Cardiac glycosides block cancer growth through HIF-1α- and NF-κB-mediated Plk1 expression

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Cardiac glycosides as inhibitors of the sodium/potassium adenosine triphosphatase (sodium pump) have been reported to block cancer growth by inducing G2/M phase arrest in many cancer cells. However, no detailed studies have been performed to distinguish between these two phases of cardiac glycoside-arrested cells. Furthermore, the underlying mechanisms involved in this cell cycle arrest process are still not known. Here, we report that bufalin and other cardiac glycosides potently induce mitotic arrest by the downregulation of polo-like kinase 1 (Plk1) expression. Live-cell imaging results demonstrate that bufalin-treated cells exhibit a marked delay in entering prophase at an early stage and are then arrested at prometaphase or induced entry into apoptosis. This phenotypic change is attributed to the downregulation of Plk1. We also show that bufalin and the knockdown of sodium pump reduce Plk1, at least in part, through downregulation of the nuclear transcription factors, hypoxia-inducible factor-1α (HIF-1α) and nuclear factor-kappa B (NF-κB). These findings suggest that cardiac glycosides induce mitotic arrest and apoptosis through HIF-1α- and NF-κB-mediated downregulation of Plk1 expression, demonstrating that HIF-1α and NF-κB are critical targets of cardiac glycosides in exerting their anticancer action.

Introduction

The sodium pump is the target for cardiac glycosides, a group of compounds including bufalin, ouabain, digoxin and oleandrin, which have been commonly used to treat heart failure for many years (1). Some of these glycosides such as ouabain as well as several other cardenolides and bufadienolides have been identified as endogenous circulating compounds in human (2,3). Recent studies have revealed that the expression levels of the sodium pump in many cancer cells (e.g. colon cancer, prostate cancer, pancreatic cancer, lung cancer and breast cancer) are higher than that of normal cells (4). Regardless of tissue origin, the higher the expression levels of the sodium pump, the more sensitive the cells become to treatment with cardiac glycosides (5). Cardiac glycosides can arrest cells at a phase with 4N DNA content in most cancer cells (6,7). The mechanisms of how cardiac glycosides control cell cycle progression are poorly understood due to the fact that both G2 and M phase cells possess the same 4N DNA content. In many experimental systems, it is difficult to distinguish these two phases of cells as well as the relationship between cell cycle arrest and cell death. However, live-cell imaging experiment can be used to distinguish between G2 and M phase as well as analyze apoptosis.

G2/M phase transition is positively regulated by mitotic kinase, polo-like kinase 1 (Plk1). Plk1 is mainly expressed from late G2 to M phase (8). In eukaryotic organisms ranging from yeast to human, Plk1 is expressed at the centrosomes, kinetochores, midbody and cytokinetic furrow during mitosis and meiosis (9,10). The critical functions of Plk1 include regulating mitotic entry, sister chromatid cohesion, spindle formation, chromosome segregation and cytokinesis, and these processes are attributed to the poleward force generated by Plk1 (11). When Plk1 function is lost in Drosophila, several proteins required for mitosis cannot be accumulated at the spindle poles (12). Consistent with this finding, in human cells, Plk1 has been reported to be involved in the recruitment of Aurora A into spindle poles for centrosome maturation and of Aurora B into kinetochores for chromosome assembly (13–15). When Plk1 is inactivated, cells are delayed in entering mitosis and are then arrested in mitosis followed by apoptosis (16). In fact, it is not known whether Plk1 is involved in cardiac glycoside-mediated G2/M phase arrest.

To date, the signal pathways for regulation of Plk1 expression are still poorly understood. The transcription factors hypoxia-inducible factor-1α (HIF-1α) and nuclear factor-kappa B (NF-κB) constitute critical signaling pathways for controlling cell survival, proliferation and metabolism. They are hyperactivated in many tumor cells (17–19). NF-κB has previously been reported to upregulate Plk1 (20). However, the interaction between HIF-1α and Plk1 is not known. Apart from confirming the previous finding that NF-κB can regulate Plk1, we have reported for the first time that HIF-1α is also involved in the regulation of Plk1 at both messenger RNA (mRNA) and protein levels. Moreover, we have demonstrated that the sodium pump is involved in such regulation.

In this study, we have systematically analyzed cardiac glycoside-mediated cell cycle progression in synchronized cells by using pharmacological and RNA interference techniques. Our data have demonstrated that cardiac glycosides can arrest cells in mitosis for a long period of time and induce some of these cells to enter apoptosis, and this action is attributed to the downregulation of Plk1 via HIF-1α and NF-κB pathways. These findings with important therapeutic implications have provided insights into the molecular mechanisms of cardiac glycosides-induced cell cycle arrest and cell death.

Materials and methods

Reagents and antibodies

Bufalin (025-15241) was purchased from Wako Pure Chemical Industries. Alexa Fluor 488 donkey anti-rabbit IgG (A21206), Alexa Fluor 488 donkey anti-mouse IgG (A21202) and Alexa Fluor 555 donkey anti-mouse IgG (A50570) antibodies were purchased from Life Technologies Corporation. Dulbecco’s modified Eagle’s medium (12800-017) and fetal bovine serum (FBS; 16000-044) were purchased from Gibco Invitrogen. McCoy’s 5A medium (M4892), digoxin (D6003), ouabain (O3125), and anti-α-tubulin antibody (T6074) were obtained from Sigma. Propidium iodide (PI)/ribonuclease (RNase) staining buffer solution (550825) was obtained from BD Pharmingen. Anti-sodium/potassium adenosine triphosphatase α-1 monoclonal antibody (sc-16043), anti-mouse IgG-horseradish peroxidase (HRP) (sc-2004) antibodies were obtained from Santa Cruz Biotechnology. Anti-sodium/potassium adenosine triphosphatase α-1 (sc-16043) and anti-mouse IgG-horseradish peroxidase (HRP) (sc-2004) antibodies were obtained from Santa Cruz Biotechnology. Anti-sodium/potassium adenosine triphosphatase α-3 monoclonal antibody (MA3-915) was from Affinity Bioreagents. Chemiluminescence HRP substrate (WBKLS0500) and anti-mitotic protein monoclonal α2 (MMP-2; phosphoSer/Thr-Pro) antibody (05-368) were from Millipore. Anti-histone H3 (9715) and anti-Plk1 (208G4) (4513) antibodies were from Cell Signal Technology.

Cell culture

The human colon cancer HT-29 and HCT-116 cells were cultured in McCoy’s 5A medium supplemented with 10% FBS and the human cervical cancer HeLa cells in Dulbecco’s modified Eagle’s medium, respectively. For G1/S boundary, cells were treated with 2 mM thymidine for 17 h and released in fresh 10% FBS medium for 9 h, followed by 15 h of treatment with 2 mM thymidine. For M phase, cells were treated with 0.33 μM nocodazole for 13h.

Immunofluorescence staining

Cells grown on slides were fixed with 3.5% formaldehyde for 10 min and then permeabilized with 0.1% Triton X-100 for 10 min. Slides were...
incubated with primary antibodies for 2 h at room temperature, followed by incubation with Alexa Fluor 488- and 555-conjugated secondary antibodies for 1 h. All antibodies were diluted with 0.5% bovine serum albumin. Images were taken with an Olympus FV1000 confocal microscope (Olympus, Center Valley, PA) using a x60, 1.35 NA oil objective. The intensity of the images was measured using the AlphaEaseFC software version 6.0.0 (Alpha Innotech, San Leandro, CA).

**Mitotic index analysis of phosphorylation of MPM-2**

The treated cells were collected, washed twice with ice-cold phosphate-buffered saline (PBS) and fixed with ice-cold 70% ethanol at 4°C for >12 h. The fixed cells were permeabilized with 0.1% Triton X-100 for 5 min, blocked with 0.5% bovine serum albumin for 15 min and incubated with anti-phospho-MPM-2 antibody at room temperature for 1 h, followed by incubation with donkey anti-mouse Alexa Fluor 488 secondary antibody for 1 h. Cells were washed with PBS for three times, incubated in PI/RNase A buffer solution for 10 min and immediately analyzed on a FACScan flow cytometer (Becton-Dickinson, San Jose, CA).

**Cell cycle and apoptosis analysis**

After treatment, cells were fixed in ice-cold 70% ethanol at 4°C for >12 h, washed twice with PBS and then stained with PI/RNase A solution for 10 min. The percentage of apoptotic cells (sub-G1) and cell cycle distribution were analyzed by flow cytometry.

**Small interfering RNA transfection**

The small interfering RNAs (siRNAs) for α1 isoform of sodium pump (sense: 5′-GGGCAGUGUUUCAGGCUAA-3′, anti-sense: 5′-UUAGCCUGAAACA CUGCC-3′) (21), NF-κB p65 (sense: 5′-GAUUGAGGAGAAACGUAA AdTdT-3′, anti-sense: 5′-UUUACGUUUCUCUAACUGdTdT-3′) (22), HIF-1α (sense: 5′-CUGAUAGCCAGCAACUUUA-3′, anti-sense: 5′-UCAG UUGCUUGGUUCAG-3′) (23) and Plk1 (sense: 5′-AAGGCGGCUUGCC A buffer solution for 10 min and immediately analyzed on a FACScan flow cytometer (Becton-Dickinson, San Jose, CA).

**Fig. 1.** Bufalin and other cardiac glycosides induce G2/M phase arrest and apoptosis in cancer cells. (A) Asynchronous populations of HT-29 and HCT-116 cells were treated with 100 nM bufalin or other cardiac glycosides (e.g. 400 nM digoxin and 400 nM ouabain) for 48 h. Cells were fixed with 70% ethanol, washed with PBS, stained with PI/RNase staining buffer for 15 min in the dark, and then immediately analyzed by flow cytometry. (B) Double-thymidine-synchronized HeLa, HCT-116 or HT-29 cells were released for 13 h in the presence of vehicle [0.1% dimethyl sulfoxide (DMSO)], bufalin (100 nM) or nocodazole (Noc., 0.33 μM) and then harvested at 10.5 h after release into 10% FBS medium. Cell cycle was analyzed by flow cytometry. All data shown are representative of three independent experiments.
Fig. 2. Bufalin-treated cells enter into G2 phase normally but are delayed in entering M phase. (A) Quantification of the percentage of cells progressed through prophase. HeLa cells stably expressing H2B-YFP were synchronized at the G1–S boundary by double-thymidine treatment. Images were taken 6h after release from thymidine block in the presence or absence of 100nM bufalin. Cells with chromosome condensation were considered as cells progressed through prophase and were shown as percentage of the total cell number (n = 113 and 133 for the control and bufalin treatment, respectively). (B) HeLa cells were synchronized by double-thymidine block and then released in the presence of vehicle (0.1% DMSO) or bufalin (100nM) for 7h. Cell cycle was analyzed by flow cytometry. (C) Western blot analysis of mitotic index phospho-histone H3 (Ser10) (p-H3) in thymidine-synchronized HeLa cells after release into 10% FBS medium for different time intervals. (D) Western blot analysis of mitotic index phospho-histone H3 (Ser10) (p-H3) in thymidine-synchronized HeLa cells 13h after release in the presence of bufalin (25, 100nM), nocodazole (Noc. 0.33 μM), a combination of these two drugs or equal amount of DMSO. (E) The levels of...
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AAGUGC-3′, anti-sense: 5′-GCACUGGCAAAGGCGCCCTT-3′) (24) were obtained from Genepharm. A non-target siRNA was used as a control with sense (5′-UCUACGAGGCACGACUU-3′) and anti-sense (5′-AACUCUCUGCCCGUAGA-3′) (21). The α3 siRNA (sc-36012) for α3 isoform of sodium pump was purchased from Santa Cruz. In brief, cells were seeded into six-well plates and incubated overnight. When cells were approximately 50% confluent, cells were transfected in McCoy’s 5A or Dulbecco’s modified Eagle’s medium with 75 nM control siRNA, 75 nM α1 siRNA, 37 nM α3 siRNA, 75 nM Plk1 siRNA or 75 nM Plk1 using DharmaFECT transfection reagent according to the manufacturer’s protocol.

Western blot analysis

For preparation of cell extracts, cells were lysed with lysis buffer [20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1 µg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride] for 1 h on ice. Cell lysates were centrifuged at 13 000g for 15 min at 4°C. The total proteins were resolved on a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membrane. The membrane was incubated with the respective primary antibody at 4°C overnight and then washed and incubated with the HRP-conjugated secondary antibody to allow detection of the appropriate bands using the chemiluminescence HRP substrate. The relative band intensity was quantified using the AlphaEaseFC software version 6.0.0.

Live-cell imaging experiments

Experiments were performed using the Olympus FV1000 confocal microscope or a Nikon fluorescence microscope. Cells were incubated in a chamber. The chamber was placed on the stage of the Olympus confocal microscope or the Nikon fluorescence microscope containing a heating block and maintained in a humidified atmosphere containing 5% CO2 at 37°C during imaging. The G1–S boundary cells were treated with or without 100 nM bufalin for up to 6 h and then taken one frame every 10 or 15 min for 11 h under the microscope. For microtubule polymerization analysis, mitotic cells were obtained by shake-off after 13 h of exposure to 0.33 µM nocodazole. The obtained mitotic cells were released in fresh 10% FBS medium at 37°C for 20 min and exposed to vehicle or bufalin for 20 min, followed by capturing one frame every 5 or 8 min for the experiment.

Fig. 3. Bufalin treatment eventually leads to prometaphase arrest or apoptosis. (A) HeLa cells stably expressing H2B-YFP were blocked at the G1–S boundary by double-thymidine treatment. Images were started 6 h after thymidine release in the presence or absence of 100 nM bufalin. Selected images containing the typical behavior of the population (e.g., start of recording, mitotic entry, mitotic exit or apoptosis, at the end of experiment) from live-cell imaging movies are shown. Time is displayed in hh:mm. The scale bar indicates 10 µm. For the full movies, see Supplementary Movies S1–S3, available at Carcinogenesis Online. Results shown are representative of >10 independent experiments. (B) Quantification of the experiment described in (A). For each cell imaged, the white bars indicate the time spent in cell division from chromosome condensation to chromosome division, and the red bars represent apoptosis.

phospho-MPM-2 (phospho-mitotic protein monoclonal #2, p-MPM-2) and DNA contents were analyzed by flow cytometry in HeLa and HT-29 cells treated as in (D). Cells were stained with phospho-MPM-2 antibody and PI before analysis. Sn, synchronized cells. All data shown are representative of more than three independent experiments.
2 h. The movie parameters included a framing rate of 10 or 15 min for thymidine-synchronized G_1–S boundary cells and of 5 or 8 min for nocodazole-synchronized mitotic cells. The movies were generated using the MetaMorph software. All images were processed by the FV10-ASW1.6 (Olympus) and the Adobe Photoshop CS2 (Adobe Systems, San Jose, CA) software.

**RNA isolation and reverse transcription–PCR**

Total RNA was isolated by Trizol (Invitrogen). Reverse transcription–PCR was performed using Impron II reverse transcriptase (Promega) according to the manufacturer’s instructions. To detect the mRNA levels of Plk1, HIF-1α, NF-κB p65 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), primers used were as follows: Plk1 forward, 5′-TTCGTGTTCGTGGTGTGGA-3′, reverse, 5′-CTCTAAAGGTTGTGGCCACT-3′; HIF-1α forward, 5′-CAGAGCAGGAAAAGGAGTCA-3′, reverse, 5′-TAGTAGCTGCATGATCGTCTG-3′; NF-κB p65 forward, 5′-GCAGAAAGAGGACATTGAGTG-3′, reverse, 5′-ACATCGGGTGGAATCATATTG-3′ and GAPDH forward, 5′-AAGGTCGGAGTCAACGATT-3′, reverse, 5′-CCATGGGTGGAATCATATTG-3′. GAPDH was used as the internal control.

**Statistical analysis**

Statistical analysis was performed using two-tailed Student’s t-test for comparison of two groups or one-way analysis of variance for comparison of more than two groups, followed by Tukey’s multiple comparison tests. For multiple testing, the P values were determined using a two-way analysis of variance with Bonferroni post-tests. All statistical analyses were carried out with the GraphPad Prism software version 5.01 (GraphPad, San Diego, CA). Data were expressed as mean ± standard error of the mean (SEM) of at least three independent experiments. A P value < 0.05 was considered statistically significant.

**Results**

**Bufalin and other cardiac glycosides induce G_2/M phase arrest and apoptosis in cancer cells**

Previous studies demonstrated that cardiac glycosides could induce cell cycle arrest at G_2/M phase and apoptosis in many cancer cells (25–29). Consistent with previous findings, we found that certain cancer cells such as HeLa and HCT-116 are more sensitive to cardiac glycosides-induced apoptosis compared with HT-29 cells, as indicated by the percentage of sub-G_1, an index of apoptotic cells (Figure 1A). This finding suggests that the susceptibility of cells to cardiac glycosides-induced apoptosis may be cell line dependent. Furthermore, we found that bufalin and other cardiac glycosides (e.g. digoxin and ouabain) could significantly induce G_2/M phase arrest in asynchronized cells (Figure 1A). Consistent with this finding, bufalin could prevent cells from passing through G_2/M phase in synchronized cells (Figure 1B). These arrested cells could re-enter into G_1 phase with 2N DNA content after removal of bufalin in a manner similar to the nocodazole-treated cells (Figure 1B). We conclude, therefore, that bufalin and other cardiac glycosides could induce G_2/M phase arrest in most cancer cells and induce apoptosis in some cells.

**Bufalin-treated cells enter G_2 phase normally but their transition into M phase is delayed**

To reveal the action of bufalin on cell cycle progression, we performed live-cell imaging experiments in HeLa cells stably expressing histone H2B-YFP. Bufalin does not affect microtubule polymerization. (A) Nocodazole-synchronized HeLa cells stably expressing H2B-YFP were released into 10% FBS medium for 20 min and then exposed to vehicle (0.1% DMSO) or bufalin (100 nM). At 40 min, cells were filmed under an Olympus confocal movie microscope. Selected single frames together with time points are shown. Time is displayed in hh:mm. The scale bar represents 10 μm. Full movies can be seen in Supplementary Movies S4 and S5, available at Carcinogenesis Online. Results shown are representative of more than five independent experiments. (B) The percentage of mitotic cells at the indicated time points was quantified. Cells were synchronized and treated with vehicle or bufalin as in (A). n ≥ 14 cells per group. Results shown are representative of more than five independent experiments. (C) The timing of mitotic exit was analyzed. For each imaged cell, the dot represents the time taken for cell division. Mitotic cells were obtained by nocodazole arrest and then treated with vehicle or bufalin as in (A). Results shown are representative of more than five independent experiments.
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Fig. 5. Bufalin-induced mitotic arrest and cell death are attributed to the downregulation of Plk1 expression. (A) Representative western blot and densitometric analysis normalized to β-actin demonstrating the effects of bufalin, digoxin and ouabain on Plk1 expression. Thymidine-synchronized HeLa or HT-29 cells were released in the presence of digoxin (400 nM), ouabain (400 nM) or bufalin (100 nM) for 9 h. The total cell lysates were blotted with the Plk1 and β-actin (loading control) antibodies. (B) Asynchronous populations of HeLa or HT-29 cells were treated with digoxin (400 nM), ouabain (400 nM) or bufalin (100 nM) for 24 h.
H2B-YFP. As shown in Figure 2A, the percentage of cells entering prophase decreased in the bufalin-treated group compared with the control group, suggesting that the bufalin-treated cells exhibit delayed entry into prophase. These delayed cells were not arrested at G1 or S phase, as indicated by the fact that these bufalin-treated cells could enter G2 phase with 4N DNA content similar to the control cells (Figure 2B). Phosphorylation of histone H3 at serine 10, a mitotic marker, is high in M phase for chromosome condensation (30). In this study, phosphorylation of histone H3 significantly increased at the beginning of 6h and then sharply decreased at 13h in HeLa cells after release from double-thymidine block (Figure 2C), whereas the amount of histone H3 protein did not significantly change during this period (Figure 2C). Therefore, we detected the p-H3 signal by western blot Carcinogenesis Online. The HeLa cells transfected with siRNA against Plk1 released into bufalin, nocodazole or a combination of these two drugs. As shown in Figure 2D, the p-H3 levels were significantly reduced in bufalin and nocodazole co-treated cells compared with the nocodazole alone treatment. These findings suggest that bufalin delays the cells from entering mitosis. As shown in Figure 2E, the percentage of cells with 4N DNA content was significantly increased in cells treated with bufalin in the presence or absence of nocodazole compared with vehicle treatment. We also found that the percentage of cells with 4N DNA content treated with a combination of bufalin and nocodazole was similar to that of cells treated with nocodazole alone. However, phosphorylation of MPM-2, another mitotic marker, was significantly reduced in cells by co-treatment with bufalin and nocodazole compared with cells treated with nocodazole alone (Figure 2E), suggesting that bufalin could prevent cells from going through mitosis. Taken together, these results indicate that bufalin delays cells from entering mitosis.

**Bufalin treatment eventually leads to prometaphase arrest or apoptosis**

To investigate the cells that are delayed mitotic entry by bufalin treatment would subsequently be arrested in mitosis, we performed fluorescent microscopy to record the mitotic progression in bufalin-treated HeLa cells stably expressing histone H2B-YFP. Control cells without bufalin treatment synchronously released from the G1–S boundary succeeded in passing through S, G2 and then M phase, as indicated by the presence of chromatid cohesion, chromosome alignment and chromosome segregation (Figure 3A; Supplementary Movie S1, available at Carcinogenesis Online). However, bufalin-treated cells entered prophase followed by apoptosis, or failed to go through metaphase until 14.5h, as characterized by the presence of sister chromatid cohesion and the absence of chromosomes alignment on the metaphase plate (Figure 3A; Supplementary Movies S2 and S3, available at Carcinogenesis Online). The time course of bufalin action on bufalin entry into mitosis was further investigated by live-cell imaging experiments.

The bufalin-treated cells either remained in mitosis with failure of chromosome segregation until the end of the experiment (16h 50min) or entered a state of apoptosis with highly condensed and fragmented chromatin from mitotic arrest (Figure 3B). However, control cells spent only 2.07±0.20h between the initiation of prophase and exit from anaphase (Figure 3B). Taken together, the cells after bufalin treatment were arrested at prometaphase and some of these cells eventually entered apoptosis.

**Bufalin does not affect microtubule polymerization**

In order to ascertain whether the bufalin-induced mitotic arrest could be attributed to the blockade of microtubule polymerization, we tracked cell cycle progression in the mitotic HeLa cells stably expressing H2B-YFP in the presence or absence of bufalin under a live-cell imaging microscope. Selected frames from live-cell imaging movies showed that bufalin treatment did not affect the progression from metaphase to cytokinesis, as chromosome segregation and chromosome decondensation in these cells are similar to control cells (Figure 4A; Supplementary Movies S4 and S5, available at Carcinogenesis Online). Consistent with this result, we did not find any significant effect of bufalin on mitosis at specific time points in many mitotic cells after nocodazole wash-out (Figure 4B). Regarding the timing of mitosis, from early prophase to late anaphase, the bufalin-treated cells spent 75.71±5.23 min to exit mitosis after nocodazole removal similar to control cells with 81.25±4.40 min (Figure 4C). It can thus be concluded that bufalin did not affect microtubule polymerization, suggesting that bufalin-induced mitotic arrest and cell death are independent of the blockade of microtubule polymerization.

**Bufalin-induced mitotic arrest and apoptosis are attributed to the downregulation of Plk1 expression**

Because the phenotypic changes in the bufalin-treated cells were similar to the Plk1-inhibited cells, we asked whether cardiac glycosides could regulate Plk1. Plk1 is well known for its function in regulating the localizations of Aurora A and Aurora B for mitotic spindles formation (13,15).

To address this question, we analyzed the Plk1 protein levels and its distribution in bufalin-treated cells. We found that bufalin and other sodium pump inhibitors digoxin and ouabain effectively reduced the protein levels of Plk1 in synchronized and asynchronized cells (Figure 5A and B). Furthermore, the distribution of Plk1 in the cardiac glycoside-treated cells was analyzed by immunofluorescence staining. In control cells, Plk1 antibody clearly stained centrosomes and kinetochores (Figure 5C). In contrast, after bufalin treatment, >80% of the mitotic cells exhibited weak Plk1 signals, loss of mitotic spindle architecture, failure to exit mitosis and generation of apoptotic bodies (Figure 5D). The intensity of Plk1 signals was reduced >6-fold at centrosomes compared with that in control metaphase cells and decreased >2-fold at kinetochores (Figure 5D and E). Consistent with this finding, knockdown of Plk1 by siRNA reduced Plk1 signals at both the centrosomes and kinetochores, prevented cells from passing through mitosis and induced apoptosis in some cells (Figure 5F, G and H). These results indicated that bufalin could prevent Plk1-mediated microtubules attachment with the centrosomes.
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Fig. 6. HIF-1α and NF-κB pathways are involved in bufalin-induced downregulation of Plk1. (A) Representative western blot and densitometric analysis normalized to β-actin demonstrating the effect of bufalin on HIF-1α and NF-κB expression. Before entering into mitosis, thymidine-synchronized HeLa or HT-29 cells were released in the presence or absence of bufalin (100 nM) for 9 h. (B) Representative western blot and densitometric analysis normalized to β-actin demonstrating the effect of sodium pump knockdown on HIF-1α and NF-κB expression. HeLa or HT-29 cells were transfected with siRNAs against HIF-1α and NF-κB.
and kinetochores. To investigate whether these observations in the cardiac glycoside-treated cells were due to inhibition of the sodium pump, we silenced the catalytic (α) subunit of the sodium pump using siRNA in both HeLa and HT-29 cells, and asked if depletion of the sodium pump could affect Plk1 expression. Consistent with previous findings (31,32), our results showed that human cervical cancer HeLa cells only expressed α1 isoform, whereas human colorectal cancer HT-29 cells expressed both upregulated α3 and downregulated α1 isoforms (Figure 5I). The knockdown efficiency of α1 siRNA and α3 siRNA was evaluated by western blot. As shown in Figure 5I, α1 was significantly reduced by siRNA transfection in HeLa cells and both α1 and α3 were significantly reduced in HT-29 cells. α1 depletion significantly inhibited the expression of Plk1 in HeLa cells (Figure 5I). However, α3 depletion, but not α1, significantly reduced Plk1 levels in HT-29 cells (Figure 5I). These results indicated that Plk1 protein expression could be regulated by the sodium pump via different α isoforms in a tissue-dependent manner. Furthermore, these results are consistent with previous reports that both α1 and α3 could act as anticancer targets of cardiac glycosides and α3 is more sensitive to cardiac glycosides than α1 (5,33). Taken together, these results demonstrated that cardiac glycoside-induced mitotic arrest and apoptosis are attributed to the downregulation of Plk1.

**HIF-1α and NF-xB pathways are involved in bufalin-induced downregulation of Plk1**

Recent studies reported that the sodium pump inhibitors cardiac glycosides could inhibit cell proliferation through inhibition of HIF-1α or NF-xB (34,35). We thus tested whether the bufalin-induced downregulation of Plk1 is dependent on HIF-1α or NF-xB. We first analyzed the effect of bufalin on HIF-1α and NF-xB expression by western blot. As shown in Figure 6A, bufalin significantly reduced the protein levels of both HIF-1α and NF-xB, consistent with the action of sodium pump knockdown (Figure 6B). These results indicated that bufalin regulated both HIF-1α and NF-xB through the sodium pump. HIF-1α and NF-xB are transcription factors of many target genes (36,37). To study whether HIF-1α or NF-xB regulates Plk1, we analyzed Plk1 at both protein and mRNA levels in HIF-1α- or NF-xB-knockdown cells. As shown in Figure 6C and D, the expression levels of Plk1 protein and mRNA were significantly reduced by both HIF-1α siRNA and NF-xB p56 siRNA in both HeLa and HT-29 cells, suggesting that transcription factors HIF-1α and NF-xB p56 could positively regulate Plk1 expression at the translational level. We further used siRNA against HIF-1α or NF-xB p56 to show that HIF-1α and NF-xB p56 are required for mitosis-to-G2 phase transition. As shown in Figure 6E, silencing of HIF-1α or NF-xB p56 could significantly prevent cells from passing through G2/M phase, suggesting that HIF-1α and NF-xB p56 are, at least in part, involved in mitosis-to-G2 phase transition. To further confirm the involvement of HIF-1α and NF-xB p56 in regulating Plk1 expression in mitosis, the subcellular distribution and the protein levels of Plk1 were analyzed in both HIF-1α- and NF-xB-knockdown cells. As shown in Figure 6F, knockdown of HIF-1α or NF-xB and knockdown of Plk1 significantly blocked the recruitment of Plk1 to the mitotic centrosomes and unattached kinetochores, and resulted in mitotic arrest or apoptosis. Quantification of these Plk1 signals showed that the intensity of staining for Plk1 at the centrosomes and kinetochores of mitotic cells was significantly reduced by HIF-1α siRNA and NF-xB siRNA (Figure 6G and H). Taken together, these results indicated that the downregulation of Plk1 by bufalin is mediated, at least in part, via both HIF-1α and NF-xB pathways.

**Discussion**

Previous studies have reported that the sodium pump inhibitors cardiac glycosides (e.g. bufalin, digoxin and ouabain) can block cell survival by arresting the cell cycle at a phase with 4N DNA content in many cancer cells. However, the exact point of cell cycle arrest and the underlying mechanism are not understood. In this study, we have monitored cell cycle progression by using live-cell imaging experiments to gain insights into the action of cardiac glycosides on mitotic progression.

Our results indicated that bufalin could induce G2/M phase arrest. Although it was reported that a tetraploid G2 arrest could be induced by nutlin-3 in p53 wild-type cell lines HCT-116 and U2OS (38), our data demonstrated that bufalin-arrested cells with 4N DNA content could pass through mitosis and re-enter into G1 phase with 2N DNA content after removal of bufalin, indicating that the cells were not arrested at tetraploid G2 phase (4N, like G2/M) by bufalin in p53-defective (e.g. HT-29 and HeLa) and in p53-positive (e.g. HCT-116) cells. This finding is consistent with previous study showing that bufalin could induce G2/M phase arrest in p53 wild-type U2OS cells (29). Therefore, bufalin-mediated cell cycle arrest at G2/M phase is independent on the p53 status.

Aurora A disrupted cells lead to monopolar spindle defects, chromosome condensation and failure to go through mitosis (39). Cells treated with Aurora B inhibitor hesperadin exhibit polyplody nuclei and misaligned chromosomes decondensation, and exit from mitosis without cytokinesis (40,41). Plk1 depletion or inhibition causes cells to arrest in mitosis with chromosome alignment failure, and most of these cells subsequently go into apoptosis or remain arrested in mitosis for a long period of time (41). In our study, bufalin-treated cells failed to go through mitosis with chromosome condensation and failure of chromosome alignment at metaphase plate. This finding is consistent with the phenotypic change in Plk1-knockdown cells, suggesting that Plk1 plays a critical role in bufalin-induced mitotic arrest and cell death.

Lin et al. (20) found that inhibition of NF-xB could increase the sensitivity of esophageal squamous cell carcinoma cells to anokysis by downregulating Plk1 expression, suggesting that NF-xB positively regulates Plk1 at the transcriptional level. Consistent with this finding, our results showed that bufalin and silencing of the sodium pump could decrease NF-xB expression. Furthermore, van Uden et al. (42) showed that tumor necrosis factor-α could increase NF-xB and then regulate HIF-1α. On the contrary, lipopolysaccharide-mediated upregulation of HIF-1α was independent of NF-xB activity (43). Taken together, these findings indicated that the expression levels of HIF-1α are not completely dependent on NF-xB activity. In our study, we
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have demonstrated that HIF-1α expression is not dependent on NF-κB activity.

There are four isoforms of α (catalytic) subunit of the sodium pump, namely α1, α2, α3 and α4. These four different gene products are expressed in a tissue-dependent manner in mammalian cells. Although α1 is widely expressed, α2 and α3 are mainly expressed in heart, neuron and muscle, and α4 is expressed in testis and spermatotoides (44). The potential anticancer property of cardiac glycosides with no or slight side effect is attributed to the high expression levels of the sodium pump in cancer cells. Yang et al. (5) investigated the effect of cardiac glycosides on the growth of both human and mouse cancer cells in relation to sodium pump α subunit. It was demonstrated that cardiac glycoside oleandrin selectively inhibited the proliferation of α3 isoform overexpressed in human pancreatic cancer cells but not in rodent cells, which predominantly express α1 isoform. This result indicated that cancer cells with a higher ratio of α3 to α1 isoform are more sensitive to cardiac glycoside treatment, suggesting that the relatively high levels of α3 with limited expression of α1 can be used to predict which human cancer cells could be treated by cardiac glycosides (5). In line with this finding, it was demonstrated that the increased expression of α1 isoform with low α3 expression correlated with the resistance of certain cancers (e.g. glioma and melanoma) to cardiac glycosides, as α1 isoform is important for tumor growth and cell survival (45). However, cardiac glycosides could also significantly inhibit the growth of human cancer cells (breast and cervical cancer cells) with high expression of α1 but not primary cells such as peripheral blood mononuclear cells and neutrophils (21,32,46). Previous study reported that all human α isoforms exhibit a similar affinity for cardiac glycosides (47). Knockdown of α1 could significantly inhibit cancer growth in α1-overexpressed cancer cells (21). These findings indicated that α1 could also be a potential target for cancer treatment. Taken together, both α1 and α3 could be employed as anticancer targets in a tissue-dependent manner (33). This conclusion agrees with our findings showing that cardiac glycosides inhibited cell proliferation in α3-overexpressed colon cancer HT-29 cells and in α1-overexpressed cervical cancer HeLa cells. Although the α isoforms have been studied for decades, the underlying reason for their tissue-specific distribution and their mode of action are not completely understood. Our findings that the mitosis-required Plk1 could be regulated by different α isoforms (e.g. α1 and α3) in a tissue-dependent manner add new insight into understanding sodium pump-mediated cell cycle progression. These findings also provide support to the notion that cardiac glycosides could inhibit various cancer cell growth via different α isoforms of the sodium pump.

In this study, it was found that cardiac glycoside-mediated mitotic arrest was attributed to the reduction of Plk1 via HIF-1α and NF-kB, which are overexpressed in many cancer cells. Our finding not only enriches our understanding of the anticancer mechanisms of cardiac glycosides but also provides an explanation to the increased cell growth and division rates of many cancer cells with an overexpressed level of sodium pump. The implication of this finding in cancer chemotherapy warrants further investigations.

Supplementary material

Supplementary Movies S1–S4 and Supplementary Material can be found at http://carcin.oxfordjournals.org/

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References

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