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Cardioprotective effect of 5-lipoxygenase gene (*ALOX5*) silencing in ischemia-reperfusion

Oleksandr O. Lisovyy, Victor E. Dosenko, Vasyl S. Nagibin✉, Lesya V. Tumanovska, Maria O. Korol, Olga V. Surova and Olexiy O. Moibenko

Department of General and Molecular Pathophysiology, Bogomoletz Institute of Physiology, Kiev, Ukraine

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It is well known that 5-lipoxygenase derivates of arachidonic acid play an important pathogenic role during myocardial infarction. Therefore, the gene encoding arachidonate 5-lipoxygenase (*ALOX5*) appears to be an attractive target for RNA interference (RNAi) application. In experiments on cultivated cardiomyocytes with anoxia-reoxygenation (AR) and *in vivo* using rat model of heart ischemia-reperfusion (IR) we determined influence of *ALOX5* silencing on myocardial cell death. *ALOX5* silencing was quantified using real-time PCR, semi-quantitative PCR, and evaluation of LTC_4 concentration in cardiac tissue. A 4.7-fold decrease of *ALOX5* expression ($P<0.05$) was observed in isolated cardiomyocytes together with a reduced number of necrotic cardiomyocytes ($P<0.05$), increased number live ($P<0.05$) and unchanged number of apoptotic cells during AR of cardiomyocytes. Downregulation of *ALOX5* expression in myocardial tissue by 19% ($P<0.05$) resulted in a 3.8-fold reduction of infarct size in an open chest rat model of heart IR ($P<0.05$). Thus, RNAi targeting of *ALOX5* protects heart cells against IR injury both in culture and *in vivo*.

Keywords: *ALOX5*, RNA interference, cardiomyocytes, ischemia-reperfusion

INTRODUCTION

Lipoxygenases constitute a family of monomeric non-heme, non-sulphur iron dioxygenases that catalyze the conversion of polyunsaturated fatty acids into conjugated hydroperoxides (Brash, 1999; Rådmark, 2002). *ALOX5* gene encodes 5-lipoxygenase (5-LO) mediating conversion of arachidonic acid (AA) to leukotriene A_4 (LTA_4) — a precursor for a variety of biologically active eicosanoids (Murphy & Gij´on, 2007). Biosynthesis of leukotrienes is accompanied by generation of prooxidants such as lipid hydroperoxides (Brash, 1999; Rådmark, 2002; Murphy & Gij´on, 2007). Combined action of eicosanoids and prooxidants determines a pathogenic role of 5-LO during IR of the heart, which is supported by experimental data indicating cardioprotective effects of 5-LO inhibitors (Hashimoto *et al.*, 1990; Kolchin *et al.*, 1990; Rossoni *et al.*, 1996; Parkhomenko *et al.*, 2000; Riccioni *et al.*, 2008).

Despite fairly detailed investigation of 5-LO activity during IR and MI, changes in *ALOX5* gene expression in heart tissue and cardiomyocytes under such conditions have not been studied. However, it is beyond any doubt that understanding of the genetic changes occurring during myocardial ischemia and ventricular remodeling is critical for developing therapies enhancing beneficial changes in gene expression and inhibiting potentially harmful ones. Gene therapy holds great potential for the ischemic heart disease treatment on many levels, including protection against IR injury, influence on cell death mechanisms, stimulation of angiogenesis, etc. Therefore, RNA interference (RNAi) technology, which is one of the most modern and specific methods of targeted gene silencing, holds much promise (Fire *et*

✉Corresponding author: Vasyl S. Nagibin, Department of General and Molecular Pathophysiology, Bogomoletz Institute of Physiology, Bogomoletz str. 4, Kiev 01024, Ukraine; tel.: +38 (044) 256 2481; fax: +38 (044) 256 2073; e-mail: nvasya@ukr.net

Abbreviations: AA, arachidonic acid; ALOX5, arachidonate 5-lipoxygenase; AR, anoxia-reoxygenation; IR, ischemia-reperfusion; LT, leukotriene; MI, myocardial infarction; 5-LO, 5-lipoxygenase; PTP-1B, protein tyrosine phosphatase 1B; RNAi, RNA interference; SHP-1, Src homology domain 2 (SH2)-containing tyrosine phosphatase-1; PBS, phosphate-buffered saline.

al., 1998; Sledz & Williams, 2005; Sugano *et al.*, 2005; Nordlie *et al.*, 2006; Song *et al.*, 2008). As far as we know, RNAi utilization for protection of heart cells against ischemic injury was described only in two published articles (Sugano *et al.*, 2005; Song *et al.*, 2008). Sugano *et al.* (2005) demonstrated that suppression of Src homology domain 2 (SH2)-containing tyrosine phosphatase-1 (SHP-1) with SHP-1-specific small interference RNAs (siRNA) markedly reduced infarct size in a rat *in vivo* model of heart IR (Sugano *et al.*, 2005). In a second paper Song *et al.* (2008) have shown that siRNA's against protein tyrosine phosphatase 1B (PTP-1B) effectively protect cardiomyocytes against hypoxia-reoxygenation injury and inhibit apoptosis of cultivated cardiomyocytes (Song *et al.*, 2008). Despite the effectiveness of cardioprotection achieved in the papers mentioned above it seems reasonable to underline that the choice of genes SHP-1 and PTP-1B was quite unexpected, the pathogenic role of the protein products encoded by those genes in heart IR being practically unstudied.

We have chosen in our study the *ALOX5* gene encoding the enzyme 5-LO as the target for RNAi silencing considering the well-known role of 5-LO in pathogenesis of MI. We found that *ALOX5* gene suppression in culture of isolated rat cardiomyocytes and in heart tissues *in vivo* provides a significant cyto- and cardioprotective effect reducing the ischemic injury range.

MATERIALS AND METHODS

Culture of rat neonatal cardiomyocytes. Neonatal cardiomyocytes were isolated from ventricular myocardium of 2-day-old Wistar rats by enzymatic digestion according to Reinecke *et al.* (1999) with some modifications (Dosenko *et al.*, 2005; 2006). The number of live and necrotic cells was determined by staining with 0.2% trypan blue and was 90–95% and 5–10%, respectively. The resulting cardiomyocytes were plated in culture dishes covered with 2% gelatin at a density of 120 000 per cm^2. Cardiomyocytes were cultivated for 1–2 days in DMEM/medium 199 (4:1), containing 7% calf serum, 4.2 mM Na_2CO_3, 15 mM Hepes and antibiotics (100 g/ml streptomycin, 0.05 mg/ml gentamycin and 100 U/ml penicillin) at 37°C in an atmosphere of 5% CO_2, 20% O_2 and 75% Ar (standard gas mixture).

RNA interference. Double-stranded scrambled (sense 5′-UGU UCA GCG AAA UAU AAC CUU-3′ and antisense 5′-UUA CAA GUC GCU UUA UAU UGG-3′) or *ALOX5*-specific siRNA (sense 5′-GUA CAG GAA GGG AAC AUU UUU-3′ and antisense 5′-UUC AUG UCC UUC CCU UGU AAA-3′) were prepared from corresponding oligonucleotides provided by Metabion (Germany) according to the manufacturer's instructions. Isolated cardiomyocytes were washed with sterile PBS and transfected with 2 μg of siRNA using Rat Cardiomyocytes Neo Nucleofector Kit (VPE-1002, Amaxa, USA) according to the manufacturer's protocol. Immediately after transfection, cells were placed in fresh medium and cultivated for 24 h.

For gene silencing *in vivo* these siRNAs were injected in the tail vein of female Wistar rats (weight 300–340 g) in a dose of 80 μg twice with a 24-hour interval.

Anoxia-reoxygenation. After 24 h of cultivation cells transfected with scrambled or *ALOX5*-specific siRNA were subjected to 30 min of anoxia followed by 60 min of reoxygenation. Anoxia was attained with an airtight jar from which O_2 was flushed with a gas mixture of 5% CO_2 and 95% Ar. Reoxygenation was realized by exchanging fresh medium and by its aeration with the standard gas mixture (Dosenko *et al.*, 2005; 2006).

Cell viability. The number of live, necrotic and apoptotic cells was determined by staining with 8.75 μM bisbenzimide (Hoechst 33342), propidium iodide and examination by fluorescence microscopy. Five-hundred cells were counted in each experiment (Tumanovs'ka *et al.*, 2004).

Caspase-3 assay. After sonication, the remaining unlysed cells, membranes, and nuclei were removed by centrifugation at 5000×*g* for 10 min. The supernatant was incubated in a buffer consisting of 100 mM Hepes (pH 7.4) and 5 mM dithiothreitol. The fluoropeptide substrates *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (DEVD-AMC) and *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoro-methylcoumarin (DEVD-AFC) were used to measure the caspase-3 activity. After a 1-h incubation with 50 μM of these fluorogenic peptides, the fluorescence of the reaction products was monitored on a Hitachi 4000 spectrofluorimeter at 380 nm excitation and 460 nm emission (for DEVD-AMC) and 380/460 (for DEVD-AFC) using free 7-amino-4-methylcoumarin (AMC) as a standard. The reaction was carried out in the absence or presence of pan-caspases inhibitor — *N*-acetyl-Asp-Glu-Val-Asp-al (100 μM). Part of hydrolysis inhibition of the respective substrates by the inhibitor was taken as the caspase-3 activity and was expressed as nM AMC per 10^6 cells/1 hour.

Ischemia-reperfusion *in vivo*. Four days after last injection of siRNA the rats underwent left anterior descending artery ligation for 1 h and following reperfusion for 3 h. Briefly after anesthesia with urethane (1500 mg/kg) and intubation, animals were ventilated with a volume-cycled rodent respirator. Ligation of the left coronary artery was performed with a 7–0 monofilament polypropylene suture (SURGIPRO II, VPF-713-X, UK), 3–4 mm from the tip of the left auricle. Reperfusion was started

after 60 min of ischemia by cutting the knot of the suture. Myocardial ischemia confirmation was obtained by registration of ECG change — ST segment elevation relatively to base line that was 41.8±5.1% in sham-operated animals and 181.8±11.2% in ischemia-reperfusion group. Sham-operated animals underwent all surgical procedures besides ligation of coronary artery. The Standing Committee on Animal Research from our institution has approved the animal study protocol.

RNA isolation and reverse transcription. RNA isolation from cardiomyocytes and heart tissue was performed using Trizol RNA-prep kit (Isogen, Russia). RNA concentration was determined using a NanoDrop spectrophotometer ND1000 (NanoDrop Technologies Inc., USA). Reverse transcription (RT) was performed using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas), 1.2–1.5 μg of total RNA and random-hexamer primer. Single-strand cDNA obtained was used for polymerase chain reaction (PCR) and real-time PCR.

PCR. Single-stranded DNA molecules obtained by RT were used for amplification of *ALOX5* gene fragment using primers: *ALOX5*-S 5′-GCC TAA GAA GCT CCC AGT GAC-3′ and *ALOX5*-A 5′-ACT GGT GTG TAC AGG GGT CAG-3′. Amplification mixture contained 5 μl of 5× PCR-buffer, 2.5 mM magnesium sulfate, 200 μM mixture of four nucleoside triphosphates, 20 pM each primer and 0.5 U of *Taq*-polymerase (AmpliSense, Russia), volume brought up to 25 μl with deionized water. Amplification program consisted of 35 cycles: denaturation — 95°C, 45 s, annealing — 64°C, 45 s and elongation 72°C, 45 s. For controlling the quality of RNA isolation and comparison of *ALOX5* gene expression intensity, we amplified also fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (a housekeeping gene). Amplified fragments were separated in 2.5% agarose gel containing ethidium bromide. Visualization and estimation of amplified fragment brightness after horizontal electrophoresis was performed using a transilluminator and Vitran software (Biocom, Russia).

Real-time PCR. We performed amplification in 10 μl of SYBR Green PCR Master Mix containing 30 pM of each primer. For amplification of *ALOX5* and 18S rRNA gene (housekeeping gene) fragments we used the following primers: *ALOX5*-S-5′-GCC TAA GAA GCT CCC AGT GAC-3′ and *ALOX5*-A-5′-ACT GGT GTG TAC AGG GGT CAG-3′, 18S-S-5′-CTT AGA GGG ACA AGT GGC G-3′ and 18S-A-5′-GGA CAT CTA AGG GCA TCA CA-3′. Sample volume was made to 20 μl with deionized water. Amplification was performed on a 7500 Fast Real-Time PCR System thermocycler. Amplification program consisted of initial AmpliTaq Gold® DNA polymerase activation step at 95°C for 10 min and following 50 cycles: denaturation (95°C for 15 s), annealing and elongation (64°C for 60 s). For control of specificity we analyzed the melting temperature of PCR product by sequential increase of temperature from 64°C to 99°C with registration of the drop in fluorescence strength of double-stranded DNA-SYBR Green complexes. We performed calculations using the 7500 Fast Real-time 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.

5-LO activity measurement in heart tissues. In spite of the fact that the primary product of AA transformation by 5-LO is LTA_4 we evaluated 5-LO activity based on LTC_4 concentration as it is formed stoichiometrically from LTA_4 by LTC_4-synthase. The amount of LTC_4 was determined in ethanolic extracts of heart tissue. The extracts were prepared by pulverizing pieces of heart tissue in liquid nitrogen. Collected ethanolic extracts were evaporated at 40°C in air bath and kept in a freezing chamber. LTC_4 determination was performed using radioimmunoassay technique and a standard assay kit (Amersham, USA). Sample radioactivity determination was performed on a liquid scintillation counter (Beckman Coulter Inc., USA).

Infarct size determination. Infarct size was determined as described (Ito *et al.*, 1997; Wolff *et al.*, 2000; Buehler *et al.*, 2002; Martire *et al.*, 2003) with two different methods – triphenyltetrazolium chloride (TTC) and propidium iodide staining. In our opinion propidium iodide staining is a more objective, accurate and up-to-date method for infarct size determination. The same conclusion was made by other researchers (Ito *et al.*, 1997; Wolff *et al.*, 2000). That is why we present only results of propidium iodide staining. Briefly, at the end of the 3-h reperfusion period, rats underwent propidium iodide intravenous injection (420 μl of 8.75 μM solution per one animal). After 20 min heart was removed and sliced perpendicularly to its long axis into four equal-thickness transverse slices. The slices were then photographed under a Nikon Eclipse E200 microscope. Tissue that showed bright red fluorescence of propidium iodide was presumed to be necrotic. Total necrotic area was digitized using computer-assisted planimetry software ImageJ 1.37 (National Institutes of Health, Bethesda, MD, USA).

Statistics. Statistical analysis was performed with Excel 2003, Origin 7.0 and 7500 Fast System SDS software. All data were expressed as the mean (S.E.) of replicate experiments performed in each assay. Statistical differences were evaluated using Stu-

dent's *t*-test. $P<0.05$ was taken to indicate a statistically significant difference.

RESULTS

ALOX5 gene expression in isolated cardiomyocytes at anoxia-reoxygenation and *ALOX5* gene silencing

The level of *ALOX5* mRNA in isolated rat cardiomyocytes deviates $7.5\times10^{-5}\pm7.5\times10^{-6}$ relative units in normal conditions and practically does not change after anoxia-reoxygenation (Fig. 1A). This shows that cardiomyocytes express *ALOX5* and thus are likely to be a source of eicosanoids derived from AA *via* the 5-LO pathway. Introduction of *ALOX5*-specific siRNA into a culture of cardiomyocytes caused a 4.7-fold downregulation of *ALOX5* mRNA level ($P<0.05$) in comparison with scrambled siRNA-treated cells (Fig. 1B).

Cell death after anoxia-reoxygenation and *ALOX5* gene silencing

The proportion of live, necrotic and apoptotic cells in control conditions is 90±0.9%, 3±0.3% and 6±0.7%, respectively (Fig. 2). After AR the percentage of live cells decreased to 79±1.3% ($P<0.05$) whereas necrotic and apoptotic cells increased to

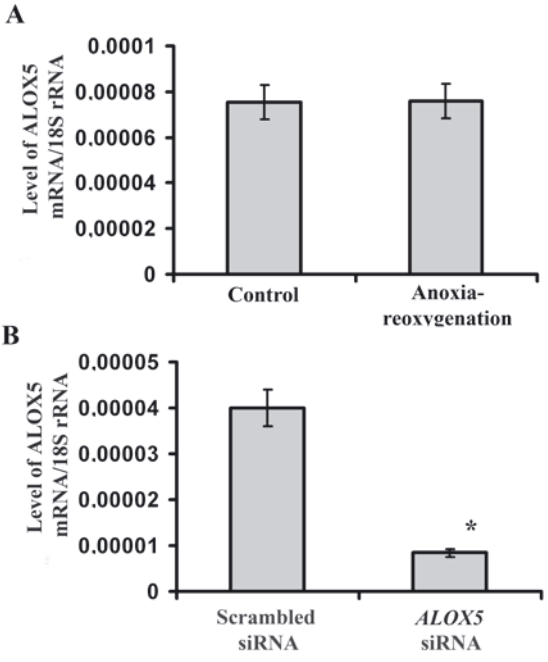

Figure 1. *ALOX5* mRNA level in isolated rat cardiomyocytes.
Effect of anoxia-reoxygenation (**A**) and *ALOX5* gene silencing with siRNA (**B**). Results, obtained by Real-time PCR, are expressed as means (±S.E.M.; bars). n = 8. $^{*}P<0.05$ compared to scrRNA application.

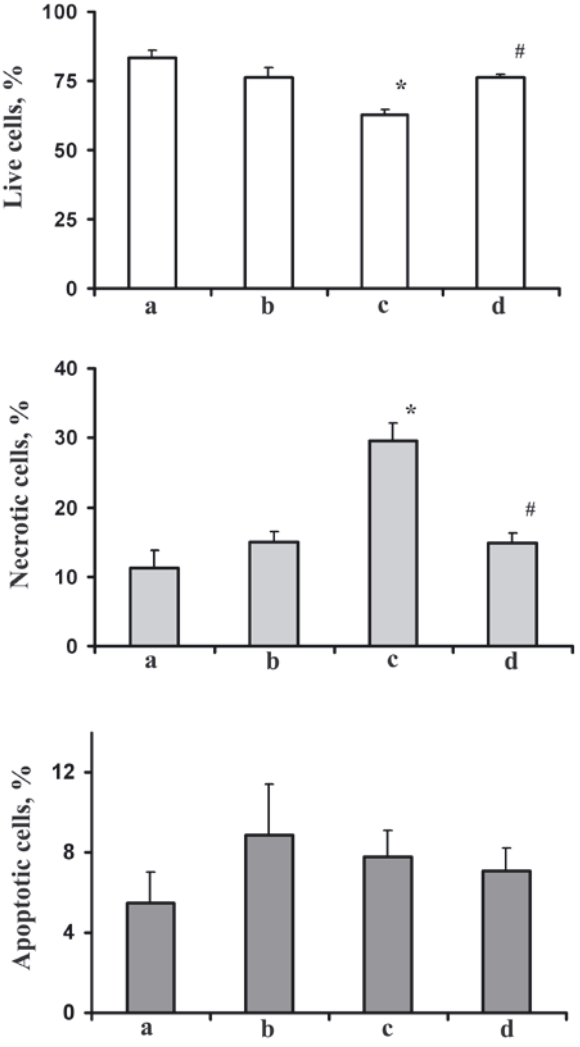

Figure 2. Fraction of live, necrotic and apoptotic cardiomyocytes after various treatments.
Cell status was determined by staining with 8.75 μM bisbenzimide (Hoechst 33342) and propidium iodide. Treatments: a, 24 h after scrRNA administration; b, 24 h after *ALOX5*-specific siRNA administration; c, AR after scrRNA administration; d, AR after *ALOX5*-specific siRNA administration. n = 10. $^{*}P_{a\ vs\ c}<0.05$, $^{\#}P_{c\ vs\ d}<0.05$.

8±1.1% ($P<0.05$) and 12±1% ($P<0.05$), respectively. Caspase activity also increased from 412.8±3.78 nM AMC per 10^6 cells/1 h in control to 506.4±10.26 nM AMC per 10^6 cells/1 h in anoxia-reoxygenation (1.2-times, $P<0.001$) with DEVD-AMC as substrate and 700.8±12.6 nM AMC per 10^6 cells/1 h and 781.8±13.2 nM AMC per 10^6 cells/1 h, respectively (1.1-times, $P<0.01$) with DEVD-AFC as substrate. Introduction of scrambled siRNA into cardiomyocytes affected the proportions of live, necrotic and apoptotic cells in the culture: 83±3.0%, 11±3.0% and 6±1.6%, respectively. Introduction of *ALOX5*-specific siRNA also induced changes in these proportions: 76±3.7%, 15±1.4% and 9±2.5%, respectively. After AR the fraction of live cells in culture transfected with

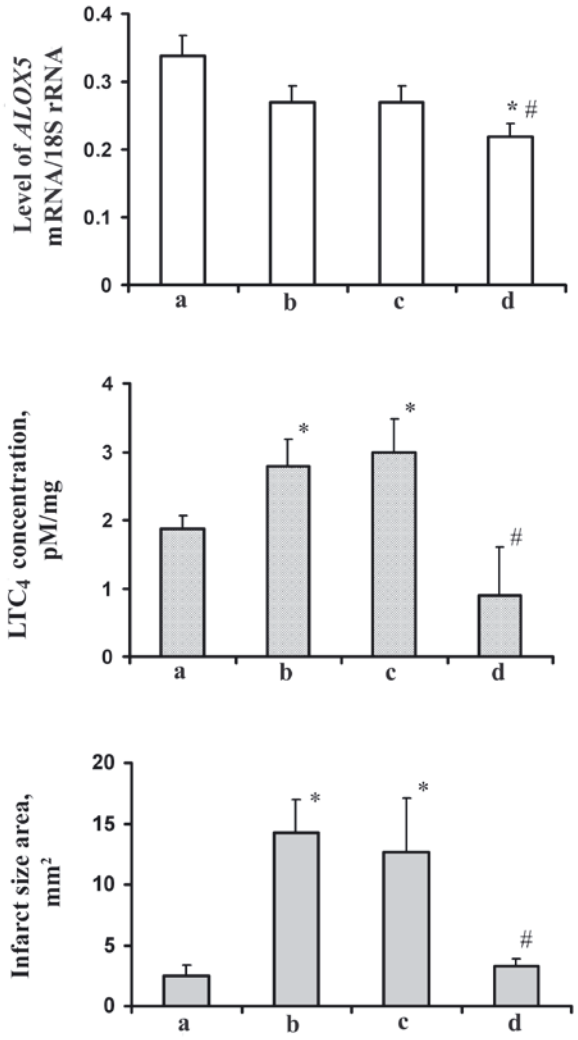

Figure 3. *ALOX5* mRNA, LTC_4 levels and infarct size in the heart after various treatments.
Treatments: a, sham-operated; b, IR; c, IR after scrRNA administration; d, IR after *ALOX5*-specific siRNA administration. Results are expressed as means (±S.E.M.; bars), n = 10. $^{*}P<0.05$ compared to sham-operated, $^{\#}P<0.05$ compared to scrRNA administration. Animals were subjected to different treatments, heart was removed and *ALOX5* mRNA level was determined by reverse transcription PCR, that of LTC_4 by radioimmunoassay and infarct area by fluorescence microscopy of heart slices.

scrambled siRNA was reduced to 63±1.9% ($P<0.05$ compared to scrambled without AR), necrotic cells increased to 30±2.6% ($P<0.05$ compared to scrambled without AR) whereas the percentage of apoptotic cells was not changed significantly. The fractions of live and necrotic cells after AR of cultures with silencing of the *ALOX5* gene were 76±1.3% ($P<0.05$ compared to scrambled group with AR) and 15±1.3% ($P<0.05$ compared to scrambled group with AR), while that of apoptotic cells was not changed (Fig. 2). The caspase activity was not changed in the culture with siRNA-induced *ALOX5* silencing after AR compared to the AR group without siRNA treatment (506.4±10.26 nM AMC per 10^6 cells/1 h *vs* 510.6±12.06 nM AMC per 10^6 cells/1 h, respectively, using DEVD-AMC, $P>0.05$, 781.8±13.2 nM AMC per 10^6 cells/1 h *vs* 726.6±28.2 nM AMC per 10^6 cells/1 h, respectively, using DEVD-AFC, $P>0.05$). Thus, RNA interference of the *ALOX5* gene diminishes necrotic and does not affect apoptotic cell death at AR in cardiomyocyte culture.

Influence of *ALOX5*-specific siRNA on gene expression and 5-LO activity in heart tissues at ischemia-reperfusion *in vivo*

Similarly as in cultured cardiomyocytes, expression of the *ALOX5* gene in heart tissue samples obtained from control (sham-operated) animals and animals subjected to heart ischemia-reperfusion *in vivo* was not significantly different (Fig. 3). In scrambled siRNA-treated animals LTC_4 level was increased 1.75-fold after ischemia-reperfusion compared to sham-operated animals (Fig. 3). *ALOX5*-specific siRNA application caused a 1.2-fold downregulation of the *ALOX5* gene after ischemia-reperfusion comparing to scrambled siRNA-treated animals ($P<0.05$) (Fig. 3). At the same time the LTC_4 level was decreased 3.3-fold ($P<0.05$) (Fig. 3).

Myocardial infarct size after heart ischemia-reperfusion and *ALOX5* gene silencing

Figure 4 presents pictures obtained by fluorescence microscopy of heart slices from one representative experiment. These pictures demonstrate a significant decrease of the necrotic area with red fluorescence in slices from myocardium of the rats with *ALOX5* gene silencing after ischemia-reperfusion. Statistical analysis of six independent experiments revealed that after IR the total necrotic area of myocardium was 5.8-fold increased comparing to sham-operated rats ($P<0.05$). At the same time, the infarct area size in animals with suppressed *ALOX5* gene was decreased 3.8-fold comparing to scrambled siRNA-treated animals ($P<0.05$) (Fig. 3).

DISCUSSION

Our data shows that *ALOX5* gene silencing decreases the frequency of necrotic cell death after anoxia-reoxygenation of cultured cardiomyocytes as well as reduces heart infarct area size at ischemia-reperfusion *in vivo*. There is evidence that lipid hydroperoxidation mediated by 5-LO may lead to key features of necrosis development — disruption of organelle and plasma membranes (Kuhn *et al.*, 1990; van Leyen *et al.*, 1998; Spiteller, 2003;

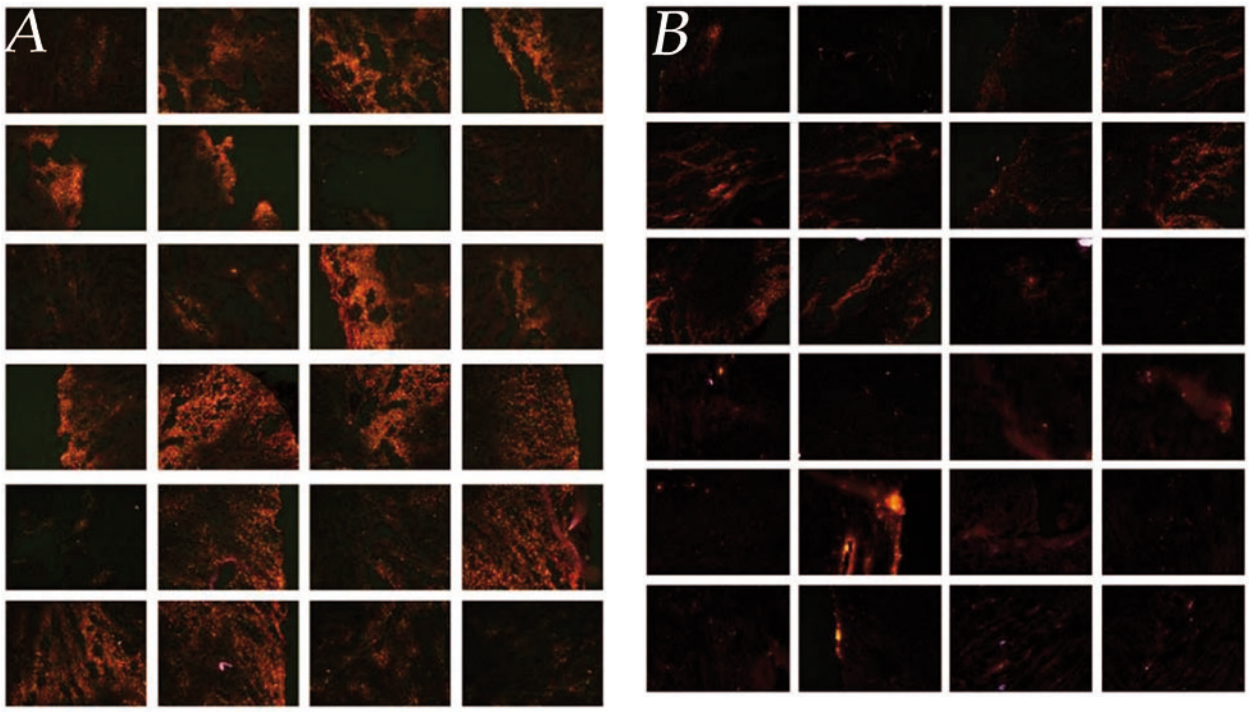

Figure 4. Myocardial necrosis after ischemia-reperfusion.
Animals were subjected to IR after scrRNA (**A**) or *ALOX5*-specific siRNA (**B**) treatment. After propidium iodide injection hearts were removed, transverse slices were viewed under a fluorescence microscope." because It represents what were done in our experiments.

Festjens *et al.*, 2006). It is also well known that leukotrienes play an important role in coronary vessel constriction and PMN leucocyte recruitment, and exert an arrythmogenic effect (Feuerstein, 1986). Therefore, down-regulation of *ALOX5* with the ensuring decrease of 5-LO protein formation reduces the possibility of realization of the above scenario. An involvement of lipoxygenases in TNFα-induced apoptosis and other apoptotic pathways has been reported several times (Maccarrone *et al.*, 2001; Spiteller, 2003). However, according to our data, *ALOX5* gene silencing does not affect the apoptotic death of cardiomyocytes. This indirectly points to an insignificant role of 5-LO and 5-LO-derived eicosanoids in the triggering of the apoptosis pathway at anoxia-reoxygenation of cardiomyocytes. Another possible mechanism which can explain the involvement of lipoxygenases in cardiomyocyte functioning and their resistance to ischemia involves activation of leukotriene receptors (CysLt1 and CysLt2) (Liu *et al.*, 2003). CysLT mRNA was found recently in heart tissues using various methods, including *in situ* hybridization, Northern blotting and RT-PCR (Lynch *et al.*, 1999; Heise *et al.*, 2000; Kamohara *et al.*, 2001; Ogasawara *et al.*, 2002). Accordingly, leukotrienes can exert autocrine action on cardiomyocytes by interaction with specific receptors. Liu *et al.* (2003) demonstrated that cysteinyl leukotrienes (CysLT) mediate an angiotensin II-evoked increase in cytosolic free Ca^{2+} concentration in neonatal rat cardiomyocytes which can be prevented by the 5-LO inhibitor AA-861 and the CysLT1-selective antagonist MK-571 (Liu *et al.*, 2003). This data allows one to assume that the protective effect of *ALOX5* silencing is related to reduced Ca^{2+} overloading of cardiomyocytes occurring at anoxia-reoxygenation. Ca^{2+}-dependent injury of cardiomyocytes, in particular with induction of necrosis, has already been described by several authors (Zimmermann *et al.*, 2001; Nakayama *et al.*, 2003).

In our opinion, RNA interference holds great potential in therapy of cardiovascular disorders, in particular MI. The advantages of this method involve the reversible nature of gene suppression and possibility of silencing multiple genes simultaneously. According to the latest data expression of genes encoding micro RNAs (mainly miR21, miR214, miR379 and miR146b) that regulate gene expression at the post-transcriptional level by either degradation or translational repression of a target mRNA, changes during experimental MI (Roy *et al.*, 2009). In this aspect determination of the most effective strategy for MI gene therapy is not straight forward. It is essential to clarify which approach will be the most effective — suppression of key regulatory genes such as the genes encoding the micro RNAs or silencing of effector genes, for instance *ALOX5*. It is conceivable that RNA interference targeting genes encoding

interfering RNAs could provide a more pronounced protective effect.

Taken together, the current study presents evidence on *ALOX5* gene expression in myocardium and in isolated cardiomyocytes. It also indicates a protective effect of *ALOX5* gene silencing *via* specific siRNA both in the heart after ischemia-reperfusion *in vivo* and in cultivated cardiomyocytes subjected to anoxia-reoxygenation.

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