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Effect of Rapamycin on Staurosporine-Induced Cardiac Myofibroblast Death

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Summary

Many cardiac diseases are associated with an excessive accumulation of myofibroblasts, characterized by increased production of extracellular matrix proteins and resistance to apoptosis, which leads to progression of fibrosis and cardiac dysfunction. Targeting the mechanisms of myofibroblast elimination is a promising strategy for treating fibrosis that requires further investigation.

The aim of this work was to determine the ability of the autophagy activator rapamycin to affect the staurosporin-induced death of cardiac myofibroblasts.

Materials and methods. *In vivo* modeling of cardiac fibrosis was performed using a mouse model of aortic arch ligation. *In vitro* studies used myofibroblasts obtained by differentiation of cardiac fibroblasts in presence of transforming growth factor beta 1 (TGFb1). To study the mechanism of myofibroblasts elimination, a cell model was developed using staurosporine, an alkaloid that can initiate apoptosis in a culture of cardiac myofibroblasts. The activity of apoptosis and autophagy was studied using immunofluorescence staining, immunoblotting, and flow cytometry.

Results. It was shown that pressure-induced cardiac overload causes the accumulation of myofibroblasts characterized by a low rate of apoptosis (annexin V+ cells in sham-operated hearts and after modeling pressure overload (0.0016 ± 0.0006% and 0.0019 ± 0.0009%; $P = 0.32$, $N = 10$), leading to marked interstitial fibrosis in the myocardium. It was found that rapamycin is able to enhance the effect of staurosporin and cause increased myofibroblast death due to autophagy-associated mechanisms (control 1.68 ± 0.66% ($N = 4$); staurosporin 65.8 ± 2.63% ($N = 4$); rapamycin + staurosporin 73.73 ± 0.67% ($n = 4$); control vs staurosporin $P < 0.0001$; control vs rapamycin + staurosporin $P < 0.0001$; staurosporin vs rapamycin + staurosporin $P = 0.0071$).

Conclusion. Rapamycin enhanced myofibroblast apoptosis induced by staurosporine, which may be related to regulation of the mTOR signaling and increased autophagy activity. The molecular mechanisms of this process require further research.

Keywords: cardiac myofibroblasts; rapamycin; staurosporine; cardiac fibrosis; autophagy

Conflict of interest. The authors declare no conflict of interest.

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Introduction

Cardiac fibrosis is an important component in the pathogenesis of most cardiovascular diseases, including myocardial infarction, congestive heart failure, cardiomyopathies, arterial hypertension, still remaining an unresolved health problem in advanced economies around the world [1, 2]. Fibrosis is characterized by excessive deposition of extracellular matrix proteins in the interstitium, leading to significant remodeling of the cardiac microenvironment, increased cardiac wall stiffness, and irreversible impairment of cardiac systolic function. In addition, disruption of the myocardial native structure creates an ideal «breeding ground» for ventricular arrhythmias with highest risk of sudden death, and accelerates the progression of heart disease [3–5]. The results of numerous scientific studies provide sufficient evidence to consider fibroblasts/myofibroblasts as the main pathological determinant in development of diseases associated with fibrosis [6]. It is well known that fibroblasts are the most important regulators of the cardiac microenvironment, ensuring the regulation of collagen synthesis/degradation, implementation of

electromechanical coupling, effectuation of a rapid response to damage (e. g., strengthening the infarcted heart wall to prevent its rupture), and exchange of secretory signals [7–9]. Meanwhile, disruption of fibroblasts elimination mechanisms, which are implemented through apoptosis, triggers a «vicious circle» of fibroblast activation, leading to their differentiation into myofibroblasts, their excessive accumulation in cardiac tissue, excessive matrix deposition, and the development of pathology [10]. This determines the high relevance of studies aimed at investigating the molecular mechanisms of myofibroblast pool regulation, and searching for compounds with the potential to induce myofibroblast elimination, as the basis of a strategy preventing progression of fibrosis. In this regard, compounds that modulate autophagy, an evolutionarily conservative mechanism for maintaining cellular homeostasis through utilization of macromolecules and organelles via the lysosomal degradation pathway, are of particular interest [11–12]. Recent studies by our and other research groups have shown, that numerous conditions associated with fibrosis are characterized by decreased autophagy, both in individual subpopulations of fibroblasts/myofibroblasts and in other cells of the cardiac microenvironment [12, 13]. Consequently, increasing autophagy activity within physiological limits may be a potential strategy for influencing myofibroblast function and the mechanisms of fibrosis development in general.

The aim of this study was to determine the potential of the autophagy activator rapamycin to affect staurosporine-induced death of cardiac myofibroblasts.

Materials and Methods

Animals. All animal procedures were approved by the Ethics Committee of the Institute of experimental cardiology of Academician E. I. Chazov National medical research center for cardiology (permit No. LA/28.07.2023 dated July 28, 2023; No. LA/26.09.2023 dated September 28, 2023), and were carried out in accordance with current international standards. The studies were conducted on male C57BL/6J mice weighing 20–23 g. Throughout all experiments, the mice were kept at a temperature of 24°C with a 12-hour day-night cycle and free access to food and water. The animals were euthanized by anesthesia with isoflurane followed by dislocation of the cervical spine.

Modeling of transverse aortic stenosis. The animals were divided into two groups: 1) sham-operated animals (N= 10); 2) animals with modeling of transverse aortic constriction (TAC) (N= 72). Tribromoethanol (avertin, intraperitoneally; 100 µl/kg) was used to anesthetize the animals. The TAC was modeled via access to thoracic cavity provided by midline sternotomy. To facilitate exposure of the aortic arch, the thymus and neck muscles were displaced in the surgical field, and a 6.0 suture thread was inserted under the aorta between the origins of the brachiocephalic and left common carotid arteries. Next, a 27G curved needle was placed on the aortic arch, over which a ligature was tied. After tightening the ligature (around the needle and aorta), the needle was quickly removed. Control mice were subjected to a simialr procedure, but without aortic arch ligation (sham-operated animals). After the procedure, the conventional layered wound closure was employed. Oral panadol syrup was used for postoperative pain relief (for 5 days). The animals were euthanized 30 days after the procedure.

Analysis of cardiac tissue cryosections after TAC modeling. For histological studies, the hearts of the mice were removed, washed with physiological solution, embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek), frozen in liquid nitrogen vapor, and used for cryosection preparation. Cryosections 7 µm thick were prepared on a Leica CM1900 cryostat, placed on slides, and stored at –70°C.

Heart sections were stained with picrosirius red to visualize collagen in accordance with previously described protocols [14–16]. The sections were washed with distilled water and placed in a hot dye solution for 10 minutes. The sections were then washed three times with a 5% acetic acid solution. After staining, the slides were washed with distilled water, dehydrated, and mounted using a xylene-based medium. The stained preparations were examined using standard light and polarized microscopy, and the images were documented using a Leica Aperio CS2 device (Leica). Morphometric analysis was performed by measuring the size of cardiomyocytes using Image J software (NIH, USA).

Assessment of autophagy-regulating proteins expression by immunoblotting. To analyze autophagy activity, samples of left ventricular cardiac tissue from control and TAC-operated animals were used. The lysate proteins were separated by SDS-electrophoresis in a 10% polyacrylamide gel on a Mini- PROTEAN 2 device (Bio-rad, USA). Electrophoresis was performed on a PVDF membrane (Millipore, USA) using a Transblot Turbo device (Bio-rad, USA). After electrotransfer, the membrane was incubated in a blocking buffer (phosphate-saline buffer containing 5% skim milk powder (AppliChem, USA). The membranes were then incubated with antibodies against LC3 II/I (Abclonal, USA), p62/SQSTM1 (Abclonal, USA), the active form of caspase 3 (cleaved caspase-3; Cell Signaling, USA) and tubulin (Cell Signaling, USA) for 12 hours at +4°C with constant stirring. The membranes were then washed three times in PBS containing 0.05% Tween-20 (each wash for 10 minutes with constant stirring). After washing, the membranes were incubated with secondary antibodies against rabbit immunoglobulins conjugated with horseradish peroxidase AffiniPure (H + L) (Jackson ImmunoResearch, USA). The mem-

branes were then washed three times for 10 minutes each in phosphate-buffered saline containing 0.05% Tween-20. Protein detection was performed using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, USA). The signal was recorded using the Fusion-SL 3500.WL gel documentation system (Vilber Lourmat, France). Densitometric analysis of luminescence intensity of the studied proteins was performed using Image J software (National Institutes of Health, USA).

Obtaining a culture of mouse cardiac myofibroblasts. Mouse cardiac tissue (intact hearts of C57BL/6J mice; *N*=160) was minced and fermented in a solution of 0.1% type II collagenase and 0.1% trypsin (10 times for 10 minutes at 37°C). The isolated cells were resuspended in modified DMEM/F12 medium containing 10% fetal calf serum and antibiotics. After 120 minutes, the floating cells were washed with PBS, and the adhered cells were used for culture expansion for 3 days. Next, the obtained cells were deprived and stimulated with recombinant TGFb1 (5 ng/ml) for 4 days to induce fibroblast differentiation towards myofibroblasts for obtaining a myofibroblast culture [17–19].

Detection of autophagosomes using Cyto-ID dye. Cyto-ID is a dye that selectively detects autophagosomes with minimal staining of lysosomes, allowing it to be used for specialized monitoring of autophagy [20]. The Cyto-ID® Green Detection Kit (Sigma, USA) was used to visualize autophagy vesicles. Mouse cardiac myofibroblasts (1×10^4) were cultured on glass slide chambers in the absence or presence of staurosporine. The sample was stained with Cyto-ID® reagent for 30 minutes at 37°C and analyzed using Image Exfluorer (LCI, South Korea).

Evaluation of myofibroblast characteristics. Immunocytochemistry was used to detect specific myofibroblast markers (collagen, smooth muscle alpha-actin). Cells were cultured in sterile culture plates with wells, washed, and fixed for 10 minutes in a 3.7% formaldehyde solution prepared in phosphate-saline buffer (pH 7.3). Next, the cells were washed three times with PBS, permeabilized in a 0.1% solution of Triton X100/PBS (10 minutes), and after washing with PBS, the cells were incubated in a «blocking» solution that prevents nonspecific binding of II antibodies (1% BSA/PBS solution, 10% serum from the animal donor of II antibodies). The slides were then stained with primary and II antibodies conjugated with Alexa Fluor 594 (Invitrogen, USA). Cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole). Phalloidin-Alexa594 (a bicyclic oligopeptide capable of selectively binding to polymerized fibrillar actin, a component of the cytoskeleton) was used to evaluate the cytoskeleton.

The «collagen gel contraction» assay was used to assess the contractile phenotype of myofibroblasts. Collagen I (2.5 mg/ml) was used as the carrier gel. The collagen gel was obtained by diluting Viscol (Imtek, Russia) with 0.1 N NaOH solutions and 10× culture medium. Cell suspension (fibroblasts and myofibroblasts) was mixed with ready-made collagen gel (at a ratio of 2×10^5cells per 1 ml of carrier gel) and seeded at 250 µl per well in a 48-well plate (Corning, USA). After gel polymerization, 0.5 ml of medium was added to each well and cultured at 37°C, 5% CO_2. After 72 hours, the change in gel area reflecting the contractile ability of the cells was recorded.

Assessment of myofibroblast apoptosis rate. The rate of myofibroblast apoptosis in heart sections of sham-operated and TAC mice was studied using the TUNEL in situ cell apoptosis kit in accordance with the manufacturer's recommendations (Rosch, USA). To assess myofibroblast apoptosis after exposure to staurosporine (50 nM) and staurosporine in combination with rapamycin (10 µM) *in vitro*, flow cytometry was used in combination with double staining with annexin V and propidium iodide in accordance with the kit manufacturer's instructions (BD Pharmingen, USA). Attached cells were collected with trypsin-EDTA solution, combined with detached cells, washed in PBS, and resuspended in binding buffer. Next, annexin V and propidium iodide were added, and the mixture was incubated at room temperature for 30 minutes before analysis. The analysis was performed by flow cytometry using BD FACS Aria III (BD Pharmingen, USA). The number of annexin V+ apoptotic cells was calculated as a percentage of the total population.

Microscopy and image analysis. Cells and cryosections of the myocardium were analyzed using an Axiovert 200 M fluorescence microscope (Carl Zeiss, USA) and AxioVision 4.8 software (Carl Zeiss, USA).

Statistical analysis. Data are presented as mean ± standard deviation (*M* ± *SD*). Statistical analysis of data was performed using GraphPad Prism software (GraphPad Software 8.3.0). Normality of distribution was tested using the Shapiro–Wilk test. Statistical comparison of *in vivo* study data was performed using Student's *t*-test. Comparison of in vitro study data was performed using one-way ANOVA with Welch's correction, and intergroup pairwise comparisons were performed using Holm–Sedak multiple comparison test. Differences were considered statistically significant at $P<0.05$.

Results

To assess the activity of apoptosis in cardiac fibroblasts, we used *in vivo* modeling of cardiac fibrosis caused by pressure-induced cardiac overload. Animals survival rate on the 30th day of follow up after TAC procedure was 14%. There were no late infection complications after TAC surgery. Signs of LV hypertrophy and marked interstitial and perivascular fibrosis, detected by picrosirius red stain, were documented in 30 days after TAC procedure (Fig. 1, *a, b*). Assessment of

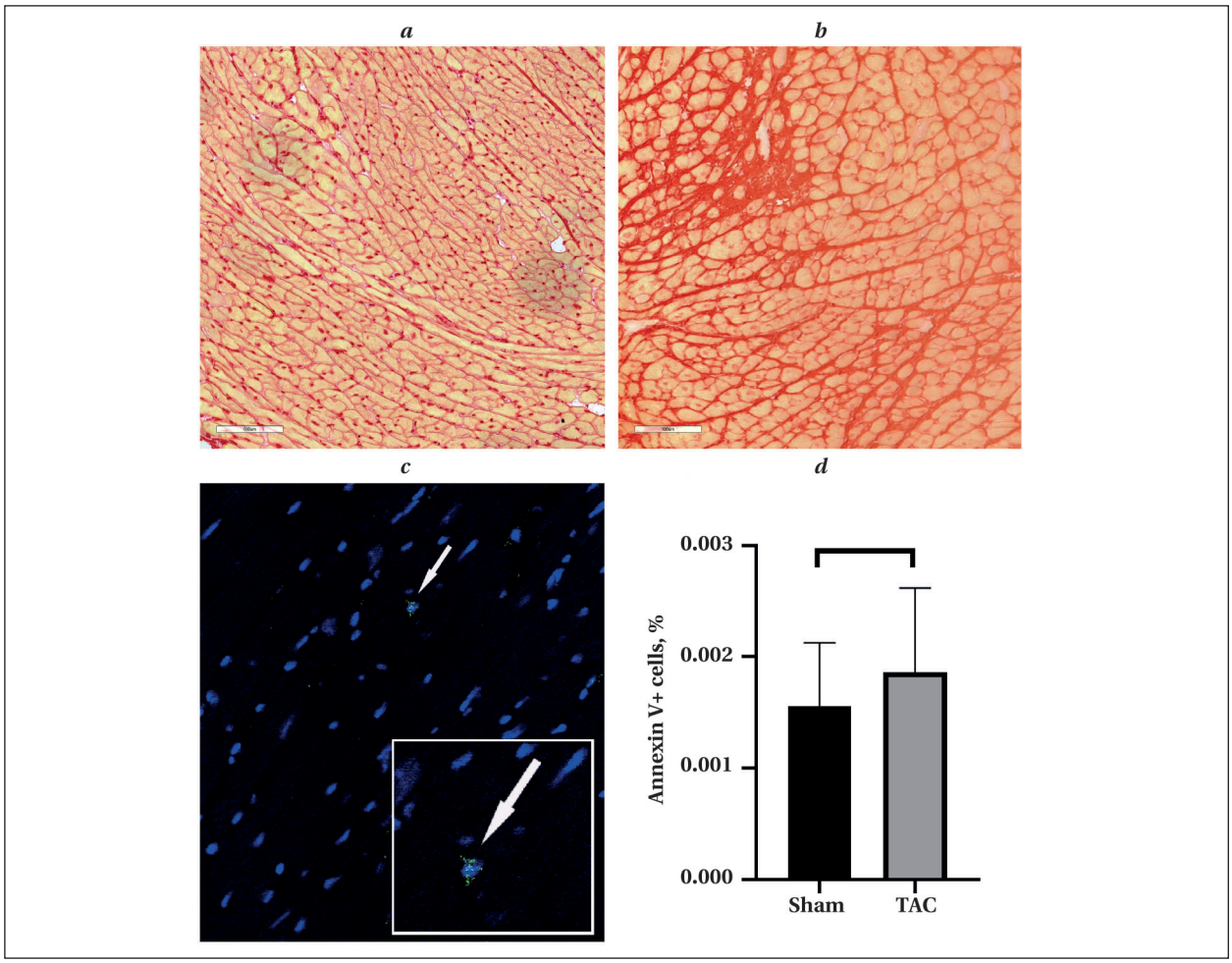

Fig. 1. The development of cardiac fibrosis was characterized by low rates of fibroblast/myofibroblast apoptosis.
Note. Representative images of heart sections from sham-operated mice *(a)* and animals after TAC *(b)*. Stained with picrosirius red. Collagen fibers are outlined in red. Representative images of annexin V+ cell detection (green in the square field), reflecting their entry into apoptosis, in the heart of a mouse after TAC modeling *(c)*. Cell nuclei are stained with DAPI. The arrow points to an annexin V+ cell. *d* — the bars represent quantitative assessment of annexin V+ cell content in sham-operated and TAC hearts. Statistical analysis was performed using Student's *t*-test; *staurosporine*=0.32, *N*=10).

apoptosis rate using the Tunel assay showed that only a few cells in the interstitium had positive staining, indicating the initiation of cell death mechanisms (Fig. 1, *c*). Meanwhile, the total number of Tunel+ cells in cardiac interstitium of animals in the sham-operated and TAC groups did not differ (Fig. 1, *d*). Therefore, fibrogenesis in the heart was characterized by low rates of fibroblast/myofibroblast death.

To clarify the mechanisms of myofibroblast resistance to apoptosis, a myofibroblast culture was obtained by exposing primary cardiac fibroblasts to the profibrotic cytokine TGFb1. TGF-β treatment led to changes in the morphology of cardiac fibroblasts: spindle-shaped cells increased in size, and a network of stress fibers formed based on polymerized actin microfilaments (Fig. 2, *a, b*). The stress fibrils included smooth muscle α-actin fibers, indicating cytoskeletal reorganization and acquisition of a contractile phenotype by the cells (Fig. 2, *b*). Indeed, treatment of cells with the profibrotic inducer TGFb1 led to the generation of cytoskeletal tensile forces that caused contraction of the collagen-formed gel (Fig. 2, *c, d*). Thus, treatment of cells with TGFb1 led to a change in cell morphology, reorganization of the cytoskeleton with the formation of a branched network of stress fibrils, increased expression of smooth muscle alpha-actin, collagen 1, and the acquisition of contractile ability, indicating their differentiation towards myofibroblasts. (Fig. 2, *b*).

To study the mechanism of myofibroblast elimination, an *in vitro* cell model was developed based on cell culture in the presence of staurosporine. Rounded cells with signs of chromatin condensation appeared just 6 hours after treating myofibroblasts with staurosporine (Fig. 3). To confirm the induction of cell death after the addition of staurosporine, we performed immunoblotting studies and found the formation of cleaved caspase 3, indicating the activation of the intrinsic apoptosis signaling pathway (Fig. 3, *d*). In addition, we found that the above

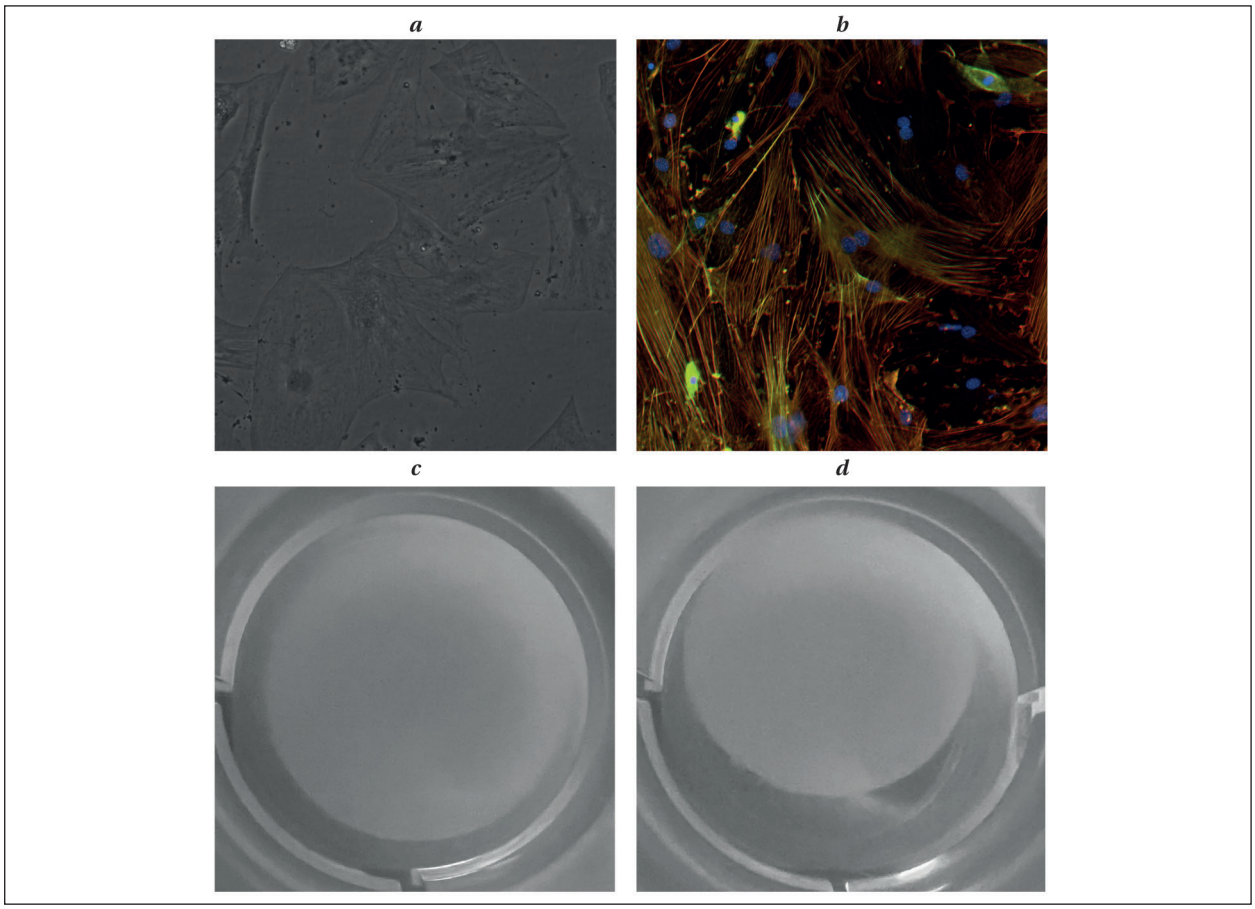

Fig. 2. Characteristics of mouse heart myofibroblasts.
Note. *a* — representative images of mouse heart myofibroblasts (phase contrast). *b* — representative images of immunofluorescence staining of myofibroblasts with antibodies to smooth muscle alpha-actin (green) and phalloidin (red); Yellow indicates co-localization of the smooth muscle alpha-actin and phalloidin signals. *c, d* — representative images of collagen gel contraction containing fibroblasts *(c)* and myofibroblasts *(d)*.

manifestations were accompanied by the accumulation of autophagosomes, as shown by the CytoID assay using specific dye, and signs of autophagy machinery activation (increased LC3II/LC3I ratio and p62/SQSTM1 degradation) (Fig. 3, *c–e*). One day after staurosporine treatment, CytoID+ vacuoles accumulated in most apoptotic cells, indicating a link between cell death and autophagy.

Next, we decided to test how addition of rapamycin, which causes an increase in autophagy activity, could influence the rate of apoptosis in myofibroblasts induced by staurosporine. We found that culturing cells in the presence of rapamycin had no effect on the viability of myofibroblasts. However, its combined use with staurosporine enhanced cell death, emphasizing the important role of increased autophagy activity as a trigger for apoptosis (control 1.68 ± 0.66% (N=4); staurosporine 65.8 ± 2.63% (N=4); rapamycin+staurosporine 73.73 ± 0.67% (N=4); control vs staurosporine $P < 0.0001$; control vs rapamycin+staurosporine $P < 0.0001$; staurosporine vs rapamycin+staurosporine p=0.0071) (Fig. 3, *e*).

Discussion

A sound evidence has been accumulated for considering myofibroblasts as key cells responsible for excessive synthesis, deposition, and remodeling of extracellular matrix proteins in cardiac fibrosis [7–10]. Despite certain success in identifying biological inducers and understanding the background of fibroblast differentiation towards myofibroblasts, the exact mechanisms that support myofibroblasts persistence in fibrotic tissues remain poorly understood.

In this study, we showed that pressure-induced cardiac overload causes accumulation of myofibroblasts population with a low rate of caspase-dependent cell death, and development of noticeable perivascular and interstitial fibrosis in the myocardium. An *in vitro* cell model was developed to study the mechanism of myofibroblast elimination. It is based on the use of staurosporine, an alkaloid that can initiate apoptosis in cardiac myofibroblast cultures through caspase-dependent and caspase-independent mechanisms [21–23]. We found that rapamycin can enhance the effect of staurosporine

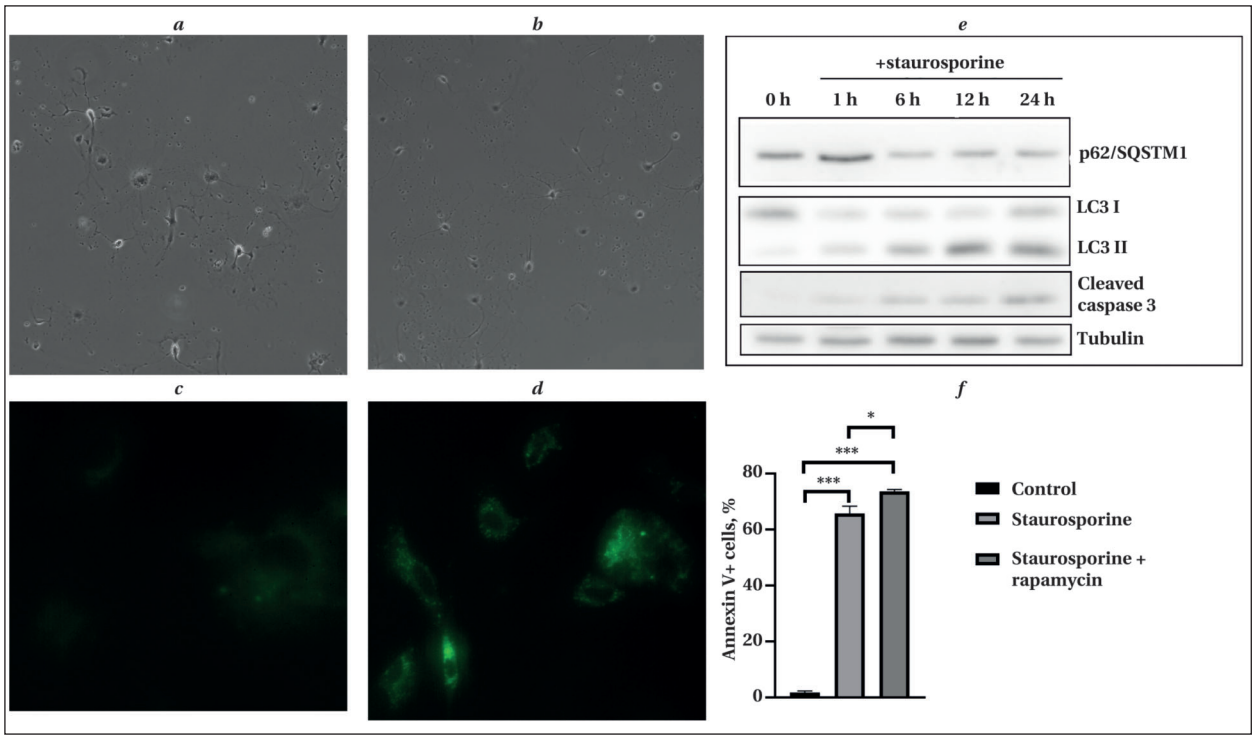

Fig. 3. Staurosporine activated autophagy and caused myofibroblast death.
Note. *a, b* — representative images of myofibroblasts (phase contrast); *a* — after treatment with staurosporine (50 nM, 24 hours); *b* — after treatment with staurosporine (50 nM, 24 hours) in combination with rapamycin (10 μM, 24 hours). *c, d* — representative images of Cyto-ID+autophagosomes (green); *c* — in control myofibroblasts; *d* — after treatment with staurosporine. *e* — representative images of membranes stained with antibodies to p62/SQSTM1, LC3 I, II, cleaved caspase 3, and tubulin, reflecting protein expression in control myofibroblasts and those treated with staurosporine (50 nM, 1, 6, 12, and 24 hours). *f* — graphs showing quantitative assessment of apoptosis rates in control myofibroblast culture, cells after treatment with staurosporine, and treatment with staurosporine in combination with rapamycin for 24 hours. Statistical analysis was performed using ANOVA with Welch's correction (*** — $P < 0.0001$; * — $P = 0.0071$; $N = 4$ in each group).

and cause increased myofibroblast death through autophagy- associated mechanisms.

Autophagy is an evolutionarily conservative process that participates in maintaining homeostatic balance between the synthesis, degradation, and recycling of organelles and proteins to meet metabolic needs and regulate cellular functions [24–26]. As an adaptive response, autophagy can alleviate stressful conditions such as hypoxia, oxidative stress, starvation, endoplasmic reticulum stress, etc. [27]. In the early stages, it is cytoprotective, but its prolonged activation can lead to cell organelle dysfunction, the initiation of the apoptotic cascade, and cell death (programmed type II cell death) [28, 29]. The serine/threonine protein kinase mTOR (mammalian target of rapamycin) plays the key role among all sensors regulating autophagy [30]. Under conditions of nutrient availability, it can inhibit the autophagosome initiation complex — ULK (Unc-51-like kinase 1 complex (ULC)). The mTOR is also involved in the regulation of autophagy-related gene transcription. It can phosphorylate the TFEB transcription factor, preventing its nuclear translocation and transcription of a number of genes associated with autophagy [31–35]. Studies conducted by our group have shown that the development of cardiac fibrosis caused by pressure-induced cardiac overload is characterized by decrease of autophagy activity in cardiac tissue. Detailed bioinformatic studies of single cell transcriptome revealed no change in the expression of genes associated with autophagy in the entire pool of cardiac fibroblasts and its individual subpopulations during the development of cardiac dysfunction [12]. This can be explained by preservation of high mTOR activity, which is responsible for reducing intracellular collagen degradation via autophagy. It mediates cell resistance to apoptosis, and impairs their ability to adapt to stress [36]. To test this hypothesis, we conducted in vitro experiments using the apoptosis inducer staurosporine. This compound was originally obtained from *Streptomyces* and was characterized by its ability to inhibit protein kinase C [37]. However, it was later found to be a non-selective inhibitor of a number of different kinases [38, 39], which gives it the potential to initiate apoptosis in most mammalian cells. In our study, we found that treatment of cells with staurosporine initiated the death of myofibroblasts. It was accompanied by simultaneous increase in the number of autophagosomes and a change in protein expression

(an increase in the LC3II/LC3I ratio and degradation of p62/SQSTM1), associated with increased autophagy activity. A possible explanation for the staurosporine-induced increase in autophagy lies in modified mTOR activity and phosphorylation of downstream translational regulatory components [40]. These include p70 S6 kinase, which phosphorylates ribosomal protein S6 and eukaryotic initiation factor (eIF)4E. Exposure to staurosporine led to a decrease in p70 S6 kinase activity, dephosphorylation of ribosomal protein S6, increased binding of 4E-BP1 to eIF4E, and a simultaneous decrease in the amount of eIF4F complexes. Thus, disruption of mTOR signaling by staurosporine initiates an early cellular response (prior to caspase cascade activation) to a stress stimulus, including that achieved through activation of autophagy, and defines the readiness for the initiation of apoptosis.

Next, we tested how additional stimulation of autophagy can affect the rate of myofibroblasts apoptosis induced by staurosporine. For this purpose, we used rapamycin (sirolimus), an antibiotic/immunosuppressant produced by the soil bacterium *Streptomyces hygroscopicus*, which induces autophagy by acting on its molecular target, mTOR (mammalian target of rapamycin) kinase [24]. In our study, the use of rapamycin significantly enhanced the proapoptotic effect of staurosporine, which may be associated with more powerful and sustained inhibition of mTOR signaling [41, 42].

Conclusion

Thus, pressure-induced cardiac overload causes accumulation of myofibroblasts, characterized by a low apoptosis rate, and the development of marked interstitial fibrosis in the myocardium. To study the mechanism of myofibroblast elimination, a cell model was developed using staurosporine, an alkaloid capable of initiating apoptosis in a culture of cardiac myofibroblasts. It was found that rapamycin can enhance the effect of staurosporine and cause increased myofibroblast death through autophagy-associated mechanisms. The molecular mechanisms of this process require further study.

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