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Induction of TRPV5 expression by small activating RNA targeting gene promoter as a novel approach to regulate cellular calcium transportation

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ABSTRACT

Aim: Promoter-targeted small activating RNAs (saRNAs) have been shown to be able to induce target gene expression, a mechanism known as RNA activation (RNAa). The present study tested whether saRNA can induce the overexpression of TRPV5 in human cells derived from the kidney and subsequently manipulate cell calcium uptake.

Main methods: Three saRNAs complementary to the TRPV5 promoter were synthesized and transfected into cells. TRPV5 expression at the RNA and protein levels was analyzed by quantitative real-time PCR and Western blotting respectively. For functional study, transcellular Ca^{2+} transportation was tested by fura-2 analysis. Dihydrotestosterone (DHT), a suppressor of cellular calcium transportation, was administered to challenge the activating effect of selected saRNA.

Key findings: One of these synthesized saRNAs, ds-2939, significantly induced the expression of TRPV5 at both mRNA and protein levels. Fura-2 analysis revealed that the intracellular Ca^{2+} concentration was elevated by ds-2939. DHT treatment reduced transmembrane Ca^{2+} transport, which was partially antagonized by ds-2939.

Significance: Our results suggest that a saRNA targeting TRPV5 promoter can be utilized to manipulate the transmembrane Ca^{2+} transport by upregulating the expression of TRPV5 and may serve as an alternative for the treatment of Ca^{2+} balance-related diseases.

Introduction

Since the discovery of RNA interference (RNAi), the therapeutic potential of RNAi has been demonstrated by numerous studies (Castanotto and Rossi, 2009). To the contrary, recent studies reported that promoter-targeted small activating RNAs (saRNAs), including synthetic saRNAs and microRNAs, can induce, instead of silencing, gene expression (Li et al., 2006; Janowski et al., 2007; Place et al., 2008), and this mechanism has been termed as RNA activation (RNAa). With its unique ability of stimulating endogenous gene expression, RNAa offers potential novel druggable targets for disease treatment. Although recent studies have demonstrated the therapeutic benefits of synthetic saRNAs in treating cancer in animal studies (Wang et al., 2012; Kang et al., 2012), few studies have examined the therapeutic potential of saRNAs for non-cancerous diseases.

Calcium homeostasis is closely associated with renal reabsorption and indispensable in the maintenance of normal physiological processes. But how Ca^{2+} makes its entrance into renal absorptive epithelia is largely unknown until the identification of transient receptor potential vanilloid 5 (TRPV5) gene which encodes a highly Ca^{2+}-selective cation channel protein (Hoenderop et al., 2005; Clapham, 2003). TRPV5 is mainly localized to the apical membrane of the distal renal tubule and plays an important role in the regulation of active transepithelial Ca^{2+} reabsorption in the kidney (Gkika et al., 2006). It has been shown that TRPV5 gene ablation in mice evokes a significant renal calcium leak, resulting in severe hypercalciuria (Hoenderop et al., 2003). Downregulation of TRPV5 has been linked to bone structure abnormalities (van Abel et al., 2006), type I vitamin D-dependent rickets (Woudenberg-Vrenken et al., 2009) and androgen-related urolithiasis tendency (Yoshioka et al., 2010). Therefore, it will be interesting to know whether restoring the expression of TRPV5 by RNAa could affect calcium transmembrane transport.

In the present study, we set out to determine the activating ability of saRNAs targeting TRPV5 promoter and to further explore the functional benefits of TRPV5 activation in regulating calcium homeostasis in cells derived from the kidney. We show that a saRNA complementary to the promoter of TRPV5 activates TRPV5 expression and antagonizes androgen-induced gene repression and transmembrane Ca^{2+} transport.

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Materials and methods

saRNA design and synthesis

Four kilobases of the human TRPV5 promoter sequence were scanned for saRNA target sites based on the rational design rules as previously reported (Huang et al., 2010). The synthesized saRNA sequences were: ds-control: 5′-UUCUCCGAACGUGUCACGUdTdT-3′, 5′-ACGUGACACGUUCG GAGAAdTdT-3′; ds-914: 5′-GGUUCCUCCUCCUCUUUAUdTdT-3′, 5′-AUAAAG AGGAGGAGGAACCdTdT-3′; ds-1704: 5′-CUCCCAGUGAGAACAUAAUdTdT -3′, 5′-AUUAUGUUCUCACUGGGAGdTdT-3′; ds-2939: 5′-GGGCAACGAAA GUGAAACUdTdT-3′, 5′-AGUUUCACUUUCGUUGCCCdTdT-3′ (Genepharma). In addition, three sequences of mutant ds-2939 were synthesized to test sequence requirement of TRPV5 activation. The mutant saRNAs were (underlined bases are mismatched): ds-2939-m3: 5′-AAACAACGAAAGUGAAACUdTdT-3′, 5′-AGUUUCACUUUCG UUGUUUdTdT-3′, ds-2939-mm: 5′-GGGCAACGAAGUGGAAACUdTdT-3′, 5′-A GUUUCCACUUCGUUGCCCdTdT-3′, ds-2939-m5: 5′-GGGCAACGAAAGUGAA UCAdTdT-3′, 5′-UGAUUCACUUUCGUUGCCCdTdT-3′ (Genepharma).

Cell culture and transfection

Human kidney tubular epithelial HK2 and embryonic kidney HEK293 cells were maintained in DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 at 37 °C. Human prostate cancer PC3 cells were cultured in DMEM + F12 medium (Gibco) also supplemented with 10% FBS. Cells were plated in growth medium at a density of 60–70% one day before transfection. Transfection of synthesized saRNAs was carried out using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Androgen treatment of cell was carried out using dihydrotestosterone (DHT) (Sigma-Aldrich).

Western blotting

Cells were collected and washed twice by cold PBS, and each well was treated with 60 μL lysis buffer (2 mmol Tris–HCl, pH 7.4, 50 mmol NaCl, 25 mmol EDTA, 50 mmol NaF, 1.5 mmol Na_3VO_4, 1% Triton X-100, 0.1% SDS, supplemented with protease inhibitors 1 mM phenylmethylsulfonylfluoride, 10 mg/L pepstatin, 10 mg/L aprotinin and 5 mg/L leupeptin) (Sigma). Protein concentrations were determined using the DCTM protein assay regents (Bio-Rad). Equal amounts of protein (50 μg) were separated on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond C; Amersham Pharmacia Biotech). Membranes were blocked in 5% non-fat dry milk in TBS-T (0.1% Tween-20, 150 mM NaCl, 25 mM Tris–HCl, pH 7.6) for 2 h at room temperature and incubated with primary antibodies appropriately diluted in TBS-T overnight at 4 °C. After three washes for 10 min with TBS-T, membranes were incubated for 1 h with secondary antibody appropriately diluted in TBS-T. Membranes were developed for chemiluminescence detection by using ECL detection kit (Pierce) and Universal Hood II image system (Bio-Rad) as recommended by the manufacturer. Quantity One software was used to quantify the intensities of bands. The primary antibodies used included those against TRPV5 (Epitomics) and GAPDH (Santa Cruz). The secondary antibodies were anti-mouse and anti-rabbit IgG conjugated with HRP (Santa Cruz). All Western blotting analyses were repeated 3 times and images of blots were analyzed using ImageJ software.

Quantitative real-time PCR

Total RNA isolated with TRIzol was reverse transcribed using PrimeScript® RT reagent Kit (Takara) according to the manufacturer's protocol. RT-PCR primers for TRPV5 and GAPDH were: TRPV5-forward: 5′-CCAGACAGAGGACCCAACCA-3′, TRPV5-reverse 5′-GGTCATCCTCCTTGTCTGAGTTCTT-3′, GAPDH-forward: 5′- GCACCGTCAAGGCTGAGAAC-3′, GAPDH-reverse 5′-TGGTGAAGACGCCAG TGGA-3′. Quantitative real-time PCR was performed using iTaq SYBR Green Supermix (Bio-Rad). PCR parameters consisted of 95 °C for 5 min, followed by 33 cycles of 95 °C for 30 s, 57 °C 30 s, and 72 °C 30 s, with a final extension at 72 °C for 5 min. All samples were processed in triplicates and all values were normalized for the GAPDH expression levels. Relative quantification of TRPV5 mRNA levels was determined by the comparative Ct method.

Immunofluorescence

Cells were plated on 24 well cell culture plates overnight and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature, washed with PBS, and permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 5 min. After blocking with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature, cells were incubated with mouse monoclonal antibodies against human TRPV5 for 12 h at 4 °C. The Alexa Fluor 488 donkey anti-mouse IgG (Life Technologies) was used as secondary antibody green fluorescence.

Intracellular Ca^{2+} measurements using Fura-2

HK2 cells were loaded in DMEM with a final concentration of Fluo-2/AM of 3 μmol/L at 37 °C in the dark for 1 h. Fura-2-loaded cells were placed on a fluorescence inverted microscope. Changes in Ca^{2+} were monitored with Fura-2 excited at 340 and 380 nm. The ratio value of the fluorescence intensity at 340 and 380 nm was recorded to determine the intracellular Ca^{2+} concentration.

Statistical analysis

Statistical significance was evaluated using one-way ANOVA with the LSD-t post-test. SPSS version 13.0 for Windows was used for statistical analysis. A P value of less than 0.05 was considered to be statistically significant.

Results

Activation of TRPV5 expression by a TRPV5 saRNA

We designed and synthesized 3 saRNAs targeting TRPV5 gene promoter at sequence positions -3085 (ds-914), -2295 (ds-1704) and -1060 (ds-2939) relative to the transcription start site of TRPV5 (Fig. 1A). A negative control saRNA (NC) which lacked homology to any known human sequences was also synthesized. All saRNAs were transfected into HK2 cell at the 40 nM concentration for 72 h. Quantitative RT-PCR analysis demonstrated a 32.5-fold increase in TRPV5 mRNA level in HK2 cells transfected with ds-2939 compared to control treatments, whereas ds-914 and ds-1704 did not significantly change TRPV5 mRNA abundance(Fig. 1B). We also evaluated TRPV5 protein levels in saRNA transfected HK2 cells. Consistent with mRNA expression induction, Western blotting results showed that TRPV5 protein level was induced by 11.6 fold in cells transfected with ds-2939 only (Fig. 1C). These results suggest that ds-2939 saRNA is a strong activator of TRPV5 mRNA and protein.

We further determined the dose–response and time course effects of ds-2939 on TRPV5 expression in HK2 cell. We first treated cells with ds-2939 at concentrations ranging from 5 nM to 80 nM and assessed TRPV5 expression 72 h later. Western blot analysis demonstrated a dose-dependent induction of TRPV5 by ds-2939 with its lowest active concentration at 5 nM and its activity plateauing at 40 nM (Fig. 1D). A concentration of ds-2939 higher than 40 nM did not induce higher expression of TRPV5 (Fig. 1D). We also assessed the time course change of TRPV5 expression from day 1 to day 6 following a single transfection of 40 nM ds-2939. During this time, cells were passaged twice. As