Role of Pannexin1 Channels in Cisplatin-Induced Apoptosis of Testicular Cancer I-10 Cells Post-print

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Abstract

Objective: To investigate the role and mechanism of pannexin1 channels in cisplatin-induced apoptosis of testicular cancer I-10 cells. Methods: MTT assay was used to detect cell viability; Annexin V/PI double staining and Hoechst 33258 staining were used to detect early and late apoptosis, respectively; chemiluminescence assay was used to detect extracellular ATP concentration; ELISA was used to detect intracellular IP3 content. Results: MTT assay showed that cell viability in the group treated with the pannexin1 channel inhibitor CBX combined with cisplatin was higher than that in the cisplatin alone group ($P<0.01$); Annexin V/PI double staining showed that the early apoptosis rate in the CBX combined with cisplatin group was lower than that in the cisplatin alone group ($P<0.001$); Hoechst 33258 staining showed that the late apoptosis rate in the CBX combined with cisplatin group was lower than that in the cisplatin alone group ($P<0.01$); chemiluminescence assay indicated that extracellular ATP concentration in the CBX combined with cisplatin group was lower than that in the cisplatin alone group ($P<0.05$); ELISA showed that intracellular IP3 concentration in the CBX combined with cisplatin group was lower than that in the cisplatin alone group ($P<0.05$). Conclusion: Pannexin1 channels are involved in cisplatin-induced apoptosis of testicular cancer I-10 cells, and the mechanism may involve the ATP/IP3 signaling pathway.

Full Text

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Abstract

Objective: To investigate the role of pannexin 1 channels in cisplatin-induced apoptosis in testicular cancer I-10 cells and the underlying mechanisms. **Methods:** MTT assay was used to assess cell viability. Annexin V/PI double staining and Hoechst 33258 fluorescence staining were employed to detect early- and late-stage apoptosis, respectively. Extracellular ATP levels and intracellular IP3 levels were measured using commercial detection kits. **Results:** I-10 cells co-treated with the pannexin 1 channel inhibitor carbenoxolone (CBX) and cisplatin (DDP) showed significantly higher viability compared with cells treated with cisplatin alone ($P<0.01$). CBX significantly reduced cisplatin-induced early-stage apoptosis ($P<0.001$) and late-stage apoptosis ($P<0.01$), and caused marked decreases in extracellular ATP and intracellular IP3 levels during cisplatin-induced apoptosis ($P<0.05$). **Conclusion:** Pannexin 1 channels participate in cisplatin-induced apoptosis in I-10 cells, possibly through the ATP/IP3 signaling pathway.

Key words: carbenoxolone; pannexin 1 channel; cisplatin; apoptosis; ATP; testicular carcinoma

Introduction

Testicular cancer is a highly malignant solid tumor that predominantly affects young men aged 20-35 years [1]. Multi-drug combination chemotherapy centered on cisplatin demonstrates good therapeutic efficacy against testicular cancer. However, adverse reactions such as bone marrow suppression and nephrotoxicity severely limit its clinical application. Therefore, identifying novel targets with high specificity that can enhance the sensitivity of testicular cancer to cisplatin is of great significance for improving clinical outcomes and reducing side effects.

Pannexins are a new family of channel proteins first reported by Panchin et al. in 2000 [2]. Six pannexin proteins assemble to form a "hexameric channel" that allows passage of molecules with relative molecular mass less than 1000, facilitating material exchange and signal transduction between intracellular and extracellular microenvironments. Pannexin channels participate in various important physiological and pathological processes, including cell apoptosis, ATP release, and calcium wave propagation [3-4]. Among the three identified subtypes (pannexin1, pannexin2, pannexin3), pannexin1 (Panx-1) has been most extensively studied.

In recent years, numerous studies have reported that during chemotherapy-induced tumor cell apoptosis, Panx-1 channels can mediate ATP release into the extracellular space [5-8]. Extracellular ATP can act on P2Y receptors on the cell membrane surface to activate the inositol trisphosphate (IP3) pathway [4]. IP3 is an important second messenger that regulates apoptosis and can induce Ca2+ release from the endoplasmic reticulum, leading to cell apoptosis [9-10]. However, the effect of Panx-1 channels on cisplatin-induced apoptosis in testicular cancer cells and whether this involves the ATP/IP3 signaling pathway have not been reported.

In this study, we used the Panx-1 channel inhibitor carbenoxolone (CBX) as a tool drug to preliminarily investigate the role of Panx-1 channels in cisplatin-induced apoptosis in testicular cancer I-10 cells and the potential mechanisms involved.

Materials and Methods

1.1 Cell Line and Culture

The mouse testicular cancer cell line I-10 was purchased from ATCC. Cells were cultured in F12 high-glucose medium supplemented with 2.5% (V/V) fetal bovine serum, 15% (V/V) horse serum, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were grown adherently in 25 cm^2 ventilated culture flasks at 37°C in a humidified incubator with 5% CO_2. Subculture was performed using 0.25% trypsin cell digestion solution (containing 0.02% EDTA) 2-3 times per week.

1.2 Reagents

F12 high-glucose medium, fetal bovine serum, and horse serum were purchased from Gibco. Cisplatin (DDP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Hoechst 33258 staining kit, and CBX were purchased from Sigma-Aldrich. FITC-Annexin V/PI double staining kit was purchased from BD Biosciences. Trypsin and ATP detection kit were purchased from Beyotime. Mouse inositol 1,4,5-trisphosphate (IP3) ELISA kit was purchased from CUSABIO. Other common reagents were of analytical grade from domestic sources.

1.3 Cell Viability Assay (MTT)

Logarithmic growth phase cells were seeded in 96-well plates at a density of 5$×10^{4}$ cells/mL with 5 replicate wells per group. When cells reached approximately 40-50% confluence, the medium was replaced with fresh drug-containing medium (using serum inactivated for 2 h [6], as nucleotidases in serum would degrade ATP). Groups included cisplatin alone and cisplatin plus CBX combination, with cisplatin concentrations of 0, 2, 4, 8, 16, 32, 64, and

128 mol/L and CBX concentration of 100 mol/L. After 24 h of culture, 15 L of MTT (5 mg/mL) was added to each well and incubated for an additional 4 h. The medium was then removed, and 150 L of DMSO was added to each well and incubated at 37°C for 30 min. Absorbance (A value) was measured at 570 nm using a microplate reader after shaking for 10 min. All sample detection procedures were performed under light-protected conditions. Cell survival rate was calculated using the formula:

Cell survival rate = (A_{{{drug}}*{{treated}}}{group} - A_{{{blank}}*{{control}}}) / (A{{{control}}*{{group}}} - A{{{blank}}_{{control}}})

1.4 Early Apoptosis Detection (Annexin V/PI)

Logarithmic growth phase cells were seeded in 12-well plates at a density of 2$×10^{5}$ cells/mL. After 24 h, the medium was replaced with fresh drug-containing medium using inactivated serum. Groups included blank control, CBX alone, cisplatin alone, and cisplatin plus CBX combination, with cisplatin concentration of 100 mol/L and CBX concentration of 100 mol/L. After 8 h, cells were digested and collected using trypsin without EDTA (centrifuged at 2000 r/min for 5 min), washed twice with pre-cooled PBS (centrifuged at 2000 r/min for 5 min), and collected at 1$×10^{5}$ to 5$×10^{5}$ cells. Cells were suspended in 100 L of Binding Buffer, then 5 L of Annexin V and 5 L of PI were added, mixed gently, and incubated at room temperature under light-protected conditions for 15 min. Finally, 400 L of Binding Buffer was added, and samples were analyzed by flow cytometry.

1.5 Late Apoptosis Detection (Hoechst 33258)

Cell seeding and drug treatment were performed as described in section 1.4. After 16 h, the culture medium was removed, cells were washed twice with PBS, fixed with 4% paraformaldehyde for 15 min, and washed twice with PBS. Each well was then incubated with 500 L of Hoechst 33258 (10 g/mL) at room temperature under light-protected conditions for 30 min. After washing away the dye with PBS, cells were observed and photographed under a fluorescence microscope. Five random fields were selected to count late apoptotic cells and calculate the apoptosis rate.

1.6 Extracellular ATP Concentration Detection

Cell seeding and drug treatment were performed as described in section 1.4. Extracellular culture medium was collected at 8, 16, and 24 h, centrifuged, and the supernatant was used as samples. In an opaque 96-well plate, 100 L of ATP detection working solution was pre-added to each well, followed by 20 L of sample per well and mixed well. Chemiluminescence was measured using a multifunctional microplate reader with 1 s excitation and sensitivity set at 200. All sample detection procedures were performed under light-protected conditions on ice.

1.7 Intracellular IP3 Content Detection (ELISA)

Cell seeding and drug treatment were performed as described in section 1.4. At 8, 16, and 24 h, culture plates were placed on ice, washed once with cold PBS, and cells were transferred to 1.5 mL centrifuge tubes. After two freeze-thaw cycles to disrupt cell membranes, samples were centrifuged at 4°C and 5000 g for 5 min. The supernatant was diluted 200-fold with PBS as samples. In the ELISA plate, 100 L of sample was added to each well and incubated for 2 h, followed by 100 L of biotin-labeled antibody working solution and incubated for 1 h. After washing 3 times, 100 L of horseradish peroxidase-labeled avidin working solution was added and incubated at 37°C for 1 h. After washing 5 times, 90 L of substrate solution was added and incubated at 37°C under light-protected conditions for 30 min. Finally, 50 L of stop solution was added, and absorbance at 450 nm was measured within 5 min using a microplate reader.

1.8 Statistical Analysis

All experimental data were obtained from at least three independent experiments and expressed as mean ± standard deviation. Statistical analysis was performed using SPSS 16.0 software. Comparisons between two groups were conducted using independent samples t-test, while comparisons among multiple groups were performed using one-way ANOVA. P<0.05 was considered statistically significant.

Results

2.1 Effect of CBX on Cisplatin Cytotoxicity

Cell viability was assessed using the MTT assay. When treated with cisplatin alone at concentrations of 16, 32, 64, and 128 mol/L, the survival rates of I-10 cells were $0.57\$\pm0.03, 0.43\pm0.03, 0.32\pm0.02, and 0.24\pm0.03, respectively. Co-treatment with cisplatin and CBX increased the survival rates to 0.71\pm0.04, 0.64\pm0.01, 0.61\pm0.01, and 0.59\pm\$0.02$ respectively. The combination of CBX and cisplatin significantly improved I-10 cell survival rates, with all P values <0.01, indicating statistically significant differences [Figure 1: see original paper].

2.2 Effect of CBX on Cisplatin-Induced Apoptosis

Cisplatin exerts its anti-tumor effects primarily by inducing apoptosis, which includes both early and late stages. Early apoptosis was detected using Annexin V/PI double staining [Figure 2: see original paper]A, lower right quadrant). The early apoptosis rate in the cisplatin alone group was $17.34\%\$\pm0.57\pm0.44\pm4.31\pm\$1.73\%$ (P<0.01).

2.3 Effect of CBX on ATP/IP3 Pathway

To verify whether CBX inhibition of cisplatin-induced apoptosis is related to the Panx-1 channel-mediated ATP/IP3 pathway, we measured ATP and IP3 levels at 8, 16, and 24 h [Figure 3: see original paper]. At 8, 16, and 24 h, extracellular ATP and intracellular IP3 levels in the cisplatin alone group were 1.54$\pm$0.15*and*1.29$\pm$0.07; 2.55$\pm$0.26*and*1.40$\pm$0.08; *and*1.20$\pm$0.07*and*1.26$\pm$0.06, *respectively.Inthecisplatinplu* respectively—all significantly lower than the cisplatin alone group ($P<0.05$).

Discussion

Cisplatin is one of the most widely used chemotherapeutic agents and remains the first-line drug for testicular cancer [11]. After entering tumor cells, the chlorine atoms of cisplatin are replaced by water molecules, forming active nucleophilic groups that can bind to DNA, thereby inhibiting DNA replication, transcription, and repair, ultimately leading to tumor cell apoptosis [12]. However, only 5-10% of cisplatin can covalently bind to DNA [13], suggesting that other mechanisms may be involved in cisplatin-induced tumor cell apoptosis. This study is the first to demonstrate that the Panx-1 channel inhibitor CBX can significantly reduce cisplatin cytotoxicity [Figure 1: see original paper] and apoptosis induction [Figure 2: see original paper] in testicular cancer I-10 cells, indicating that Panx-1 channels may play an important role in the anti-tumor effects of cisplatin on I-10 cells.

Recent studies have shown that Panx-1 channels are involved in cell death processes in various contexts. Draganov demonstrated that ivermectin-induced tumor cell death requires Panx-1 channel participation [14]. Cheung reported that Panx-1 channels are involved in osteocyte apoptosis following micro-damage [15]. Yang' s research indicated that Panx-1 channel mediation is required for endotoxic shock-induced cell death [16].

Panx-1 channels are hemichannels located on the cell membrane, and the Panx-1 proteins that form these channels are expressed in various tissues including brain, heart, liver, lung, and testis [17], with a long half-life of 32 h [18]. Our results show that cisplatin treatment of testicular cancer I-10 cells leads to massive ATP release, which is significantly inhibited by the Panx-1 channel inhibitor CBX [Figure 3A: see original paper], suggesting that Panx-1 channels may be the primary pathway for ATP release. The carboxyl terminus of Panx-1 protein contains a small autoinhibitory domain composed of 46 amino acids, so under basal conditions, Panx-1 channel opening is minimal. Boyd-Tressler' s research suggests that during apoptosis induced by various chemotherapeutic drugs (anti-Fas, staurosporine, etoposide, doxorubicin), massive ATP release occurs because caspase-3 cleaves the autoinhibitory domain at the carboxyl terminus of Panx-1 protein, thereby irreversibly activating Panx-1 channels [6]. Cisplatin can activate caspase-3 during tumor cell apoptosis [19-20], which may be the mechanism by which cisplatin induces ATP release in I-10 cells.

Extracellular ATP is a signaling molecule that can act on purinergic receptors on the cell surface to cause increased intracellular IP3 levels [4]. Our study shows that cisplatin treatment of testicular cancer I-10 cells also leads to increased intracellular IP3 levels, which can be suppressed by CBX [Figure 3B: see original paper]. This may be because CBX inhibits Panx-1 channel-mediated ATP release, thereby suppressing ATP-induced increases in intracellular IP3 levels. IP3 is an important second messenger that regulates apoptosis; it acts on IP3 receptors to cause Ca2+ release from the endoplasmic reticulum [21-23]. Eugenin' s study [9] demonstrated that in astrocytes infected with HIV, IP3 from dying cells diffuses through gap junctions to neighboring cells, causing increased Ca2+ concentration in uninfected cells, followed by mitochondrial Ca2+ accumulation and cytochrome C release, ultimately leading to apoptosis of uninfected astrocytes. Therefore, we speculate that increased intracellular IP3 levels may be one of the mechanisms by which cisplatin induces I-10 cell apoptosis.

In summary, our study demonstrates that Panx-1 channels participate in cisplatin-induced apoptosis in testicular cancer I-10 cells, possibly through mediating the ATP/IP3 signaling pathway. This provides new insights for targeting Panx-1 channels to enhance cisplatin chemotherapy efficacy against testicular cancer. However, specific drugs that can enhance Panx-1 channel activity have not yet been identified, and the regulation of Panx-1 protein synthesis and metabolism in cells remains unclear—these could be the focus of future research.

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