The role of the voltage-gated potassium channel, Kv2.1 in prostate cancer cell migration

Hyun Woo Park, Min Seok Song, Hun Ju Sim, Pan Dong Ryu, So Yeong Lee*

Laboratory of Veterinary Pharmacology, College of Veterinary Medicine and Research Institute for Veterinary Science, Seoul National University, Seoul, 08826, Korea

* Corresponding Author:

So Yeong Lee, DVM, PhD

Laboratory of Veterinary Pharmacology

College of Veterinary Medicine, Seoul National University,

1 Gwanak-ro, Gwanak-gu, Seoul, 08826, Korea

Tel: + 82-2-880-1283

Fax: +82-2-879-0378

E-mail: leeso@snu.ac.kr

Materials and Methods

Cell culture

WPMY-1 is the immortalized prostate cell (1); LNCaP (LNCaP.FGC) is an androgen-dependent cell line derived from a lymph node metastasis of prostate carcinoma with a low metastatic potential (2); DU145 is an androgen-independent cell line derived from CNS metastasis of prostate carcinoma with a moderated metastatic potential (3); and PC-3 is an androgen-independent cell line derived from a bone metastasis of grade IV prostate adenocarcinoma with a high metastatic potential (4).

WPMY-1 cells were cultured in DMEM (Welgene, Daegu, Korea) whereas LNCaP, DU145, and PC-3 cells were in RPMI1640 (Welgene) containing 10% fetal bovine serum (FBS) and 1% antibiotic antimycotic solution at 37 ℃ and 5% CO_2.

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was prepared using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The isolated RNA (2 μg) with random hexamers and moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) was used to synthesize the cDNA. The PCR reaction was performed using 2 μL cDNA, 1x GoTaq® Green Master Mix (Promega), and specific target primers (Kv2.1 forward: 5′-GAATGTCCGCCGCGTGGTCCA-3′, reverse: 5′-CTTGGCTCTCTCCAGAGCCTC-3) under the following reaction conditions: initial denaturation at 95 ℃ for 5 min and then 35 cycles at 94 ℃ for 40 s, annealing at 60 ℃ for 40 s, and an extension at 72 ℃ for 1 min followed by a final extension at 72 ℃ for 7 min. The PCR products were loaded on 1.6% agarose gel for electrophoresis.

Real-time RT-PCR

Real-time RT-PCR was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using a GAPDH as a reference. The real-time RT-PCR reaction was performed with 2 μl cDNA, 1x SYBR Green Master Mix (Applied Biosystems), and 10 pM primers (Kv2.1 forward: 5′-GTCTCTGGGCTTCACTTTGC-3′, reverse: 5′-ACTTGGTGTCGTCCTCATCC-3′) under the following reaction conditions: initial step at 95 ˚C for 10 s, and then 40 cycles of cycling processes (95 ˚C for 5 s and 60 ˚C for 30 s). The single product synthesized by the paired primers was subjected to a dissociation curve analysis.

Western blot analysis

The cells were lysed using radioimmunoprecipitation assay buffer (Sigma-Aldrich) and fractionated using of a subcellular protein fractionation kit for cultured cells (Thermo Scientific, Waltham, MA, USA) into cytosol, membrane, and nuclear proteins according to the manufacturer's instructions; Bicinchoninic acid protein assay kit (Thermo Scientific) was used to measure protein concentration.

The quantified protein was loaded on a 10% acrylamide gel for SDS-PAGE and then transferred onto a nitrocellulose membrane (GE Healthcare, Chicago, IL, USA). 1x TBS-Tween 20 containing 5% nonfat milk (BD, Franklin Lakes, NJ, USA) was used to block non-specific antibody binding, and the membranes onto which the proteins were transferred were probed overnight with commercial primary antibodies targeting the proteins Kv2.1, Na-K ATPase (Abcam, Cambridge, UK), GAPDH, and -actin (Santa Cruz Biotechnology, CA, USA). The membranes probed with primary antibodies were incubated with horseradish peroxidase conjugated goat anti-rabbit or anti-mouse secondary antibodies (GenDEPOT, Katy, TX, USA) and visualized using WesternBright™ Quantum™ (Advansta, San Jose, CA, USA). An ImageQuant™ LAS 4000 image analyzer (GE Healthcare) was used to visualize the immune complexes.

Patch-clamp recordings

The WPMY-1 and PC-3 cells prepared for patch-clamp recordings were incubated for 24 h in 12-well plates, containing 12 mm coverslips. Patch pipettes were pulled from the borosilicate glass capillaries (1.5/1.0 OD/ID (mm), World Precision Instruments, Sarasota, FL, USA), resulting in a pipette resistance ranging from 2 MΩ to 5 MΩ. The internal pipette solution (in mM) consisting of 145 KCl, 3 $MgCl_2$, 10 HEPES, 3 EGTA, and 2 Mg-ATP (pH 7.2, adjusted with KOH) and the bath solution (in mM) containing 145 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES (pH 7.4 adjustment with NaOH) were used for the recordings. The Kv channel currents were recorded in the whole-cell configuration using an Axoclamp 200B amplifier (Axon Instruments, Foster City, CA, USA). Electric signals were filtered at 1 kHz and were digitized at 10 kHz using an analog - digital converter (Digidata 1550B; Axon Instruments) and the pClamp software (version 11.0; Axon Instruments). The voltage-clamp mode was performed at −60 mV voltage holding according to the following protocol: the cells were exposed to a hyperpolarizing pulse of −100 mV for 100 ms and the membrane currents were activated by voltage pulses for 2000 ms within the range of −100 to 70 mV at 10 mV increments, followed by a pulse of −30 mV for 500 ms. We used the average value of the currents from 0.2 s to 0.5 s to construct current density-voltage curves. We calculated the membrane capacitance of PC-3 using the capacitive transient current induced by hyperpolarizing step (−5 mV), starting from a holding potential. Membrane capacitance was calculated by integrating the area of the capacitive transient at the onset of the pulse and then dividing the integrate by the amplitude of the pulse (−5 mV) (5).

Transfection with small interference RNA (siRNA)

Cells were transfected with Kv2.1 siRNA (Santa Cruz Biotechnology) and Lipofectamine® RNAiMAX (Invitrogen, Carlsbad, CA, USA). Transfection was performed following the manufacturer's instructions. The PC-3 cells were plated in 24-well plates for the viability test and 100 mm for the migration assay. The vehicle control group was treated by Lipofectamine® RNAiMAX only. The incubation time was 48 h and then cells were moved into a 24-well culture insert (SPL, Gyeonggi-do, Korea).

Cell viability test

The viability rate of the WPMY-1 and PC-3 cells were determined using a cell counting kit-8 (CCK-8) (Dojindo, Rockville, MD, USA) according to the manufacturer's instructions. WPMY-1 and PC-3 cells were seeded on a 24-well plate. The following day, the cells were treated with 4 mM N-acetylcysteine (NAC, pH 7.4, Sigma-Aldrich) for 12 h, ascorbic acid (sigma) for 12 h or with 600 nM stromatoxin-1 (ScTx-1; Alomone) for 24 h in serum-free media. CCK-8 was added for 2 h, and the absorbance at 450 nm was detected using an Infinite® F50 microplate reader (Tecan, Männedorf, Switzerland).

NAC and ascorbic acid-induced ROS reduction

PC-3 cells were cultured into a 6-well plate for a whole-cell prep and in 100 mm dish for subcellular fractionation. When the cells had sufficiently grown, the PC-3 cells were incubated for 12 h with serum-free medium containing 4 mM NAC or 40 μM ascorbic acid to induce ROS reduction. The control (SFM) group was incubated for 12 h with serum-free medium without NAC or ascorbic acid as serum may alter the redox state (6).

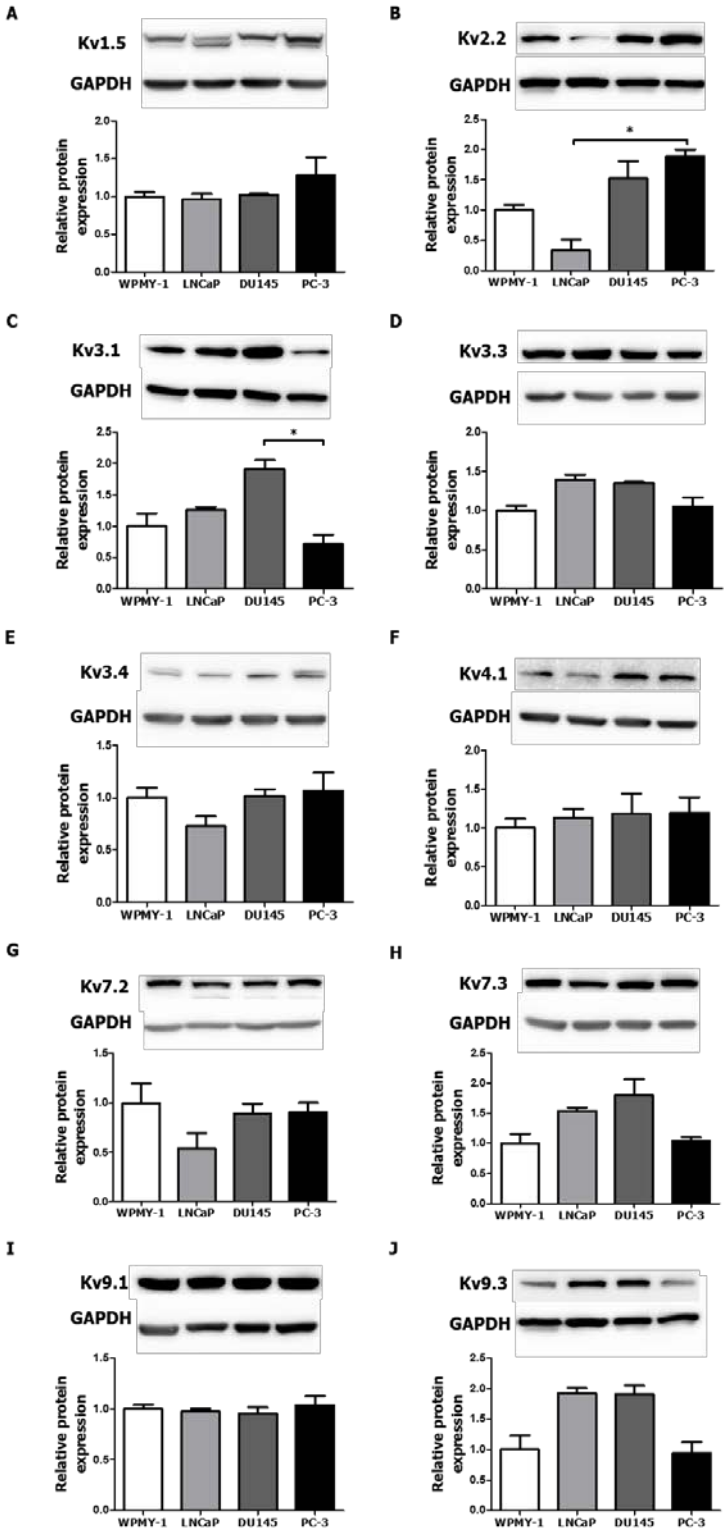
Cell migration

Cell migration was confirmed with a 24-well culture insert for a transwell migration assay. WPMY-1 (6×10^4) and PC-3 (1.0×10^5) cells were placed on the upper layer of a cell-permeable membrane by using an serum-free medium, and a medium containing 10% FBS was placed below the membrane. The ScTx-1 group was treated with 600 nM ScTx-1 for 10 min. After a 48 h incubation, the cells that migrated through the membrane stained with Hemacolor® rapid staining (Sigma-Aldrich). The siRNA and their control group of migrated cells were stained with diff-quick staining (Sysmex, Kobe, Japan). All of the data were counted using ImageJ software (NIH).

Statistical analysis

Data are presented as mean ± standard error. Unpaired t-test with welch's correction was employed for the analysis of two groups of data, and the Kruskal - Wallis test with Dunn's Multiple Comparison Test was used to analyze more than two groups of data (GraphPad Prism version 5.0, San Diego, CA, USA).

Figure S1



Supplementary figure 1. S1. Relative protein expression levels of Kv channels in prostate cells (A) Kv1.5 (100 kDa), (B) Kv2.2 (120 kDa), (C) Kv3.1 (58 kDa), (D) Kv3.3 (75 kDa), (E) Kv3.4 (70 kDa), (F) Kv4.1 (60 kDa), (G) Kv7.2 (60 kDa), (H) Kv7.3 (75 kDa), (I) Kv9.1 (65 kDa), and (J) Kv9.3 (90 kDa). All experiments were performed triplicate and the data represent the mean ± standard error. * $p < 0.05$.

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