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Correction of vascular hypercontractility in spontaneously hypertensive rats using shRNAs-induced delta protein kinase C gene silencing

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ABSTRACT

Potassium conductance in vascular smooth muscle (VSM) is known to be altered in arterial hypertension. High level of protein kinase C (PKC) activity is a common feature for hypertension of different genesis. The main goal of this study was to investigate the efficacy of the RNA interference (RNAi) technique targeting PKC delta-isoform gene as a possible pharmacological tool to restore vasodilator potential in spontaneously hypertensive rats (SHR). Experimental design of the study comprised RNAi and patch-clamp techniques, RT-PCR analysis and standard acetylcholine test. Total outward currents and acetylcholine-induced endothelium-dependent relaxant responses were blunted in SHR. BK_{Ca} alpha subunit mRNA expression in SHR was unchanged whereas K_V and K_{ATP} mRNA expression appeared significantly increased. PKC inhibitor, chelerythrine (100 nM), restored potassium channels activity in SHR. PKC-delta-isoform protein expression and PKC-delta-isoform mRNA expression are 2.5–4 fold increased in VSM from SHR. PKC gene silencing with the short hairpin RNAs (shRNAs)-plasmid delivery system administered intravenously led to an increment in maximal amplitude of acetylcholine-relaxation, restored outward K^+ currents and PKC-delta-isoform mRNA and protein expression. Arterial blood pressure in SHR was normalized following shRNAs administration. We conclude that BK_{Ca} channels are likely to be the most PKC-dependent member of K^+ channels family responsible for vascular hypercontractility in SHR while K_v and K_{ATP} channels may constitute a reserve mechanism for the maintenance of vasodilator potential under BK_{Ca} channelopathy. It is likely that RNAi technique is a good therapeutic approach to inactivate PKC gene and to normalize vascular functions and high arterial blood pressure in SHR.

1. Introduction

Arterial hypertension development is a complex and multi-factorial process that involves a number of functional systems that contribute to vascular malfunction development. It is well known that both vascular smooth muscle (VSM) contraction and relaxation are closely coupled to membrane potential, which, in turn, is mainly determined by potassium channels activity. The data obtained clearly indicate that potassium conductance is altered in essential hypertension (Cox et al., 2001). It is known also that a high level of protein kinase C (PKC) activity in vascular wall (Soloviev and Bershtein, 1992; Soloviev et al., 1998, 2005) and related overproduction of reactive oxygen species (ROS) (Lyle and Griendling, 2006) are typical features for different types of arterial hypertensions.

The large conductance Ca^{2+}-activated K^+ channels (BK_{Ca} also termed as Slo1, or MaxiK) is the predominant K^+ channel species in most arteries, and are known to play an essential role in vascular function *via* its involvement in changes of membrane polarization (Nelson and Quayle, 1995; Ghatta et al., 2006). BK_{Ca} channels differ from all other K^+ channels due to their high sensitivity to both $[Ca^{2+}]_i$ and voltage. Inhibition of BK_{Ca} channels produces membrane depolarization and subsequent vasoconstriction, and their dysfunction plays an important role in the pathogenesis of a number of vascular diseases including pulmonary and systemic hypertension (Korovkina and England, 2002).

BK_{Ca} channels comprised of alpha pore-forming (BKalpha) subunits and regulatory beta subunits (BKbeta1) play a pivotal role in VSM relaxation. When the gene for BKbeta1 is knocked out, the result is usually an increased vascular tone and hypertension

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development (Grimm and Sansom, 2010). Nevertheless, the role of BK_{Ca} in vascular malfunction at arterial hypertension is not decisive since the hypertension associated with BKbeta1 gene deletion occurs also because of enhanced fluid retention (Grimm and Sansom, 2010).

One of the main mechanisms underlying increased VSM contractility at arterial hypertension may be due to an increased PKC activity (Soloviev et al., 1992, 2005; Dempsey et al., 2000; Kizub et al., 2010). It is also known that this enzyme appears tightly involved in plasmolemmal ion permeability. For instance, it has been shown that PKC regulates activity of K^+ channels (Aiello et al., 1996, Boland and Jackson, 1999). There is a strong evidence that PKC modulates BK_{Ca} channels function providing to increasing of vascular tone in both physiological and pathophysiological (hypertension and irradiation) conditions (Taguchi et al., 2000; Barman et al., 2003; Kizub et al., 2010).

It is known that RNA-interference (RNA_i) using small interfering RNAs (siRNAs, a double-stranded RNA molecule having 21–23 bp) represents a powerful tool for silencing a target gene in gene therapy (Kim et al., 2005). Given the ability to knock down essentially any gene of interest, RNAi technique using siRNAs generates a great deal of interest in both basic and applied pharmacology. The current challenge in pharmacology now is to identify RNAi target sites in cells, to construct a "naked" siRNA or a shuttle vector encoding short hairpin RNAs (shRNAs) plasmid complex to ensure the prolonged expression in animal tissues. The shRNAs are more efficient than siRNAs on the induction of gene silencing and appear as an extremely powerful and popular gene silencing agent (Cheng and Chang, 2007).

The main goal of this study was to investigate the efficacy of RNA interference (RNAi) technique targeting PKC delta-isoform gene as a possible pharmacological tool to restore vasodilator potential in spontaneously hypertensive rats (SHR).

2. Materials and methods

2.1. Isolation of vascular smooth muscle cells

All animal studies were performed in accordance with the recommendations of the European Convention for the protection of Vertebrate Animals used for Experimental and other Scientific Purposes and approved by the Institutional Animal Care and Use Committee of all institutions listed.

Isolated smooth muscle cells were dispersed from rat thoracic aorta obtained from mature male healthy Wistar rats (WKY) and spontaneously hypertensive rats (SHR) (250–300 g) by papain treatment. Briefly, the rats were anesthetized with ketamine (45 mg/kg b.w., i.p.) and xylazine (5 mg/kg b.w., i.p.). Segments of the thoracic aorta (1.0–1.5 cm-long) were excised and cleaned of adipose and connective tissue. The aorta was then cut into small pieces ($1.5 \times 1.5\ mm^2$) in a cold low-Ca^{2+} solution containing (mM): 140 NaCl; 6 KCl; 3 $MgCl_2$; 10 glucose; and 10 HEPES for 15 min. The vascular tissues were transferred to a fresh low-Ca^{2+} solution containing: 0.2 mg/ml papain (11.55 U/mg), 0.3 mg/ml dithiothreitol, and 0.3 mg/ml bovine serum albumin. The tissues were then stored at +5 °C for 18 h and then incubated for 15 min at 37 °C. The tissues were then washed (2–3 min) twice in a fresh low-Ca^{2+} solution to remove the papain. Cells were dispersed by agitation using a glass pipette, and placed in normal Krebs bicarbonate buffer. Aliquots of the myocytes were stored at +5 °C and remained functional for at least 5 h.

2.2. Electrophysiology

The whole-cell patch clamp method was used to study whole-cell K^+ currents (voltage clamp mode). Data acquisition and voltage protocols were performed using an Axopatch 200B Patch-Clamp amplifier and Digidata 1200B interface (Axon Instruments Inc., Foster City, CA, USA) coupled to a computer equipped with pClamp software (version 6.02, Axon Instruments Inc., Foster City, CA, USA). Membrane currents were filtered at 2 kHz and digitized at a sampling rate of 10 kHz. The reference electrode was an Ag–AgCl plug electrically connected to the bath.

At the beginning of each experiment, the junction potential between the pipette solution and bath solution was electronically adjusted to zero. No leakage current subtraction was performed on the original recordings, and all cells with input resistances below 1 GΩ were excluded from further analysis. Macroscopic current values were normalized as pA/pF. The membrane capacitance of each cell was estimated by integrating the capacitive current generated by a 10 mV hyperpolarizing pulse after electronic cancellation of pipette-patch capacitance using Clampfit software (version 6.02, Axon Instruments Inc., Foster City, CA, USA). All electrophysiological experiments were carried out at room temperature (20 °C). Patch pipettes were made from borosilicate glass (Clark Electromedical Instruments, Pangbourne Reading, England) and backfilled with intracellular solution (mM): 140 KCl, 2 $MgCl_2$, 1 Na_2ATP, 10 HEPES, 2 EGTA, and 1 $CaCl_2$, adjusted to pH 7.2 with KOH, resulting in a free [Ca^{2+}] of approximately 170 nM. Pipettes had resistances of 2.5–5.0 MΩ. The standard Krebs external solution contained (mM): 133 NaCl, 5.0 KCl, 16.3 $NaHCO_3$, 1.38 NaH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgCl_2$, and 7.8 glucose at pH 7.4.

2.3. Contractile recording experiments, acetylcholine test

Experiments were performed on the segments of thoracic aortas obtained from healthy rats as well from SHR and SHR treated with shRNAs targeted to PKC delta isoform. Following euthanasia, a 2 cm long segment of the thoracic aorta was dissected and cleaned of both connective and adipose tissues and then this segment was cut into 1 to 1.5 mm width rings. The vascular rings were prepared with special care in order to keep the endothelium intact. All procedures were performed at room temperature in nominally Ca^{2+}-free solution. Aortic rings were mounted isometrically under a resting tension of 10 mN in a flowing tissue bath, between a stationary stainless steel hook and an isometric force transducer (AE 801, SensoNor A/S, Norten, Norway) coupled to a AD converter Lab-Trax-4/16 (World Precision Instruments, Inc., Sarasota, USA). Experiments were made at 37 °C in modified Krebs bicarbonate buffer solution of the following composition (in mM): 133 NaCl, 4.7 KCl, 16.3 $NaHCO_3$, 1.38 NaH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, and 7.8 glucose at pH 7.4.

Following the equilibration period, the rings were exposed several times to norepinephrine (NE, 10 μM) until reproducible contractile responses were obtained. Then, the acetylcholine in a range of concentrations from 10^{-9} to 10^{-5} M was added to the bath solution on the plateau of NE-induced contraction. Acetylcholine test was performed on the 7th and 30th days after RNA-interference.

2.4. Arterial blood pressure measurements

Systolic arterial blood pressure was measured in non-anesthetized rats using tail cuff sphygmomanometer S-2 (Hugo Sachs Electronic, Germany).

2.5. Western blot analysis

Briefly, following SDS-PAGE on 10% acrylamide gel, proteins were transferred on nitrocellulose membrane (Thermo Scientific) according to the protocol (Abcam). The membrane was blocked with 5% non fat dry milk in PBS-T (in mM, 150 NaC, 50 $NaH_2PO_4 \cdot H_2O$, 0,1% Tween-20) for 1 h to prevent nonspecific antibody binding. After washing with PBS-T (3 ×), the nitrocellulose