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Cardiac glycoside-induced cell death and Rho/Rho kinase pathway: Implication of different regulation in cancer cell lines

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ABSTRACT

Previously, we demonstrated that the Rho/ROCK pathway is involved in ouabain-induced apoptosis in HUVEC. In the current work, we investigated whether the Rho/ROCK pathway is functional during cardiac glycosides-induced cytotoxic effects in cancer cell lines, as well as in non-tumor cells. For that purpose, we evaluated the role of ROCK activation in bleb formation and cell migration over upstream and downstream effectors in addition to ROCK cleavage after cardiac glycosides treatment. All three cardiac glycosides (ouabain, digoxin and bufalin) induced cell death in HeLa and HepG2 cells and increased the formation of blebbing in HeLa cells. In contrast to our previous study, ROCK inhibitor Y27632 did not prevent bleb formation. Observation of ROCK II cleavage after ouabain, digoxin and oxaliplatin treatments in HeLa and/or HepG2 cells suggested that cleavage is independent of cell type and cell death induction. While inhibiting cleavage of ROCK II by the caspase inhibitors z-VAD-fmk, z-VDVAD-fmk and z-DEVD-fmk, evaluation of caspase 2 siRNA ineffectiveness on this truncation indicated that caspase-dependent ROCK II cleavage is differentially regulated in cancer cell lines. In HeLa cells, ouabain induced the activation of ROCK, although it did not induce phosphorylation of ERM, an upstream effector. While Y27632 inhibited the migration of HeLa cells, 10 nM ouabain had no effect on cell migration. In conclusion, these findings indicate that the Rho/ROCK pathway is regulated differently in cancer cell lines compared to normal cells during cardiac glycosides-induced cell death.

1. Introduction

Cardiotonic glycosides (i.e., digitalis) are defined as specific inhibitors of an integral membrane protein, Na^+/K^+ ATP-ase (NKA) [1]. There are two types of cardiac glycosides: cardenolides, such as ouabain, digoxin and digitoxin, and bufadienolides such as bufalin. They have been prescribed for the treatment of congestive heart failure since William Withering reported on the use of *Digitalis purpurea* extract in 1785 [2]. Since digitalis was discovered in mammals endogenously [3], many studies have been performed to elucidate the role of these compounds in physiological and pathophysiological processes. Most of the studies involved the effect of cardiac glycosides in several cardiovascular pathologies. However, recent studies indicate that ouabain and other cardiac glycosides have some important cytotoxic and antimigratory effects in cancer cell lines.

At micromolar concentrations, digitalis interact with NKA by inhibiting the pumping functions of the enzyme [4]. In addition, low doses of digitalis activate several intracellular signaling pathways by modulating the interaction between NKA and neighbouring proteins within caveolae, resulting in activation of Src and the concomitant transactivation of EGF receptors [5,6].

Cardiac glycosides have apoptotic, antiproliferative and antimigratory activities against a variety of cancer cells. The inhibitory effect of digitalis on cell proliferation is thought to rely on the inhibition of angiogenesis, topoisomerase I and II [7]. It is known that cardiac glycosides induce apoptosis developed by death receptors and inhibit expression of antiapoptotic proteins such as Bcl-xL and Bcl-2 and transcription factors such as NF-kB and activator protein 1 (AP-1) [7].

At physiological concentrations, ouabain prevents migration of human lung cancer H292 cells through the downregulation of regulatory proteins, such as focal adhesion kinase (FAK), ATP-dependent tyrosine kinase (Akt), and cell division cycle 42 (Cdc42) [8]. It is established that bufalin has inhibitory effects on cell migration and invasion in T24 bladder cancer by modulating the levels of tight junction-associated factors and preventing the activation of matrix metalloproteinases [9].

Small G protein, Rho and its the most studied effectors, Rho kinases (ROCK I and ROCK II), are involved in several cellular functions including smooth muscle contractions, non-muscle cell contractions and cell migration [10–12]. It has been shown that the

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Rho/Rho kinase pathway has pivotal roles in the development and metastasis of cancer cells. Activation of the Rho/Rho kinase pathway is required for the amoeboid movement of cancer cell invasion [12]. In addition, elevated expression of ROCK I and ROCK II was observed in human breast cancer [13] and hepatocellular carcinoma [14], colon [15], and bladder cancer [16], respectively. Moreover, mutations have been shown at three different points of the ROCK I gene in primary human breast cancer cells and lung cancer cell lines [17,18]. Two somatic mutations in the ROCK II gene were identified in primary gastric carcinoma and melanoma cells as well [18]. Most of these mutations result in elevated kinase activation of both ROCK I and II and lead to cancer progression by increasing cell motility and decreasing adhesion of cells.

The role of the Rho/Rho kinase pathway in cell migration varies according to the cell type and migration conditions. Nakayama et. al. presented that migration of endothelial cells, macrophages, and smooth muscle cells were prevented by treatment with the Rho kinase inhibitor Y27632 in a dose-dependent manner, whereas fibroblast and epithelial cell migrations were not affected [19]. In the same study, preventing cell migration with myosin II inhibitor, in a similar way as the inhibitor of Rho kinase, did suggest that the role of Rho kinase on cell migration occurs through the regulation of myosin II activity [19]. In another study, it was demonstrated that the Rho A/ROCK pathway is sufficient for negatively regulating integrin-mediated adhesion and for the retraction of the tail of cells during migration of monocytes [20].

Overall, it is known that cardiac glycosides have inducible effects on cytotoxicity, inhibitory effects on cancer cell migration, and the Rho/Rho kinase pathway is involved in the metastasis of cancer cells. However, the role of the Rho/Rho kinase pathway in cardiac glycosides-induced cell death and migration in cancer has yet to be elucidated.

In our previous work, we showed for the first time in endothelial cells that ouabain induces proteolytic cleavage of ROCK I and ROCK II, resulting from caspase 3 and caspase 2 activation, respectively [21]. We also determined that ouabain-induced ROCK II cleavage generated a new fragment of approximately 140 kDa, which was not demonstrated in the previous studies [21]. In that study, it was also determined that pretreatment of endothelial cells with a specific ROCK inhibitor (Y27632) before ouabain exposure inhibits apoptotic membrane blebbing and the ouabain-induced cell to cell detachment in ouabain-induced apoptosis [21]. These results demonstrate that the Rho/ROCK pathway is involved in cardiac glycoside-induced apoptotic effects.

Although there have been several studies on the mechanisms of action of cardiac glycosides cytotoxic effects, less well-known is the relationship between cardiac glycosides and the Rho/Rho kinase pathway in the mechanisms of cell death and migration in cancerous cells. Therefore, we aimed to evaluate the possible roles of the Rho/ROCK pathway in two different cancer cell lines, HeLa and HepG2, in response to cardiac glycosides during cell death and migration by answering the following questions: 1. Does the Rho/ROCK pathway, which is involved in ouabain-induced membrane blebbing in endothelial cells, mediate the formation of blebs in cancer cell lines? 2. Does ouabain have any effect on HeLa cell migration? 3. If so, is the Rho/ROCK pathway involved in that effect? 4. Is the new 140 kDa fragment of ROCK II generated in ouabain-treated endothelial cells specific for non-tumor cells? 5. Or, do the different apoptotic stimuli trigger the production of this fragment?

2. Materials and methods

2.1. Cell culture

HeLa and HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin at 37 °C with 5% CO_2. When cells reached 70–80% confluence, various drugs at indicated times and concentrations were applied to cells. These drugs include cardiac glycosides ouabain (Sigma), digoxin (Tocris) and bufalin (Tocris), pan-ROCK inhibitor Y27632 (Tocris), Rho A inhibitor CT04 (Cytoskeleton), pan-caspase inhibitor z-VAD-fmk (Tocris), caspase 3 inhibitor z-DEVD-fmk (Tocris), caspase 2 inhibitor z-VDVAD-fmk (Tocris).

2.2. Real time monitoring of cytotoxicity and cell migration (xCelligence assay)

An xCELLigence Real-Time Cell Analyzer (RTCA) DP system (Roche) was used to monitor the viability and migration of cells after treatment of cardiac glycosides and oxaliplatin, as previously described [21,22]. Briefly, 5×10^4 cells were plated per well of an E-plate. Cell growth and/or viability was monitored every 15 min. The next day existing medium was replaced with fresh medium. When cells were in log phase, cells were treated with ouabain (1 nM–1 μM), digoxin (10 nM–10 μM), bufalin (10 nM–10 μM), and a standard chemotherapeutic drug, oxaliplatin (30 μM–1 mM). HeLa and HepG2 cells were monitored for another 24 h and 60 h, respectively. IC_{50} values of drugs were evaluated by calculating the area under the curve based on a percentage of untreated drug groups.

To evaluate the effects of Rho and Rho kinase inhibition on cardiac glycosides-induced cytotoxicity, the RTCA DP system was again used. After plating 5×10^4 cells, the next day the medium was changed. Before the treatment of cardiac glycosides, cells were exposed to either 10 μM Y27632 for 30 min or 0.5 μg/ml Rho inhibitor CT04 for 2 h. After the pretreatment, cells were treated with 1 μM ouabain, 1 μM digoxin and 300 nM bufalin. After incubation, the slope of the original trace from the RTCA DP system as an indicator of cell death rate was calculated and analyzed.

HeLa cell migration was performed using xCELLigence real-time cell migration. 160 μl DMEM containing 10% FBS was added to each well of the lower chamber as a chemoattractant agent. 4×10^5 HeLa cells were seeded into each well of the upper chamber in serum-free medium. The CIM-plates were left in an incubator for 30 min to allow cell attachment. When the cell index reached to ~1, the cells were incubated with drugs as indicated. Next, migrated cells were monitored every 15 min for 48 h using the RCTA DP system.

2.3. Effects of cardiac glycosides on cell morphology by phase contrast and immunofluorescence microscopy

Phase contrast microscoby was used to evaluate the morphological cellular changes. After drug treatment images of cells were captured, and blebbing cells were counted and expressed as the percentage of total number of cells.

For fluorescence staining, cells were grown on μ-slide (Ibidi). At the end of drug treatment, cells were washed with 500 μl PBS and fixed with 2% paraformaldehyde for 15 min. Then cells were washed with PBS 3 times followed by 0.1% Triton X-100 permeabilization for 5 min. For blocking 1% BSA in PBS was used for 45 min. For immunostaining cells were then incubated in pERM (1:50, Cell Signaling) primary antibody for 2 h at room temperature. Monolayers washed with PBS 3 times and incubated with FITC-conjugated goat anti-rabbit IgG secondary antibody (1:50, Pierce) for 1 h at room temperature. For F-actin staining, 50 μg/ml Rhodamine-phalloidin (Sigma) for 45 min and for nucleic acid staining 0.1 μg/ml 4′-6-diamidino-2-phenylindole (DAPI) (Sigma) for 5 min was used. Immunofluorescence was visualized using a Leica DMIL inverted fluorescence microscope and recorded with a Leica (DFC 420C) digital camera.