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Na^+/K^+-ATPase as a target for cardiotonic steroids and cisplatin

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ABSTRACT : The sodium (Na^+)/potassium (K^+)-ATPase is an ion pump located on the surface of all animal cells. It pumps three sodium ions out of the cell while pumping two potassium ions into the cell, hydrolyzing one ATP molecule as the driving force for the reaction. Na^+/K^+-ATPase forms and maintains the electrochemical gradient in cells, which provides the basis for the excitability of nerve and muscle tissues and contributes to the osmotic regulation of cell volume. In addition, the electrochemical Na^+ gradient is the driving force for the secondary transport of nutrients such as amino acids, sugars, and drugs. Recently, the Na^+/K^+-ATPase has been studied as an important target for cancer treatment, as it has been implicated in the development and progression of many cancers. Na^+/K^+-ATPase forms a phosphoenzyme intermediate (EP) during ATP hydrolysis.

Cardiotonic steroids have been used to treat congestive heart failure and arrhythmias, and recently their anti-cancer activities have been reported. Ouabain, a specific inhibitor of Na^+/K^+-ATPase, is a cardiotonic steroid that binds to EP, inhibiting its dephosphorylation and the release of inorganic phosphate.

Cisplatin is one of the most potent anti-tumor agents. Many studies have examined the relationship between cisplatin and Na^+/K^+-ATPase from the viewpoint of cisplatin accumulation and the prevention of nephrotoxicity. It has been suggested that the transport of cisplatin into cells is mediated by the Na^+/K^+-ATPase and that Na^+/K^+-ATPase activity is inhibited by cisplatin, although the underlying mechanism remains unclear.

In this review, we evaluate the mechanisms underlying inhibition of Na^+/K^+-ATPase by cisplatin. We also summarize the structure, function, and enzymatic reaction of Na^+/K^+-ATPase, as well as the potential for the pump to serve as a target for ouabain and cisplatin. Finally, we will describe experiments conducted by our group showing the mechanism of Na^+/K^+-ATPase inhibition by cisplatin, and the combined effects of ouabain and cisplatin on cancer cell viability.

Key Words : Na^+/K^+-ATPase, cardiotonic steroid, ouabain, cisplatin

1. Na^+/K^+-ATPase

The sodium (Na^+)/potassium (K^+)-ATPase[1-5], also known as the Na-K pump, is found in the plasma membrane of all animal cells, and is responsible for translocating three sodium ions outside the cell and two potassium ions into the cell, hydrolyzing one ATP molecule as the driving force for this reaction (Fig. 1). The Na^+/K^+-ATPase is most abundantly expressed in ion-transporting epithelia such as the kidneys, and in excitable tissues such as the brain and cardiac muscle. It is composed of α and β subunits in equimolar ratios. The α is the catalytic subunit, which contains the binding sites for Na^+, K^+, ATP, and cardiotonic steroids, and the β subunit is the regulatory subunit. There are four isoforms of the α subunit (α1-α4) and three isoforms of the β subunit (β1-β3). A third type of subunit, FXYD, is found in some cells. Seven different FXYD proteins (FXYD1 to FXYD7) have been identified, each of which has distinct functional effects on the transport characteristics of the Na^+/K^+-ATPase. FXYD2, the γ subunit of Na^+/K^+-ATPase, is the best-studied FXYD protein. The different

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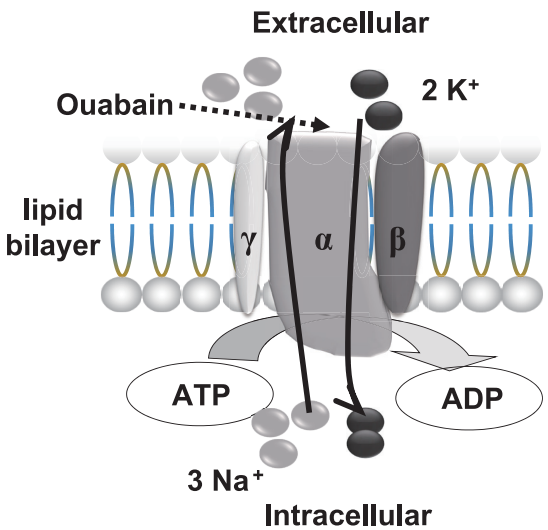

Fig. 1 Na^+/K^+-ATPase pump.

α and β subunits and FXYD proteins are selectively expressed in various normal tissues in a species-dependent manner, but their normal expression pattern is altered in a tissue-specific manner in cancer cells. The Na^+/K^+-ATPase contributes substantially to maintenance of the membrane potential of cells, which provides the basis for the excitability of nerve and muscle tissues, and contributes to the osmotic regulation of cell volume. In addition, the electrochemical Na^+ gradient is the driving force for the secondary transport of nutrients such as amino acids, sugars, and drugs.

2. Na^+/K^+-ATPase as an ion transporter and its enzymatic reaction

The Na^+/K^+-ATPase forms and maintains the electrochemical gradient in cells. The mechanism of its enzymatic cycle was established by Post and Albers[1-7], so it is often referred to as the Post-Albers cycle (Fig. 2). Na^+/K^+-ATPase has two conformational states, E1 and E2. It binds Na^+ and ATP in its E1 conformation, and is then phosphorylated on an aspartate residue, resulting in the occlusion of three Na^+ ions and their subsequent release to the extracellular side. This new conformational state (E2-P) binds K^+ with high affinity, leading to dephosphorylation of the Na^+/K^+-ATPase and occlusion of two K^+ ions (K)E2. Then K^+ is released into the cytosol after ATP binds to the enzyme with low affinity. In the absence of K^+, E2-P is directly dephosphorylated and inorganic phosphate is released, making it Na-dependent. In the absence of Na^+, (K)E2 hydrolyzes p-nitrophenylphosphate (*p*NPP) and releases *p*-nitrophenol

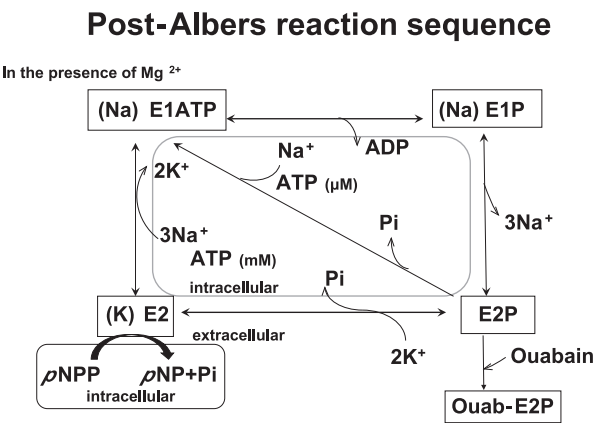

Fig. 2 Post-Albers reaction.

(*p*NP) and inorganic phosphate, facilitating the detection of K-dependent *p*NPPase activity. Cardiotonic steroids, such as ouabain, bind to E2-P and form ouabain-binding E2P (Ouab-E2P), which is stable and rarely dephosphorylated. Ouabain and other cardiac glycosides are normally potent Na^+/K^+-ATPase inhibitors, but it has been reported that exogenous cardiac glycosides, specifically ouabain, increases Na^+/K^+-ATPase activity at low concentrations (nanomolar) *in vitro*.

3. Na, K-ATPase as a signal transducer

In the past 20 years, studies have shown that Na^+/K^+-ATPase interacts with neighboring membrane proteins, forms signalosomes, and transduces messages downstream using intracellular signaling pathways[1-5, 8]. It has been suggested that there are two pools of Na^+/K^+-ATPase within the plasma membrane, with two distinct functions. One is the classical pool that functions as an energy-transducing ion pump, and the other is the signal transducing pool of enzymes that are restricted to caveolae. Signaling pathways including Src kinase, epidermal growth factor receptor, and mitogen-activated protein kinase are rapidly activated by the interaction of cardiotonic steroids with the Na^+/K^+-ATPase, independent of changes in intracellular Na^+ and K^+ concentrations. It has been proposed that the interaction of cardiotonic steroids with Na^+/K^+-ATPase may also affect cell adhesion and migration.

4. Cardiotonic steroids as Na^+/K^+-ATPase inhibitors

Many plants contain cardiotonic steroids such as

digoxin and ouabain and cardiotonic steroids are also found in animals, mainly in toads[4, 8]. Recently it was found that mammalian tissues and body fluids contain digitalis-like compounds such as digoxin, ouabain, and bufadienolide family members[1-5, 8]. Endogenous ouabain release from adrenal glands is regulated by adrenalin and angiotensin II, suggesting that ouabain concentration in the blood changes rapidly upon hormonal stimulation. These endogenous digitalis-like compounds inhibit Na^+/K^+-ATPase and modify the role of Na^+/K^+-ATPase as a signal transducer, leading to activation of such processes as cell proliferation, heart contractility, and hypertension. Cardiotonic steroids and cardiotonic glycosides bind to the extracellular surface of the Na^+/K^+-ATPase, resulting in its inhibition and an increase in intracellular sodium concentrations, which in turn, leads to decreased function of the Na/Ca exchanger and an increase in intracellular calcium concentration in myocardinal cells. This is reportedly the mechanism underlying the positive inotropic effects of cardiotonic steroids. Cardiotonic steroids have long been used for the treatment of congestive heart failure due to their positive inotropic agents.

5. Cardiotonic steroids as potential anti-cancer agents

Epidemiological studies conducted during the late 20th century revealed that very few patients maintained on cardiotonic steroid treatment for heart problems died from cancer[8]. Since then, there has been growing interest in using cardiotonic steroids as anti-cancer agents. In addition, it has been suggested that the interaction of endogenous digitalis-like compounds with Na^+/K^+-ATPase might be associated with tumor growth. Furthermore, several studies have reported the altered expression of Na^+/K^+-ATPase subunits in different cancer types compared to corresponding normal tissues. These considerations suggest the possibility of cardiotonic steroids as anti-cancer agents.

6. Cisplatin as anti-cancer agents

Cisplatin is one of the most potent anti-tumor agents, displaying clinical activity against a wide variety of solid tumors including testicular, lung, ovarian, cervical, and head and neck tumors[9, 10]. In the last four decades, platinum-based compounds such as cisplatin have received much attention because of their potential anti-tumor activity and increasing application in cancer therapy. Their interactions with DNA are responsible for the anti-tumor activity, but their toxicity has been ascribed to their interactions with the thiol groups of proteins. The anti-tumor activities of platinum-based compounds are associated with many severe toxic side effects caused by protein structural alterations and enzymatic changes that are implicated in their mechanism of action. Thus, the reactions of cisplatin with bionucleophiles other than DNA have biological importance because these interactions play central roles in modulating the activity of platinum-based anti-tumor drugs. Many studies have investigated the relationship between cisplatin and Na^+/K^+-ATPase from the standpoint of drug accumulation and prevention of nephrotoxicity[11, 12]. Several studies have suggested that transport of cisplatin into cells is mediated by the Na^+/K^+-ATPase, and that Na^+/K^+-ATPase activity is inhibited by cisplatin. Furthermore, the toxicity of platinum-based anti-cancer drugs, such as cisplatin and chloroplatinic acid, is related to inhibition of Na^+/K^+-ATPase activity. The relationship between cisplatin and Na^+/K^+-ATPase is complicated, as cisplatin disrupts the source of its own translocation energy. Thus, it is important to elucidate the mechanism underlying inhibition of Na^+/K^+-ATPase activity by cisplatin for its optical clinical use.

7. Mechanism underlying the inhibition of Na^+/K^+-ATPase activity by cisplatin

We studied the mechanism underlying the inhibition of Na^+/K^+-ATPase activity by cisplatin using enzymes prepared from Ca 9-22 cells, which were derived from human squamous cell carcinoma of the gingiva[13] or purified from rabbit brain[14]. When the pre-incubation time of Na^+/K^+-ATPase with cisplatin was constant, the activity decreased in a concentration-dependent manner, and depending on the pre-incubation time at each indicated cisplatin concentration. When the same experiments were conducted on ice, the inhibitory effect decreased by almost 50%. Pre-incubation of cisplatin with water did not influence the inhibitory effect. However, co-incubation with 2-mercaptoethanol (2-ME) led to recovery of almost 75% of its activity. Similar to other heavy metals, platinum chloride also appeared to be a non-competitive inhibitor of Na^+/K^+-ATPase activity, and compared with cisplatin, was a more potent inhibitor of

Na^+/K^+-ATPase[13, 14]. The inhibition of Na^+/K^+-ATPase activity by cisplatin depends on its concentration, pre-incubation time, and temperature, but not on hydration time[14]. We tested the effects of cisplatin on the partial reactions of the enzyme, Na-dependent ATP hydrolysis, and K-dependent *p*-NPP hydrolysis activities to determine which step in the Na^+/K^+-ATPase reaction is inhibited by cisplatin. Cisplatin inhibited both activities depending on its concentration and the pre-incubation time, whereas Na-dependent ATP hydrolysis activity was inhibited at lower concentrations. Formation of a phosphoenzyme intermediate (EP) of Na^+/K^+-ATPase was also inhibited by cisplatin depending upon its concentration and the pre-incubation time. An eight-fold higher concentration of 2-ME (4 mM) than cisplatin (0.5 mM) prevented inactivation of the enzyme by cisplatin, and inhibition of Na^+/K^+-ATPase by pre-treatment with cisplatin was recovered almost completely with 2-ME treatment. These results suggest that the active form of cisplatin inhibits Na^+/K^+-ATPase activity by inhibiting EP formation, and that inhibition by cisplatin is arrested by addition of a thiol group.

We hypothesized that inhibition of kidney Na^+/K^+-ATPase activity by platinum-containing anti-cancer drugs may be related to their nephrotoxicity. To test this hypothesis, we studied the effects of cisplatin, nedaplatin and carboplatin on Na^+/K^+-ATPase purified from pig kidneys and human renal proximal tubule epithelial cells[15]. All of the tested drugs decreased cell viability and inhibited Na^+/K^+-ATPase activity depending on both their concentrations and the pre-incubation time. The intensity to decrease live cells and to inhibit the activity was rated as cisplatin > nedaplatin > carboplatin. The conformation of Na^+/K^+-ATPase affected cisplatin-induced inhibition of ATPase activity. The inhibition of activity upon pre-treatment with cisplatin was recovered by treatment with 2-ME, cysteine, a reduced form of glutathione, and sodium thiosulfate. These results suggest that inhibition of the Na^+/K^+-ATPase by platinum-containing anti-cancer drugs is related to their nephrotoxicity, and that some thiol compounds can recover the activity and may lower nephrotoxicity.

8. Regulation of cisplatin sensitivity in oral squamous carcinoma cells by Na^+/K^+-ATPase activity

Cisplatin is one of the major chemotherapeutic drugs, but tumor cells acquire resistance, limiting its use. One of the main causes of resistance is reduced drug accumulation. We investigated what regulates intracellular cisplatin accumulation[16] using six types of oral squamous carcinoma cells. Assessment of cisplatin sensitivity was determined by measuring ATP levels in cells. Intracellular cisplatin levels, copper accumulation, and cisplatin efflux was measured. The specific activity of the Na^+/K^+-ATPase and copper-transporting P-type ATPase (Cu-ATPase) was detected. The role of ouabain, the specific Na^+/K^+-ATPase inhibitor, in intracellular cisplatin accumulation was evaluated and Western blot analysis of Na^+/K^+-ATPase α and β subunits, P-glycoprotein, and Cu-transporting ATPases ATP7A and ATP7B was performed. Among the cells, human oral squamous cell carcinoma HSC-3 and BHY cells were the most cisplatin-sensitive and cisplatin-resistant, respectively. Compared to BHY cells, HSC-3 cells exhibited increased cisplatin accumulation, increased Na^+/K^+-ATPase activity, and increased expression of the α and β subunits and ATP7A and ATP7B ATPases. There were no marked differences in specific Cu-ATPase activity between cells and both cells did not express P-glycoprotein. Treatment with ouabain markedly reduced intracellular cisplatin accumulation in both cell lines. These results indicate that Na^+/K^+-ATPase activity regulates intracellular cisplatin accumulation, and the Cu-ATPase only plays a marginal role, if any, in cisplatin transport.

9. Change in sensitivity of cisplatin-resistant oral cancer cells to platinum-based compounds

To further study the mechanism underlying the cisplatin resistance of cancer cells and the role of Na^+/K^+-ATPase, we tested the sensitivity of cisplatin-resistant oral cancer cells to cisplatin, carboplatin, nedaplatin (anti-cancer agent), and ouabain[17]. We used oral cancer cells H1 and KB, and their cisplatin-resistant cell lines H1R and KBR[18, 19]. Cell viability was evaluated by measuring intracellular ATP content. Viability of the parent and resistant cells decreased depending upon the concentrations of cisplatin, carboplatin, and nedaplatin, but resistant cells needed higher concentrations to decrease cell number compared to parent cells. The 50% inhibitory concentrations of the anti-cancer agent were from low to high concentration in the order of cisplatin, nedaplatin, and carboplatin, suggesting that cisplatin-resistant cells

were cross-resistant to nedaplatin and carboplatin. The viability of both parent and resistant cells also decreased depending on the ouabain concentrations. We tested the combined effects of cisplatin and ouabain on cell viability of both parent and resistant cells. The ouabain-dependent decrease in viable cells was protected in the presence of low cisplatin concentrations, and cisplatin-dependent decreases in cell number were affected by the presence of ouabain. The combined effects of cisplatin and ouabain differed among the parent and resistant cells. As described above, both ouabain and cisplatin inhibited Na^+/K^+-ATPase activity and altered cell growth and function. Moreover, it was recently reported that cardiotonic steroids are anti-cancer candidates and also affect cisplatin-induced cell death[20, 21]. Thus, we propose the complicated interaction of ouabain and cisplatin in cancer cells, although more studies are necessary to confirm this hypothesis (Figs. 3 and 4).

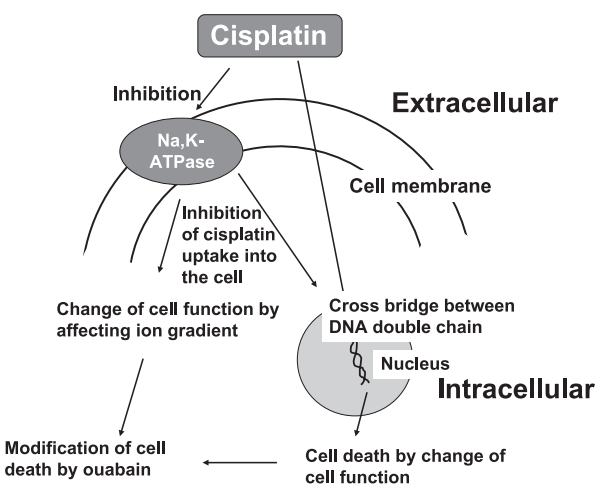

Fig. 3 Effects of cisplatin on Na^+/K^+-ATPase and cell viability.

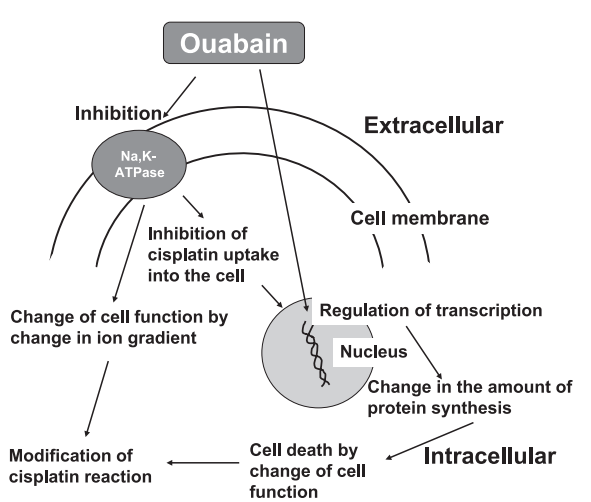

Fig. 4 Effects of ouabain on Na^+/K^+-ATPase and cell viability.

Conclusions

In addition to the classical role of the Na^+/K^+-ATPase as a sodium and potassium ion transporter, this enzyme also works as a signal transducer and is implicated in the development and growth of cancer. Ouabain, a cardiotonic steroid, and cisplatin, an anti-cancer drug, inhibit Na^+/K^+-ATPase activity and affect its functions. As a result, ouabain modifies the anti-cancer effects of cisplatin and is also an anti-cancer drug candidate. Cisplatin alters its own anti-cancer effects as well as those of ouabain by inhibiting Na^+/K^+-ATPase activity.

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