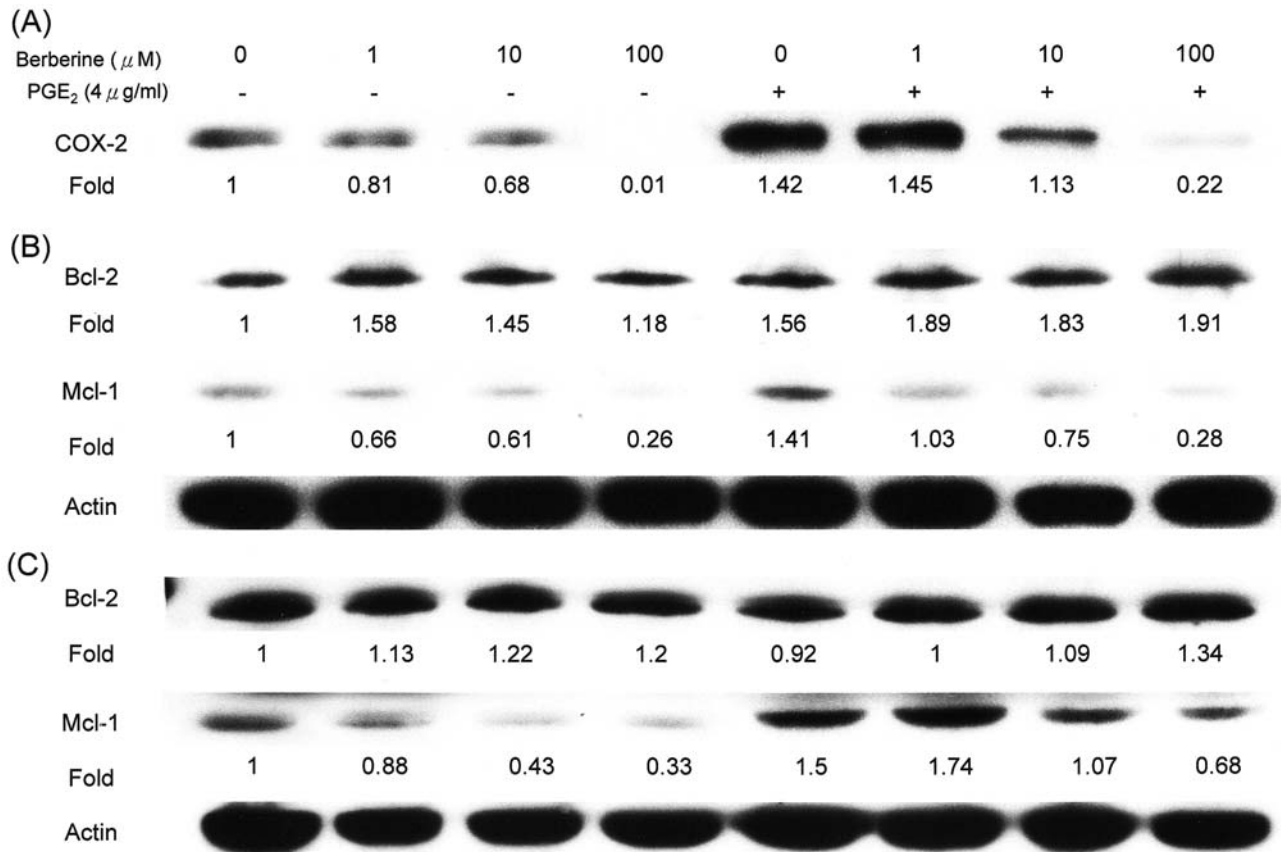
Figure 3. *The effect of berberine on the expression of apoptotic proteins in KB cells. Protein samples from KB cells treated with berberine for 3 h were probed with COX-2 antibody (A), protein lysates from cells treated with berberine for 3 h or 6 h (B and C, respectively) were probed with Bcl-2 or Mcl-1 antibody. Actin was used as loading control.*
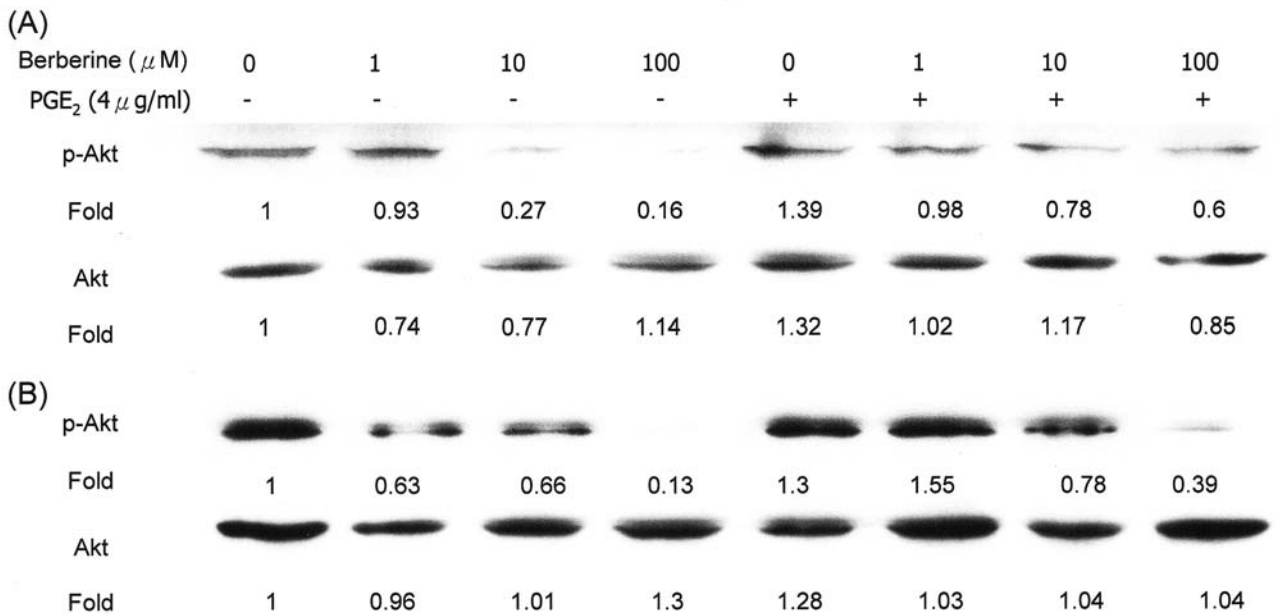

Figure 4. *Berberine inhibited the phosphorylation of Akt in KB cells. Protein lysates from cells treated with berberine for 3 h or 6 h (A and B, respectively) were probed with Akt or phospho-Akt (Ser 473) antibody.*

fold of control, respectively). Treatment with PGE$_2$ (4 μg/ml) for 3 h resulted in an increase of COX-2 expression in KB cells, however, the increased expression was still inhibited by berberine (Figure 3A).

The expression of Bcl-2, a major member of the apoptotic protein family, in KB cells was not inhibited by berberine in the presence or absence of PGE$_2$ at either 3 h or 6 h treatment (Figure 3B, 3C). In contrast, berberine treatment of KB cells for 3 h down-regulated Mcl-1, another apoptotic protein, with or without the addition of PGE$_2$. The inhibitory effect was less profound in the presence of PGE$_2$ than the treatment group without PGE$_2$ (Figure 3B). After berberine treatment of KB cells for 6 h, the inhibitory effect of berberine on Mcl-1 expression was similar to that found at 3 h, with or without the addition of PGE$_2$ (Figure 3C).

Berberine inhibited Akt phosphorylation. Expression of Akt and phosphorylated Akt were examined for possible involvement in the berberine-induced down-regulation of Mcl-1 in KB cells. Berberine treatment in the presence or absence of PGE$_2$ for 3 or 6 h had no effect on the total Akt expression in KB cells. However, berberine treatment of KB cells for 3 h or 6 h dose-dependently inhibited the phosphorylation of Akt (Ser 473). Although PGE$_2$ treatment significantly up-regulated phosphorylaed Akt at 6 h, berberine clearly inhibited the phosphorylation of Akt (Figure 4A, 4B).

Discussion

Apoptosis is a morphologically and biochemically distinct form of cell death which is considered to be "cellular suicide". Many studies reported that both COX-2 expression and prostanoids play an important role in apoptosis (22,23). COX-2 has been shown to be induced by nuclear factor-ÎB (NF-ÎB) and activator protein-1 (AP-1) transcription factors (24), parallel to p53/Bax pro-apoptotic expression (25), or the activation of peroxisome proliferator-activated receptor-γ (PPARγ) (26). In this study, berberine treatment resulted in a time- and dose-dependent growth inhibition of KB cells (Figure 1), increased caspase-3 activity and characteristic apoptotic cell morphology (Figure 2). However, this result is distinct from the results caused by specific COX-2 inhibitors (27).

In this study, PGE$_2$ not only stimulated the proliferation of KB cells (Figure 1C), but also increased the COX-2 expression (Figure 3A). These stimulations were blocked by berberine, suggesting that apoptosis is associated with COX-2 expression and COX-2-derived PGE$_2$ synthesis. Mechanistic studies indicated that COX-2 may regulate apoptosis by increasing PGE$_2$ synthesis. Endogenous or exogenous PGE$_2$ prevent apoptosis, perhaps through the cytochrome C-dependent pathway (17), increase intracellular cyclic adenosine monophosphate (cAMP) levels through

induction of inhibitor of apoptosis protein-2 (IAP-2) (28), or induce early growth response factor-1 (EGR-1) by EP4 prostanoid receptors *via* the phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinases (29). Our results clearly showed that berberine down-regulated Mcl-1, but not Bcl-2 expression, and that the stimulatory effect of PGE$_2$ on Mcl-1 did not reverse the suppression of berberine effect (Figure 3B, 3C). This effect is different from PGE$_2$-induced Bcl-2 expression and resisted the apoptosis caused by COX-2 inhibitor (18). Moreover, berberine prevented the phosphorylation of Akt in the presence or absence of PGE$_2$ (Figure 4). The observation that Akt phosphorylation and Mcl-1 expression were inhibited concomitantly agrees well with the finding of Lin *et al.*, who indicated that modulation of the level of Mcl-1 was dependent on the PI3K/Akt pathway (21).

The anti-tumor activity of berberine had been correlated with induced apoptosis by formation of complexes with DNA (3), induction of topoisomerase II-mediated DNA cleavage (30), or inhibited tumor growth and lymphatic metastasis *via* repressed AP-1 activity (31). Using oral cancer cells, we have found that berberine reduced the binding of AP-1 to the response element in KB cells, which resulted in decreased COX-2 expression and PGE$_2$ synthesis (5). In this study, we further showed that the decreased level of PGE$_2$ by berberine is related to decreased Akt phosphorylation and Mcl-1 expression. These results together suggest that the anti-tumor or chemo-preventive potential of berberine is complex and is worthy of further investigation.

Acknowledgements

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