Sustained Oligomycin Sensitivity Conferring Protein Expression in Cardiomyocytes Protects Against Cardiac hypertrophy Induced by Pressure Overload via Improving Mitochondrial Function

Yingying Guo,^{1,†} Kailiang Zhang,^{1,†} Xu Gao,¹ Zhou Zhou,¹ Zhiheng Liu,¹ Kevin Yang,² Kai Huang,³ Qinglin Yang,^{4,*} and Qinqiang Long^{1,*}

¹Division of Cardiology, Department of Internal Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China;

²Department of Nutrition Sciences, University of Alabama at Birmingham, Birmingham, Alabama, USA; ³Department of Cardiovascular Diseases, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China; ⁴Cardiovascular Center of Excellence and Department of Pharmacology, Louisiana State University Health Science Center, New Orleans, Louisiana, USA.

[†]These authors contributed equally to this work.

Cardiac hypertrophy is a major risk factor for congestive heart failure, a leading cause of morbidity and mortality. Abrogating hypertrophic progression is a well-recognized therapeutic goal. Mitochondrial dysfunction is a hallmark of numerous human diseases, including cardiac hypertrophy and heart failure. F₁F_o-ATP synthase catalyzes the final step of oxidative energy production in mitochondria. Oligomycin sensitivity conferring protein (OSCP), a key component of the F₁F_o-ATP synthase, plays an essential role in mitochondrial energy metabolism. However, the effects of OSCP-targeted therapy on cardiac hypertrophy remain unknown. In the present study, we found that impaired cardiac expression of OSCP is concomitant with mitochondrial dysfunction in the hypertrophied heart. We used cardiac-specific, adeno-associated virus-mediated gene therapy of OSCP to treat mice subjected to pressure overload induced by transverse aortic constriction (TAC). OSCP gene therapy protected the TAC-mice from cardiac dysfunction, cardiomyocyte hypertrophy, and fibrosis. OSCP gene therapy also enhanced mitochondrial respiration capacities in TAC-mice. Consistently, OSCP gene therapy attenuated reactive oxygen species and opening of mitochondrial permeability transition pore in the hypertrophied heart. Together, adeno-associated virus type 9-mediated, cardiac-specific OSCP overexpression can protect the heart via improving mitochondrial function. This result may provide insights into a novel therapy for cardiac hypertrophy and heart failure.

Keywords: OSCP, cardiac hypertrophy, mitochondria, OXPHOS, mPTP

INTRODUCTION

CARDIAC HYPERTROPHY is an independent risk factor that increases the incidence and mortality of various cardiovascular diseases in clinical practice. Long-term pathological hypertrophy may initially accommodate hemodynamic overload, but often decompensates to heart failure.¹ The myocardium is among the major consumers of adenosine 5'-triphosphate (ATP) in the body. More than 95% of ATP produced by the normal myocardium comes from oxidative phosphorylation (OXPHOS) in mitochondria.² Mitochondria make up about 30% of the mass of cardiomyocytes, playing crucial roles not only in energy metabolism but also signaling and cell fate determination.³ Many cell and animal studies have consistently shown that mitochondrial

dysfunction plays a pivotal role in the pathological development of cardiac hypertrophy and heart failure.^{4–7}

Mitochondrial energy generation is the process of electron transfer through four mitochondrial electron carriers: complex I, complex II, complex III, and complex IV. The accumulated proton motive force then drives the ATP synthase (complex V) to catalyze the phosphorylation of adenosine-5'-diphosphate (ADP) to ATP.⁴ The mitochondrial ATP synthase is mainly composed of two parts: the F₁ subunit protruding from the inner membrane and the F_o subunit embedded in the membrane. F₁ and F_o are connected by two handle-like subunits, the central rotor shaft and the peripheral stator. In mammalian mitochondrial enzymes, the peripheral stem consists of oligomycin

*Correspondence: Dr. Qinqiang Long, Division of Cardiology, Department of Internal Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Avenue, Wuhan, China 430030. E-mail: qqlong@tjh.tjmu.edu.cn; Prof. Qinglin Yang, PhD, Cardiovascular Center of Excellence and Department of Pharmacology, Louisiana State University Health Science Center, 533 Bolivar Street 4th Floor, Room 416, New Orleans, LA 70112, USA. E-mail: qyang1@lsuhsc.edu

sensitivity conferring protein (OSCP), b, d, and F6 subunits. OSCP is directly linked to the α and β subunits of F1 and b subunits of Fo, and the binding of OSCP represents the final step in the assembly of complex V.^{8,9}

OSCP, encoded by the nuclear gene ATP5O, is highly conserved across species and it contains 180–190 amino acids with a molecular weight of ~23 kDa; however, the roles of OSCP in animal tissue have only just begun to emerge. OSCP is considered essential to coupling the proton motive force from the proton gradient to ATP synthase activity (see review Ref.¹⁰). OSCP may also interact with cyclophilin D (CypD), a key regulator of mitochondrial permeability transition pore (mPTP),^{11,12} but it remains controversial whether OSCP is a crucial part of the mPTP.¹³ OSCP null cells show slow growth with decreased mitochondrial biogenesis and declined OXPHOS rate.¹³ OSCP has also been found to be reduced in brains from patients with Alzheimer's disease¹⁴ and hearts from patients with heart failure.¹⁵ Overall, OSCP merits additional research. OSCP ensures the structural and functional coupling between Fo and F1 of ATP synthase, and plays an important role in maintaining the structural and functional integrity of ATP synthase. In the past, most of our understanding on the functions of OSCP has been at the *in vitro* and cellular level. While these studies define the biochemical and biophysical roles of OSCP, studies remain scarce concerning its role in pre-clinical animals. In 2006, a study showed that cardiac OSCP levels were reduced in swine models of myocardial infarction and heart failure.¹⁶ We speculate that the loss of OSCP in the heart, an organ with high demand for energy, attributes significantly to the cardiac pathological development under mechanical stress, such as pressure overload. We aimed to test a hypothesis that therapeutic interventions enhancing cardiac OSCP expression should be protective in the heart under pressure overload condition.

In this study, we found that OSCP mRNA expression and protein were reduced in hypertrophied hearts induced by pressure overload in mice. Adeno-associated virus (AAV)-mediated cardiac-specific overexpression of OSCP protected the heart from cardiac dysfunction, hypertrophy, and remodeling. Furthermore, OSCP overexpression in the heart improved the integrity of the mitochondrial structure and function. Therefore, the present study provides a potential innovative therapeutic strategy for the treatment of cardiac hypertrophy and heart failure.

MATERIALS AND METHODS

Animal study

All animal experiments were approved by the Animal Research Committee of Tongji Medical College of Huazhong University of Science and Technology. C57BL/6J mice (Beijing Huafukang Biotechnology Co., Ltd) were raised in the animal health facilities of Tongji Medical

College, with facility certificate and mouse quality certificate. All animal studies were carried out in accordance with the National Institute of Health's "Guidelines for the Care and Use of Laboratory Animals" and approved by the Animal Experimental Ethics Committee of Tongji Medical College. Mice were housed in temperature-controlled cages under a 12-h light/dark cycle and given free access to water and normal chow.

AAV generation

The open reading frame of mouse Atp5o (NM_138597) was cloned into the pAAV vector under control of a cardiac troponin T (cTNT) promoter and was subsequently used for AAV generation. AAVs were generated by DesignGene Biotechnology (Shanghai, China). Adeno-associated virus type 9 (AAV9) encoding green fluorescent protein (GFP) was used as control. The AAV9 vector backbone was a kind gift from Prof. Daowen Wang (Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology) and Prof. William T. Pu (Boston Children's Hospital, Harvard Medical School).

Transverse aortic constriction

The transverse aortic constriction (TAC) surgery is performed as described below. Male mice (6–7 weeks old, 19–24 g body weight) were intraperitoneally injected with 1% sodium pentobarbital (0.1 mL/20 g). The anesthetized mice were placed on the surgical board after being depilated through the chest. The endotracheal tube was inserted and connected to the mouse ventilator. During the operation, a thoracic incision was made. The blood vessels were bluntly separated, and a 7–0 silk thread was ligated on the 27G blunt needle between the innominate artery and the left common carotid artery. Immediately after ligation, the needle was removed to achieve a narrowing effect. The chest was sutured with a 4–0 silk thread and the skin was sutured with 6–0 Prolene. The mice were placed in a warm cage at 37°C until completely recovered from anesthesia. Sham-operated mice also underwent the same procedure but without ligation of the transverse aorta. Mice were injected with AAV 3 days after TAC and sacrificed 4 weeks later.

Echocardiographic assessment

Echocardiography was performed in mice anesthetized with 1.5% isoflurane as previously described^{17,18} using a Vevo 1100 Imaging System (Visual Sonics, Toronto, Canada) equipped with a 30 MHz linear-array transducer. Heart rate was maintained at about 450 beats per minute. Left ventricular (LV) end-systolic diameter and LV end-diastolic diameter, the percentage of fractional shortening (FS, %), ejection fraction (EF, %), and other parameters were measured from the M-mode and two-dimensional images obtained in the long- and short-axis views by the corresponding matching software. The Doppler tracing was

measured at the posterior end of the aortic arc with or without ligation. All measurements were performed from leading-edge according to the American Society of Echocardiography guidelines.

Human heart samples

The samples from explanted hearts used in this study were obtained from three patients who had received heart transplants (diagnosed as dilated cardiomyopathy) at the Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, and three age-matched healthy subjects from traffic accidents. All study participants provided informed written consent and the study was approved by the Institutional Ethics Committee of Tongji Hospital and conducted in accordance with the principles of the Helsinki Declaration. Myocardial posterior wall samples were obtained within 2 h from accidental death or heart transplantation. Immediately after tissue procurement, the samples were stored in liquid nitrogen and kept at -80°C ; samples were later subjected to western blot.

Mitochondrial isolation

Cardiac mitochondria were isolated from male C57BL/6J mice. Briefly, blood and main vasculature were dissected, and heart tissue was minced on ice, then suspended in buffer A (250 mM sucrose, 10 mM Tris/Cl, 0.5 mM ethylenediaminetetraacetic acid), and homogenized using a 2 mL Potter-Elvehjem teflon/glass homogenizer. The resulting samples were centrifuged at 1,000 *g* for 10 min, and the supernatant with mitochondria was poured into another ice-cold tube, followed by centrifugation at 8,000 *g* for 5 min. The mitochondrial-enriched sediments were resuspended in buffer A. Mitochondrial protein content was determined by the Lowry method.

Assessment of mitochondrial respiration

Mitochondrial respiration was measured as previously described.¹⁹ Briefly, 200 μg of freshly isolated mitochondria was measured in 2 mL of Mir05 mitochondrial respiration medium (3 mM MgCl_2 , 60 mM lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4 , 20 mM HEPES, 110 mM D-sucrose, 1 g/L BSA, and 0.5 mM EGTA) using an Oroboros 2k-oxygraph (Oroboros Instruments, Innsbruck, Austria). Mitochondrial respiration was stimulated by basal substrates (5 mM pyruvate, 2 mM malate, 10 mM glutamate, and 1 mM ADP) for complex I activity. Next, 10 mM succinate was added to measure combined respiration rates of complex I and complex II. After that, 2 μg /mL oligomycin was added for the estimate of the overall mitochondrial-related respiration. Furthermore, carbonyl cyanide p-trifluoromethoxyphenylhydrazone was added to determine the maximal coupling respiration. Finally, the addition of 2.5 μM antimycin A allowed for the measurement of nonmitochondrial oxygen consumption.

Mitochondrial swelling assay

After mitochondrial isolation from mouse heart, 50 μg of fresh mitochondria was resuspended in assay buffer: 150 mM KCl, 5 mM HEPES, 2 mM K_2HPO_4 , 5 mM glutamate (pH 7.3), and 250 μM CaCl_2 was used to induce mitochondrial swelling. The mPTP opening inhibitor cyclosporine A was added as negative control. The absorbance value was read at 540 nm with a spectrophotometer, and the reading was made every 15 s, for a total of 600 s. Analysis was done after data collection.

Detection of reactive oxygen species production

Dihydroethidium (DHE; Beyotime Institute of Biotechnology, Haimen, Jiangsu, China) was applied to frozen section samples (5- μm -thick sections). The heart sections were stained with 5 μM DHE and incubated in a light-protected humidified chamber at 37°C for 30 min. Fluorescence intensity was examined by fluorescence microscopy (Nikon DXM1200 fluorescence microscope), and images were analyzed with the ImageJ software. We performed DHE staining on frozen heart sections from three mouse hearts of each group. We measured four different regions of interest (ROI) selected from different tissue areas in one image. Every selected ROI was at least 200 μm apart. The corresponding mean fluorescent intensity of DHE staining was measured using the ImageJ program (National Institutes of Health, USA).

Total RNA extraction and real-time PCR

Total RNAs were extracted using the RNAsimple Total RNA Kit (TIANGEN, Beijing, China). Total RNA (1 μg) was reverse-transcribed into cDNA using a cDNA Synthesis Kit (TRANSGEN, Beijing, China) according to the manufacturer's protocol. Quantitative real-time PCR (qPCR) analyses were carried out using the StepOne Real-Time PCR system (Applied Biosystems) to determine transcript levels of target genes. Expression of each gene was normalized to actin or 36B4. The sequences of the primers are listed as follows: mOSCP-For: CAAGCGC ACCGTCAAAGTG, mOSCP-Rev: GCACCGTCTTTAA CTCAGAGAG; ANP-For: GGGGTAGGATTGACAG GAT, ANP-Rev: CGTGATAGATGAAGGCAGGAA; BNP-For: GGGAGAACACGGCATCAT, BNP-Rev: GC CATTTCCTCCGACTTT; 36B4-For: TGGAGACAAGG TGGGAGCC, 36B4-Rev: CACAGACAATGCCAGGA CGC. At least three independent experiments were conducted to ensure the reproducibility of data.

Western blotting

The frozen cardiac tissues were lysed in the RIPA buffer (Applygen Technologies, Inc., Beijing, China). The BCA Protein Assay Kit (Boster Biological Engineering Co., Ltd, Wuhan, China) was used to measure protein concentrations. Thirty micrograms of protein samples was separated by 10% sodium dodecyl sulfate/polyacrylamide

gel electrophoresis and transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA). Furthermore, 5% nonfat dried milk was used to block the membrane for 2 h at room temperature and then incubated with a primary antibody (OSCP 1:1,000, sc-365162; Santa Cruz, USA) at 4°C overnight. After being washed with Tris-buffered saline with 0.1% Tween 20, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody anti-mouse IgG (1: 2,500; Boster Biological Engineering Co., Wuhan, China) and a peroxidase-conjugated secondary antibody anti-rabbit IgG (1:10,000; Santa Cruz) for 1 h at room temperature. Enhanced chemiluminescence reagents were used to expose the bands. Equivalent protein loads were verified and normalized using GAPDH (1:1,000; Santa Cruz) and actin (1:1,000, A4700; Sigma-Aldrich, USA) blots. Finally, bands were then quantified by densitometry using ImageJ software.

Histological analysis

Heart tissue samples were fixed in 4% paraformaldehyde for immunohistochemistry. Samples were embedded in paraffin, cut into 4- μ m-thick sections, and stained with hematoxylin and eosin (H&E) or Masson's trichrome blue in Wuhan Servicebio Technology Co., Ltd., China. Images were taken with a light microscope. Quantitative analysis of the cardiomyocyte cross-sectional area was conducted by measuring 100–150 cardiomyocytes from four to six mouse hearts per group. Fibrotic area was measured based on Sirius Red-stained area to total myocardial area from 30 to 50 randomly chosen frames from three mice per group.

Transmission electron microscopy

Heart tissue was removed from the animal and immediately rinsed in phosphate-buffered saline (PBS). The tissue was placed in a culture dish with 0.5% glutaraldehyde and 0.2% tannic acid in PBS and cut 1 mm cubes and then transferred to modified Karnovsky's fixative (4% formaldehyde and 2.5% glutaraldehyde containing 8 mM CaCl_2 in 0.1 M sodium cacodylate buffer, pH 7.4). Samples were washed with PBS and postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h to produce osmium black. Samples were then dehydrated through a graded series of ethanol and embedded in Epon/SPURR resin (Thermo Fisher Scientific) that was polymerized overnight at 65°C. Sections were examined at different magnifications with an HT7700 transmission electron microscope (HITACHI, Japan).

Statistical analyses

Data for two-group comparisons were analyzed with the nonparametric Student's *t*-test; otherwise, data were analyzed by one-factor or mixed two-factor analysis of variance and multiple comparisons test using the GraphPad Prism 6 software (GraphPad Software, Inc.). The

values of quantitative results are expressed as mean $\pm$ SEM. Differences between groups and treatments were regarded as significant at $p < 0.05$.

RESULTS

OSCP expression is downregulated in pressure overload-induced hypertrophied hearts

As a key component of the ATP synthase, OSCP is expressed constitutively in a variety of organs, including the heart. However, it remains unknown if cardiac OSCP expression is altered in hypertrophied hearts. To determine the potential change of OSCP in the heart, we examined the changes in OSCP transcript and protein in myocardial tissue from mice with TAC-induced cardiac hypertrophy. qPCR and western blot revealed that the expression of OSCP was markedly declined in the hypertrophied hearts at both the transcript and protein levels (Fig. 1A, B). Western blot also showed that OSCP was decreased in samples from failing hearts from patients compared with the healthy hearts from donors (Fig. 1C). These results recognize that OSCP expression declined in the pathological hearts at either the hypertrophic or failure stage.

Cardiomyocyte-specific overexpression of OSCP attenuates cardiac hypertrophy in pressure overload hypertrophied hearts

We next investigated whether a sustained expression of OSCP in the heart could prevent pathological progression in mice with TAC-induced pressure overload. We used a gene therapeutic approach using an AAV9 containing a chicken cTNT promoter (AAV9-cTNT-OSCP), which allows the overexpression of OSCP specifically in cardiomyocytes of the mouse heart. Mice treated with AAV9-cTNT-GFP served as controls. Three days after the sham operation and TAC, 2.1×10^{11} vg/mouse of AAV-GFP or AAV-OSCP virus was injected through the tail vein (Fig. 2A). Four weeks later, mice were sacrificed and the heart samples were collected. The results confirmed that OSCP transcripts (Fig. 2B) and protein expression (Fig. 2C) both were increased significantly after AAV9-cTNT-OSCP injection compared with AAV9-cTNT-GFP control. The echocardiographic examination showed no change in cardiac function in mice injected with AAV9-cTNT-OSCP after sham operation. To verify that the TAC surgical ligation was successful, Doppler was used to detect blood flow velocity in the aortic arch at 4 weeks after TAC or sham surgery (Fig. 3A). Cardiac function in TAC mice injected with AAV9-cTNT-OSCP was significantly improved compared with mice injected with AAV9-cTNT-GFP (Fig. 3A), as both EF% (Fig. 3B) and short-axis shortening rate (FS%; Fig. 3C) were maintained in AAV9-cTNT-OSCP-treated TAC mice. While TAC pressure overload induced a substantial increase of interventricular septum (IVS) and posterior wall

Figure 1. Detection of OSCP expression in hypertrophied and failing hearts. **(A)** Real-time PCR analysis of OSCP mRNA level in hearts following 4 weeks of sham or TAC. **(B)** Western blot of OSCP and actin (loading control) levels in hearts following 4 weeks of sham or TAC. **(C)** Western blot of OSCP and GAPDH in healthy and failing human hearts. Data are expressed as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$ compared with sham operation and normal donor. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; OSCP, oligomycin sensitivity conferring protein; TAC, transverse aortic constriction. Color images are available online.

Figure 2. Cardiac specific overexpression of OSCP 4 weeks after tail vein injection of AAV9-cTNT-OSCP in C57BL/6J mice. **(A)** Experimental protocol of TAC surgery and gene therapy. **(B)** Real-time PCR analysis of myocardial OSCP transcript expression. **(C)** Western immunoblot analysis of cardiac OSCP expression in TAC and sham mice after AAV9-cTNT-OSCP and AAV9-cTNT-GFP injection, respectively. Data are expressed as mean $\pm$ SEM, $n = 5$, * $p < 0.05$. Color images are available online.

Figure 3. Echocardiographic parameters in mice with pressure overload. **(A)** Representative M-mode (*top*) and Doppler tracings of blood flow (*bottom, dot line*) echocardiography tracings obtained from mice at the midpapillary level. Echocardiographic measurement of EF% **(B)** and FS% **(C)**. Data are expressed as mean $\pm$ SEM, $n=7-10$, $*p<0.05$. ns, not significant. EF, ejection fraction; FS, fractional shortening. Color images are available online.

thickness (LVPW) at both systole and diastole in AAV9-cTNT-OSCP and AAV9-cTNT-GFP groups, IVS and LVPW dimensions at systole and diastole in TAC mice were decreased with AAV9-cTNT-OSCP (Table 1). The TAC hearts were substantially enlarged compared with sham hearts, but enlargement was less pronounced in AAV9-cTNT-OSCP- than AAV9-cTNT-GFP-treated mice (Fig. 4A). The ratios of heart-to-body weight (HW/BW) and heart weight to tibial length (HW/TL) were reduced in AAV9-cTNT-OSCP- compared with AAV9-cTNT-GFP-treated mice (Fig. 4B, C). Real-time PCR assay showed that the transcript expression of atrial natriuretic peptide and B-type natriuretic peptide was significantly more elevated in the hearts of AAV9-cTNT-GFP- than the AAV9-cTNT-OSCP-treated group (Fig. 4D). In addition, histological staining of H&E, Masson's trichrome, and Sirius red on heart sections showed a smaller cross-sectional area of cardiomyocytes (Fig. 4E, F) and less pronounced fibrosis (Fig. 4E, G) in

AAV9-cTNT-OSCP-treated mice than in AAV9-cTNT-GFP-treated mice after TAC. Transmitted electron microscopy imaging of heart sections showed improved mitochondrial integrity in cardiomyocyte ultrastructure of AAV9-cTNT-OSCP- compared with AAV9-cTNT-GFP-treated hearts (Fig. 4E). After TAC, AAV9-cTNT-GFP hearts showed mitochondrial network disruption with loss of matrix, vacuolization, swelling, and a reduced mitochondrial crista compared with AAV9-cTNT-OSCP hearts (Fig. 4E). These results indicate that OSCP gene therapy protects the heart against pressure overload hypertrophy and pathological development.

Sustained cardiac OSCP expression maintains mitochondrial respiration capacities in mouse hearts subjected to pressure overload

As a key component of ATP synthase, OSCP may affect the biological activity of ATP synthase. Therefore, we examined the rate of mitochondrial OXPHOS in

Table 1. Echocardiographic parameters of left ventricular function

Parameters	Sham		TAC	
	AAV9-cTNT-GFP	AAV9-cTNT-OSCP	AAV9-cTNT-GFP	AAV9-cTNT-OSCP
Peak gradient, mmHg			57.57 ± 6.94**	61.67 ± 3.14
IVSd, mm	0.90 ± 0.06	0.91 ± 0.033	1.21 ± 0.04**	1.45 ± 0.13
IVSs, mm	1.37 ± 0.14	1.40 ± 0.06	1.52 ± 0.04	1.69 ± 0.12
LVIDd, mm	4.12 ± 0.14	2.65 ± 0.14	4.04 ± 0.09	3.80 ± 0.06 [#]
LVIDs, mm	2.66 ± 0.17	2.83 ± 0.08	3.20 ± 0.17*	2.56 ± 0.14 ^{##}
LVPWd, mm	0.81 ± 0.02	0.83 ± 0.02	0.92 ± 0.09	1.03 ± 0.10
LVPWs, mm	1.13 ± 0.08	1.10 ± 0.02	1.10 ± 0.09	1.37 ± 0.15
LVIDd VOL, μ L	72.35 ± 4.05	72.54 ± 2.86	71.18 ± 3.93	60.19 ± 2.02 [#]
LVIDs VOL, μ L	26.57 ± 4.63	27.74 ± 1.57	39.71 ± 5.68	23.51 ± 2.70 [#]
Stroke volume, μ L	45.78 ± 0.73	45.69 ± 1.34	31.47 ± 3.28*	36.68 ± 1.67
Cardiac output, mL/min	23.23 ± 1.40	23.51 ± 0.46	17.44 ± 1.67*	19.58 ± 1.12
Heart rate (bpm)	497.0 ± 17.06	487.7 ± 20.17	552.3 ± 11.57	501.2 ± 18.79
Body weight (g)	25.39 ± 2.16	27.42 ± 0.62	26.36 ± 1.76	25.47 ± 2.24

n = 7–11 in each group. Data are expressed as mean ± SD.

p* < 0.05, *p* < 0.01 compared with the corresponding group injected with GFP virus after sham operation. [#]*p* < 0.05, ^{##}*p* < 0.01 compared with group injected with GFP virus after TAC operation.

GFP, green fluorescent protein; IVSd, diastolic interventricular septum; IVSs, systolic interventricular septum; LVIDd, left ventricular internal diameter in diastole; LVIDs, systolic left ventricular internal diameter; LVPWd, left ventricular diastolic posterior wall thickness; LVPWs, left ventricular systolic posterior wall thickness; TAC, transverse aortic constriction; VOL, volume.

OSCP-overexpressed mice with or without TAC-induced cardiac hypertrophy. Mitochondrial respiration on samples extracted from sham and TAC-mice were assessed using the Oroboros oxygraph 2k system (Fig. 5A). Routine mitochondrial respiration was determined after the concomitant addition of malate (2 mM) and pyruvate (5 mM). Interestingly, routine mitochondrial respiratory capacity was upregulated by OSCP overexpression compared with controlled mitochondria, this difference was significant after TAC (Fig. 5B). Maximum phosphorylation respiration capacity (OXPHOS CI+CII) and maximum electron transport system-uncoupled respiratory capacity (ETS CI+CII) were elevated in mitochondria from AAV9-cTNT-OSCP-treated hearts (Fig. 5C, D). The proton leak (LEAK CI+CII) respiration, measured after oligomycin addition, was significantly increased in mitochondria from AAV9-cTNT-OSCP hearts compared with those of control (Fig. 5E). The respiratory control rate, an indicator of the structural integrity of the inner mitochondrial membrane and OXPHOS efficiency, was also increased accordingly in mitochondria from AAV9-cTNT-OSCP hearts (Fig. 5F). These results suggest that OSCP overexpression can sustain mitochondrial OXPHOS in TAC-induced cardiac hypertrophy to protect myocardial function.

Sustained cardiac OSCP expression reduces reactive oxygen species in mouse hearts subjected to pressure overload

We next investigated if sustained cardiac OSCP expression in the hypertrophied heart would also minimize reactive oxygen species (ROS) production from the impaired mitochondria in the hypertrophied heart. The frozen heart sections stained with DHE showed that the intensity of positive staining was substantially increased in hypertrophied hearts, but DHE staining of OSCP-overexpressed hearts was substantially reduced compared with those of GFP-controlled hearts (Fig. 6A, B). Therefore, these findings support that OSCP overexpression may help reduce ROS production in the heart under the pressure overload condition, which may contribute to protecting the hypertrophied heart.

Sustained cardiac OSCP expression in pressure overload heart suppresses the mPTP opening

The protective effects of OSCP on mitochondrial integrity were also evidenced upon calcium overload, a situation that induces the opening of the mPTP, leading to failure of osmotic pressure control, water influx, and swelling of mitochondria.²⁰ Isolated cardiac

Figure 4. AAV9-mediated OSCP overexpression improves cardiac dysfunction in pressure overload-induced hypertrophied hearts. Three days after TAC or sham operation, the mice were subjected to AAV injection and maintained for 4 more weeks (*n* = 8–12). **(A)** Representative images of the hearts from sham and TAC mice. Scale bar, 1 mm. **(B)** Heart weight to body weight ratios (HW/BW), **(C)** Heart weight to tibial length ratios (HW/TL). **(D)** Real-time PCR assessment of natriuretic peptide A (ANP) and B (BNP) transcript expression normalized to 36B4 on samples from experimental groups (*n* = 3–7). Data are expressed as mean ± SEM, **p* < 0.05. **(E)** Cardiac histology, fibrosis assessment, and TEM in mice 4 weeks after TAC. The cross-sectional area of cardiomyocytes was obtained from heart sections with H&E staining. Fibrosis was assessed on heart sections stained with Masson's trichrome and Sirius red. Scale bar, 200 μ m. The quantified fibrosis data were based on Sirius red. Representative images of LV TEM assessment. The arrowhead indicates mitochondria. Scale bar: 5 μ m (upper) and 1 μ m (lower). **(F, G)** Statistical analysis of the results of H&E staining and Masson's trichrome staining measured using ImageJ software. Data are presented as the mean ± SEM, **p* < 0.05, ***p* < 0.01, ****p* < 0.001. AAV, adeno-associated virus; SEM, standard error of the mean; TEM, transmission electron microscope. Color images are available online.

Figure 5. AAV9-mediated OSCP overexpression improves mitochondrial respiration capacities in mice suffering TAC-induced cardiac hypertrophy. **(A)** Representative profiles of six different experiments in each group. **(B)** Basal mitochondrial respiration capacities (routine). **(C)** Maximal phosphorylating respiration capacity via convergent input through complexes I and II (OXPHOS CI+CI). **(D)** The maximal uncoupled respiratory capacity of the electron transport system (ETS CI+CI). **(E)** Inhibition of the phosphorylation system by oligomycin (LEAK CI+CI). **(F)** RCR. Data are presented as the mean $\pm$ SEM, $n=6$, * $p<0.05$, ** $p<0.01$. OXPHOS, oxidative phosphorylation; RCR, respiratory control ratio. Color images are available online.

mitochondria demonstrated a steady loss of light scattered by the mitochondrial membrane structure after the addition of 250 mM calcium. As shown in the TAC-induced cardiac hypertrophy model, overexpression of OSCP in cardiomyocytes increased resistance of mitochondrial membranes to calcium ions and inhibited the opening of mPTP, but showed no change in sham groups (Fig. 7A, B). This finding indicates that OSCP may protect mitochondrial membrane integrity.

DISCUSSION

The heart is highly dependent on energy, which is mostly derived from mitochondrial OXPHOS.² The mitochondrion is not only the primary energy generator but also a key determinant of signaling and cell fate.³ Mitochondrial dysfunction is one of the main players in the pathological development of cardiac hypertrophy and heart failure.⁴⁻⁷ The current study uncovered that

Figure 6. AAV9-mediated OSCP overexpression reduces myocardial reactive oxygen species levels in mice subjected to TAC-induced cardiac hypertrophy. **(A)** Representative images of DHE staining (red) on frozen heart sections. **(B)** Quantification of fluorescence density ($n=3$). Values are presented as the mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$. DHE, dihydroethidium. Color images are available online.

OSCP, a key component of the mitochondrial ATP synthase, is reduced in hypertrophied and failing hearts. More importantly, by improving mitochondrial respiration, gene therapy that restores the cardiomyocyte-specific expression of OSCP is sufficient to at least partly mitigate the cardiac pathological progression in mice subjected to pressure overload.

ATP synthase catalyzes the final step of energy production in the mitochondria. Alterations of ATP synthase activity could have a remarkable influence on mitochondrial respiration. Studies suggest that OSCP, as a key component of the mitochondrial ATP synthase, is pivotal in controlling the OXPHOS flux and, subsequently, mitochondrial function. Therefore, the reduction of OSCP in the pathological hearts should contribute significantly to mitochondrial dysfunction found in these hearts. OSCP

deficiency in yeast and cultured mammalian cells has major consequences.^{13,21} However, the *in vivo* consequences of OSCP deficiency in animal remain unclear. Reduced cardiac OSCP expression in failing hearts from patients has been reported.¹⁵ Cardiac expression of OSCP was decreased in the marginal zone of the endocardium after 6 weeks of ligating the left anterior descending coronary artery of the pig heart.¹⁶ In this study, we showed that TAC-induced cardiac hypertrophy can cause a decrease in OSCP transcript and protein expression. We have attempted to generate an OSCP KO mouse line using the CRISPR/Cas9 technology. However, no homozygous mice were born, probably due to embryonic lethality. Interestingly, we did not see overt phenotype in the heterozygous OSCP KO mice (data not shown), suggesting that partial OSCP deficiency alone is not sufficient to cause

Figure 7. AAV9-mediated OSCP overexpression reduces mPTP opening in isolated cardiac mitochondria. **(A)** Mitochondrial swelling was induced by adding 50 μM calcium. Dynamic changes in absorbance at 540 nm (OD_{540}) reflecting changes in mitochondrial swelling. **(B)** Calculation of mitochondrial swelling in experimental mice. Values are expressed as mean $\pm$ SEM, $n=5$, ** $p < 0.01$. mPTP, mitochondrial permeability transition pore. Color images are available online.

major cardiac phenotype. Therefore, the downregulation of cardiac OSCP in the hypertrophied and failing heart may only contribute to the pathological progression under conditions of persistent pathological stress.

Mitochondria isolated from a failing myocardium show impaired respiratory ability, various defects in the electron transport chain complex, and reduced OXPHOS.^{22,23} Complex I and II dysfunction precedes the transition from compensatory cardiac hypertrophy to heart failure.²⁴ Proteomic studies suggest that TAC-induced cardiac hypertrophy in mice may be associated with declines in complexes I, III, IV, and V.²⁵ At 20 weeks after TAC surgery, three subunits (b, e, g subunits) of complex V were observed to decline, and five subunits (F6, alpha, beta, gamma, O subunits) were increased in isolated mitochondria of Sprague Dawley rats.²⁵ Therefore, mitochondrial dysfunction is a crucial pathogenesis in the progression of cardiac hypertrophy and heart failure. In the current study, we affirmed the hypothesis that gene therapy that replenishes the TAC hearts with OSCP can mitigate the pathological progress via improving the mitochondrial structure and function. OSCP may optimize the efficiency of ATP synthase stability and activity and hence improve upstream mitochondrial respiration with reduced ROS by-products. It has been recognized in recent years that the ATP synthase also plays a crucial role in maintaining the mitochondrial cristae structure.^{26,27} OSCP overexpression may also protect the pathological heart by maintaining the integrity of the mitochondrial cristae. Therefore, the key mechanisms underlying the protective role of sustained OSCP expression in the hypertrophied heart should largely be derived from the crucial role of OSCP in optimizing ATP synthase integrity and activity, and hence, the upstream flow of OXPHOS flux.

While other studies only show the decline of OSCP expression in pathological hearts, our current findings indicate that both OSCP transcript and levels were declined in TAC-induced hypertrophic hearts. These results suggest that the decline of cardiac OSCP in the hypertrophied heart may be related to transcriptional repression, but the underlying mechanisms are unclear. It is likely that one of the upstream signals related to cardiac pathological growth may be responsible for the repressed transcript expression of OSCP. Further studies are warranted to uncover this puzzle.

mPTP is a theoretical pore in the inner mitochondrial membrane that abruptly increases permeability to solutes less than 1.5 kDa. Excessive opening is often linked to cell death. The mPTP opening leading to cell death occurs during the pathological progression of the heart.²⁸ While CypD is recognized as the key regulator of mPTP opening, the exact components of the mPTP remain unclear,²⁹ but the ATP synthase has been proposed as one of the top

candidates.^{30–36} Recent studies suggest that CypD interacts with OSCP and uncouples OSCP from the ATP synthase, resulting in mPTP opening and reduced mitochondrial OXPHOS.^{37,38} However, this hypothesis has been recently challenged with the observation of no mPTP changes in OSCP KO cells.¹³ OSCP^{-/-} HAP1 cells did show reduced growth rate, decreased mitochondrial DNA copy number, and declined OXPHOS rate and complexes I, III, and IV proteins.¹³ In this study, we sought to determine if OSCP overexpression could reduce mPTP opening by out-matching CypD and did find that OSCP overexpression substantially reduced mPTP opening in the hypertrophied heart. Since ATP can also inhibit mPTP opening,³⁹ the current result is insufficient to prove if ATP synthase somehow forms mPTP because the OSCP-related upregulation of ATP may also block further mPTP opening. Further study is needed to establish the identification of mPTP. However, it is clear that a sustained level of OSCP is required to prevent excessive mPTP opening in the pathological heart.

The loss of OSCP in cells leads to decreased mtDNA.¹³ Mutations or depletion of mtDNA can cause a decrease in the activity of these enzymes leading to mitochondrial dysfunction. We investigated if the protective effect of OSCP overexpression in cardiomyocytes is related to upregulating mtDNA expression. Our results showed no significant change in the expression of mtDNA in our TAC mice, thus excluding the possibility of OSCP-related mtDNA upregulation. It is plausible that only partial OSCP deficiency may not be sufficient to result in mitochondrial mtDNA replication in the heart.

In summary, the results of this study indicate that loss of OSCP may contribute to the progression of cardiac hypertrophy. Gene therapy that helps to maintain cardiac expression of OSCP during cardiac hypertrophy can improve mitochondrial function and improve the prognosis of heart failure. The results of this study can provide new therapeutic targets for the treatment of cardiac disorders from pressure overload stress.

ACKNOWLEDGMENTS

A scientist development award from the American Heart Association National Center and an Innovative Basic Science from the American Diabetes Association.

AUTHOR DISCLOSURE

No competing financial interests exist.

FUNDING INFORMATION

This work was supported by grants from the National Natural Science Foundation of China (81500312 and 81570366).

REFERENCES

- Go AS, Mozaffarian D, Roger VL, et al. Heart disease and stroke statistics—2013 update: a report from the American Heart Association. *Circulation* 2013;127:e6–e245.
- Ventura-Clapier R, Garnier A, Veksler V. Energy metabolism in heart failure. *J Physiol* 2004;555:1–13.
- Barth E, Stammeler G, Speiser B, et al. Ultrastructural quantitation of mitochondria and myofilaments in cardiac muscle from 10 different animal species including man. *J Mol Cell Cardiol* 1992;24:669–681.
- Facundo H, Brainard RE, Caldas FRL, et al. Mitochondria and cardiac hypertrophy. *Adv Exp Med Biol* 2017;982:203–226.
- Bache RJ, Zhang J, Murakami Y, et al. Myocardial oxygenation at high workstates in hearts with left ventricular hypertrophy. *Cardiovasc Res* 1999;42:616–626.
- Ingwall JS. Energy metabolism in heart failure and remodelling. *Cardiovasc Res* 2009;81:412–419.
- Torrealba N, Aranguiz P, Alonso C, et al. Mitochondria in structural and functional cardiac remodeling. *Adv Exp Med Biol* 2017;982:277–306.
- Murphy BJ, Klusch N, Langer J, et al. Rotary substates of mitochondrial ATP synthase reveal the basis of flexible F1-Fo coupling. *Science* 2019; 364:eaaw9128.
- Weber J. ATP synthase: subunit-subunit interactions in the stator stalk. *Biochim Biophys Acta* 2006;1757:1162–1170.
- Antoniol M, Giorgio V, Fogolari F, et al. The oligomycin-sensitivity conferring protein of mitochondrial ATP synthase: emerging new roles in mitochondrial pathophysiology. *Int J Mol Sci* 2014;15:7513–7536.
- Devenish RJ, Prescott M, Boyle GM, et al. The oligomycin axis of mitochondrial ATP synthase: OSCP and the proton channel. *J Bioenerg Biomembr* 2000;32:507–515.
- Gauba E, Guo L, Du H. Cyclophilin D promotes brain mitochondrial F1FO ATP synthase dysfunction in aging mice. *J Alzheimers Dis* 2017;55: 1351–1362.
- He J, Carroll J, Ding S, et al. Permeability transition in human mitochondria persists in the absence of peripheral stalk subunits of ATP synthase. *Proc Natl Acad Sci U S A* 2017;114: 9086–9091.
- Beck SJ, Guo L, Phensy A, et al. Deregulation of mitochondrial F1FO-ATP synthase via OSCP in Alzheimer's disease. *Nat Commun* 2016;7:11483.
- Liu J, Wang C, Murakami Y, et al. Mitochondrial ATPase and high-energy phosphates in failing hearts. *Am J Physiol Heart Circ Physiol* 2001;281: H1319–H1326.
- Hu Q, Wang X, Lee J, et al. Profound bioenergetic abnormalities in peri-infarct myocardial regions. *Am J Physiol Heart Circ Physiol* 2006;291:H648–H657.
- He L, Kim T, Long Q, et al. Carnitine palmitoyltransferase-1b deficiency aggravates pressure overload-induced cardiac hypertrophy caused by lipotoxicity. *Circulation* 2012;126:1705–1716.
- Wang P, Liu J, Li Y, et al. Peroxisome proliferator-activated receptor {delta} is an essential transcriptional regulator for mitochondrial protection and biogenesis in adult heart. *Circ Res* 2010;106: 911–919.
- Long Q, Huang L, Huang K, et al. Assessing mitochondrial bioenergetics in isolated mitochondria from mouse heart tissues using oroboros 2k-oxygraph. *Methods Mol Biol* 2019;1966:237–246.
- Elrod JW, Molkentin JD. Physiologic functions of cyclophilin D and the mitochondrial permeability transition pore. *Circ J* 2013;77:1111–1122.
- Prescott M, Bush NC, Nagley P, et al. Properties of yeast cells depleted of the OSCP subunit of mitochondrial ATP synthase by regulated expression of the ATP5 gene. *Biochem Mol Biol Int* 1994; 34:789–799.
- Beer M, Seyfarth T, Sandstede J, et al. Absolute concentrations of high-energy phosphate metabolites in normal, hypertrophied, and failing human myocardium measured noninvasively with (31)P-SLOOP magnetic resonance spectroscopy. *J Am Coll Cardiol* 2002;40:1267–1274.
- Stanley WC, Recchia FA, Lopaschuk GD. Myocardial substrate metabolism in the normal and failing heart. *Physiol Rev* 2005;85:1093–1129.
- Griffiths ER, Friehs I, Scherr E, et al. Electron transport chain dysfunction in neonatal pressure-overload hypertrophy precedes cardiomyocyte apoptosis independent of oxidative stress. *J Thorac Cardiovasc Surg* 2010;139:1609–1617.
- Dai DF, Hsieh EJ, Liu Y, et al. Mitochondrial proteome remodelling in pressure overload-induced heart failure: the role of mitochondrial oxidative stress. *Cardiovasc Res* 2012;93:79–88.
- Paumard P, Vaillier J, Couly B, et al. The ATP synthase is involved in generating mitochondrial cristae morphology. *EMBO J* 2002;21:221–230.
- Quintana-Cabrera R, Quirin C, Glytsou C, et al. The cristae modulator Optic atrophy 1 requires mitochondrial ATP synthase oligomers to safeguard mitochondrial function. *Nat Commun* 2018;9:3399.
- Miura T, Tanno M. The mPTP and its regulatory proteins: final common targets of signalling pathways for protection against necrosis. *Cardiovasc Res* 2012;94:181–189.
- Karch J, Molkentin JD. Identifying the components of the elusive mitochondrial permeability transition pore. *Proc Natl Acad Sci U S A* 2014; 111:10396–10397.
- Alavian KN, Beutner G, Lazrove E, et al. An uncoupling channel within the c-subunit ring of the F1FO ATP synthase is the mitochondrial permeability transition pore. *Proc Natl Acad Sci U S A* 2014;111:10580–10585.
- Giorgio V, von Stockum S, Antoniel M, et al. Dimers of mitochondrial ATP synthase form the permeability transition pore. *Proc Natl Acad Sci U S A* 2013;110:5887–5892.
- Bonora M, Bononi A, De Marchi E, et al. Role of the c subunit of the FO ATP synthase in mitochondrial permeability transition. *Cell Cycle* 2013; 12:674–683.
- Halestrap AP. The C ring of the F1Fo ATP synthase forms the mitochondrial permeability transition pore: A critical appraisal. *Front Oncol* 2014;4:234.
- Bonora M, Bravo-San Pedro JM, Kroemer G, et al. Novel insights into the mitochondrial permeability transition. *Cell Cycle* 2014;13:2666–2670.
- Bernardi P, Di Lisa F, Fogolari F, et al. From ATP to PTP and back: A dual function for the mitochondrial ATP synthase. *Circ Res* 2015;116:1850–1862.
- Giorgio V, Fogolari F, Lippe G, et al. OSCP subunit of mitochondrial ATP synthase: role in regulation of enzyme function and of its transition to a pore. *Br J Pharmacol* 2019;176:4247–4257.
- Giorgio V, Bisetto E, Soriano ME, et al. Cyclophilin D modulates mitochondrial F0F1-ATP synthase by interacting with the lateral stalk of the complex. *J Biol Chem* 2009;284:33982–33988.
- Chinopoulos C, Adam-Vizi V. Modulation of the mitochondrial permeability transition by cyclophilin D: moving closer to F(0)-F(1) ATP synthase? *Mitochondrion* 2012;12:41–45.
- Hagen T, Lagace CJ, Modica-Napolitano JS, et al. Permeability transition in rat liver mitochondria is modulated by the ATP-Mg/Pi carrier. *Am J Physiol Gastrointest Liver Physiol* 2003;285:G274–G281.

Received for publication January 13, 2020;
accepted after revision August 11, 2020.

Published online: September 15, 2020.