

Age-associated murine cardiac lesions are attenuated by the mitochondria-targeted antioxidant SkQ1

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Summary. Age-related changes in mammalian hearts often result in cardiac hypertrophy and fibrosis that are preceded by inflammatory infiltration. In this paper, we show that lifelong treatment of BALB/c and C57BL/6 mice with the mitochondria-targeted antioxidant SkQ1 retards senescence-associated myocardial disease (cardiomyopathy), cardiac hypertrophy, and diffuse myocardial fibrosis. To investigate the molecular basis of the action of SkQ1, we have applied DNA microarray analysis. The global gene expression profile in heart tissues was not significantly affected by administration of SkQ1. However, we found some small but statistically significant modifications of the pathways related to cell-to-cell contact, adhesion, and leukocyte infiltration. Probably, SkQ1-induced decrease in leukocyte and mesenchymal cell adhesion and/or infiltration lead to a reduction in age-related inflammation and subsequent fibrosis. The data indicate a causative role of mitochondrial reactive oxygen species in cardiovascular aging and imply that SkQ1 has potential as a drug against age-related cardiac dysfunction.

Key words: Mitochondria-targeted antioxidant, Myocardial fibrosis, Cardiac hypertrophy, Leukocyte infiltration

Introduction

Mammalian heart is prone to significant senescent-associated functional decay (Ruben et al., 1992;

Hagiwara et al., 1996; Percy and Barthold, 2007; Taylor, 2012; Shioi and Inuzuka, 2012). One of the most important cardiac pathologies in adult (>9 month) mice is spontaneous cardiomyopathy (Price and Papadimitriou, 1996; Hagiwara et al., 1996; Taylor, 2012). This fatal disease may influence animal lifespan either directly or due to induced atrial thrombosis (Son, 2003; Taylor, 2012). The morphological features of this disease in myocardium include focal necrosis of cardiomyocytes, focal interstitial fibrosis, and moderate infiltration of neutrophils, lymphocytes, and macrophages (Faccini et al., 1990; Price and Papadimitriou, 1996; Elwell and Mahler, 1999; Frith et al., 2007; Greaves, 2007; Taylor, 2012). Formally, compensatory hypertrophy and diffuse myocardial fibrosis is typical for old mice, though these features are not listed among the mandatory morphological signs of cardiomyopathy (Elwell and Mahler, 1999). The incidence of spontaneous cardiomyopathy varies among different lines of animals and also depends on both gender and housing conditions (Price and Papadimitriou, 1996; Brayton, 2006). The pathogenesis of this disease has been poorly studied; however, some authors have noted an important role of reactive oxygen species (ROS) in the development of this disease (Price and Papadimitriou, 1996).

Mitochondria are essential intracellular organelles functioning mainly as ATP suppliers, but under some conditions they may also produce destructive ROS. The role of mitochondrial ROS (mtROS) in the pathogenesis of heart disease is poorly understood, although increased mtROS level is typical for a number of cardiac diseases (Tsutsui et al., 2011; Maulik and Kumar, 2012; Madamanchi and Runge, 2013).

Mitochondria-targeted antioxidants of the SkQ

family were recently synthesized and used successfully in the treatment and/or prevention of many animal disease models, including heart pathology (Bakeeva et al., 2008; Skulachev et al., 2011). The SkQ1 compound was also shown to increase the lifespan of BALB/c and C57BL mice (Anisimov et al., 2011). However, the molecular and cellular mechanisms of SkQ1 action are not well understood. In this study, we investigated the possible molecular mechanisms of the cardioprotective action of SkQ1 in aging mice using gene expression profiling.

Materials and methods

Animals

Studies were performed on two mouse strains: BALB/c and C57Bl/6. BALB/c mice were kept in the SPF vivarium of the Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry (Moscow), and C57Bl/6 mice were housed in the Wenner-Gren Institute, Stockholm University (Stockholm).

A total of 209 BALB/c mice (90 males and 119 females) were housed until natural death and examined post-mortem with full necropsy and histopathology for pathological evaluation. The data concerning longevity were published previously (Anisimov et al., 2011).

Also, eight special groups of female BALB/c mice were studied after sacrifice:

1) three groups (control, 1 nmol/kg SkQ1, 30 nmol/kg SkQ1) consisting of 7 14-month-old females each were used for gene expression study;

2) three groups (control, 1 nmol/kg SkQ1, 30 nmol/kg SkQ1) consisting of 14 or 15 24-month-old females each were used for heart morphometric study;

3) two control groups of younger females (3 months of age): one consisting of 7 animals (used for gene expression study) and the other of 15 animals (for heart morphometric study).

The animals were sacrificed by CO₂ inhalation. All of the mice were free from any visible signs of infections at the time of sacrifice.

Seventeen untreated C57BL/6 mice (9 males and 8 females) and 17 SkQ1-treated C57BL/6 animals (7 males and 10 females) were sacrificed in accordance with the ethical requirements because of moribund appearance and examined with diagnostic necropsy and histopathology.

All procedures were in compliance with European Directive-2010 of FELASA.

SkQ1 treatment

BALB/c mice received SkQ1 at the doses of 1 or 30 nmol/kg body weight per day by addition to their drinking water throughout their lifespan. C57Bl/6 mice received SkQ1 at the dose of 1400 nmol/kg body weight per day by addition to their drinking water, starting at the age of 14-16 weeks and continuing throughout their lifespan (106-110 weeks).

Necropsy and histopathological examination

Deceased mice underwent full necropsy for cause of death and elucidation of incidental lesions. Hearts and samples of other organs were collected and fixed with 10% buffered formalin and processed routinely for paraffin embedding. Four micron sections were stained with the hematoxylin-eosin technique. Van Gieson, Alizarin Red, von Kossa, and other staining protocols were also used if necessary. Diagnoses of cardiac pathologies were determined using criteria published in well-known guides (Faccini et al., 1990; Price and Papadimitriou, 1996; Elwell and Mahler, 1999; Frith et al., 2007; Greaves, 2007; Taylor, 2012).

Sacrificed females were dissected, the weight of the heart was measured, and specimens of hearts were taken for histopathological examination and gene expression analysis. After the formalin fixation and paraffin-embedding procedure, 4 μm sections of hearts were stained with the hematoxylin-eosin and van Gieson protocols. Van Gieson-stained sections were used for evaluation of cardiac fibrosis by the measurement of percent area of red-stained collagen fibers with AxioVision 3.0 (Carl Zeiss) and Image J software.

All examinations and evaluations were conducted on blinded slides.

The data were statistically evaluated using the χ^2 , Kruskal-Wallis test, and t-test using Statistica 6.0 software (probability (p) value of less than or equal to 0.05).

RNA extraction and oligonucleotide microarray

RNA was isolated from mouse heart using the Qiagen RNeasy Mini Kit (Qiagen, Inc. Valencia CA) according to the manufacturer's protocol. RNA was quantified using NanoDrop, and quality was assigned using the Agilent Total RNA 6000 chip. RNA pools obtained from hearts from each group of mice were composed of 1 microgram of purified RNA. Four hundred nanograms of total RNA was amplified using the Illumina® TotalPrep™ RNA Amplification Kit (Ambion). Amplified RNA was hybridized with MouseRef-8 v2.0 Expression BeadChips (Illumina) targeting ~25,600 RefSeq transcripts according to the Illumina protocol. Data acquisition and analysis were done using the GenomeStudio Software (Illumina) and the Gene Expression Module.

KEGG analysis of transcriptional pathways

After identifying genes that were significantly regulated by SkQ1 administration, the gene lists were analyzed using gene ontology (GO) and signaling pathway analysis (using pathways defined by the Kyoto Encyclopedia of Genes and Genomes (KEGG) (<http://www.genome.jp/kegg/>)). We performed these two analyses on specific up- or downregulated gene sets using WebGestalt (<http://bioinfo.vanderbilt.edu/webgestalt/>), a web-based gene-set analysis toolkit that

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is a part of Onto-Tools (<http://vortex.cs.wayne.edu/projects.htm>). This application allows the expression frequency of a specific gene in the experimental set to be compared to its expression frequency in the background rodent gene set that is maintained at Onto-Tools or to another user gene set. Functional clustering of genes into GO groups yields two indices that describe the degree of gene-set enrichment (R), i.e. how over-represented this GO group is in the input set compared to what one would expect from a complete background genome set, and also the probability that this enrichment would occur by chance. We used a cutoff of at least two genes needing to be present to fully populate a GO term group and a probability (p) value of less than or equal to 0.05. Similar criteria were used for the clustering of genes into functional signaling pathways (derived from KEGG).

Results

SkQ1 prevents spontaneous cardiomyopathy in BALB/c and C57BL/6 mice

Several types of heart lesions were revealed from the pathological examination of spontaneously deceased BALB/c and C57BL/6 mice. Definitive diagnosis of cardiomyopathy was made based on signs such as necrosis, focal fibrosis, and inflammatory changes in the myocardium predominating in the left ventriculus. Also, background myocardial (only for C57BL/6 mice) and epicardial (only for BALB/c mice) mineralization and sporadic atrial thrombosis were observed (Fig.1).

Old BALB/c males have high frequency of cardiomyopathy (found in one fourth of all males examined). In contrast to BALB/c males, females have very low number of cardiomyopathy incidents (Table 1). We found that SkQ1 decreased the incidence of cardiomyopathy in BALB/c male mice (1 and 30 nmol/kg/day) and in all C57BL/6 mice (1400 nmol/kg/day) (Table 1).

SkQ1 did not influence on development of atrial thrombosis, myocardial and epicardial mineralization (data not shown).

SkQ1 decreases age-related heart hypertrophy and diffuse fibrosis in female BALB/c mice

Sacrificed old BALB/c mice had no signs sufficient

to make definitive diagnosis of cardiomyopathy. However, the enlargement of hearts and slight but diffuse fibrosis were detected in specimens from old mice. Moreover, the relative area of collagen fibers in the myocardium was significantly greater in old mice ($2.77 \pm 0.22\%$) than in young mice ($1.85 \pm 0.18\%$). Diffuse myocardial fibrosis in two SkQ1-treated groups was less than in the control mice (Fig. 2).

Statistical significance ($p < 0.01$) was revealed for both groups of 24-month-old mice consuming either 1 or 30 nmol SkQ1/kg per day compared with the control group of 24-month-old mice. Also, SkQ1 was found to retard development of such trait of senescence as increase of heart mass with age. SkQ1 attenuated this effect by almost 50% ($p < 0.05$) (Fig. 3).

Effects of oral SkQ1 administration on gene expression

We applied microarray analysis of RNA transcripts from hearts of control and SkQ1-treated BALB/c mice. We found 220 probe sets were differentially expressed by a minimum of $\pm 50\%$ on SkQ1 treatment with a false-positive discovery rate < 0.05 in both groups of mice administered 1 and 30 nmol/kg body weight per day (Fig. 4). Of these, 68 showed decreased expression and 152 increased expression on SkQ1 treatment.

However, no specific biological processes were identified using KEGG clustering analysis. The pathways that were most influenced are listed in Table 2. The impact factor is calculated for each pathway incorporating parameters such as the normalized fold change of the differentially expressed genes, the statistical significance of the set of pathway genes, and the topology of the signaling pathway, thus providing biologically meaningful results (Draghici et al., 2007).

It is noteworthy that SkQ1 treatment affected pathways related to different aspects of cell-to-cell contact and adhesion (i.e. "Adherens junction", "Leukocyte transendothelial migration", and "Cell adhesion molecules"). Since SkQ1 is a mitochondria-targeted substance, we assume that SkQ1 administration may influence gene expression associated with mitochondria. However, among all differentially expressed probe sets, only one had mitochondrial localization.

Since age-related heart inflammation and subsequent fibrosis may be greatly influenced by changes in the inflammatory cytokines, chemokines and/or their

Table 1. Incidence of spontaneous age-related cardiomyopathy (%) in spontaneously deceased untreated and SkQ1-treated mice (* $p < 0.05$; ** $p < 0.01$).

SkQ1, nmol/kg per day	BALB/c, females	BALB/c, males	C57BL/6, (males and females)
0	2.9 (N=35)	25.9 (N=27)	47.1 (N=17)
1	6.7 (N=45)	6.3* (N=31)	-
30	5.1 (N=39)	3.2** (N=32)	-
1400	-	-	5.9* (N=17)

Table 2. Pathway list ranked by impact factor ($p < 0.05$) calculated for differentially expressed transcripts in BALB/c mice: control group vs. combined group consuming 1 and 30 nmol/kg SkQ1 per day.

Pathway name	Input genes in pathway (%)
Adherens junction	4
Leukocyte transendothelial migration	10
Cell adhesion molecules (CAMs)	7
T cell receptor signaling pathway	12
Phosphatidylinositol signaling system	10

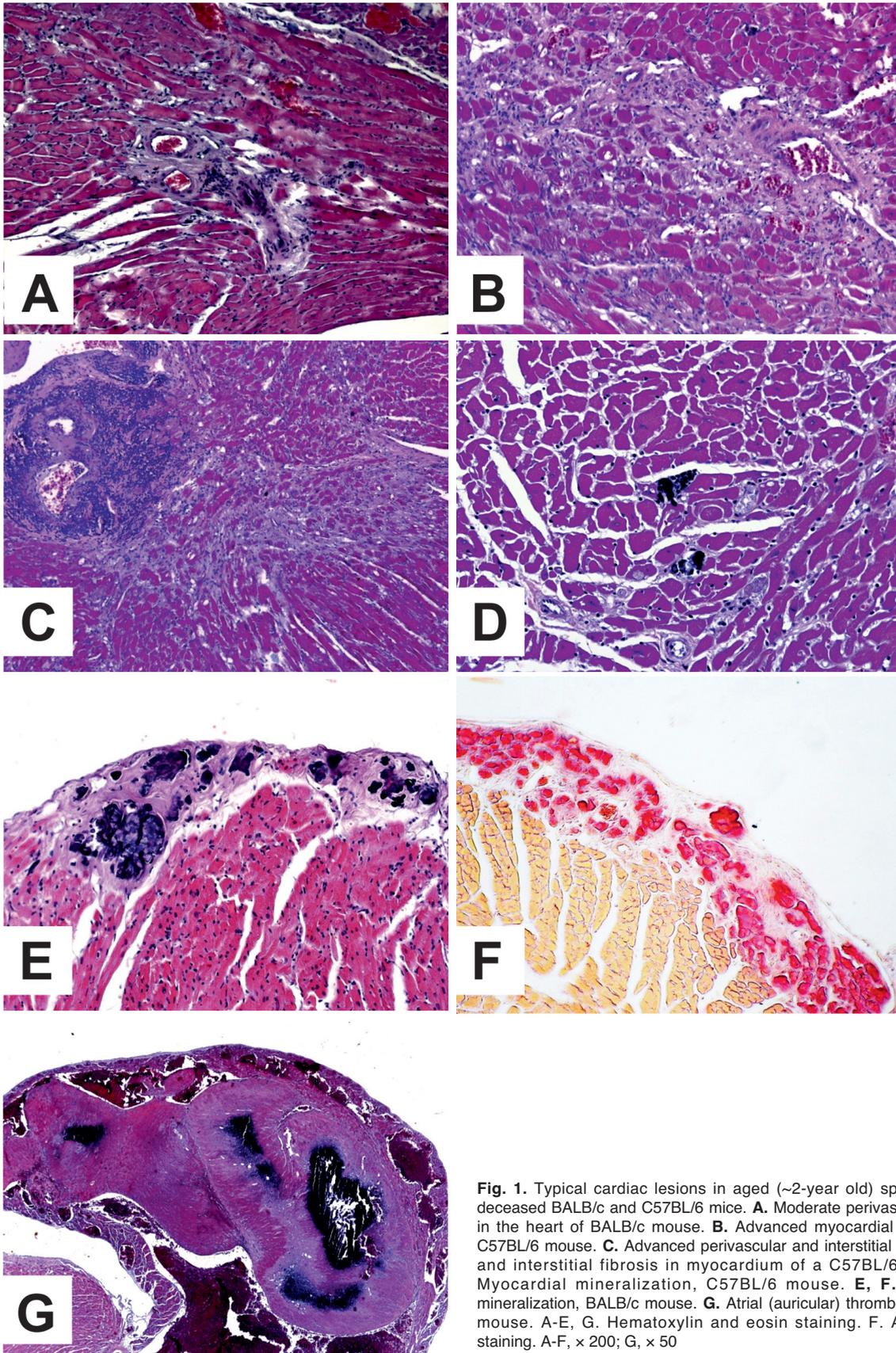


Fig. 1. Typical cardiac lesions in aged (~2-year old) spontaneously deceased BALB/c and C57BL/6 mice. **A.** Moderate perivascular fibrosis in the heart of BALB/c mouse. **B.** Advanced myocardial fibrosis in a C57BL/6 mouse. **C.** Advanced perivascular and interstitial inflammation and interstitial fibrosis in myocardium of a C57BL/6 mouse. **D.** Myocardial mineralization, C57BL/6 mouse. **E, F.** Epicardial mineralization, BALB/c mouse. **G.** Atrial (auricular) thrombosis, BALB/c mouse. A-E, G. Hematoxylin and eosin staining. F. Alizarin Red staining. A-F, $\times 200$; G, $\times 50$

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receptors we additionally focused on the inflammatory-related genes whose ontological criteria shared the following syntax: inflammatory response (Gene Ontology id: GO:0006954). The results of our analysis are presented in Table 3.

Discussion

Cardiovascular aging is a complex, multistage process that has been studied intensively in recent years.

According to the free radical theory of aging, an increased production of reactive oxygen species (ROS) is implicated both in the aging process and the development of age-related cardiovascular diseases (Harman, 1956). Numerous data have confirmed increased ROS production in aging heart and vessels (reviewed in (Zhang and Gutterman, 2007)). Among all ROS sources in cells, those derived from mitochondria are suggested to play a causative role in age-related pathologies (Skulachev et al., 2011). The recently

Table 3. Inflammatory response genes significantly affected by SkQ1 treatment. *Upregulated genes in SkQ1-treated animals are indicated as positive values, and vice versa.

ORF	Gene	Function	Fold change*
SCGB1A1	Secretoglobin, family 1A, member 1 (uteroglobin)	Negative regulation of cytokines production	-9.6
CXCL15	Cxcl15 chemokine (C-X-C motif) ligand 15	Chemotaxis for neutrophils	-6.6
CHIA	Chia1 chitinase, acidic 1	Chitin hydrolysis, TH2 inflammatory responses	-3.7
AGER	Advanced glycosylation end product-specific receptor	Advanced glycation end-product receptor; positive regulator of inflammatory response	-3.3
CHI3L3	Chitinase-like 3	Beta-N-acetylhexosaminidase	-3.1
REG3G	Regenerating islet-derived 3 gamma	Positive regulation of wound healing	-2.9
HC	Hemolytic complement	Complement activation; positive regulator of chemokine secretion	-2.4
HP	Haptoglobin	Hemoglobin utilisation	-2.1
PSTPIP1	Proline-serine-threonine phosphatase-interacting protein 1	Negative regulation of CD2-triggered T cell activation	2.5
GATA3	GATA binding protein 3	Transcription factor mediating regulation of Th1 and Th2 cell differentiation	2.6
PIK3CD	phosphatidylinositol 3-kinase catalytic delta polypeptide	Mediates many aspects of immune responses	2.7
CD28	CD28 antigen	Co-stimulatory signal of T cells, required for their activation	2.9
NFKBID	nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, delta	Negative regulation of I-kappaB kinase/NF-kappaB signaling	2.9
PIK3CG	Phosphoinositide-3-kinase, catalytic, gamma polypeptide	Assembly of adherens junctions, and NK cells cytotoxicity	3.1
PBK	PDZ binding kinase	Activation of lymphoid cells	4.2
RASGRP1	RAS guanyl releasing protein 1	Regulation of T-cells and B-cells development, homeostasis and differentiation	5.1
CCL25	Ccl25 chemokine (C-C motif) ligand 25	Chemotaxis for thymocytes, macrophages, and dendritic cells	6.2
LAT	Lat linker for activation of T cells	Activation of the T-cell antigen receptor signal transduction pathway	6.7

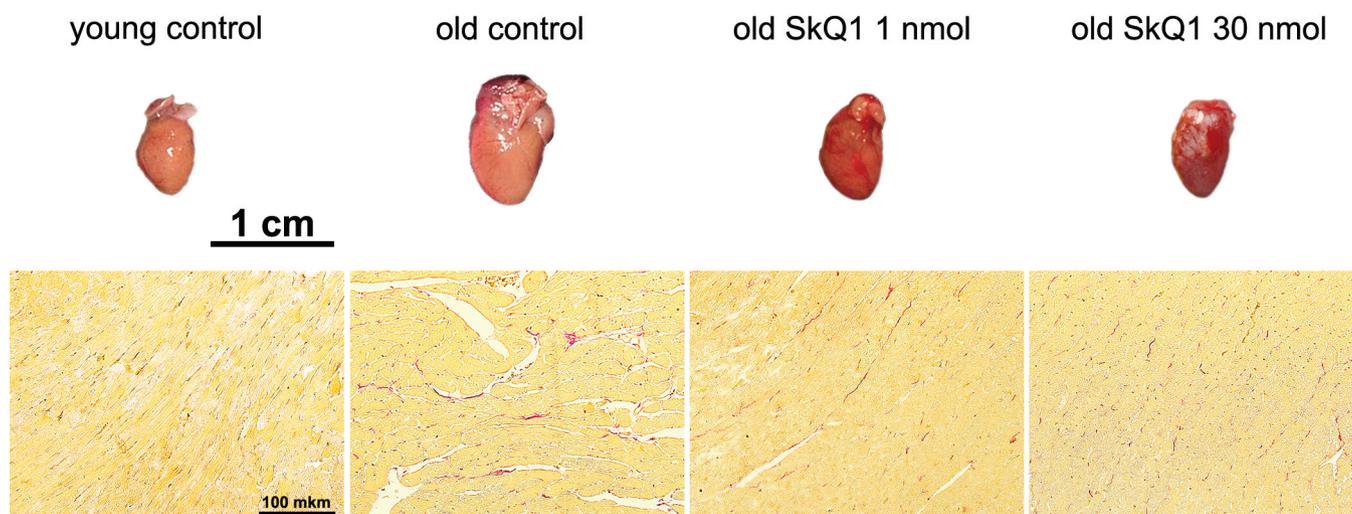


Fig. 2. Gross (upper panel) and histological (lower panel, Van Gieson picrofuchsin staining for collagen fibres (red)). Appearance of young (3 month) and old (24 month) female mice hearts from control and SkQ1-treated groups. x 400

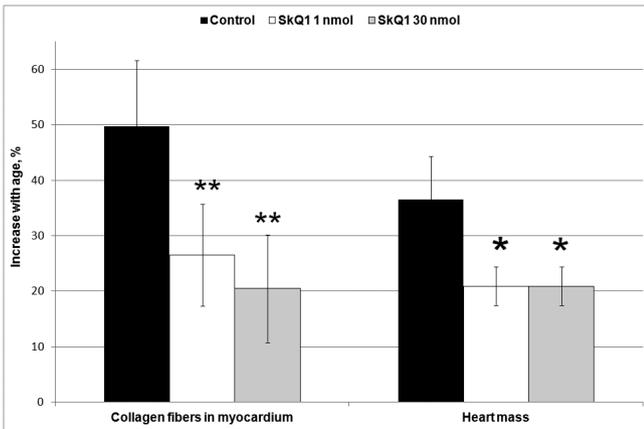


Fig. 3. SkQ1 decreases development of fibrosis and retards age-dependent increase in heart mass of female BALB/c mice. Asterisks above the experimental bars indicate significance between the experimental group and control group (* $p < 0.05$; ** $p < 0.01$).

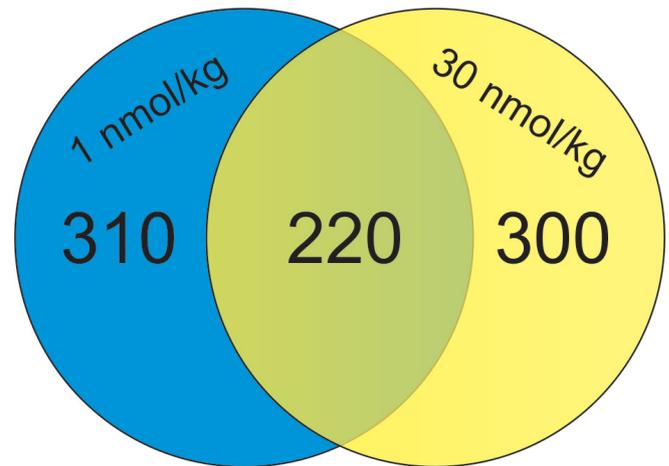


Fig. 4. Number of differentially expressed transcripts in heart tissue altered by SkQ1 administration.

developed mitochondria-targeted antioxidants have already demonstrated efficacy against multiple age-associated pathologies (Bakeeva et al., 2008; Skulachev et al., 2011). Administration of SkQ1 was also shown to increase median lifespan of BALB/c mice (Anisimov et al., 2011). In the current work, we describe cardio-protective effects of SkQ1 and analyze possible molecular mechanisms using microarray analysis. We found that SkQ1 treatment significantly diminished the incidence of senile cardiomyopathy, as well as the rate of myocardial hypertrophy and cardiac diffuse fibrosis. Cardiac lesions are known as one of the main causes of age-related death of mice (Elwell and Mahler, 1999; Son, 2003; Taylor, 2012). It is possible that increased median lifespan of mice is connected to diminished cardiac pathologies and myocardial dysfunction.

Some known mechanisms of age-related cardiomyopathy pathogenesis might be the target of SkQ1 action: necrosis and apoptosis of cardiomyocytes, leucocyte adhesion and migration (inflammatory response of endothelium), inhibition of intracellular regeneration (hypertrophy), fibroblast proliferation, and collagen synthesis, and, finally, migration of fibroblast progenitors to myocardium. Apoptotic and necrotic death of cardiomyocytes might theoretically be influenced by SkQ1 treatment, since SkQ1 was shown to reduce ROS-induced cell death (Antonenko et al., 2008). However, the available microarray data do not support or refute this hypothesis.

A number of studies have revealed the importance of inflammation as a major risk factor underlying aging and age-related diseases (Chung et al., 2009). The inflammatory process involves the delivery of blood soluble elements into a site of injury, followed by a more prolonged leukocyte infiltration. The latter includes a series of subsequent steps mediating capturing, rolling, adhesion, penetration of the endothelium and the

subendothelial basement membrane, and migration to the site of injury (Muller, 2003). Intercellular adhesion molecule 1 (ICAM-1) is involved in the firm adhesion of leukocytes to endothelial cells through interactions with integrins of the leukocytes. The penetration process depends on successful disruption of endothelial adherens junctions composed of cadherins, catenins, and other structural proteins.

We applied gene expression analysis to study molecular mechanisms of SkQ1 action in mouse hearts. We have shown that SkQ1 treatment did not result in a remarkable change in the gene expression profile. Nevertheless, the top three of all influenced pathways were related to different aspects of cell-to-cell contact and adhesion (Table 3.). These results are in good agreement with previous studies on SkQ1 action *in vitro*. SkQ1 was shown to upregulate expression of cadherins responsible for adherens junction formation (Agapova et al., 2008). Cadherins are transmembrane proteins essential for maintaining tissue structure and vascular permeability (Tepass et al., 2000). It was shown that stabilization of endothelial junctions and/or cadherin-catenin complex strongly inhibited leukocyte transmigration (Wittchen et al., 2005; Schulte et al., 2011).

In our experiments, ICAM-1 mRNA expression showed a slight (~40%) reduction. However, two-fold reduced expression of ICAM-1 mRNA was observed independently using real-time PCR analysis in the aortas of SkQ1-treated mice, thus supporting our data (Zinovkin et al., 2014). In turn, reduced ICAM-1 expression may also lower leukocyte adhesion and further infiltration in the tissues of SkQ1-treated mice.

In order to investigate SkQ1 effect on the cardiac inflammatory processes, we additionally focused on the genes involved in the inflammatory response (Table 3). Only 18 differentially expressed inflammatory genes

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were identified in the mice hearts. The complicated pattern in the inflammatory gene changes does not allow making a reliable conclusion about involvement of a specific inflammatory pathway in the observed cardiac lesions.

Transmigration of leukocytes is a key step in the inflammatory process, which is often followed by persistence of inflammation and proliferation of cells with a fibroblast-like phenotype and subsequent fibrosis (Cieslik et al., 2011). It is quite possible that reduced myocardial fibrosis observed in SkQ1-treated mice is a consequence of reduced leukocyte extravasation. However, myocardial fibrosis is also thought to be influenced by the endothelial-mesenchymal transition and recruitment of mesenchymal progenitor cells (Zeisberg et al., 2007; Sopol et al., 2011). Both processes are dependent on expression of extracellular protein (adhesion) complexes and may potentially be targeted by SkQ1.

Cardiac hypertrophy is believed to be an attempt to compensate for the increased stress on the aging heart. Reduction of cardiac hypertrophy in SkQ1-treated mice might be explained by reduced tissue damage preventing this compensation. In line with this, the use of another mitochondria-targeted antioxidant, MitoQ, attenuated cardiac hypertrophy in spontaneously hypertensive rats (Graham et al., 2009). However, contrary to our data, the prolonged MitoQ treatment did not result in a particular process or pathway in mouse hearts (Rodriguez-Cuenca et al., 2010).

Based on the results of our microarray analysis, we suppose that reduced inflammation and leukocyte infiltration in cardiac tissue of SkQ1-treated mice may be due to the modulation of intercellular pathways resulting in the stabilization of intercellular junctions and reduction of cell adhesion molecule exposure. This may lead to reduced leukocyte transmigration and inflammation. It is noteworthy that these pathways appear to be sensitive to mitochondrial ROS (mtROS) production, since SkQ1 has shown high efficacy as a mitochondria-targeted ROS scavenger (Skulachev et al., 2011).

Mitochondrial ROS have been implicated in the pathogenesis of various cardiovascular diseases such as atherosclerosis, hypertension, and diabetes (reviewed in (Zhang and Gutterman, 2007)). In genetic knockout mice lacking the mtROS-scavenging enzyme manganese superoxide dismutase, the mice developed cardiomyopathy within the first weeks of birth (Li et al., 1995; Lebovitz et al., 1996). The detrimental role of mtROS in the process of heart aging is further supported by overexpression of catalase targeted to mitochondria, thus protecting transgenic mice from cardiac aging and prolonging lifespan (Dai et al., 2009). All these data suppose a causative role of mtROS in cardiovascular aging.

Thus, the mitochondria-targeted antioxidant SkQ1 was shown to have cardioprotective activity in aging mice, most likely due to lowering of mtROS production,

resulting in reduced effector cell migration and prevention of cardiac hypertrophy and fibrosis. However, to confirm the data obtained with microarray analysis, further proteomic and functional studies are required.

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