

CIC-3 is Involved in NPPB-Induced Apoptosis in DU145 Prostate Cancer Cells

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Abstract

NPPB3-(4-5-nitro-2-(3-phenylpropylamino) benzoic acid, as one of the most commonly used chloride channel blockers, is very essential for chloride channel studies. However, the role of NPPB in mediating apoptosis is uncertain. The purpose of this study was to investigate the apoptosis mechanism of NPPB in human cancer DU145 cell lines. Cell viability was examined by MTT assay. JC-1 fluorescent probe was used for detecting I membrane potential. Cell apoptosis was determined by flow method (FCM). The expression of apoptosis-related protein Caspase-3, Cleaved caspase-3, PARP, Cleaved PARP, Bcl-2, Mcl-1, Bcl-xl, Bcl-w and Bax was detected by western blotting. Cell proliferation rate was remarkably inhibited 2 h after treating with NPPB(50,100 μmol/L) (p<0.01); Flow cytometry method analysis showed that the apoptosis rate in each treated group were significantly higher, especially for the promotion of early apoptosis rate effect was significantly (P<0.05); Images of JC-1 showed NPPB led to the decrease of intracellular mitochondrial membrane potential; Western blot results showed that caspase-3/PARP signaling pathway was activated, while the expression of cleaved caspase-3, cleaved PARP was significantly increased (P<0.01).PARP, as substrate of caspase-3, was significantly decreased (P<0.05).Bcl-2, Bcl-xl compared with the control group had no significant difference, and anti-apoptotic protein Mcl-1 (P<0.05) and Bcl-w (P<0.01) decreased significantly, pro-apoptotic protein Bax (P<0.01) were obviously upregulated. Our findings indicated that NPPB could increase apoptosis in DU145 cell lines via mitochondria-related apoptotic pathway, and following results presented that CIC-3 Chloride Channels involved in the process of NPPB induced DU145 cells apoptosis.

Keywords: CIC-3; NPPB; Apoptosis; Human prostate cancer DU145 cell lines; Mitochondria-related apoptotic pathway

Introduction

Prostate cancer (PCa) is one of the most commonly diagnosed malignant cancer in men, with 220,800 (26%) incident cases in US alone. 27,540 PCa cases was reported in the United States in 2015, and it was also predicted as the second leading cause of cancer deaths among men [1]. For advanced and metastatic tumors, were always compromised by the appearance of hormone refractory cancer cells, eventually leading to the recurrence of cancer.

Especially, the therapeutic DU145 human prostate cell is a classical cell line of prostatic cancer [2]. Therefore, it is important to explore its molecular mechanisms and pathophysiologic mechanism during prostate apoptosis.

Ion channels, especially chloride channel, are associated with cell proliferation, apoptosis, migration and other cellular events [3]. Over-expression of anti-apoptotic oncoprotein Bcl-2 in androgen-dependent prostate cancer epithelial cells resulted in the increase of swelling-activated Cl⁻ current and the enhancement of endogenous

expression of CIC-3 protein [4]. It prompted a potential mechanism between CIC channel and apoptotic mediation of prostate cancer.

NPPB3-(4-5-nitro-2-(3-phenylpropylamino) benzoic acid, as one of the most commonly used chloride channel blockers, is very essential for chloride channels studies. Some studies had demonstrated that chloride channel blockers could reduce nasopharyngeal carcinoma cell apoptosis which induced by 5-Fu [5].

However, some other studies had reported that chloride channel blockers NPPB could induce in laryngo carcinoma Hep-2 cells [6] and increase infarct apoptosis in rabbit myocardium [7]. Therefore, the mechanism of chloride channels blocker NPPB in mediating apoptosis remained uncertain.

In this study, we investigated the role of chloride channels blocker NPPB during mediating apoptosis in human prostate cancer cellular line DU145 cells. Through the research, we provided the foundation to clarify the relationship between the chloride channel and prostate cancer cells apoptosis.

Materials and Methods

Cell culture

Human prostate cancer cellular line DU145 cells were purchased from Shanghai Institute of Cell Biology. Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Cell culture medium was prepared with RPMI 1640 medium supplemented with 10% (vol/vol) FBS and 1% penicillin/streptomycin (Life Technologies). In the treatment groups, culture medium was replaced every 24 h with, containing desired concentrations of chemicals.

Cell viability assay

Cells were seeded in 96-well plates at the density of 4,000 cells per well with 100 µL media. After treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to cells (1 mg/mL final concentration), and cells were allowed to grow at 37°C for another 3 h. MTT containing media were removed, and MTT precipitate was dissolved in 100 µL DMSO. The optical absorbance was determined at 570 nm using a microplate reader (iMark; Bio-Rad).

Mitochondrial membrane potential (MMP) measurement

A JC-1 mitochondrial membrane potential assay kit (Sigma-Aldrich, St.Louis, Missouri, USA) was used to detect changes in mitochondrial membrane potential. The detected process was performed following the manufactory's instruction. JC-1 is a fluorescent lipophilic cationic probe. Normal mitochondria formed J-aggregates, emitting red fluorescence, while the damaged mitochondrion was determined by the increase ratio of green- cell.

Immunofluorescence

Cells were seeded on a seeding glass. After treating with different culture medium, samples were fixed with paraformaldehyde. TritonX - 100 was used to crack cell membrane, antibody CIC-2 and CIC-3 taped with (fluorescein isothiocyanate, FITC) or PI was added and incubated for 30 min. Staining results were observed with fluorescence microscope.

Flow cytometry assay

An annexin V-FITC and propidium iodide (PI) double staining kit (Invitrogen, Carlsbad, CA, USA) was used to analyze cellular apoptosis. Airway epithelial cells were seeded into 6-well plates (5 × 10⁵ cells/well) and treated with denatonium at different concentrations and for 2 h.

The cells were digested with trypsin (Gibco® Trypsin-EDTA, Invitrogen, Carlsbad, CA, USA), washed with PBS for three times, suspended in 500 µL binding buffer and, finally, incubated with 5 µL of FITC-conjugated Annexin-V and 5 µL of PI for 15 min at room temperature in dark. Then, samples were analyzed by flow cytometry.

Quantitative real-time -PCR (qRT-PCR) assay

CIC-1 to CIC7 mRNA levels were measured in prostate cancer cell lines DU145 by qRT-PCR. Total RNA was extracted using TRIzol (Life Technologies), and reverse transcription was performed from 3 µg total RNA using oligo(dT) and RevertAid Reverse Transcriptase (Thermo Scientific) according to the supplier's instructions.

Quantitative PCR was performed with SuperReal PreMix SYBR Green (TIANGEN) using an Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies). Relative cDNA level was calculated by the comparative.

CT (cycle threshold) method. PCR primers included (5' to 3')

CIC-1 sense (GCATTTGGAAGGCTGGTAGG),

CIC-1 antisense (TGTGGAGACTGTGTGGGAGAC),

CIC-2 sense (GGGAGTGGTCTGAAAGAAT),

CIC-2 antisense (CCCAAAGAGGGAGAGGAAGT),

CIC-3 sense (CCTGGCTGCTGATGTTATGA),

CIC-3 antisense (CTGAGGGCAAATCCCACTAA),

CIC-4 sense (GCCTGTTTGGTGGCATTTAG),

CIC-4 antisense (GGCAAGTGTTCAGCGTCAT),

CIC-5 sense (AATGAAGCCAAGCGGAGAG),

CIC-5 antisense (ACCAGAGCAGCGAAGAAAGA),

CIC-6 sense (GGCACAACCTGACAAATGAA),

CIC-6 antisense (ATCTGAGCAAACGGGTGAGT),

CIC-7 sense (GGCATCTCCCTGTCCTACCT),

CIC-7 antisense (GCAGTCATCAGCACCAACAT),

GAPDH sense (CTCGTGGAGTCTACTGGTGT),

GAPDH antisense (GTCATCATACTTGGCAGG TT).

Western blot assay

Cell pellets were lysed using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific) and resolved by SDS/PAGE. The isolated proteins were separated on polyacrylamide gels and transferred to PVDF membranes.

After blocking with 5% bovine skim milk, the membranes were incubated with antibodies specific for human Caspase-3(CST), Cleaved caspase-3(CST), Cleaved PARP(CST), Bcl-2(CST), Bak(CST), Bcl-xl(CST), Bcl-w(CST), CIC-3 (Abcam), CIC-2 (Abcam) and α -tubulin(Sigma) at 4°C overnight and were then incubated with rabbit anti-rabbit or anti-mouse IgG at room temperature for 1 hour. Membranes were visualized on a ChemiDoc XRS+ System (Bio-Rad) using Immobilon Western Chemiluminescent HRP Substrate (Millipore).

CIC-2 or CIC-3 silencing assay

For silencing, Lipofectamine RNAiMAX Reagent (Life Technologies) was used for transfection according to the manufacturer's instructions. Cells were transfected with 50 nM scrambled or siRNAs (Ribobio Co., Ltd.) for 24 h followed by downstream western blotting and qRT-PCR experiments.

Statistical analysis

The data were expressed as the means \pm SEM of at least three independent experiments. p value <0.05 vs. Control was considered statistically significant.

Results

NPPB inhibited cell survival and proliferation in DU145 cells

The cytotoxic effect of NPPB on human prostate cancer DU145 cells was examined with varying concentrations of NPPB and times by MTT assay. The result showed that the viability of DU145 was reduced by NPPB (Figure 1A). There was a dramatically decrease in the proliferation of cells with increasing doses of NPPB (0-100 $\mu\text{mol/L}$) in DU145 cells.

In particular, both 50 $\mu\text{mol/L}$ and 100 $\mu\text{mol/L}$ dose of NPPB could obviously inhibit DU145 cell proliferation. By comparing the proliferation rate in NPPB groups (0-100 $\mu\text{mol/L}$) at 0.5 h, 2 h and 8 h, the greatest inhibition effect was observed at 2 h (Figure 1B, $p < 0.01$). These observations indicated that NPPB could inhibit the proliferation and survival of DU145 cells.

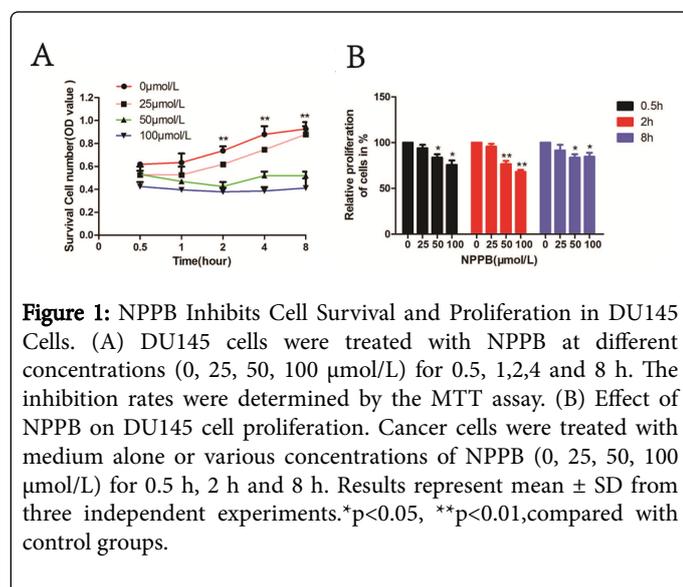


Figure 1: NPPB Inhibits Cell Survival and Proliferation in DU145 Cells. (A) DU145 cells were treated with NPPB at different concentrations (0, 25, 50, 100 $\mu\text{mol/L}$) for 0.5, 1, 2, 4 and 8 h. The inhibition rates were determined by the MTT assay. (B) Effect of NPPB on DU145 cell proliferation. Cancer cells were treated with medium alone or various concentrations of NPPB (0, 25, 50, 100 $\mu\text{mol/L}$) for 0.5 h, 2 h and 8 h. Results represent mean \pm SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$, compared with control groups.

NPPB induced early apoptosis in DU145 cell lines

To further determine the effect of NPPB on DU145 cells, the induction of DU145 apoptosis by NPPB was first examined by flow cytometry analysis, stained with annexin V and PI. The result showed that NPPB (50 $\mu\text{mol/L}$, 100 $\mu\text{mol/L}$) caused a remarkable increase in early cell apoptosis (Figure 2A).

Thus, aiming to demonstrate the effect of NPPB in inducing apoptosis in DU145 cell, the expression of Caspase-3, cleaved caspase-3 and PARP, cleaved PARP was measured by western blot after treatment with NPPB (50 $\mu\text{mol/L}$, 100 $\mu\text{mol/L}$) at 2 h.

As the result showed, the expression of cleaved caspase-3 and cleaved PARP were up-regulated under the effect of NPPB (Figure 2B and 2C). These result suggested the increase of caspase activity and mitochondrial damage may relate to NPPB-induced apoptosis in DU145 cells.

NPPB induced mitochondrial membrane potential in prostate cancer DU145 cells

To further examine whether NPPB induced mitochondrial damage in prostate cancer DU145 cells, a JC-1 mitochondrial membrane

potential assay kit was used to detect changes in mitochondrial membrane potential.

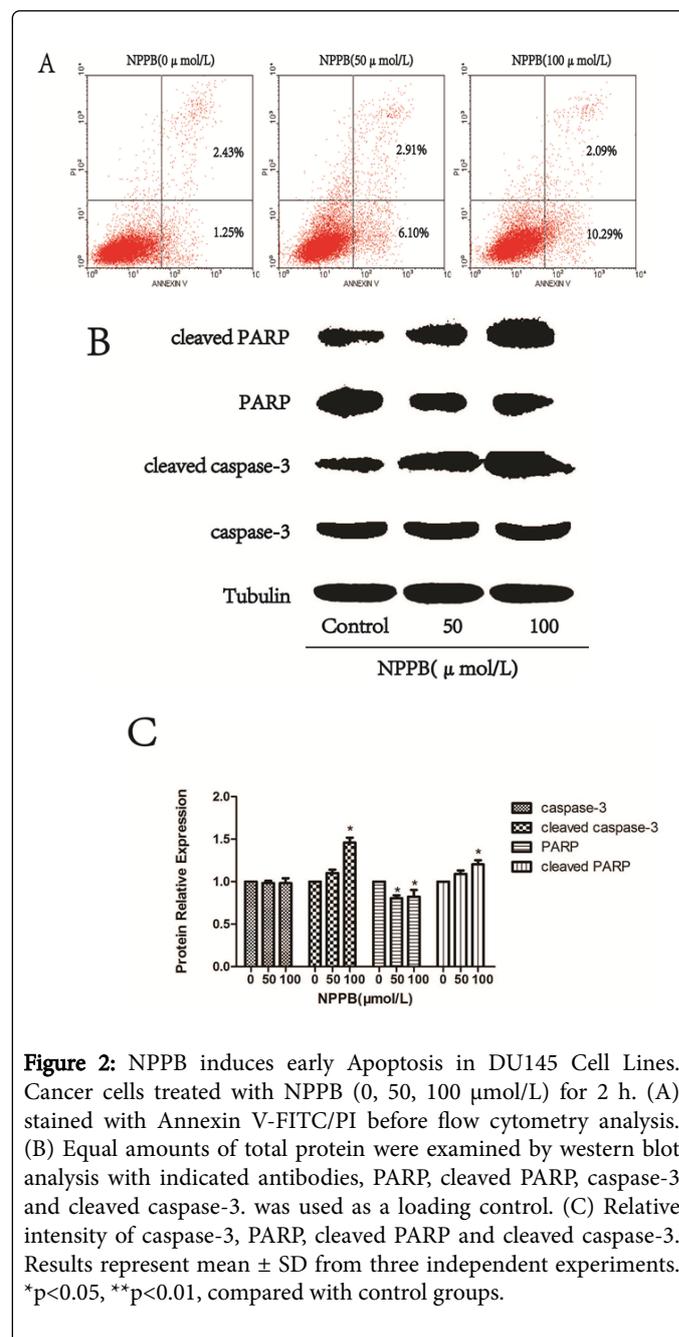


Figure 2: NPPB induces early Apoptosis in DU145 Cell Lines. Cancer cells treated with NPPB (0, 50, 100 $\mu\text{mol/L}$) for 2 h. (A) Stained with Annexin V-FITC/PI before flow cytometry analysis. (B) Equal amounts of total protein were examined by western blot analysis with indicated antibodies, PARP, cleaved PARP, caspase-3 and cleaved caspase-3. was used as a loading control. (C) Relative intensity of caspase-3, PARP, cleaved PARP and cleaved caspase-3. Results represent mean \pm SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$, compared with control groups.

As the result showed in Figure 3A and its quantitative chart in Figure 3B, cells in the control group appeared to have normal mitochondria, while DU145 cells in NPPB treatment group (50, 100 $\mu\text{mol/L}$) for 2 h) showed more green fluorescence. These results suggested NPPB induced prostate cancer DU145 cells apoptosis via promoting mitochondrial damage.

NPPB induced mitochondrial apoptosis pathway in DU145 cells by regulating Bcl-w, Mcl-1 and Bax

Previous result suggested that mitochondrial apoptosis pathway may be the apoptosis pathway in human prostate cancer DU145 cell line after induced by NPPB. Thus, we measured the expression of the B-cell lymphoma 2 (Bcl-2) family of s Bcl-2, Mcl-1, Bcl-xl, Bcl-w and Bax in DU145 cells by western blot after treatment with NPPB.

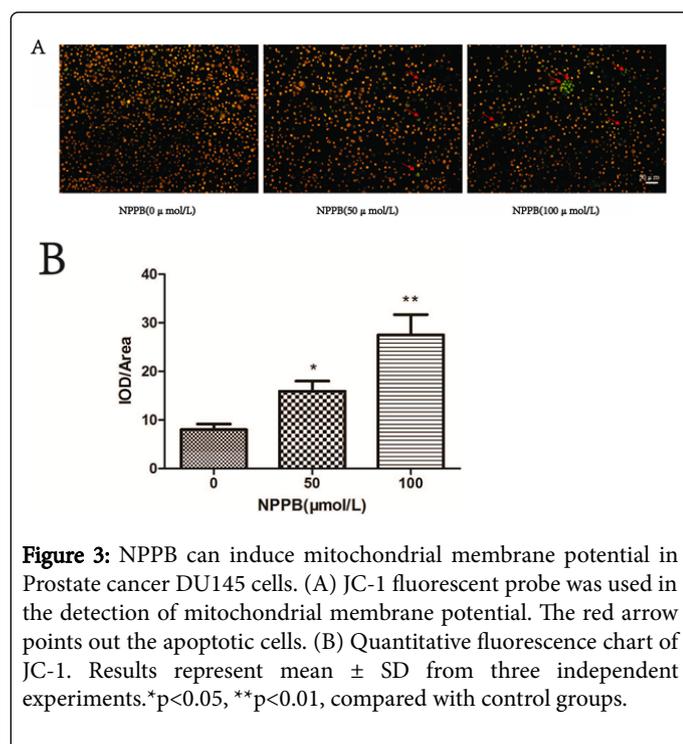


Figure 3: NPPB can induce mitochondrial membrane potential in Prostate cancer DU145 cells. (A) JC-1 fluorescent probe was used in the detection of mitochondrial membrane potential. The red arrow points out the apoptotic cells. (B) Quantitative fluorescence chart of JC-1. Results represent mean \pm SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$, compared with control groups.

The results showed that the expression of antiapoptotic proteins Bcl-w and Mcl-1 were significantly decreased, and pro-apoptosis protein Bax is obviously increased (Figure 4A and 4B). These results suggested that NPPB triggers the mitochondrial pathway, regulating Bcl-2 family proteins Bcl-w, Mcl-1 and Bax to induce apoptosis in DU145 cells.

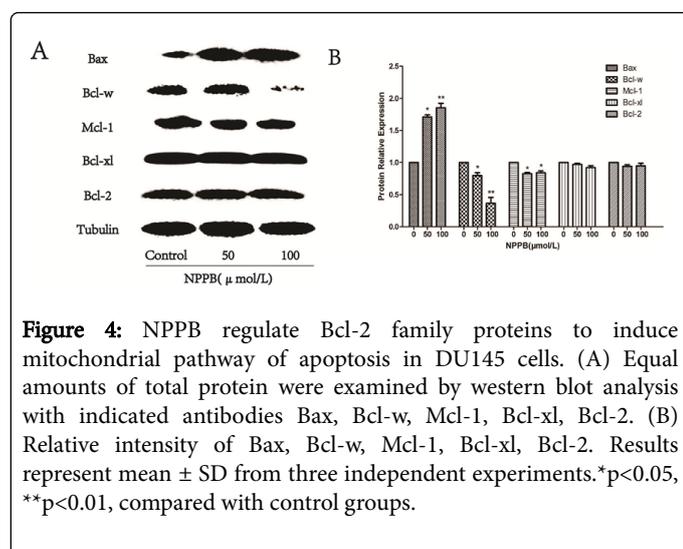


Figure 4: NPPB regulate Bcl-2 family proteins to induce mitochondrial pathway of apoptosis in DU145 cells. (A) Equal amounts of total protein were examined by western blot analysis with indicated antibodies Bax, Bcl-w, Mcl-1, Bcl-xl, Bcl-2. (B) Relative intensity of Bax, Bcl-w, Mcl-1, Bcl-xl, Bcl-2. Results represent mean \pm SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$, compared with control groups.

CIC-2 and CIC-3 are involved in the process of NPPB induced DU145 cells apoptosis

We found that the chloride channel blockers NPPB could induce the mitochondrial-apoptosis in DU145 cells. Based on this, we through the qRT - PCR to detect the mRNA expression of the family of CIC, CIC-2 and CIC-3 are evident in DU145 cells, the expression of other members of the family of CIC almost no expression (Figure 5A and 5B). We further detected CIC-2, CIC-3 on the distribution of the DU145 by immunofluorescence staining (Figure 5C). Results presented that CIC-2 and CIC-3 had large expression in cytoplasm and cell membrane.

Then, We knockdowned the expression of CIC-2, CIC-3, (Figure 5D-5 G) to test whether the effect on the cell apoptosis (Figure 5H-5J). The result of Western Blotting and flow cytometry exposed that knockdowned CIC-2, CIC-3 could promote cell apoptosis. We further tested Bcl2 family protein expression changes in knockdowned CIC-3 cells (Figure 5K).

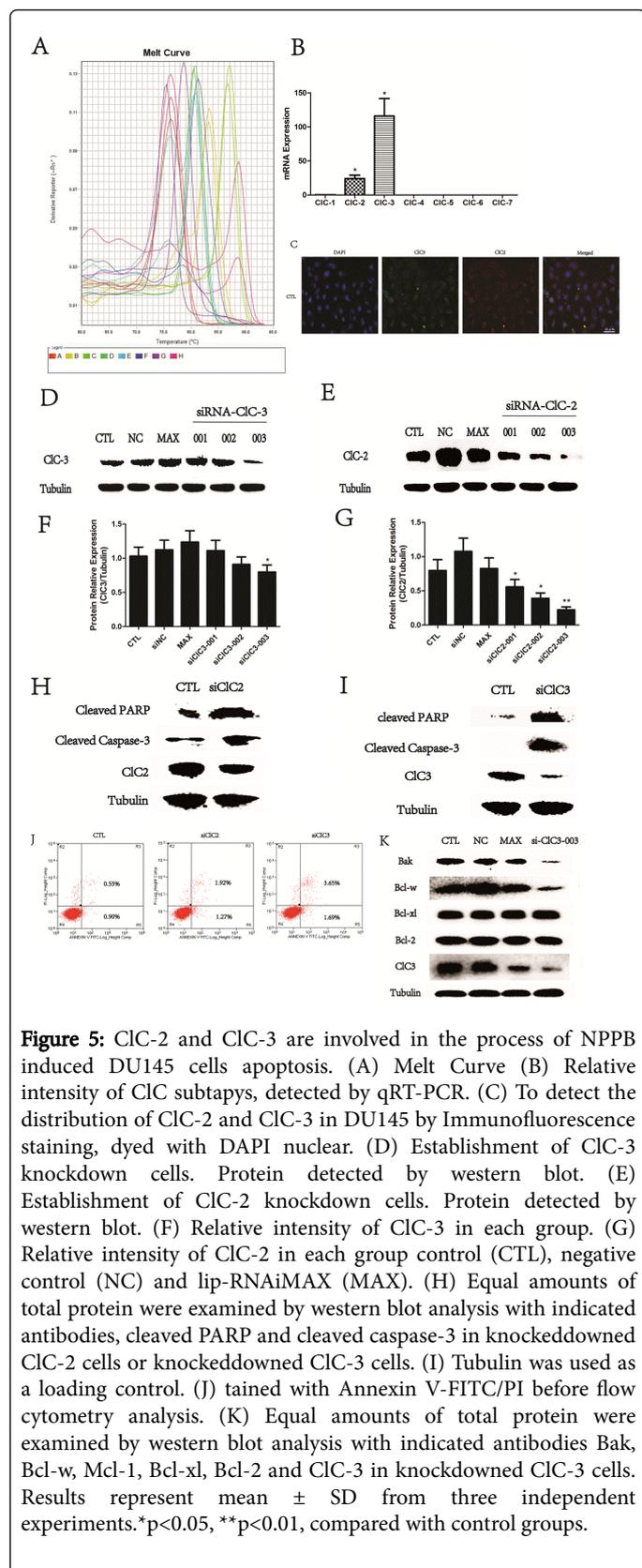
To sum up, we determined the CIC in DU145 cells, CIC-2, CIC-3 chloride channel significantly higher than other CIC chloride channel subtypes expression, and knockdowned CIC-2 or CIC-3 could accelerate cell apoptosis, especially the CIC-3 could promote cell apoptosis by regulating the Bcl2 family proteins.

Discussion

Prostate cancer (PCa) is still one of the leading causes of cancer deaths among men. Although certain mechanisms playing significant roles in PCa apoptosis have been questioned for years, the exact mechanisms of the PCa apoptosis are still mostly unknown. Chloride channels are among the most promising targets to develop therapeutic approaches, it is important to clarify the chloride channels mechanisms associated with PCa apoptosis.

In this study, We evaluated the proliferation inhibition and apoptosis promotion effects of chloride channel blocker NPPB in human prostate cancer DU145 cells, along its mechanism of action. The MTT assay results showed that the viability of DU145 was remarkably reduced by NPPB. The results of flow cytometry showed that NPPB caused a significantly increase in cell early apoptosis. And then our results showed that the expression of cleaved caspase-3 and the expression of cleaved PARP were up-regulated after affected with NPPB.

The results of JC-1 suggested that mitochondrial pathway of apoptosis may be the apoptosis pathway in human prostate cancer DU145 cell line after induced by NPPB. We subsequently found that NPPB can significantly down-regulate anti-apoptosis proteins Bcl-w and Mcl-1 expression, upon-regulate pro-apoptosis protein Bax expression. The expression of Bcl-2 and Bcl-xl have no significant difference. It is revealed that NPPB induces prostate cancer DU145 cell apoptosis by inhibiting anti-apoptosis proteins Bcl-w and Mcl-1 expression and increase pro-apoptosis Bax expression, and activate the following mitochondrial apoptotic pathway downstream until the caused apoptosis.



The cytotoxic effect of NPPB on human prostate cancer DU145 cells were examined with varying concentrations of NPPB and times by

MTT assay. The results showed that the viability of DU145 was reduced by NPPB. Annexin V was a protein with high affinity for the phospholipid phosphatidylserine (PS) exposed from inner layer to external environment in apoptotic cells. Caspase-3, as an inactive zymogen in cells, was a key protease that was cleaved and activated during the early stage of apoptosis by extrinsic and intrinsic pathways. The cleaved caspase-3 was thus regarded as a marker of early apoptosis [8-10]. PARP was a substrate for caspase-3. Study confirmed that PARP activation through the destabilization of mitochondrial outer membranes promotes the release and nuclear translocation of Apoptosis-Inducing Factor (AIF) and Endonuclease G leading to apoptosis [11,12]. The induction of apoptosis by NPPB was firstly examined by flow cytometric analysis of DU145 cells stained with annexin V and PI. Western Blotting were then used to determined the expression of apoptotic s, caspase-3 and PARP. These results suggested the increasing caspase activity and mitochondrial damage may relate to NPPB-induced apoptosis in DU145 cells.

To forward ensure whether NPPB induces mitochondrial damage in Prostate cancer DU145 cells, a JC-1 mitochondrial membrane potential assay kit was used to detect changes in mitochondrial membrane potential. In live, non-apoptotic cells, the orange fluorescence intensity was increased as the mitochondrial potential dye was accumulated in the mitochondria. In apoptotic and dead cells, stain intensity was decreased due to the collapse of the mitochondrial membrane potential (MMP). The results of JC-1 suggested that mitochondrial pathway of apoptosis may be the apoptosis pathway in human prostate cancer DU145 cell line after induced by NPPB. Mitochondria integrated the pro-apoptotic signaling environment via the B-cell lymphoma 2 (Bcl-2) family of proteins to regulate cell apoptosis [13,14]. Certainly, Bcl-2, Mcl-1, Bcl-xl, Bcl-w and Bax were most important apoptosis-related genes. Bcl-2, Mcl-1, Bcl-xl, Bcl-w were cell death inhibitors, whereas Bax was a pro-apoptosis protein. Thus, we measured the expression of Bcl-2, Mcl-1, Bcl-xl, Bcl-w and Bax in DU145 cells by western blot after treatment with NPPB. These above results implied that NPPB induces apoptosis in DU145 cell lines by promoting a style of mitochondrial damage, and further affects the viability of DU145 cells.

In qPR-PCR, we found that CIC-2 and CIC-3, especially the later, have more expression than other chloride channel in DU145 cell line. To determine whether CIC-2 and CIC-3 function is relevant to NPPB action, we performed functional studies using siRNA. The results showed that inhibition of the expression of CIC channel protein significantly decreased the expression of the anti-apoptosis protein bcl-w, and proceed to inhibit proliferation and induce apoptosis in human prostate cancer DU145 cell line. These results shows that the CIC-3 Chloride Channels is involved in the process of NPPB induced DU145 cells apoptosis.

In conclusion, the chloride channel blocker NPPB inhibit proliferation and induce apoptosis in DU145 cell line via blocking the CIC chloride channels, especially CIC-2 and CIC-3, and proceed to activate the mitochondria-related apoptotic pathway. Further studies are needed to reveal the definite interactions of CIC chloride channel and the mitochondria-related apoptotic pathway.

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