Differential Response of Akt to Cyclic AMP Modulates Drug Sensitivity

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Although Akt is known to be associated with drug resistance, its role in cyclic AMP (cAMP)-related inhibition of cell proliferation is not clear. Here, we report that Akt modulates the sensitivity of hepatocellular carcinoma cells to cAMP. Treatment of hepatocellular carcinoma cell lines (HepG2 and Bel-7402) with cAMP inhibited proliferation, with HepG2 cells showing lower sensitivity to cAMP. Biochemical studies showed that cAMP increased FBS-stimulated Akt phosphorylation in HepG2 cells, but completely inhibited FBS-stimulated Akt phosphorylation in Bel-7402 cells, suggesting that the differential response of Akt to cAMP in these two cell lines might contribute to their differential sensitivity. LY294002, a phosphatidylinositol 3-kinase inhibitor that inhibits FBS-stimulated Akt phosphorylation, restored the sensitivity of HepG2 cells to cAMP and API-2 (Akt/protein kinase B signaling inhibitor-2) also showed similar effect. These results collectively indicate that the response level of Akt to cAMP may play a critical role in determining drug sensitivity.

Key words Akt; cyclic AMP; hepatocellular carcinoma; proliferation; drug sensitivity

MATERIALS AND METHODS

Chemicals Forskolin, 3-isobutyl-1-methylxanthine (IBMX), H89, and API-2 (Akt/protein kinase B signaling inhibitor-2) were purchased from Calbiochem (San Diego, CA, U.S.A.). LY294002 and 8-bromo-cAMP were purchased from Sigma (St. Louis, MO, U.S.A.). Antibodies against phospho-MAPK (p42/44), MAPK (p42/44), phospho-Akt (Ser473), Akt and PTEN were purchased from Cell Signaling (Beverly, MA, U.S.A.).

Cell Culture HepG2 and Bel-7402 cells were obtained from the Cell Bank of the Shanghai Institute for Biological Sciences (Shanghai, China). The cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified CO2 incubator containing 5% CO2 and 95% air.

Cell Proliferation Assay Cell proliferation was measured using the sulforhodamine B (SRB) assay. Briefly, cells were plated in 96-well plates at 5000 cells/well. After 24 h, the cells were incubated with different concentrations of 8-bromo-cAMP for the indicated times, and then fixed with 10% trichloroacetic acid and stained with 0.4% SRB (dissolved in 1% acetic acid). Bound dye was eluted with 100 μl of 10 μM Tris–HCl (pH 10.5), and optical absorbance was measured at 560 nm.

Western Blot Analysis Bel-7402 and HepG2 cells were plated in 6-well plates at a density of 2×105 cells/well. After drug treatment, the cells were washed twice with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) and total cell lysates were collected in SDS sample buffer (50 mM Tris–HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% Bromophenol Blue, 10% glycerol). Cell lysates containing equal amounts of protein were separated by SDS-
PAG and transferred to polyvinylidene difluoride membranes. The membranes were blocked in 5% non-fat milk in TBST (Tris-buffered saline with 0.1% Tween 20, pH 7.6), incubated with the appropriate primary antibody at 4°C overnight, and then exposed to a secondary antibody for 2 h at room temperature. Immunoreactive proteins were visualized using an enhanced chemiluminescence system (Pierce, Rockford, IL, U.S.A.).

Statistical Evaluation of Proliferation Values Descriptive statistics, such as mean and standard error, were used to summarize the results. The Student’s t-test and ANOVA test were used for univariate analysis. Statistical significance was defined by a two-tailed p-value <0.05.

RESULTS

cAMP Differentially Inhibits Proliferation of Bel-7402 and HepG2 Cells To determine the effect of cAMP on proliferation of HCC cells, we incubated Bel-7402 and HepG2 cells with 8-Br-cAMP, a cell permeable analogue of cAMP. Treatment with 8-Br-cAMP dose-dependently inhibited proliferation of both Bel-7402 and HepG2 cells, but HepG2 cells were more resistant to cAMP than Bel-7402 cells, showing IC50 values of 35.5 mM and 4.7 mM, respectively (Fig. 1). Forskolin, an activator of adenyl cyclase, also inhibited the proliferation of HepG2 and Bel-7402 cells in the presence of IBMX, a phosphodiesterase inhibitor. Similar to our findings with cAMP, HepG2 cells showed less sensitivity to forskolin than did Bel-7402 cells (data not shown).

Akt Shows Different Levels of Basal Phosphorylation in HepG2 and Bel-7402 Cells To examine the role of Akt in the differential sensitivity of HepG2 and Bel-7402 cells to cAMP, we first assessed the levels of phosphorylated Akt under basal culture conditions (complete medium supplemented with 10% FBS) in the two cell lines. Interestingly, Western blot analysis revealed that the level of phosphorylated Akt in Bel-7402 cells was 5-fold higher than that in HepG2 cells, suggesting that endogenous Akt activity is not a determinant of HCC sensitivity to cAMP. As controls, levels of non-phosphorylated Akt (total Akt), non-phosphorylated MAPK, and PTEN were also determined and were found to be similar between the two cell lines (Fig. 2).

Akt Shows Differential Responses to cAMP in Bel-7402 vs. HepG2 Cells Given that Akt plays a negative role in drug sensitivity, it seemed unlikely that the difference in basal Akt activity between these two cell lines contributed to the differential sensitivity to cAMP. Thus, we next investigated the activating phosphorylation of Akt in response to cAMP in HepG2 and Bel-7402 cells. Serum-starved cells were stimulated with FBS, which is a most common mitogen in cell culture and contains growth factors including insulin, insulin-like growth factor. FBS treatment time-dependently stimulated Akt phosphorylation in both HepG2 and Bel-7402 cells, with no significant difference in the extent and pattern of stimulation between the two cell lines. In contrast, when cells were treated with cAMP and then stimulated with FBS, we observed that cAMP inhibited FBS-stimulated Akt phosphorylation in Bel-7402 cells, but enhanced FBS-stimulated Akt phosphorylation in HepG2 cells in a time-dependent manner. We then tested the effects of forskolin, an adenylate cyclase activator capable to increasing intracellular cAMP level, on FBS-stimulated Akt phosphorylation in these two cell lines. Similarly, forskolin treatment significantly enhanced FBS-stimulated Akt phosphorylation in HepG2 cells but significantly inhibited FBS-stimulated Akt phosphorylation in Bel-7402 cells (Fig. 3). MAPK, another important protein kinase in FBS-stimulated signaling, showed similar responses to cAMP treatment in HepG2 and Bel-7402 cells, indicating that the observed differential response was Akt-specific. Interestingly, the opposing effects of forskolin on both cells were blocked by H89 (Fig. 4), a cAMP-dependent protein kinase (PKA) specific inhibitor, but not by protein kinase C inhibitor chelerythrine chloride (data not shown), indicating that cAMP modulates Akt activation through PKA.

The PI3K Inhibitor, LY294002, Rescued the Sensitivity of HepG2 Cells to cAMP To test whether the observed differential response of Akt to cAMP affects drug sensitivity in the two cell lines, we examined whether a PI3K inhibitor (LY294002) could restore the sensitivity of HepG2 cells to cAMP. As expected, LY294002 dose-dependently inhibited FBS-stimulated Akt phosphorylation; 0.1 μM LY294002 was enough to significantly inhibit FBS-stimulated Akt phosphorylation (Fig. 5A) without inhibiting the proliferation of HepG2 cells (Fig. 5B). LY294002 at the concentration of 0.1 μM enhanced the HepG2’s sensitivity to that seen in Bel-7402 cells (IC50=4.0 μM), but had no significant effect of Bel-7402 cells’ sensitivity (Fig. 6). Higher concentration of LY294002 such as 1 μM enhanced the sensitivity of both HepG2 and Bel-7402 cells to cAMP (data not shown). These results col-
lectively demonstrate that the response of Akt affects the cell sensitivity to cAMP. To further confirm that Akt is directly involved in the cAMP sensitivity, we examined the effect of API-2, an Akt inhibitor without inhibiting PI3K or PDK, on the sensitivity of these two cell lines to cAMP. As shown in Fig. 7A, API-2 dose-dependently inhibited the FBS-stimulated phosphorylation of Akt. More importantly, API-2 significantly potentiated the sensitivity of both HepG2 and Bel-7402 cells to cAMP, which was also observed in cells treated with higher concentration (1 μM) of LY294002 (Fig. 7B). These results further demonstrate Akt functions to attenuate the cAMP action on cell proliferation.

**DISCUSSION**

Previous studies have shown that Akt is involved in mediating cell sensitivity to chemotherapeutics, including cytotoxins, all trans-retinoic acid, and tamoxifen. To our knowledge, this is the first study demonstrating that Akt plays a critical role in cell sensitivity to cAMP, an important second intracellular messenger, in HCC cells. Our findings indicate that cAMP inhibited the proliferation of both Bel-7402 and HepG2 cells, with HepG2 cells showing less sensitivity to cAMP. The relative resistance of HepG2 cells did not result from endogenous Akt activity, but rather could be attributed to a heightened Akt response to cAMP as indicated by four lines of evidence. First, Bel-7402 cells had high basal Akt activity, but were more sensitive to cAMP compared to HepG2 cells, which had lower basal Akt activity, suggesting that endogenous Akt levels did not determine the cell sensitivity to cAMP. Second, cAMP treatment potentiated FBS-stimulated Akt phosphorylation in HepG2 cells, and had a relatively lower effect on HepG2 cell proliferation. Third, the
PI3K inhibitor, LY294002, inhibited cAMP-induced potentiation of FBS-stimulated Akt phosphorylation in HepG2 cells, and this inhibitory effect restored the sensitivity of HepG2 cells to cAMP. Fourth, Akt inhibitor API-2 potentiates cAMP inhibition of both HepG2 and Bel-7402 cells, indicating that Akt indeed functions to attenuate cAMP action on cell proliferation. These findings collectively show for the first time that the Akt response, rather than its endogenous activity, modulates cell sensitivity to cAMP. Future work will be required to determine whether this effect is specific to cAMP, or if this novel mechanism is applicable to other agents that modulate Akt activity.

It has been reported that constitutive Akt activity promotes chemotheraphy resistance and cell survival in breast cancer cell lines and non-small cell lung cancer (NSCLC).\(^{1,2,6,7}\) However, our results revealed that while Bel-7402 cells had 5-fold higher endogenous Akt activity than did HepG2 cells, Bel-7402 cells were more sensitive to cAMP. This discrepancy could be due to differences in the utilized chemotherapeutics (cAMP vs. cytotoxins) or differences in the utilized cells (breast and non-small cell lung cancer cells vs. HCC cells). However, our results clearly show that the Akt response to cAMP treatment is a critical factor in modulating the sensitivity of hepatocellular carcinoma cells to cAMP. Considering the importance of Akt in cell resistance and survival, it is not surprising that a positive Akt response decreases cell sensitivity to cAMP.

Although many human HCCs have mutations in exon 5 and exon 8 of the PTEN gene,\(^{13}\) we did not find an association between high endogenous Akt activity and changes in the PTEN expression levels, indicating that PTEN may not play a role in the relative resistance of HepG2 cells to cAMP.

It has been reported that ERK is involved in regulating the growth of human HCC cells.\(^{12}\) Our data showed that phosphorylation of MAPK was similarly increased by cAMP in both cell lines (Fig. 2), suggesting that MAPK did not contribute to the relative tolerance observed in this work.

Clinical trials are currently underway to investigate the antitumor activity of 8-Cl-cAMP, and theophylline and aminophylline (which can elevate intracellular cAMP) have been reported to synergize with gemcitabine or cisplatin to induce programmed cell death in cell lines derived from human ovarian, prostate and lung cancers, and also in granulosa cell line transformed by SV40 and Ras oncogene.\(^{14}\) Thus, the fact that Akt promotes resistance to cAMP will have important implications in upcoming subsequent preclinical and clinical studies. Future investigations are needed to elucidate the mechanism of cAMP effect on proliferation and the relative sensitivity between different HCC cells.

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