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Fact or fancy: Does the high level of PKC-delta gene expression contribute to potassium channels malfunction at arterial hypertension and diabetes?

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Arterial hypertension development is a complex and multifactorial 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 [1]. It is known also that a high level of protein kinase C (PKC) activity in vascular wall [2] and related overproduction of reactive oxygen species (ROS) [3] are typical features for both essential arterial hypertension and diabetes.

One of the main mechanisms underlying an increased VSM contractility at arterial hypertension may be an increased PKC activity [4]. It is also known that this enzyme appear tightly involved in plasmolemmal ion permeability. For instance, it has been shown that PKC regulates activity of K^+ channels, which play an essential role in vascular function via its involvement in changes of membrane polarisation [5]. There is strong evidence that PKC modulates K^+ channels activity providing to increasing of vascular tone in both physiological and pathophysiological conditions [4, 6]. A crucial role in the regulation of smooth muscle cells (SMCs) plasma membrane polarization belongs to Ca^{2+}-activated K^+ (BK_{Ca}) channels, which are predominant K^+ channels in vascular SMCs and appeared to have disturbances in subunits expression and regulation and thus become dysfunctional at hypertensive and diabetic conditions [7].

Vascular tone depends on normal and coordinated function of the main vascular wall components – endothelial (ECs) and smooth muscle cells (SMCs). A considerable number of clinical studies demonstrated that endothelium-dependent vasodilatation is abnormal in patients with 1 and 2 type of diabetes [8]. Among numerous pathophysiological pathways, which contribute to the development of diabetic vascular dysfunction, activation of regulatory enzyme protein kinase C (PKC) requires special attention. Being chronically activated by hyperglycemia, PKC has many targets in ECs and SMCs and regulates such vascular functions as cell growth, angiogenesis, vascular cells permeability, Ca^{2+}-sensitization and SMCs contractility [9]. PKC-δ isoform expressed in SMCs is known to play crucial role in a number of pathological conditions [10]. Thus, PKC appeared to be a good target for pharmacological intervention in diabetic conditions.

RNA-interference (RNAi) is rapidly emerging as an essential gene-silencing tool. It is known that RNA-interference 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 [11]. Given the ability to knock down essentially any gene of interest, RNAi via siRNAs has generated a great deal of interest in both basic and applied pharmacology. The siRNAs appear as an extremely powerful and popular gene silencing agent.

Thereby, the aim of this study was to identify weather PKC-δ gene silencing using siRNAs restores K^+ channels func-

tion in VSM damaged following arterial hypertension and diabetic development.

Materials and methods. 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 with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects and/or animals.

Induction of diabetes. Diabetes was induced in male Wistar (WKY) rats (180–200 g) by a single intraperitoneal injection of streptozotocin (STZ, 65 mg/kg) dissolved in buffer solution contained 0,9 % NaCl and 0,3 % citrate Na, pH 4.6.

Isolation of vascular smooth muscle cells. Isolated smooth muscle cells were obtained from rat thoracic aorta at mature male healthy WKY, spontaneously hypertensive rats (SHR), rats with STZ-induced diabetes, SHR and diabetic rats after siRNA targeted to PKC-δ isoform administration (250–300 g). Animals were killed by cervical dislocation following ketamine (45 mg/kg b.w., IP) and xylazine (5 mg/kg b.w., IP) anesthesia. The thoracic aorta (1,5–2,0 cm-long) was removed, cleaned of adipose and connective tissues and cut into rings (width 1,0–1,5 mm) in a cold nominally Ca^{2+}-free solution containing (in mM): 140 NaCl; 5,9 KCl; 2,5 $MgCl_2$; 11,5 glucose; 10 5-N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES), pH 7,4. Then the vascular tissues were transferred to a fresh nominally Ca^{2+}-free solution containing 2 mg/ml collagenase type IA (417 U/mg), 0,5 mg/ml protease type XXV, and 2 mg/ml bovine serum albumin. The tissues were then incubated for 33 min at 37 °C. After incubation, the tissues were washed (2–3 min) twice in a fresh nominally Ca^{2+}-free solution to remove the enzymes. Cells were dispersed by agitation using a glass pipette, and then transferred into buffer solution which content was described above. Aliquots of the myocytes were stored at +4 °C and remained functional for at least 5 hours.

Electrophysiological studies. To study whole-cell integral outward potassium currents (voltage clamp mode) the whole-cell patch clamp technique in the amphotericin B (250 μg/ml) perforated-patch configuration was used. Data acquisition and voltage protocols were performed using an Axopatch 200B Patch-Clamp amplifier and Digidata 1200 B 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 capacity 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 8.0, 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 (in mM): 140 KCl; 10 NaCl; 1,2 $MgCl_2$; 10 HEPES; 11,5 glucose; 2 ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA); 1 $CaCl_2$ adjusted to pH 7,3 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 (in mM): 140 NaCl; 5,9 KCl; 1,2 $MgCl_2$; 2,5 $CaCl_2$; 11,5 glucose; 10 HEPES, pH 7,4.

Integral outward currents were elicited by 10 mV steps pulses with 300 ms duration between -100 and +70 mV from

holding potential of -60 mV. Holding potential was maintained at -60mV in all experiments. Macroscopic current values were normalized as pA/pF.

Delta isoform PKC gene knockdown by RNA interference. The silencing siRNAs for PKC - δ (PRKCD-S-5' - GGA AAG GUA CUU UGC AAU CUU - 3'; PRKCD-A-5' - AGA UCU UUU GUU UCU GAG UUU - 3') genes were synthesized by Metabion (Martinsried, Germany) and injected intravenously via the tail vein.

One-stranded siRNAs were made double-stranded by annealing. Before injection solutions of appropriate sense and antisense oligonucleotides were dissolved in annealing buffer with the following composition (mM): 30 HEPES-KOH; 100 KCl; 2 $MgCl_2$; 50 NH_4Ac; pH 7,4. Equal volumes of sense and antisense oligonucleotides solutions were mixed in the tube and then two times smaller volume of annealing buffer was added. Then, using thermocycler «GeneAmp System 2700» this solution was heated to 90 °C and incubated for 1 min and then during 45 min was cooled to room temperature. Annealed siRNAs were injected (40 μg / per rat) twice via tail vein with 24 hours interval.

Total mRNA isolation and real time (RT)-polymerase chain reaction (PCR). Total RNA was isolated from rat aorta using a Trizol RNA-prep kit (Isogen, Russian Federation) according to the manufacturer's protocol. RNA concentration was determined with the use of a NanoDrop spectrophotometer ND 1000 (NanoDrop Technologies Inc., Wilmington, DE, USA). Reverse transcription was performed using a RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, USA), using 200–300 ng of total RNA and a random hexamer primer. Single-chain DNA was used for real-time PCR (RT-PCR).

We performed amplification in 10 μl of SYBR Green PCR Master Mix containing 30 pM of each primer. For amplification mRNA delta PKC and β-actin (housekeeping gene) fragments we used next primers: PKC delta sense 5′ - CTG GAA TAG TGA GCT CCC AGA C - 3′ and antisense 5′ - ATC ACC AGT CAC CCA CTC TTC T - 3′; β-actin sense 5′ - TGT TAC GTC GCC TTG GAT TTT GAG - 3′ and antisense 5′ - AAG AGA GAG ACA TAT CAG AAG C - 3′. Primers were synthesized by Metabion (Martinsried, Germany). Sample volume was driven to 20 μl with deionized water. Amplification was performed on thermocycler 7500 Fast Real-Time PCR System. Thermal cycling conditions comprised an initial denaturation and AmpliTaq Gold® DNA polymerase activation step at 95 °C for 10 min, followed by the treatments at 95 °C for 20 s, and at 65 °C for 45 s and for 50 cycles, followed by the dissociation step. For control of specificity we performed dissociation stage-sequential increase of temperature from 65 °C to 95 °C with registration of the drop in the double-stranded DNA-SYBR Green complexes fluorescence strength.

We performed calculations using the 7500 Fast System SDS software provided. The CT (cycle threshold) is defined as the number of cycles required for the fluorescence signal to exceed the detection threshold. The expression of the target gene relative to the housekeeping gene as the difference between the threshold values of the two genes was calculated.

Chemicals. Streptozotocin, norepinephrine, BSA, amphotericin B, collagenase, protease, dihydroethidium and all constituents of the Krebs solution were purchased from Sigma Chemicals Co. (St. Louis, MO, USA).

Statistical analysis and data presentation. Electrophysiological and PCR data analysis were performed using Origin 7.5 (Microcal Software, Northampton, MA, USA.) software.

Data are shown as means ± S.E.M., n indicates the number of cells or preparations tested; each cell or preparation used for a specific experimental protocol was obtained from different animals. Multiple comparisons were performed using one way analysis of variance (ANOVA). If any significant difference was found the Tukey's multiple comparison test was applied. Differences were considered to be statistically significant when p was less than 0,05.

Results and discussion. In the present study we have tested the hypothesis that PKC-δ is involved in vascular dis-

orders development, i.e. decreased integral outward potassium currents in vascular SMCs. Potassium channels are known to play an essential role in vascular function regulation and their dysfunction contributes in pathogenesis not only pulmonary and systemic hypertension but diabetes and atherosclerosis [12].

At the day of experiment, spontaneously hypertensive rats showed blood pressure levels with average meaning of 158 ± 2 mm Hg (n = 12, p < 0,05). Diabetic rats showed a high blood glucose levels with average meaning of 30,0 ± 0,5 mM/l, (n = 12, p < 0,05) while age-matched control rats demonstrated 7,0 ± 1,6 mM/l (n = 12, p < 0,05).

It is clear that arterial hypertension is a multifactorial process that involves a number of functional systems those contribute to dominancy of constrictor influences over dilatation producing this way vascular hypercontractility and vascular tone elevation. VSM contraction is tightly coupled to membrane potential, which, in turn, is determined mainly by potassium channels activity.

It is well known that PKC is a group of regulatory enzymes that plays a key role in many cellular functions and affects a number of signal transduction pathways [13]. Among at least 12 PKC family members, lots of PKC isoforms are expressed in vascular tissues: PKC-α, PKC-β_1, PKC-β_2, PKC-γ, PKC-ε, PKC-η, PKC-ζ, PKC-δ, and PKC-ι/λ [13]. There is strong evidence of PKC-δ activation and/or overexpression in diabetes and while hypertension development [14, 15].

The data obtained in our study using RT-PCR experiments clearly demonstrate a significant diabetes-induced increase in PKC-δ isoform mRNA relative level in diabetic aortas as compared to controls. We have investigated the expression of PKC-δ isoform in vascular tissues in SHR and diabetic rats before and after its targeted disruption using siRNAs. Fig. 1 clearly demonstrates that mRNA delta-isoform expression measured using RT-PCR analysis was significantly higher in blood vessels from rats with arterial hypertension than those from WKY. Administration of siRNAs specific to

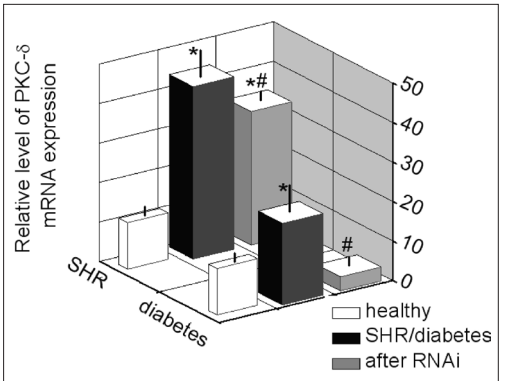

Fig. 1. Relative levels of mRNA PKC-δ isoform expression in vascular tissues of healthy WKY rats (n = 6), SHR and diabetic rats (n = 6) before and after PKC-δ isoform gene silencing using siRNAs (n = 6)

*β-Actin was used as an internal control. *p < 0,05 vs. control values; #p < 0,05 vs. SHR/diabetic values.*

PKC-δ isoform genes resulted in significant decrease of relative level of its mRNA in SHR and diabetic rat aortas.

It is became clear also that one of the main mechanisms underlying increased VSM contractility is the increased PKC activity in vascular tissues [2]. It has been established that vascular tone elevation following ionizing irradiation is due to PKC-mediated increase in myofilaments Ca^{2+}-sensitivity [2] and PKC-dependent BK_{Ca} channelopathy [4].

The whole-cell patch clamp recordings were performed using aortic SMCs from SHR, diabetic rats on 3rd month of diabetes development; diabetic rats on 7th day after PKC-δ siRNAs administration and age-matched control healthy WKY.

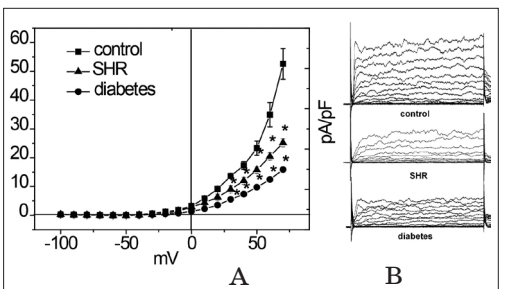

Fig. 2. Current-voltage relationships and original traces of integral outward potassium currents in isolated SMCs obtained from the thoracic aortas of healthy, SHR and diabetic rats

*The currents were elicited by 10-mV step pulses with 300-ms duration between -100 and +70 mV from a holding potential of -60 mV. Data are shown as mean ± SEM. *p < 0,05 vs. control values.*

Fig. 2 shows that the integral outward currents are decreased in aortic SMCs from SHR and diabetic rats as compared to controls, 25 ± 1 pA/pF, 16 ± 1 pA/pF and 53 ± 5 pA/pF ($n = 12$, $p < 0,05$), respectively.

Inhibited total outward currents in aortic SMCs from SHR appears significantly increased on 7th day as the result of a targeted disruption of PKC-δ isoform encoding gene using siRNAs from 25 ± 2 pA/pF to 36 ± 3 pA/pF ($n = 12$, $p < 0,05$) (Fig. 3). Also we observed a significant increase in integral outward current amplitudes on 7th day after PKC-δ isoform gene silencing to 24 ± 3 pA/pF ($n = 12$, $p < 0,05$) in comparison with diabetic values.

The data obtained in our experiments clearly demonstrate a significant increase in integral outward potassium currents in isolated vascular SMCs obtained from diabetic rats after administration of siRNAs targeted to PKC-δ genes.

It was shown that the augmentation of integral outward K^+-current in spontaneously hypertensive rats after PKC-delta knockdown is due to BK_{Ca} function restoration [15]. Inhibition of BK_{Ca} channels in vascular SMCs produces membrane depolarization, elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]i$), resulting in SMCs contraction and related vasoconstriction. Multiple studies demonstrate that BKCa are regulated by protein kinases and PKC inhibits the activation of these channels in different types of arterial smooth muscle [16].

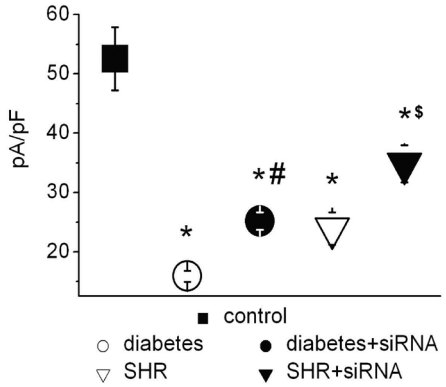

Fig. 3. Currents density in isolated SMCs obtained from the thoracic aortas of healthy, SHR and diabetic rats after PKC-δ isoform gene silencing using siRNAs

*The currents were elicited by 10-mV step pulses with 300 ms duration between -100 and +70 mV from a holding potential of -60 mV. Data are shown as mean ± SEM. *p < 0,05 vs. control values; $p < 0,05 vs. SHR values; #p < 0,05 vs. diabetic values.*

Recently it was revealed, that PKC regulation of SMCs BK_{Ca} activity is ambiguous. It is known that PKC inhibits the open-state probability of BK_{Ca} channel α-subunits, that depends on a phosphorylation of two distinct serines in the C terminus of the channel protein.

In conclusion, the main finding of this study shows that PKC-δ gene overexpression really decreases K^+ channels activity and leads to vascular malfunction at essential hypertension and diabetes while RNAi technique is a good therapeutic approach to restore normal K^+ channels function.

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Т. В. Новохацька, К. І. Клименко, В. Є. Досенко, А. І. Соловйов

Правда чи вигадка: чи насправді підвищений рівень експресії гена ПКС-дельта призводить до дисфункції калієвих каналів за артеріальної гіпертензії та діабету?

Мета дослідження – ідентифікувати, чи насправді за розвитку артеріальної гіпертонії та цукрового діабету підвищений рівень експресії гена протеїнкінази С (ПКС) дельта призводить до порушення активності та функціонування калієвих каналів в гладеньком'язових клітинах аорти щура. Дослідження було проведено із застосуванням наступних методик: індукція діабету (стрептозотоцин, 65 мг/кг), виділення гладеньком'язових клітин з аорти щура, перфорованого петч-клемпу в модифікації «ціла клітина», РНК-інтерференції за допомогою малих інтерферуючих РНК (міРНК) та полімеразної ланцюгової реакції в реальному часі (РЧ-ПЛР). Результати РЧ-ПЛР продемонстрували значне підвищення рівня експресії гена дельта ізоформи ПКС у тварин як за артеріальної гіпертензії, так і за цукрового діабету порівняно зі здоровими щурами. Застосування методу РНК-інтерференції для пригнічення експресії гена ПКС-δ викликає тривалий та стабільний інтерференційний ефект, що проявляється в суттєвому зменшенні рівня експресії мРНК ПКС-δ на 7 добу в обох групах. Встановлено, що введення міРНК призводить до суттєвого збільшення щільності інтегральних трансмембранних струмів вихідного напряму в ГМК судинної стінки за рахунок відновлення провідності крізь калієві канали різної провідності. Отримані дані свідчать, що дельта ізоформа ПКС залучена до розвитку каналопатії калієвих каналів аорти щура за розвитку як гіпертензивних станів, так і цукрового діабету. Результат дослідження чітко вказує на те, що надмірна експресія гена дельта ізоформи ПКС насправді знижує активність K^+-каналів та призводить до розвитку судинної дисфункції за артеріальної гіпертензії та діабету, тоді як застосування техніки РНК-інтерференції може бути хорошим терапевтичним підходом для відновлення нормального функціонування K^+-каналів.

Ключові слова: дельта ізоформа протеїнкінази С, калієві канали, цукровий діабет, артеріальна гіпертензія, РНК-інтерференція

Т. В. Новохацкая, К. И. Клименко, В. Е. Досенко, А. И. Соловьев

Правда или вымысел: действительно ли повышенный уровень экспрессии гена ПКС-дельта приводит к дисфункции калиевых каналов при артериальной гипертензии и диабете?

Цель исследования – идентифицировать, действительно ли при развитии артериальной гипертензии и сахарного диабета повышенный уровень экспрессии гена протеинкиназы С (ПКС) дельта приводит к нарушению активности и функционирования калиевых каналов в гладкомышечных клетках аорты крысы. Исследование было проведено с применением следующих методик: индукция диабета (стрептозотоцин, 65 мг/кг), выделение гладкомышечных клеток из аорты крысы, перфорированного пэтч-клэмпа в модификации «целая клетка», РНК-интерференции с помощью малых интерферирующих РНК (миРНК) и полимеразной цепной реакции в реальном времени (РВ-ПЦР).

Результаты РВ-ПЦР продемонстрировали значительное повышение уровня экспрессии гена дельта изоформы ПКС у животных, как при артериальной гипертензии, так и при сахарном диабете по сравнению со здоровыми крысами. Применение метода РНК-интерференции для подавления экспрессии гена ПКС-δ вызывает длительный и стабильный интерференционный эффект, проявляющийся в существенном уменьшении уровня экспрессии мРНК ПКС-δ на 7 сутки в обеих группах. Установлено, что введение миРНК приводит к существенному увеличению плотности интегральных трансмембранных токов выходящего направления в ГМК сосудистой стенки за счет восстановления проводимости через калиевые каналы. Полученные данные свидетельствуют, что дельта изоформа ПКС вовлечена в развитие каналопатии калиевых каналов аорты крысы при развитии как гипертензивных состояний, так и сахарного диабета. Результат исследования четко указывает на то, что чрезмерная экспрессия гена дельта изоформы ПКС действительно снижает активность K^+-каналов и приводит к развитию сосудистой дисфункции при артериальной гипертензии и диабете, тогда как применение техники РНК-интерференции может быть хорошим терапевтическим подходом для восстановления нормального функционирования K^+-каналов.

Ключевые слова: дельта изоформа протеинкиназы С, калиевые каналы, сахарный диабет, артериальная гипертензия, РНК-интерференция

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