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The BK_{Ca} channels deficiency and high protein kinase C activity as a main reasons for radiation-induced vascular hypercontractility

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Introduction

One of the main clinical manifestations of ionized irradiation in case of nuclear accidents or radiation beam therapy in patients with neoplasm is a local (cardiac ischemia and arrhythmias) or generalized (arterial hypertension) vasospasm development which seriously complicates the course and the treatment of the main disease. It was clearly demonstrated that even single dose non-fatal whole-body ionized irradiation of rats in dose of 5–6 Gy led to a sustained arterial blood pressure elevation and abnormalities in vascular contractility [1]. One of the leading causes of radiation-dependent deaths in bomb survivors from Hiroshima and Nagasaki [2] and survivors of Chernobyl accident [3] is associated with cardiovascular unwanted events.

Having this fact established we have focused on underlie mechanisms. For instance, we demonstrated that an increased contractility of blood vessels following irradiation may be due to selective inhibition of EDRF (NO) – dependent, but not EDHF – dependent, relaxation in irradiated vascular tissues [4].

Then, we tested a possible involvement of large conductance calcium- and voltage-activated potassium channels (BK_{Ca} channel), which appear to be a possible radiation target in vascular system. It is known that BK_{Ca} channelopathy is involved in a number of vascular disorders including diabetes and arterial hypertension [5, 6]. We have shown recently that BK_{Ca} channels play an important role in radiation-induced vascular abnormalities. The data obtained clearly indicate the decrease of outward current density in both endothelial [7] and smooth muscle cells (SMCs) accompanied with diminished BK_{Ca} mRNA expression [8].

It is well known that the native BK_{Ca} channel is composed by at least two distinct integral membrane subunits: the pore-forming (ion-conducting) α-subunit (KCNMA1) and a regulatory β-subunit (KCNMB1-4). The α-subunit is the minimal molecular component for BK_{Ca} channel activity and a functional channel can be formed with four α-subunits. Mammalian BK_{Ca} channel α-subunit is encoded by a single gene called KCNMA1, which was formerly named as Slowpoke, Slo or Slo1 [9, 10]. That is why we have focused our studies to α-subunit changes under irradiation.

Taking into account the molecular structure of BK_{Ca} and the ability of small interference RNAs (siRNAs) knockdown essentially any gene of interest [11–13], we supposed that knockdown of BK_{Ca} channel α-subunit in rat's vascular tissues is a good model for radiation-induced vascular malfunction and related arterial hypertension development in rats.

In contrast to the numerous studies demonstrating that irradiation depresses endothelium-dependent vasorelaxation, other investigations have shown that irradiation enhances vascular contractility in an endothelium-independent manner [14]. This information suggests that, in addition to the indirect effect through a decrease in endothelial-dependent relaxation, a direct effect on vascular contractility may be important in the vascular anomalies induced by irradiation.

Vascular smooth muscle is known to contract in response to an increase in cellular free calcium ($[Ca^{2+}]_i$). It is well

known that the increase in $[Ca^{2+}]_i$ activates the calmodulin dependent myosin light chain (MLC) kinase which catalyzes the phosphorylation of the 20,000 dalton MLC. MLC phosphorylation in turn activates the myosin molecule, which can then interact with actin and contract. In addition to this primary regulatory pathway, several modulatory pathways exist in smooth muscle that can alter the magnitude of force that is developed for any given level of cellular Ca^{2+}. Alterations in myofilament Ca^{2+} sensitivity can be either positive or negative depending on the pathways stimulated. For increases in myofilament Ca^{2+} sensitivity, two primary hypotheses have been proposed: G-protein dependent activation of Rho kinase and protein kinase C dependent phosphorylation of a cellular protein called CPI-17. Both proposals have a common end-point, that being inhibition of the MLC phosphatase resulting in a greater level of MLC phosphorylation for any given level of Ca^{2+} and activity of the MLC kinase.

Hypertension has been clearly shown to be associated with an increase in vascular reactivity. In turn, the enhanced reactivity during hypertension has been shown to be due in part to an increase in myofilament Ca^{2+} sensitivity [15]. Both an increase in myofilament Ca^{2+} sensitivity and a decrease in BK_{Ca} activity may result from a high protein kinase C (PKC) activity. Taken together, this suggests the possibility that alterations in PKC may be a unifying hypothesis in the irradiation induced hypertensive state and alterations in vascular function.

Thus, *the main goal of this study* was to clarify whether siRNAs administration, targeted to BK_{Ca} encoding gene, is able to alter the form and function of BK_{Ca} and this way produce vascular abnormalities and hypertension development in a manner similar to ionized irradiation. *The next goal* of this study was to test the hypothesis that the increase in vascular responsiveness that results following whole body irradiation is due to a change in the PKC dependent increase in myofilament Ca^{2+} sensitivity in the vascular smooth muscle cell.

Materials and methods. *Animals.* 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 Committees. Experiments were performed on 6–8 weeks male Wistar rats (weight 250–300 g) housed under controlled environmental conditions (21 °C, 12–12 h light-dark cycle) and free access to water and standard rodent chow.

KCNMA1 gene knockdown by RNA interference. The double-stranded silencing siRNA for KCNMA1 gene sense 5'-GUA CAG GAA GGG AAC AUU UUU-3', antisense 5'-AAA UGU UCC CUU CCU GUA CUU-3' and nonsilencing (scrambled, scrRNA) RNA: sense 5'-UGU UCA GCG AAA UAU AAC CUU-3', antisense 5'-GGU UAU AUU UCG CUG AAC AUU-3' were prepared from corresponding oligonucleotides provided by Metabion (Germany) according to the manufacture's instructions. For KCNMA1 gene knockdown *in vivo* these double-strand RNAs were injected in tail vein of rats (80 μg/kg) twice with 24-h interval. The molecular and functional changes (expression of mRNA BK_{Ca} channels α subunit, K^+ channels activity, acetylcholine-induced, endothelium-dependent vasorelaxation and norepinephrine-induced vasoconstriction, arterial systolic blood pressure) were analyzed 7 days after first siRNA injection.

It is clear that delivery of siRNAs to target cell is a serious problem and naked siRNAs administrated intravenously may be unstable in serum being degraded by endo- and exonucleases. Nevertheless, the recent data obtained [16] indicate that naked siRNAs in many studies demonstrate significant RNA-interference effect while chemical or other type modification of siRNAs can increase stability of these molecules *in vitro*, but can not demonstrate an increment in their efficacy *in vivo*. We suppose that the main experimental criteria of the siRNAs specific effectiveness is the real difference in the mRNA expression in tissues obtained from the animals treated with

specific siRNAs as compared to the healthy rats or the rats that were treated with scr-siRNAs.

Whole-body animal γ-irradiation. Rats were exposed to a 6 Gy dose of ionizing irradiation for 7.5 min. The dose of 6 Gy was used based on our previous studies showing that this dose induced vascular effects while maintaining survival of the animals throughout experimental period [1, 8, 17]. Whole-body irradiation was performed with gamma rays delivered at a rate of 0.80 Gy · min^{-1} from a ^{60}Co source (TGT ROCUS M, Russian Federation) positioned 50 cm from the animal. During irradiation, animals were restrained in a plastic box specifically designed for this study and the radiation beam was focused on the animal's chest. There was no change in housing, standard food, or drink following irradiation. All animals survived the 30 day experimental period following the exposure to irradiation.

RNA isolation and real-time 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 (Fermentas, Lithuania), using 1.2–1.5 μg of total RNA and a random hexamer primer. Single-chain DNA was used for polymerase chain reaction (PCR) and real-time PCR.

We performed amplification in 10 μl of SYBR Green PCR Master Mix containing 30 pM of each primer. For amplification mRNA BK_{Ca} channels (housekeeping gene) fragments we used next primers:

BK_{Ca} α subunit sense 5'-tac ttc aat gac aat atc ctc acc ct-3' and antisense 5'-acc ata aca acc acc atc ccc taa g-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'.

L-type Ca: sense -5`- tgacagtgagagctttgaggag -3` antisense – 5`- tctagtcctcctcgaaccagag -3`

Kv 1.5. Up: sense -5`- ttcatcaaggaagaggagaagc-3`, antisense – 5`- agtgtcctaggcaagagtggag-3`

Kir 6.2. Up: sense -5`- agaaaccctcat cttcagcaag -3`, antisense – 5`- aatctcgtcagccagataggag -3`

Kir 6.1. Up: sense -5`- acgatatctcagccactgacct -3`, antisense – 5`- ctcctcctcatggagttgtttc-3`

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 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. We calculated the expression of the target gene relative to the housekeeping gene as the difference between the threshold values of the two genes.

Isolation of rat thoracic aorta smooth muscle cells. Smooth muscle cells were isolated from rat thoracic aorta by collagenase treatment. Briefly, the rats were anesthetized with ketamine (37.5 mg/kg b.w., IP) and xylasine (5 ml/kg b.w. IP) and approximately 1.0–1.5 cm of the thoracic aorta was excised and cleaned of connective tissue. The aorta was then cut into small pieces (1.5 × 1.5 mm) in a cold low-Ca^{2+} solution containing (in mM): NaCl 140, KCl 6, $MgCl_2$ 3, D-glucose 10, HEPES 10 (pH 7.4). The vascular tissues were transferred to a fresh low-Ca^{2+} solution containing: 2 mg/ml collagenase type IA (417 U/mg), 0.5 mg/ml pronase E type XXV, and 2 mg/ml bovine serum albumin. The tissues were then incubated for 30 min at 37 °C. After incubation, the tissues were washed (2–3 min) twice in a fresh low-Ca^{2+} solution to remove

the enzymes. Cells were dispersed by agitation using a glass pipette, and then were placed in normal Krebs bicarbonate buffer. Aliquots of the myocytes were stored at +4 °C and remained functional for at least 4 h.

Electrophysiology studies. The whole-cell patch clamp technique in the amphotericin B (250 μg/mL) perforated-patch configuration was used to study whole-cell potassium 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 with computer equipped with pClamp software (version 6.02, Axon Instruments Inc., USA). Membrane currents were filtered at 2 kHz and digitized at a sampling rate of 10 kHz. The reference electrode was Ag-AgCl plug electrically connected to the bath. Macroscopic current values were expressed as current density in 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., 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): KCl 140, NaCl 10, MgCl2 1.2, $CaCl_2$ 2.5, HEPES 10, D-glucose 11.5, adjusted to pH 7.3 with KOH. Pipettes had resistances of 2.5–5.0 MΩ. The standard Krebs-HEPES external solution contained (in mM): NaCl 140, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.5, HEPES 10, D-glucose 11.5, pH 7.4.

Ca^{2+}-free, high K^+ (135 mM) solution was prepared replacing NaCl with equimolar concentration of KCl and removing Ca^{2+} from bath solution.

Measurement of Contractile Force. Segments of thoracic aorta (1.5 cm-long) were obtained as described above, cleaned of both connective and adipose tissue, and cut into 1 to 1.5 mm width rings. All procedures were performed at room temperature in a nominally Ca^{2+}-free physiological salt solution.

Experiments were performed on the thoracic aorta rings obtained from healthy, irradiated adult rats and animals after siRNAs and scr-siRNAs treatment. The vascular tissues were prepared with special care in order to keep the endothelium intact for the following vasodilatation study. If necessary endothelium was removed using 15 min incubation in Krebs bicarbonate buffer with saponin (0.1 mg/ml). Aortic rings were mounted isometrically in a 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). The rings were equilibrated for 1 h with a resting tension of 10 mN. Experiments were made at 37 °C in modified Krebs bicarbonate buffer solution of the following composition (in mM): NaCl 133, KCl 4.7, $NaHCO_3$ 16.3, NaH_2PO_4 1.38, $CaCl_2$ 2.5, $MgCl_2$ 1.2, HEPES 10, D-glucose 7.8 at pH 7.4. Following the equilibration period, the aortic rings were exposed several times to norepinephrine (10^{-5} M) until reproducible contractile responses were obtained. To test endothelial functional activity and integrity, the relaxant responses to acetylcholine (10^{-9}–10^{-5} M) were examined on the plateau of the norepinephrine (10^{-6} M) – induced contraction. Contractile abilities of VSM were estimated reproducing dose-response curves to norepinephrine.

Measurement of $[Ca^{2+}]_i$:

Experiments for the simultaneous measurement of $[Ca^{2+}]_i$ and contractile force were carried out in a 500 μl tissue chamber mounted on the stage of a fluorescence microscope LUMAM-2 (Russia) equipped with epifluorescence collection. The aortic rings were mounted isometrically between a stationary stainless steel hook and a force transducer (AE 801, SensoNor A/S, Norten, Norway). Except for during the Fura-2AM loading procedure, the rings were continuously perfused with preheated Krebs solution at 35 °C at a rate of 2.0 ml per minute. The rings were loaded with 10 M Fura 2-AM in a physiological solution of the follow-

ing composition (mM): 122 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 11.6 HEPES, 11.5 glucose, and a pH of 7.3–7.4. The loading solution also contained 2.5% DMSO and 5 mg/ml Pluronic F-127. Loading continued for 2 hours at room temperature. The tissues were then mounted in a tissue chamber for measurement of force and $[Ca^{2+}]_i$ and equilibrated in normal physiological salt solution for at least 30 min.

Fura-2 fluorescence was excited at 340 and 380 nm wavelength (λ) and recorded at 510 nm emission wavelengths from a central region (approximately 0.5 mm in diameter) on the blood surface of the aorta ring. The fluorescence emitted from the tissue was collected by a photomultiplier through a 510 nm filter. The results of $[Ca^{2+}]_i$ measurements are presented as the ratio (R) of 510 nm emission fluorescence intensity $[I_{510}(\lambda)]$ at λ = 340 nm and λ = 380 nm excitation signals: $R = I_{510}(340)/I_{510}(380)$. $[Ca^{2+}]_i$ was determined, as described by Grynkiewicz et al., using the formula $[Ca^{2+}]_i$ (nM) = $K_d \cdot [(R - R_{min})/(R_{max} - R)] \cdot (S_{f2}/S_{b2})$, where K_d (224 nM at 37 °C) is the dissociation constant of Fura-2 for Ca^{2+}; R is the ratio of fluorescence of the sample at 340 to that at 380 nm; R_{min} and R_{max} represent the ratios of fluorescence at the same wavelengths in the presence of zero and saturating Ca^{2+} respectively; and S_{f2}/S_{b2} is the ratio of fluorescence of Fura-2 at 380 nm in zero Ca^{2+} to that in saturating Ca^{2+} respectively. Prior to initiating experiments, it was determined that contractions induced by high KCl were not affected by Fura-2 loading.

Solutions. All tissues were stored in a physiological salt solution of the following composition (in mM): 122 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 15.5 $NaHCO_3$, 1.2 KH_2PO_4, and 11.5 glucose, pH 7.3–7.4. Nominally Ca^{2+}-free solutions were prepared by simply omitting $CaCl_2$. Ca^{2+}-free/EGTA solutions used for removal of cellular calcium were prepared by omitting $CaCl_2$ and including 1 mM ethylene glycol-bis-(β-aminoethyl ether)-N, N, N', N',-tetraacetic acid (EGTA). Depolarizing KCl solutions were prepared by the equimolar exchange of KCl for NaCl.

Solutions for experiments performed using permeabilized muscle contained (in mM): 3.2 MgATP, 2 free-Mg^{2+}, 12 phosphocreatine, 0.5 sodium azide, 30 N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), pH 6.9 at 21 °C. Unless otherwise stated, creatine phosphokinase (15 U/ml) and calmodulin (0.5 μM) were added to all solutions. Desired free-Ca^{2+} levels (expressed as pCa) were obtained by mixing stock solutions containing K_2EGTA and K_2CaEGTA. The total EGTA concentration was 4 mM. Ionic strength was adjusted to 150 mM with potassium propionate.

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

Chemicals. Collagenase type IA, pronase E type XXV, bovine serum albumin, paxilline, tetraethylamonium, glibenclamide, norepinephrine, acetylcholine as well all the constituents of the Krebs solution were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Fura-2 AM was obtained from Molecular Probes, Inc. (Eugene, OR, USA). All other compounds were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (0.1 % final concentration) was used as a solvent for phorbol dibutirate. This concentration did not affect the results.

Statistics and analysis. The data are shown as means ± S. E. M., and n indicates the number of cells, preparations or animals tested. Data analysis was performed using Origin 7.5 (OriginLab Corporation, Northampton, MA, USA) software. Half-maximally effective concentration (EC_{50}) values were expressed as pD_2 (-log EC_{50}). To determine the maximal responses and EC_{50}, concentration-response curves were fit to the dose-response equation: $F = F_{max}/[1+10\hat{}((\log EC_{50} - \log C)\cdot h)]$, were F and F_{max} are actual and maximal relaxation (constriction) responses, h is the Hill slope, C is actual concentration. Multiple comparisons were made using one way analysis of variance (ANOVA) and if any significant difference was found, the Tukey's multiple comparison tests were performed. Arterial pressure data were analyzed by Student's t-test for paired observations. Differences were considered to be statistically significant when P was less than 0.05.

Results

1. Irradiation and KCNMA1 gene silencing lead to similar decrease in BK_{Ca} mRNA expression and potassium outward current density. Figure 1 shows that both irradiation and siRNAs targeted to KCNMA1 gene administered in total dose of 80 μg/kg (twice with 24-h interval, intravenously) produced a 2.5–3.5-fold decrease in BK_{Ca} α-subunit mRNA level in aortic SM as compared to tissues obtained from healthy rats and the rats treated with a scrambled siRNAs (scr-siRNAs). For instance, BK_{Ca} mRNA level decreased from (0.013 ± 0.003) relative units in SM treated with a scr-siRNAs to (0.005 ± 0.002) relative units in SM treated with siRNAs targeted to KCNMA1 gene ($n = 11$, $P < 0.05$).

Thus, the expression profile of BK_{Ca} mRNA transcripts in SM appeared to be significantly decreased in KCNMA1 silencing SM similar to irradiated tissues when the level of BK_{Ca} expression has been significantly decreased 30 days after irradiation (Figure 1 A).

The next series of experiments was performed to study potassium channel activity in SMC from control, irradiated and scr-siRNAs/siRNAs-administered animals. To measure K^+ channel activity, we used whole-cell perforated patch-clamp technique using freshly isolated thoracic aorta SMCs. To induce outward currents SMCs were held at the resting potential of -60 mV and then stimulated with increasing depolarizing 300-ms voltage steps from -100 mV to +70 mV.

As shown in Figure 2 A, B total outward potassium currents stimulated by depolarized voltage step were (30 ± 1), (11 ± 1) and (13 ± 1) pA/pF at +70 mV in control, irradiated (6 Gy) and KCNMA1 gene knockdown SMC, respectively ($n = 12–24$, $P < 0.05$, see Figure 2 legend for detail). Scr-siRNAs was without visible effect on outward potassium currents in SMC as compared to control.

After that we identified the different component of total potassium current following irradiation and KCNMA1 gene silencing (Figure 3 A, B). It is become clear that total outward current significantly decreased as the result of irradiation and KCNMA1 gene silencing mainly due to inhibition of paxilline-sensitive component, i.e. the current carried through BK_{Ca} channels.

This conclusion is based on the next evidence. Paxilline (500 nM), a selective inhibitor of BK_{Ca} channels [29, 42], added to the external bathing solution significantly decreased outward K^+ current density in the aortic cells obtained from control and scr-siRNAs-treated rats while this effect was six-four-fold attenuated in irradiated and siRNA-treated VSM cells. Paxilline-sensitive components were (25 ± 2), (5 ± 1), (26 ± 4) and (9 ± 1) pA/pF at +70 mV in intact, irradiated, scr-siRNAs and siRNAs-treated SMC, respectively ($n = 12$, $P < 0.05$).

External paxilline application combined with tetraethylammonium (TEA, 10 mM), non-selective inhibitor of potassium channels, led to additional inhibi-

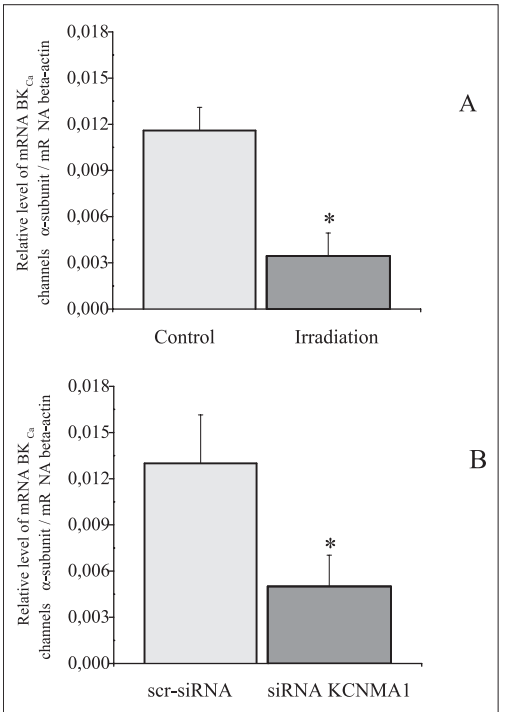

Figure 1. Expression of mRNA BK_{Ca} channels α-subunit in rat thoracic aorta smooth muscle after irradiation (A) and KCNMA1 gene silencing (B). A, aortic total RNA was isolated from 5 control rats and 6 rats on 30^{th} days after irradiation impact. β-actin was used as an internal control; B, aortic total RNA was isolated from 6 control rats (scr-siRNAs) and 7 rats on 7^{th} day following siRNAs injection. β-actin was used as an internal control. The asterisks indicate the statistical significance as compared to control and scr-siRNAs ($P < 0.05$)

tion of outward potassium current but there was no significant difference between TEA action in the cells obtained from intact, irradiated and scr-siRNAS/siRNAs treated SM cells (Figure 3 A, B). There were no differences in residual TEA-and paxilline-insensitive components of outward current in all groups of animals (Figure 3 A, B) at the potential +70 mV.

To identify the glibenclamide-sensitive component of outward current more precisely, we used the protocol described by Mishra and Aaronson [18]. Figure 4 shows that when superfusion solution was changed from standard 5.9 mM K^+ medium to Ca^{2+}-free, high K^+ (135 mM) and TEA (2 mM) containing solution (at this solution content reversal potential for K^+ shifted to 0 mV and other potassium currents are suppressed), an inward currents of about – (6.4 ± 0.4) pA/pF and – (4.7 ± 0.4) pA/pF ($P < 0.05$, $n = 6$) were recorded under normal condition and after irradiation, respectively. Glibeclamide (10 μM) significantly suppressed this current in both intact and irradiated SMCs, indicating

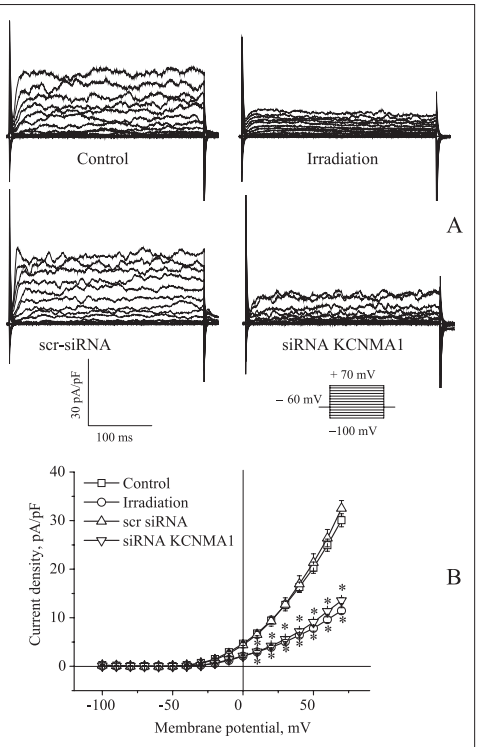

Figure 2. Effects of siRNAs – induced KCNMA1 gene silencing on total outward currents in aortic SMCs in comparison to non-fatal whole body irradiation. Outward membrane currents were elicited by a series of 10 mV pulses with 300 ms duration from -100 mV to +70 mV from a holding potential of -60 mV. A – original traces, B – current-voltage relationship of total currents recorded in isolated rat thoracic aorta SMCs obtained before and after irradiation impact and siRNAs treatment. Data shown are mean ± SEM; experiments were carried out on 24 cells from 12 control rats, 17 cells from 12 irradiated animals, 12 cells from 6 rats after scr-siRNAs and 15 cells from 7 rats after siRNA KCNMA1 injection. The asterisks indicate statistical significance as compared to control tissues and scr-siRNAs ($P < 0.05$)

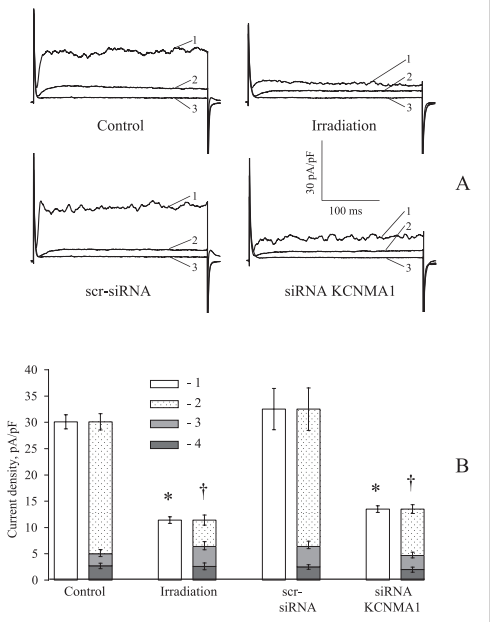

Figure 3. Effects of paxilline (500 nM) and tetraethylammonium (TEA, 10 mM) on outward potassium currents in thoracic aorta SMCs obtained from control, irradiated and treated with siRNAs rats at potential +70 mV. A – original traces showing total outward currents in vascular SMCs obtained from control, irradiated and siRNAs-treated rats before (1) and after paxilline (2) and paxilline combined with TEA (3) application in the external bath solution. B – diagrams showing the effects of irradiation and siRNAs-induced KCNMA1 gene silencing on components of total outwards potassium current (1): paxilline-sensitive component (2), TEA-sensitive component (3) and residual current (4) in the aortic SMCs. Columns are means ± S.E.M., n = 12. The asterisks indicate statistical significance as compared to control tissues ($P < 0.05$). The dagger indicates a statistically significant difference ($P < 0.05$) between paxilline-sensitive components of control currents and after irradiation and KCNMA1 gene silencing

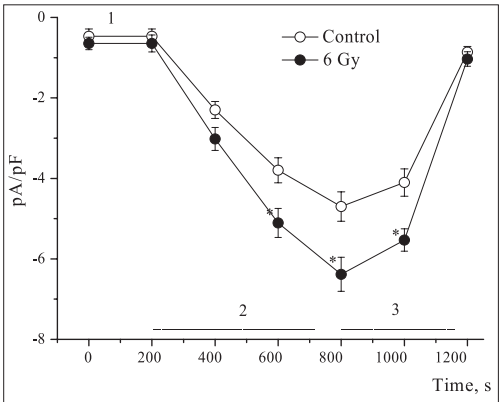

Figure 4. Effect of ionized irradiation on the resting glibenclamide (10 μM) – sensitive current in thoracic aorta SMCs. Currents were elicited by replacement of normal external solution with Ca^{2+}-free, high K^+ (135 mM) solution containing 2M TEA and 10 μM glibenclamide before and after irradiation. Horizontal bar 1 – superfusion with standard external solution; bar 2 indicates superfusion with Ca^{2+}-free, high K^+ (135 mM) solution containing 2mM TEA; bar 3 – glibenclamide (10 μM) was added to the superfusate

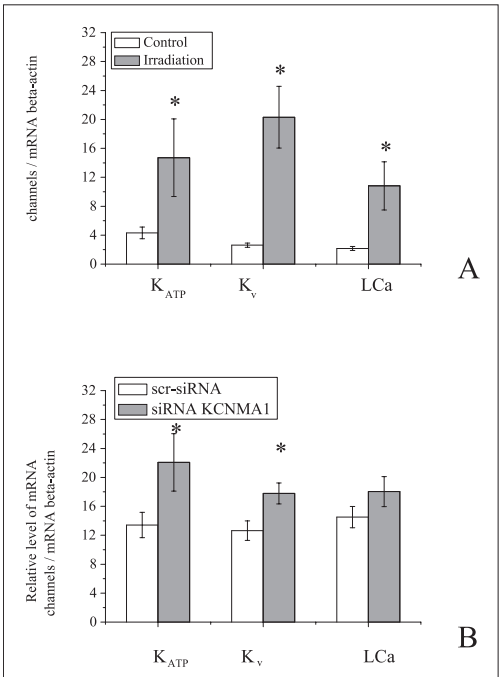

Figure 5. mRNA expression of K_V, K_{ATP} and L-type Ca^{2+} channels (LCa) in aortic SMCs obtained from control, irradiated, siRNAs and scr-siRNAs-treated rats.
A, aortic total RNA was isolated from 11 control rats and 12 rats on 30^{th} days after irradiation impact. β-actin was used as internal control; B, aortic total RNA was isolated from 6 control rats (scr-siRNAs) and 12 rats on 7^{th} day following siRNAs injection. β-actin was used as internal control. The asterisks indicate the statistical significance as compared to control ($P < 0.05$)

K_{ATP}-dependent, glibenclamide-sensitive component of outward current not only suppressed but appears to be increased significantly following irradiation.

2. *KCNMA1 gene silencing increases mRNA expression of K_V, K_{ATP} and L-type Ca^{2+} channels.* To investigate the effect further it would be interesting to know whether mRNA expression of other members of voltage-dependent potassium channels family will be affected following KCNMA1 gene silencing. Taking into account functional association between voltage-dependent calcium and K^+ channels, L-type Ca^{2+} channels mRNA expression was studied additionally. Figure 5 shows that mRNA expression of K_V and K_{ATP} channels in SM with silencing KCNMA1 gene significantly increased from (12.6 ± 1.3) to (17.8 ± 1.4) relative units and from (13.4 ± 1.8) to (22.1 ± 4.0) relative units ($P > 0.05$, $n = 12$), respectively, while L-type Ca^{2+} channels mRNA level demonstrated non-significant tendency to increment – from (14.5 ± 1.5) to (18.0 ± 2.1) relative units ($P > 0.05$, $n = 10$).

In contrast to siRNAs administration, the levels of mRNA expression of K_V and K_{ATP} channels on 30^{th} days after irradiation were increased significantly: K_V from (2.6 ± 0.03) to (20.3 ± 4.3) ($P < 0.05$), K_{ATP} from (4.3 ± 0.8) to (14.7 ± 5.4) ($P < 0.05$). The level of L-type Ca^{2+} channels expression had increased from (2.20 ± 0.03) to (10.8 ± 3.3) relative units ($P < 0.05$).

3. *KCNMA1 gene silencing changes SM responses to acetylcholine and norepinephrine.* Figure 6 shows original tracing demonstrating the results of standard acetylcholine test in control and scr-siRNA-treated groups of animals as compared to irradiated and siPNAs-treated rats. Relaxations of the rat thoracic aortas were obtained by cumulative additions of acetylcholine in doses of 10^{-9}–10^{-5} M.

Original recordings combined with dose-responses curves (Figure 7 A) clearly indicate that the maximal amplitude of endothelium-dependent SM relaxation

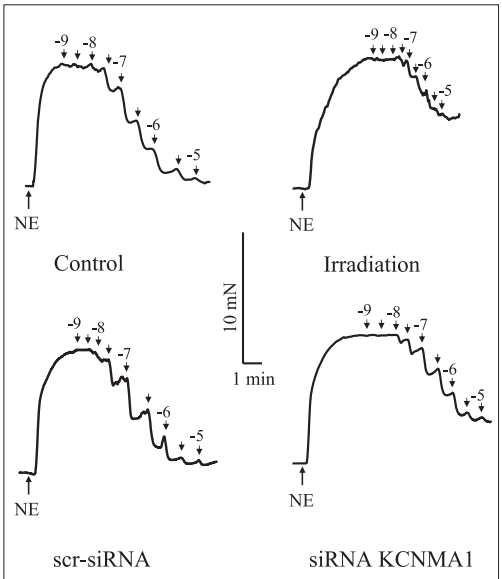

Figure 6. Representative tracings showing the endothelium-dependent relaxation by acetylcholine (ACh) in doses from 10^{-9} to 10^{-5} M in the rat thoracic aortas obtained from control and scr-siRNA treated rats in comparison to irradiated and siRNAs-treated rats. Aortic rings were pre-contracted with 10 μM norepinephrine. Dots represent additions of increasing concentration of the Ach with 0.5 steps in log C (M) units

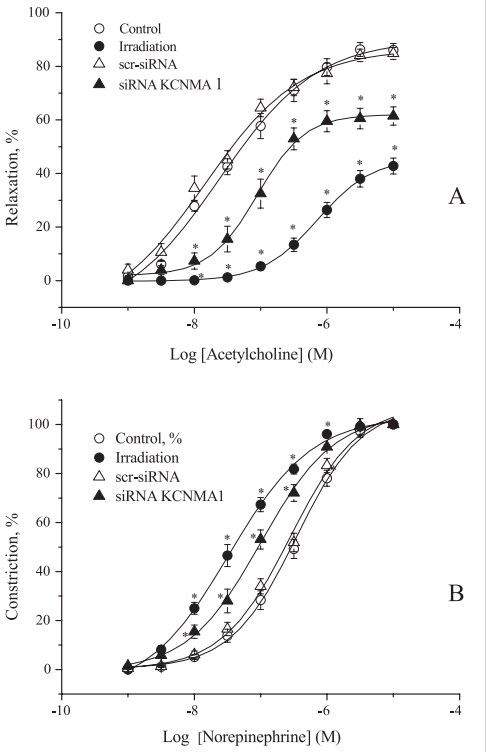

Figure 7. Effects of non-fatal whole body irradiation and siRNAs KCNMA1 gene silencing on endothelium-dependent relaxation in intact rat thoracic aorta rings (A), and dose-response curves to norepinephrine (B) in denuded rat thoracic aortas obtained from control, irradiated and scr-siRNsA/siRNAs treated rats. Relaxations are expressed as percent decrease in tension evoked by 10 μM norepinephrine, contractions are expressed as percent of maximal tension evoked by 10 μM norepinephrine. Experiments were carried out on 15 rings from eight control rats, 18 rings from nine irradiated animals, 12 aortic rings from seven rats with siRNAs treatment and 12 aortic rings from six scr-siRNAs treated rats. Values shown are means ± S.E.M., the asterisks indicate statistical significance as compared to control tissues ($P < 0.05$). Concentration-response curves were fitted to the dose-response equation

had decreased from 84–86 % in control groups (healthy and scr-siRNAs treated rats) to (43 ± 3) % and (61 ± 3) % in irradiated and KCNMA1 silencing tissues, respectively (n = 14–18, $P < 0.01$).

Moreover, both irradiation and KCMA1 gene silencing SM produced an increased sensitivity in denuded aortic SM to norepinephrine (Figure 7 B) – mean values of pD_2 (-log EC_{50}) are (6.5 ± 0.05) control, (7.50 ± 0.05) irradiated, (6.60 ± 0.05) scr-siRNAs and (7.0 ± 0.04) in KCMA1 gene silencing SM, respectively, suggesting increased SM contractility following both irradiation and KCMA1 gene silencing (n = 12–18, $P < 0.001$).

4. *Arterial blood pressure (BP) in irradiated and KCNMA1 silencing rats*. Figure 8 shows the effect of whole body irradiation (6 Gy) on arterial blood pressure measured in non-anesthetized healthy and scr-siRNAs-treated rats in comparison to irradiated rats and rats with knockdown of KCMA1 gene. Systolic BP had considerably increased on 30th day of post-irradiation from (113 ± 2) mmHg in control to (139 ± 6) mmHg (n = 15, $P < 0.05$) in irradiated animals. In contrast to irradiated rats, the rats with KCNMA1 gene knockdown demonstrated insignificant BP increment from (115 ± 2) mmHg (scr-siRNAs treated animals) to (122 ± 6) mmHg (n = 12, $P > 0.05$).

It is important to note that despite the KCMA1 gene silencing was without significant effect on BP level in experimental group of rats in total, some animal's

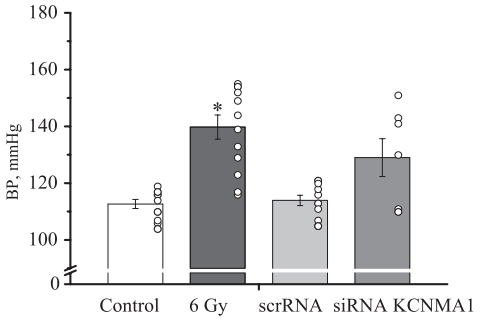

Figure 8. Effect of whole body γ-irradiation (6 Gy) impact and siRNAs administration on systolic blood pressure measured using a cuff tail sphygmomanometer in non-anesthetized rat. Measurements were made in healthy (n = 12), irradiated (n = 12), scr-siRNAs (n = 12) and siRNAs – treated rats (n = 7). Columns are means ± S. E. M. The asterisks indicate statistical significance as compared to control animals (P < 0.05)

demonstrated well expressed tendency to BP elevation.

5. *Intact vascular strips from control and irradiated animals*. The first series of experiments performed were designed to determine the effects of whole body irradiation on vascular contractile sensitivity. Figure 9 A shows the results of concentration-response curves from the cumulative addition of KCl. The addition of KCl (8–120 mM) produced concentration-dependent increases in force development in the thoracic aorta from both control and irradiated animals. The experiments were performed on the 9th and 30th days post-irradiation. Thoracic aortic rings obtained from irradiated animals on the 9th day post-irradiation were significantly more sensitive to the addition of KCl as compared to aortic rings from control animals. Maximum force development was similar in aortic rings from the two animals groups (7.6 ± 0.8) mN control; (8.6 ± 1.2) mN irradiated; $P < 0.05$, $n = 12$). The similar results were obtained on the 30th day of post-irradiation period (maximal force development was (10.0 ± 1.1) mN, $P < 0.05$, $n = 12$).

Similar experiments using the cumulative addition of phenylephrine showed increased sensitivity as evidenced by a shift to the left in the stimulation response curve in vascular rings from animals subjected to whole body irradiation as compared to those from control animals (Figure 9 B). Based on these results, whole body γ-irradiation produces a general increase in contractile sensitivity in response to all forms of stimulation, whether in response to membrane depolarisation or agonist activation.

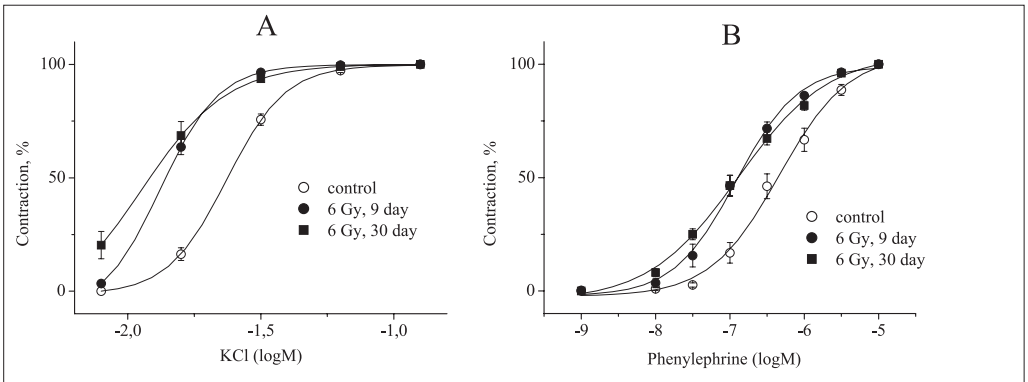

Figure 9. Effect of whole body irradiation (6 Gy) on KCl – and phenylephrine-induced contractions of rat thoracic aorta. A – Cumulative concentration-response curves for KCl were obtained with aortic rings from healthy (○) and irradiated rats on the 9th (●) and 30th (■) days of post-irradiation. Data shown are means ± SE. Curve fits of mean values were performed using the following: $F = F_{max}/[1 + exp(logEC_{50} - log[Ca^{2+}]i) \cdot h]$, where F and F_{max} are actual and maximal force, h is the Hill coefficient, EC_{50} is 50 % effective concentration. Mean value of pD_2 (-log EC_{50}) are: (1.70 ± 0.03) control, and (1.90 ± 0.05) ($P < 0.01$, $n = 12$) and (1.90 ± 0.03) ($P < 0.01$, $n = 12$), irradiated (9th and 30th days, respectively). B – cumulative concentration-response curves to phenylephrine obtained on the thoracic aorta rings from healthy and irradiated rats. Mean value of pD_2 are: (6.30 ± 0.07) control, and (6.92 ± 0.05) ($P < 0.01$, $n = 12$) and (6.89 ± 0.05) ($P < 0.01$, $n = 12$), irradiated (9th and 30th days, respectively). Contractions are expressed as a per cent of the tension evoked by maximal KCl (120 mM) or phenylephrine (10^{-5} M) concentration

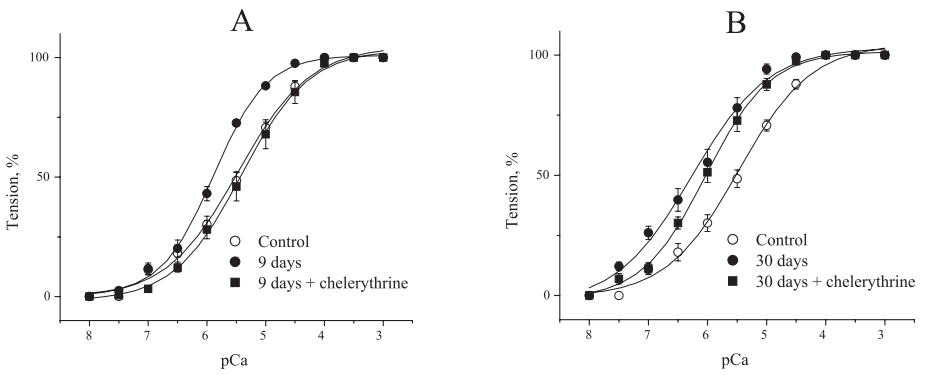

Figure 10. Force development in β-escin permeabilized rat aortic rings as a function of calcium concentration in the presence and absence of chelerythrine. Aortic rings from control (○) and irradiated (●) animals were subjected to the cumulative addition of calcium. Irradiated animals were studied on the 9th (A) and 30th (B) days of post-irradiation period after exposure γ-radiation (6 Gy). In addition, the calcium response of irradiated tissues was examined in the presence of 1 μM chelerythrine(■). The values are means ± SE. Data are normalized to force developed at pCa 5.0. Curves shown were fit to the Hill equation. See Figure 1 for details. Mean value of pD_2 (or pCa_{50} as generally accepted for permeabilized smooth muscle) for the 9th day of post-irradiation were: (5.60 ± 0.09) control; (5.98 ± 0.06) irradiated (P < 0.01, n = 12), and (5.33 ± 0.15) irradiated + chelerythrine (P < 0.05, n = 8, comparison with irradiated). The data for the 30th day of post-irradiation were: (5.60 ± 0.09) control, (6.48 ± 0.15) irradiated (P < 0.01, n = 12), and 5.99 ± 0.09, (P < 0.01 n = 8, comparison with irradiated) irradiated + chelerythrine

6. *Experiments on chemically permeabilized vascular strips from control and irradiated animals.* Although there exists many mechanisms by which irradiation could increase vascular sensitivity, the two most probable are alterations in calcium metabolism in response to stimulation or a direct effect on the contractile proteins. One of the best models to use to differentiate between these two possibilities is the chemically permeabilized strip. This preparation allows one to examine the contractile response under conditions of constant intracellular $[Ca^{2+}]_i$. The data shown in Figures 10 and 11 were obtained from experiments performed on β-escin permeabilized aortic strips. All forces shown were normalized to the maximal

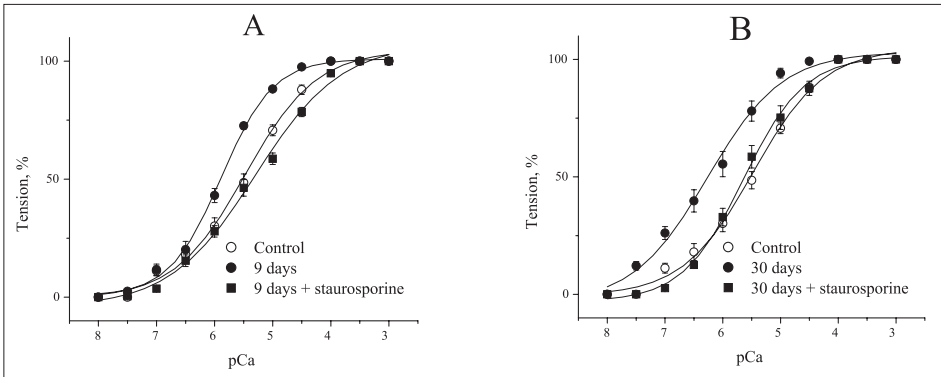

Figure 11. Force development in β-escin permeabilized rat aortic rings as a function of calcium concentration in the presence and absence of staurosporine. Aortic rings from control (○) and irradiated (●) animals were subjected to the cumulative addition of calcium. Irradiated animals were studied on the 9th (A) and 30th (B) days of post-irradiation period after exposure γ-radiation (6 Gy). In addition, the calcium response of irradiated tissues was examined in the presence of 0.1 μM staurosporine (■). The values are means ± SE. Data are normalized to force developed at pCa 5.0. Curves shown were fit to the Hill equation. See Fig. 1 for details. Mean value of pD_2 (or pCa_{50} as generally accepted for permeabilized smooth muscle) for the 9th day were: 5.60 ± 0.09 control; (5.98 ± 0.06) irradiated (P < 0.01, n =12), and (5.30 ± 0.10) irradiated + chelerythrine (P < 0.05, n =6, comparison with irradiated). The data for the 30th day of post-irradiation were: (5.60 ± 0.09) control, (6.48 ± 0.15) irradiated (P < 0.01, n = 9), and (5.53 ± 0.08) irradiated + chelerythrine (P < 0.05, n = 7, comparison with irradiated)

Ca^{2+}-dependent force attained in each vascular preparation. The results shown in these figures clearly demonstrate that on the 9th and 30th days of post-irradiation period a significant shift to the left in the calcium-tension relationship was seen denoting an increase in myofilament calcium sensitivity. For instance, on the 9th day after a single whole body γ-irradiation, an increased myofilament Ca^{2+} sensitivity of (0.38 ± 0.09) pCa units was observed in the series of experiments performed to generate Figure 10 and Figure 11.

In an attempt to uncover potential mechanism(s) responsible for the γ-irradiation induced increase in myofilament Ca^{2+} sensitivity, we performed experiments using inhibitors of PKC. Figure 10 shows the results of experiments using chelerythrine (10^{-6} M) and Figure 11 shows the results of experiments using staurosporine (10^{-7} M). Both inhibitors reversed the increase in myofilament Ca^{2+} sensitivity induced by single whole body γ-irradiation on the 9th and 30th days of post-irradiation period. For instance, on the 9th day post-irradiation chelerythrine shifted the Ca^{2+} concentration response curve (0.65 ± 0.10) μM to the right whereas staurosporine shifted the curve (0.68 ± 0.08) μM to the right. Of particular interest was the total lack of effect of the PKC inhibitors on the calcium responsiveness of permeabilized aortic tissues from control animals. This is an especially important finding given the potential for non-specific inhibition of other kinases by these inhibitors, such as the MLC kinase.

Phorbol dibutirate (PDB, 10^{-7} M), a potent PKC activator, shifted the pCa-tension response relationship for skinned smooth muscles to the left in a healthy tissues and was without effect in irradiated thoracic aorta rings (Figure 12).

7. *Simultaneous measurements of contractile force and $[Ca^{2+}]_i$.* The next series of experiments were designed to simultaneously measure stimulation-induced changes in intracellular calcium concentration and force in the intact aortic tissue from control and irradiated animals. It is important to note that in these experiments the endothelial cells were mechanically removed from the area in which fura-2 calcium fluorescence was measured. Therefore, fluorescent changes were derived specifically from the vascular smooth muscle cells and not influenced by any potential changes in the endothelium. The goal of these experiments was to determine if the irradiation induced change in myofilament calcium sensitivity demonstrated in the permeabilized preparation could also be demonstrated in an intact tissue where calcium is mobilized by normal physiological pathways.

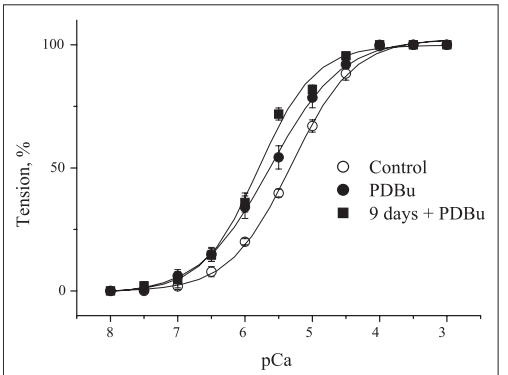

Figure 12. Effect of phorbol dibutirate (PDB, 10^{-7} M) on force development in healthy and irradiated β-escin permeabilized rat aortic rings as a function of calcium concentration. ○ – control, ● – PDB-treated healthy tissues, ■ – PDB-treated irradiated tissues. Mean value of pD_2 (or pCa_{50} as generally accepted for permeabilized smooth muscle) for the 9th day of post-irradiation were: (5.3 ± 0.03) control, (5.63 ± 0.11) PDB administration ($P < 0.05$, $n = 6$), and (5.81 ± 0.04) irradiated + PDB ($P < 0.01$, $n = 6$, comparison with irradiated)

Intact aortic preparations were stimulated with 60 mM KCl and the increase in force and preceding increase in $[Ca^{2+}]_i$ measured simultaneously. Representative tracings from such experiments are shown in Figure 13. Membrane depolarization by 60 mM KCl produced a slightly smaller increase in $[Ca^{2+}]_i$ in aorta from irradiated as compared to that from control animals. In contrast, aorta from irradiated animals produced significantly more force than tissues from control animals.

The compilation of several simultaneous force and calcium measurements are shown in Figure 14. As expected, a positive correlation exists between force developed at different KCl concentrations in the organ bath and $[Ca^{2+}]_i$. Similar to

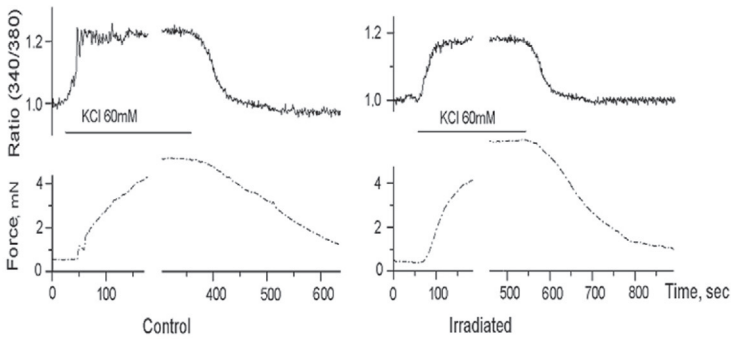

Figure 13. Representative recordings of the effect of irradiation (6 Gy) on KCl-induced increase in intracellular free Ca^{2+} concentration ($R_{340/380}$) and tension in intact rings of rat thoracic aorta. KCl-mediated muscle contraction (lower panels) and increment in ($R_{340/380}$) (upper panels) were measured simultaneously in the same aortic ring. Aortic rings from both animal groups produced similar increases in cellular $[Ca^{2+}]_i$ in response to 60 mM KCl. Aortic rings from irradiated animals produced significantly more force in response to 60 mM KCl as compared to rings from control animals

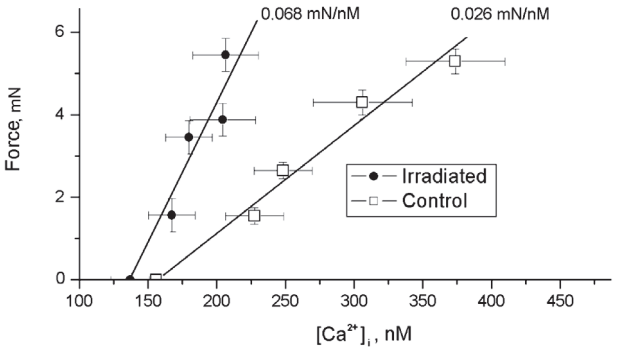

Figure 14. Effect of irradiation (6 Gy) on the KCl-mediated $[Ca^{2+}]_i$-tension relationship in the thoracic aorta. Several experiments of the type shown in Figure 4 were used to develop the $[Ca^{2+}]_i$-tension relationship shown in this figure. Rings loaded with Fura-2 were subjected to the cumulative addition of KCl and levels of force and the simultaneous measurement of $[Ca^{2+}]i$ recorded. The results are shown as (□ : control) and (■ : irradiated). The slope (mN tension/nM $[Ca^{2+}]i$) was significantly increased in irradiated tissues as compared to control, indicating that Ca^{2+} sensitivity of the myofilaments had increased

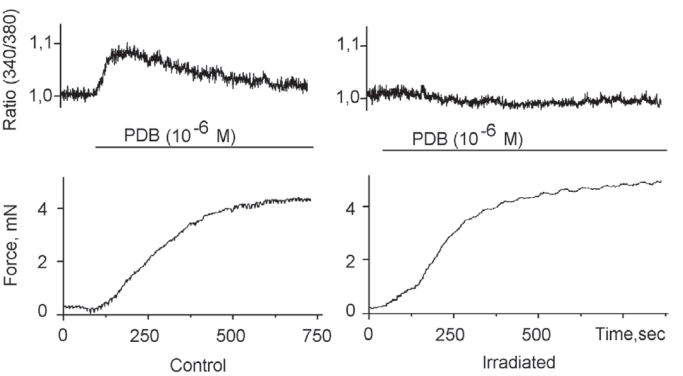

Figure 15. Typical recordings of effect of phorbol dibutirate (PDB, 10^{-6} M) on intracellular calcium concentration ($R_{340/380}$) and contractile force development in both healthy and irradiated thoracic aorta tissues. Force and $R_{340/380}$ (ratio of F_{340} to F_{380}) were measured in the same aortic ring

the results obtained from the permeabilized arterial preparations, greater levels of force are developed at any given $[Ca^{2+}]_i$ in tissues from irradiated animals as compared to those from control animals. Aortic rings obtained from irradiated animals produced 0.068 mN force/nM Ca^{2+} whereas arteries from control animals produced 0.026 mN force/nM Ca^{2+}.

Stimulation of intact aortic smooth muscles with PDB (10^{-6} M) produced a sustained progressively developing tonic contraction (it took 26–28 min before a maximal tension development was established while an increment in $[Ca^{2+}]_i$ was transient (Figure 15 A). In contrast, in irradiated tissues (Figure 15 B) the amplitude of PDB-induced contraction was higher than that seen in intact tissues and there was no any elevation in $[Ca^{2+}]_i$.

Discussion

Using RNA-interference (RNAi) phenomenon and physiological techniques we tried to identify the role and the place of BK_{Ca} channels in vascular tone maintenance following irradiation. The main finding of this study is radiation-induced suppression of the BK_{Ca} channels form and function accompanied with compensatory increment in mRNA expression and activity of other members of potassium channels family in irradiated vascular tissues. Another interesting phenomenon is the absence of significant increment in L-type Ca^{2+} channels mRNA expression in siRNAs knock-down rats as compared to irradiated rats that demonstrated an increased level of mRNA expression for this type of ionic channels.

It is well known that force development in VSM is closely coupled to membrane potential, which, in turn, is determined by potassium channels activity. The large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) is the predominant K^+ channel species in most arteries and is known to play an essential role in vascular function via its involvement in changes of VSM membrane polarisation [9, 19–21]. BK_{Ca} channels differ from all other K^+ channels due to their high sensitivity to $[Ca^{2+}]_i$ and voltage. Inhibition of BK_{Ca} produced 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, diabetes and atherosclerosis complications [22, 23].

Our preliminary data clearly indicate that BK_{Ca} appear to be the most vulnerable member of potassium channel family involved in radiation-induced arterial hypertension development [8]. The data obtained in this study indicate that KV and K_{ATP} channels are likely to be resistant to oxidative stress, and this way may constitute a reserve mechanism for the maintenance of tissue blood supply under radiation-induced BK_{Ca} channelopathy.

The data presented by Zhang and Bolton [24] suggest the existence of two type of ATP-sensitive potassium channels, one of them (termed as MK channel) was relatively insensitive to ATP in inside-out patch experiments using rat portal vein SMCs. Generally accepted that glibenclamide is widely used for K_{ATP} channels separation and identification. It has been shown also [18] that so called glibenclamid-sensitive K^+ channels exist in rat thoracic aorta myocytes and appear to have an important role in maintenance of the resting membrane potential while classical K_{ATP} channels are closed at normal intracellular ATP concentration, and play a little role in the maintenance of membrane potential.

The situation with possible similarity and/or difference between classical K_{ATP} and glibenclamide-sensitive K^+ channels, termed also as MK channels, remains yet puzzling. Nevertheless, taking into account that the role of K_V channels in aortic SMCs membrane potential and tone regulation is negligible under basal condition (near a membrane potential of -60 mV), we have studied glibenclamide-sensitive members of potassium channels family before and after irradiation. The data obtained clearly demonstrated that irradiation led to significant increase in current carrying through glibenclamide-sensitive potassium channels, indicating existing compensatory component of outward current in SMCs under irradiation.

It is likely that relative role of this compensatory mechanisms in irradiated vascular tissues is insufficient to overcome an inhibitory effect of irradiation on vasodilator potential of vascular wall. The aim of future pharmacological interventions under irradiation is to intensify the expression of this mechanism.

The main reason to provide this study were the data in which we have observed a significant and sustained elevation in arterial blood pressure in irradiated rats [1]. The clinical studies postulated also that the most serious health problem in the population exposed to radiation following Chernobyl disaster was diseases of cardiovascular system and hypertension especially [25] but underlie mechanisms of this phenomenon remained unknown. Later we have shown that radiation impact inhibits endothelium-dependent relaxation in irradiated blood vessels [4] and decreases both BK_{Ca} current density and the level of expression in α- and β_1- subunit that composed BK_{Ca} channel [8], i.e. it is became clear that irradiation leads to decrease in vasodilator potential of vascular wall.

The similar radiation-induced reduction in BK_{Ca} activity that are important in regulating the driving force for calcium entry and therefore for regulation of NO synthesis we observed earlier in endothelial cells [7]. Nevertheless, the question yet remains whether radiation-induced BK_{Ca} channelopathy in endothelial and smooth muscle cells really contributes to radiation-induced vascular hypercontractility and related arterial hypertension development.

Discovery of the RNAi phenomenon let us an instrument to test this hypothesis. RNAi using small interfering RNAs (siRNAs, a double stranded RNA molecule having 21–23 bp) has recently provided a powerful tool for silencing a target gene by means highly sequence-specific degradation of mRNA [11, 26, 27]. It is likely that this technique represents an ideal instrument to dissect the function of different splice variants of ion channel encoding genes.

We clearly understand that delivery of siRNAs to target tissues is a serious problem since the siRNAs must reach the target organ and must enter to the cytoplasm of target cells. Well known that unmodified, naked siRNAs are relatively unstable in serum, as they rapidly degraded by endo- and exonucleases. Nevertheless, the review presented [16] clearly indicates that naked siRNA in many studies demonstrate significant RNA-interference effect. On the other hands, chemical or other type modification of siRNA can increase stability of these molecules *in vitro*, but can not demonstrate an increment in their efficacy *in vivo*. The main evidence of the siRNAs specific effectiveness in our experiments is the significant difference in the ionic channels mRNA expression in vascular tissues of the animals that obtained specific siRNAs as compared to the rats that were treated with scr-siRNAs.

Thus, to provide evidence of BK_{Ca} involvement into radiation-induced vascular malfunctions, we used the siRNAs ability to downregulate specific BK_{Ca} mRNA transcript expression. RT-PCR experiments clearly indicated that siRNAs to KCNMA1 gene being administered to experimental animals led to 3-fold decrease in BK_{Ca} α-subunit mRNA level in aortic SMC as compared to tissues obtained from both control rats and the rats treated with a scr-siRNAs. The similar effects on BK_{Ca} mRNA expression in vascular tissues were seen in irradiated animals.

These data tightly correlate to inhibition of outward potassium currents carried through BK_{Ca} channels in rat thoracic aorta SMCs treated with siRNAs and this effect of siRNAs administration, in turn, is a very similar to radiation-induced inhibition in BK_{Ca} activity.

Experiments with a standard acetylcholine (Ach) test clearly demonstrated that the silencing of KCNMA1 gene led to decrease in amplitude of maximal Ach-induced relaxation in rat thoracic aortas and accompanied with increased contractile responses of denuded vascular rings to norepinephrine in a radiation-like manner. It is known that BK_{Ca} indirectly involved in nitric oxide production in endothelial cells and they are very important for valuable endothelium-dependent relaxation.

The data obtained [7] demonstrate radiation-induced suppression of BK_{Ca} in endothelial cells which, in turns, leads to the shift of reversal potential toward more positive values. It means that irradiated endothelial cells are depolarized as compared to non-irradiated cells. It produces a significant reduction in the driving force for Ca^{2+}, resulting in a decrease in NO synthesis, and producing a blunted endothelium-dependent relaxation.

Having established that the silencing of KCNMA1 gene in endothelial cells and SMCs significantly suppressed vasodilator potential of vascular wall, i.e. decreased endothelium-dependent vasorelaxation with concomitant increment in endothelium-independent vascular contractility, we next investigated *in vivo* the effect of KCNMA1 gene silencing on arterial blood pressure in comparison with irradiated animals.

Surprisingly, but we have failed in our attempts to induce sustained and significant elevation of arterial blood pressure in the group of rats treated with siRNAs targeted to KCNMA1 gene. Nevertheless, some of the rats with silencing KCNMA1 gene demonstrated pronounced increase in arterial blood pressure. The data suggest that BK_{Ca} channelopathy may be involved, at least partially, not only to radiation-induced local vascular malfunctions but to generalized vasospasm development although it is not the main reason for triggering of arterial hypertension.

The most likely that the lack of hypertensive effect of KCNMA1 gene silencing is due to the absence of L-type Ca^{2+} channels over expression in vascular tissues following siRNAs administration in contrast to that we seen after irradiation.

The next possible explanation for this surprising discovery might be the absence of BK_{Ca} in rat myocardium. As mentioned by some authors [19, 28, 29] BK_{Ca} channel ubiquitous and present in virtually all tissues except in myocardium. However, the problem appears to be more complex than meets the eye. For instance, Hu et al. [30] and Wang et al. [31] postulated the presence of BK_{Ca} in the cardiac inner mitochondrial membrane, and the data presented by Imlach et al. [32] indicate that BK_{Ca} channels located in mice and rats heart are involved in the regulation of heart rate.

Taking into account that BK_{Ca} are comprised of a α- and β_1-subunits we produced selective siRNAs-induced α-BK_{Ca} gene silencing. It is possible to suggest that selective loss of BK_{Ca} α-subunit is not sufficient to cause sustained hypertension. Xu et al. [33] studied BK_{Ca} β_1-subunit knocked out mice and their results showed that these animals are not hypertensive although this type of BK_{Ca} deficiency increased reactivity of mesenteric arteries to norepinephrine. Another group of investigators [34] concluded that the hypertension associated with BK_{Ca} β_1 knockout occurs because of enhanced fluid retention due to BK_{Ca} β_1 localization in adrenal glands where it controls aldosteron production and in less degree because of vascular dysfunction.

In intermediate conclusion, KCNMA1 gene silencing in rats alters the form and function of the BK_{Ca} channel similar to irradiation, and this type of ionic channels may contribute to related vascular abnormalities. Nevertheless, it is unlikely that BK_{Ca} can operate as a crucial factor for radiation-induced arterial hypertension. It is clear that the underlying mechanism producing the alterations of vascular function under irradiation is multifaceted and trigger mechanics leading to hypertension development under irradiation involve both multiple vascular and cardiac functional changes.

The results in the present study also clearly demonstrate that single whole body γ-irradiation significantly increases the myofilament calcium sensitivity of the vascular smooth muscle cell independent of any action on the endothelium. Moreover, our results strongly suggest that the mechanism by which irradiation increases myofilament calcium sensitivity is through a PKC dependent pathway.

In the present study we examined the relationship between Ca^{2+} and tension development in vascular smooth muscle using both permeabilized and intact preparations, where $[Ca^{2+}]_i$ can either be directly controlled or directly measured, respectively. Both approaches have dis-

tinct advantages as well as disadvantages. Chemical permeabilization of the cell membrane has been commonly used to estimate the sensitivity of myofilaments to Ca^{2+} in a variety of smooth muscle preparations. This preparation allows the precise control of the intracellular environment however there is also the potential for the loss of important regulatory elements within the cell. The simultaneous measurement of $[Ca^{2+}]_i$ and tension in the intact tissue allows one to determine directly the relationship between the two parameters during normal cellular calcium handling. Therefore this approach allows estimation of myofilament Ca^{2+} sensitivity in smooth muscle with unimpaired excitation-contraction coupling processes and retained regulatory targets for second messenger pathways. On the other hand, intracellular events cannot be controlled and small localized changes in calcium may not be measured. Combining the two preparations allows for the measurement of force during both the controlled and physiological changes in calcium concentration.

Alterations in myofilament calcium sensitivity have been shown to underlie, at least in part, the changes in smooth muscle function that occur in response to hypertension, asthma, benign prostatic hyperplasia and now based on our results, in response to γ-irradiation. The question then becomes, what are the mechanisms underlying the increase in myofilament calcium sensitivity that we observed following radiation exposure? Myofilament calcium sensitivity may be altered by influencing the enzymes that initiate or modulate actin and myosin interactions or by direct actions on the contractile proteins. Although theoretically possible, the second suggestion is not supported by experimental evidence. The first possibility is supported by a vast amount of literature [35]. Two pathways have been proposed for the enhancement of myofilament calcium sensitivity in smooth muscle. The first is the G-protein dependent activation of Rho kinase, which phosphorylates the MLC phosphatase resulting in a decrease in activity. The second is a PKC dependent pathway whereby PKC phosphorylates and activates a MLC phosphatase inhibitor, CPI-17. The endpoint for both pathways is down-regulation of the MLC phosphatase and therefore an increase in MLC phosphorylation levels at any given $[Ca^{2+}]_i$.

Our results would suggest that exposure to γ-irradiation increases myofilament calcium sensitivity by augmenting the PKC pathway. This is based on the findings that the increase in calcium sensitivity in vessels from irradiated animals was reversed by either staurosporine or chelerythrine, and PKC activator, phorbol diester had no effect on pCa-force relationship in irradiated tissue while shifted this one to the left in healthy aortic rings. It is important to point out again that neither antagonist had any effect on the calcium sensitivity of vessels from control animals. Thus, non-specific actions of these PKC inhibitors were apparently not an issue in this study. Moreover, the calcium concentration curves in the permeabilized tissues were performed in the absence of GTP and activation of the intact tissues was in response to membrane depolarization. Neither stimulation regimen would be expected to significantly activate G-proteins. Importantly, PKC has been shown to be activated in KCl stimulated intact vascular smooth muscle [36] and PKC is retained and can be activated in permeabilized vascular tissues [37].

Simultaneous measurements of $[Ca^{2+}]_i$. and force support this point of view. The amplitude of PDB-induced contraction in irradiated tissues was, at least, not smaller or even greater than in intact preparations while not accompanied with an increment in $[Ca^{2+}]_i$. suggesting that myofilament calcium sensitivity had increased mainly due to high PKC activity.

If, as we speculate, PKC activity is enhanced following irradiation, then are there potential mechanisms that could produce this end result? It is well known that following irradiation, the content of oxygen derived free radicals, including superoxide anion, hydroxyl anions, and others, increases significantly in living cells and biological liq-

uids. These reactive oxygen species are associated with radiation-induced cytotoxicity and are responsible for radiation-induced lipid peroxidation and related vascular lesions. We therefore hypothesize that irradiation induces a state of oxidative injury on vascular tissue. A possible role for oxidative stress in radiation-induced vascular dysfunction is supported by the protective effect of the antioxidant, α-tocopherol in rabbit thoracic aorta [4]. α-Tocopherol was shown to prevent the loss of endothelium-dependent relaxation when administered as a single dose shortly after radiation exposure. Nevertheless, this problem remains yet unsolved and takes more convincing evidence.

Radiation has been shown to have a direct effect on altering the expression of PKC. Radiation therapy dose-dependently increased PKC in mouse lymphocytes [38]. Interestingly, differential responses were found in the radiation induced expression of α, δ, ζ isoforms of PKC. In addition, it is known that radiation induces the translocation of PKC from the cytosol to the membrane as a result of reactive oxygen formation producing membrane lipid peroxidation [39].

Thus radiation not only increases the expression of specific PKC isoforms but also the activity of PKC mediated by reactive oxygen species. These findings are relevant to our present study as the α isoform of PKC has been implicated in calcium dependent PKC induced contractions of smooth muscle and the novel PKC isoform ε of which ζ is also a member has been implicated in calcium-independent PKC induced contractions.

It should be born in mind that vascular dysfunction including an increased PKC or Rho − kinase activities under irradiation is mediated by multiple mechanisms and maybe, at least partially, related to changes in NO metabolism. It is well known that phorbol esters may inhibit NO release, and thus an elevation of PKC activity might interfere with NO synthesis. In turn, it is likely that NO induces rat aorta dilatation via inhibition of Rho-kinase. Just recently it has been shown [41] that NO possesses the ability to relax smooth muscle via protein phophatases activation. It known that NO-generating agents may decrease phosphotransferase activity of purified PKC with IC_{50} $7.5 \cdot 10^{-5}$ M and this effect was blocked by NO scavenger oxyhemoglobin and reversed by dithiothreitol [41]. In case when NO-dependent component of relaxation is abolished resulting irradiation, PKC activity in vascular wall could be elevated and contractile force development related to increased myofilament calcium sensitivity might be increased too. At the same time experiments on skinned smooth muscle clearly demonstrate that direct effect of radiation on PKC-related mechanism of calcium myofilament sensitivity take place.

Therefore, we propose the hypothesis that whole body γ-irradiation results in the production of reactive oxygen species, which alters vascular function by an effect on both the endothelial layer and the smooth muscle cell. In the smooth muscle cell, specific isoforms of PKC are up regulated and activated, which produces an apparent increase in myofilament calcium sensitivity and BK_{Ca} expression and function. The combination of decreased NO release from damaged endothelial cells and hypercontractility of the vascular smooth muscle cells act in concert to produce increases in arterial blood pressure and vasospasm in response to normally occurring levels of endogenous agents.

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A. I. Soloviev

The BK_{Ca} channels deficiency and high protein kinase C activity as a main reasons for radiation-induced vascular hypercontractility

It is likely that large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels channelopathy tightly involved in vascular malfunctions and arterial hypertension development. In the present study, we compared the results of siRNAs-induced α-BK_{Ca} gene silencing and vascular abnormalities produced by whole-body ionized irradiation in rats. The experimental design comprised RT-PCR and patch clamp technique, thoracic aorta smooth muscle (SM) contractile recordings using intact and chemically permeabilized rat thoracic aortic rings combined with cytosolic Ca^{2+} ($[Ca^{2+}]_i$) measurements, arterial blood pressure (BP) measurements on the 30th day after whole body irradiation (6 Gy) and following siRNAs KCNMA1 gene silencing *in vivo*. The expression profile of BK_{Ca} mRNA transcripts in SM was significantly decreased in siRNAs-treated rats in a manner similar to irradiated SM. In contrast, the mRNA levels of K_v and K_{ATP} were significantly increased while L-type calcium channels mRNA transcripts demonstrated tendency to increment. The SMC obtained from irradiated animals and after KCNMA1 gene silencing showed a significant decrease in total K^+ current density amplitude. Paxilline (500 nM)-sensitive components of outward current were significantly decreased in both irradiated and gene silencing SMC. KCNMA1 gene silencing increased SM sensitivity to norepinephrine while Ach-induced relaxation had decreased. The silencing of KCNMA1 had no significant effect on BP while radiation produced sustained arterial hypertension. Therefore, radiation alters the form and function of the BK_{Ca} channel and this type of channelopathy may contribute to related vascular abnormalities. Nevertheless, it is unlikely that BK_{Ca} can operate as a crucial factor for radiation-induced arterial hypertension.

Irradiation significantly shifted pCa-tension in β-escin permeabilized smooth muscle on the 9th and 30th days post-irradiation to the left suggesting that myofilament Ca^{2+} sensitivity had increased. Inhibitors of protein kinase C (PKC), chelerythrine and staurosporine, had no effect on the pCa-tension curves in control permeabilized tissues but significantly shifted the curves to the right in permeabilized tissues on the 9th and 30th days post-irradiation. Phorbol dibutirate (PDB, 10^{-7} M), a potent PKC activator, shifted the pCa-tension response relationship for skinned smooth muscles to the left in a healthy tissues and was without effect in irradiated vascular rings. Simultaneous measurements of contractile force and $[Ca^{2+}]_i$ showed that the coefficient of myofilament Ca^{2+} sensitivity defined as a ratio of force change to $\Delta[Ca^{2+}]_i$ significantly increased following γ-irradiation. Stimulation of intact aortic smooth muscles with PDB (10^{-6} M) produced a sustained progressively developing tonic contraction while an increment in $[Ca^{2+}]_i$ was transient. In contrast, in irradiated tissues the amplitude of PDB-induced contraction was even higher than that seen in intact tissues and there was no any elevation in $[Ca^{2+}]_i$. These data strongly support the hypothesis that the sensitivity of vascular smooth muscle myofilaments to Ca^{2+} is increased following irradiation and this effect is dependent also on activation of PKC.

Therefore, we propose the hypothesis that whole body γ-irradiation results in the production of reactive oxygen species, which alters vascular function by an effect on both the endothelial layer and the smooth muscle cell. In the smooth muscle cell, specific isoforms of PKC are up regulated and activated, which produces an apparent increase in myofilament calcium sensitivity and BK_{Ca} expression and function. The combination of decreased NO release from damaged endothelial cells and hypercontractility of the vascular smooth muscle cells act in concert to produce increases in arterial blood pressure and vasospasm in response to normally occurring levels of endogenous agents.

Key words: smooth muscle contractility, RNA interference, BK_{Ca} channels, irradiation, calcium sensitivity, contractile proteins, hypertension

А. І. Соловйов

Порушення функції Ca^{2+}-залежних калієвих каналів великої провідності та висока активність протеїнкінази С – головні причини розвитку гіперскоротливості судин, викликаної впливом радіації

Цілком ймовірно, що порушення провідності кальцій-активованих калієвих каналів великої провідності (BK_{Ca}) тісно пов'язане з розвитком судинних порушень і розвитком артеріальної гіпертензії. У цьому дослідженні ми порівняли результати глушіння гена, що кодує експресію BK_{Ca} у щурів, за допомогою техніки РНК-інтерференції з судинними порушеннями, викликаними впливом іонізуючої радіації в дозі 6 Грей. В експериментах використовували методи RT-PCR та patch-clamp, реєстрацію скоротливої активності гладеньких м'язів (SM) з використанням інтактних і хімічно скінірованих аортальних кілець з одночасним виміром внутрішньоклітинної концентрації кальцію $[Ca^{2+}]_i$, вимірювання артеріального тиску (АТ) на 30-й день після опромінення. Профіль експресії транскриптів мРНК BK_{Ca} в SM був значно знижений у щурів, які отримували siRNAs і опромінених тварин. Навпаки, рівні мРНК K_v і K_{ATP} були значно збільшені, а транскрипти мРНК кальцієвих каналів L-типу продемонстрували тенденцію навіть до збільшення. Клітини SM, отримані від опромінених тварин і після глушіння гена KCNMA1, показали значне зменшення загальної амплітуди щільності вихідного струму K^+. У разі впливу паксилліну (блокатор BK_{Ca}) – чутливі компоненти вихідного струму значно зменшувалися як за опромінення, так і в разі глушіння гена KCNMA1. Глушіння гена KCNMA1 збільшило чутливість SM до норадреналіну, у той час як амплітуда ацетилхолін-індукованого розслаблення SM зменшилася. Глушіння KCNMA1 суттєво не впливало на рівень АТ, у той час як радіація викликала стійку артеріальну гіпертензію. Отже, випромінювання змінює експресію та функцію каналу BK_{Ca}, і цей тип каналопатій може сприяти виникненню судинних аномалій. Проте малоймовірно, що BK_{Ca} каналопатія може бути єдиним і вирішальним фактором розвитку пострадіаційної артеріальної гіпертензії.

Опромінення зміщує криву pCa-напруга в гладенькому м'язі на 9-й і 30-й дні після опромінення вліво, що свідчить про підвищення Ca^{2+}-чутливості міофіламентів. Інгібітори протеїнкінази С (PKC), хелеритрин і стауроспорин не впливали на криві pCa-напруга в контрольних скінірованих SM, але значно зрушили криві вправо на 9-й і 30-й дні після опромінення. Форбол дибутират (PDB, 10^{-7} моль/л), потужний активатор PKC, змістив криву pCa-напруга вліво в здорових тканинах і не впливав на опромінені судинні кільця. Одночасні вимірювання скорочувальної сили і $[Ca^{2+}]_i$ показали, що коефіцієнт Ca^{2+} – чутливості скорочувальних білків, який визначається як відношення приросту сили до змін $[Ca^{2+}]_i$, значно збільшується після опромінення. Стимуляція інтактних SM аорти PDB (10^{-6} моль/л) викликала стійке прогресуюче тонічне скорочення, в той час як приріст в $[Ca^{2+}]_i$ був короткочасним. Навпаки, в опромінених тканинах амплітуда індукованого PDB скорочення була навіть вище, ніж в інтактних. У цьому разі не було ніякого підвищення в $[Ca^{2+}]_i$. Ці дані підтверджують гіпотезу про те, що після опромінення збільшується чутливість міофіламентів SM судин до Ca^{2+}, і цей ефект обумовлений активацією PKC.

Можна вважати, що γ-опромінення призводить до утворення реактивних форм кисню й активації PKC. Це призводить до збільшення кальцієвої чутливості міофіламентів і зниження експресії та функції BK_{Ca}. Усе це разом узяте здатне призвести до гіперскоротливості клітин SM судин і викликати розвиток вазоспазму та збільшення АТ.

Ключові слова: скоротливість гладеньких м'язів, РНК-інтерференція, BK_{Ca} канали, опромінення, кальцієва чутливість, скорочувальні білки, гіпертензія

А. И. Соловьев

Нарушение функции Ca^{2+}-зависимых калиевых каналов большой проводимости и высокая активность протеинкиназы С – главные причины развития гиперсократимости сосудов, вызванной воздействием радиации

Вполне вероятно, что нарушение проводимости кальций-активируемых калиевых каналов большой проводимости (BK_{Ca}) тесно связано с развитием сосудистых нарушений и развитием артериальной гипертензии. В настоящем исследовании мы сравнили результаты глушения с помощью техники РНК-интерференции гена, кодирующего экспрессию BK_{Ca} у крыс, с сосудистыми нарушениями, вызванными воздействием ионизирующей радиации в дозе 6 Грей. В экспериментах использовали методы RT-PCR и patch-clamp, регистрацию сократительной активности гладких

мышц (SM) с использованием интактных и химически скинированных аортальных колец с одновременным измерением внутриклеточной концентрации кальция $[Ca^{2+}]_i$, измерение артериального давления (АД) на 30-й день после облучения. Профиль экспрессии транскриптов мРНК BK_{Ca} в SM был значительно снижен у крыс, получавших siRNAs и облученных животных. Напротив, уровни мРНК K_v и K_{ATP} были значительно увеличены, а транскрипты мРНК кальциевых каналов L-типа продемонстрировали тенденцию даже к увеличению. Клетки SM, полученные от облученных животных и после глушения гена KCNMA1, показали значительное уменьшение общей амплитуды плотности выходящего тока K^+. При действии паксиллина (блокатора BK_{Ca}) чувствительные к нему компоненты выходящего тока значительно уменьшались как при облучении, так и при подавлении гена KCNMA1. Глушение гена KCNMA1 увеличило чувствительность SM к норэпинефрину, в то время как амплитуда ацетилхолин-индуцированного расслабления SM уменьшилась. Глушение KCNMA1 не оказало существенного влияния на уровень АД, в то время как радиация вызывала устойчивую артериальную гипертензию. Следовательно, излучение изменяет экспрессию и функцию канала BK_{Ca}, и этот тип каналопатии может способствовать возникновению сосудистых аномалий. Тем не менее, маловероятно, что BK_{Ca} каналопатия может действовать как единственный и решающий фактор развития пострадиационной артериальной гипертензии.

Облучение сдвигало кривые pCa-напряжение в SM на 9-й и 30-й дни после облучения влево, что свидетельствует о повышении Ca^{2+}-чувствительности миофиламентов. Ингибиторы протеинкиназы С (PKC), хелеритрин и стауроспорин не влияли на кривые pCa-напряжение в контрольных скинированных SM, но значительно сдвинули кривые вправо на 9-й и 30-й дни после облучения. Форбол дибутират (PDB, 10^{-7} моль/л), мощный активатор PKC, сдвинул отношение pCa-напряжение влево в здоровых тканях и не оказывал влияния на облученные сосудистые кольца. Одновременные измерения сократительной силы и $[Ca^{2+}]_i$ показали, что коэффициент Ca^{2+}-чувствительности сократительных белков, определяемый как отношение прироста силы к изменениям $[Ca^{2+}]_i$, значительно увеличивается после облучения. Стимуляция интактных SM аорты PDB (10^{-6} моль/л) вызывала устойчивое прогрессирующее тоническое сокращение, в то время как приращение в $[Ca^{2+}]_i$ было транзиторным. Напротив, в облученных тканях амплитуда индуцированного PDB сокращения была даже выше, чем в интактных тканях. При этом не было никакого повышения $[Ca^{2+}]_i$. Эти данные подтверждают гипотезу о том, что после облучения увеличивается чувствительность миофиламентов SM сосудов к Ca^{2+}, и этот эффект обусловлен активацией PKC.

Можно полагать, что γ-облучение приводит к образованию реактивных форм кислорода и активации PKC. Это приводит к увеличению кальциевой чувствительности миофиламентов и снижению экспрессии и функции BK_{Ca}. Все это вместе взятое способно привести к гиперсократимости клеток SM сосудов и вызвать развитие вазоспазма и увеличение АД.

Ключевые слова: сократимость гладких мышц, РНК-интерференция, BK_{Ca}-каналы, облучение, кальциевая чувствительность, сократительные белки, гипертензия

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