Sarcalumenin alleviates stress-induced cardiac dysfunction by improving Ca\(^{2+}\) handling of the sarcoplasmic reticulum

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Calcium cycling; Heart failure; Pressure overload; SERCA; Protein stability

Aims Sarcalumenin (SAR) is a Ca\(^{2+}\)-binding protein expressed in the longitudinal sarcoplasmic reticulum (SR) of striated muscle cells. Although its Ca\(^{2+}\)-binding property is similar to that of calsequestrin, its role in the regulation of Ca\(^{2+}\) cycling remains unclear.

Methods and results To investigate whether SAR plays an important role in maintaining cardiac function under pressure overload stress, SAR-knockout (SAR-KO) mice were subjected to transverse aortic constriction (TAC). To examine the relation of SAR with cardiac type of SR Ca\(^{2+}\) pump, SERCA2a, we designed cDNA expression using cultured cells. We found that SAR expression was significantly downregulated in hypertrophic hearts from three independent animal models. SAR-KO mice experienced higher mortality than did wild-type (WT) mice after TAC. TAC significantly downregulated SERCA2a protein but not mRNA in the SAR-KO hearts, whereas it minimally did so in hearts from WT mice. Accordingly, SR Ca\(^{2+}\) uptake and cardiac function were significantly reduced in SAR-KO mice after TAC. Then we found that SAR was co-immunoprecipitated with SERCA2a in cDNA-transfected HEK293T cells and mouse ventricular muscles, and that SERCA2a-mediated Ca\(^{2+}\) uptake was augmented when SAR was co-expressed in HEK293T cells. Furthermore, SAR significantly prolonged the half-life of SERCA2a protein in HEK293T cells.

Conclusion These findings suggest that functional interaction between SAR and SERCA2a enhances protein stability of SERCA2a and facilitates Ca\(^{2+}\) sequestration into the SR. Thus the SAR-SERCA2a interaction plays an essential role in preserving cardiac function under biomechanical stresses such as pressure overload.

1. Introduction

A growing body of evidence has demonstrated that impaired Ca\(^{2+}\) re-uptake into the sarcoplasmic reticulum (SR) underlies the pathogenesis of cardiac dysfunction and heart failure.\(^1,2\) Since the SR Ca\(^{2+}\)-ATPase type 2a (SERCA2a) plays a principal role in Ca\(^{2+}\) sequestration into the cardiac SR, decreases in the expression and activity of SERCA2a may directly lead to cardiac dysfunction.\(^3,4\) Therefore, it is important to explore the molecular mechanism for regulating SERCA2a expression and activity in the heart.

Sarcalumenin (SAR) is an SR luminal glycoprotein responsible for Ca\(^{2+}\) buffering in skeletal and cardiac muscles.\(^5,6\) Although the Ca\(^{2+}\)-binding property and structural characteristics of SAR are similar to calsequestrin (CSQ),\(^7,8\) another well-characterized SR Ca\(^{2+}\)-binding protein, their subcellular localization is different within the SR.\(^8,9\) CSQ is condensed at the terminal cisternae of the SR, where the Ca\(^{2+}\) release channels, ryanodine receptors (RyRs), are co-localized.\(^9\) In contrast, SAR is predominantly distributed in the longitudinal SR, where SERCA and phospholamban (PLN) are located.\(^2\) CSQ is probably anchored to the SR membrane through direct binding to RyRs and other intra-membrane proteins, such as triadin and junctin.\(^7\)
Importantly, besides buffering $\text{Ca}^{2+}$ in the SR, CSQ2 directly modulates $\text{Ca}^{2+}$ release from the SR through the interaction with RyR2 in the heart. The different localization of CSQ from that of SAR is presumably due to the difference in their anchoring proteins. Previous studies have demonstrated that SAR is co-localized and interacts with SERCA1, a SERCA subtype, in the fast-twitch skeletal muscles. It remains unknown, however, whether SAR similarly interacts with SERCA2a and thus modulates its function in the heart.

We have recently demonstrated that the gene disruption of SAR in mice (SAR-knockout, SAR-KO) resulted in mild impairments in $\text{Ca}^{2+}$ transients and cardiac function. Mutant skeletal muscle from SAR-KO mice exhibited a significant decrease in the expression of SERCA1 protein, but an unchanged mRNA expression of SERCA1 when compared with control muscle. Therefore, we hypothesized that SAR modulates the stability of SERCA proteins through its protein–protein interaction in the SR luminal side. Because SR $\text{Ca}^{2+}$ handling widely influences cardiac pathology, SAR may have a protective effect on SERCA2a activity when the heart is exposed to biomechanical stresses such as pressure overload. Therefore, the present study was undertaken to examine whether SAR is another protein, in addition to PLN, which can interact with SERCA2a and regulates its function, presumably in an analogous manner with CSQ in regulating the channel function of RyRs. We were particularly interested in examining whether SAR plays a role in the prevention of heart failure induced by pressure overload.

2. Methods

2.1. Animal preparation and transverse aortic constriction

All animal care and study protocols were approved by the Animal Ethics Committee of Yokohama City University School of Medicine and the investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). SAR-KO mice and C57BL/6J wild-type controls (WT, 20–24 weeks of age) from the same genetic backgrounds were used in the present study. Pressure overload was produced by transverse aortic constriction (TAC) for 4 weeks, as we described previously. Pressure overload was performed by transverse aortic constriction (TAC) for 4 weeks, as we described previously. Transthoracic echocardiography and in vivo haodynamics were performed in sedated mice 4 weeks after surgery, as described previously. Further, for the examination of SAR protein levels in stress-loaded hearts, we also prepared two well-characterized experimental models of cardiac hypertrophy: (i) transgenic mice with a ventricle-specific overexpression of the activated H-Ras-v gene (Ras-TG) that were kindly provided by Dr Yibin Wang at the University of California, San Diego. We confirmed all constructs by direct DNA sequencing analysis.

2.2. Construction of sarcalumenin and sarcoplasmic reticulum $\text{Ca}^{2+}$-ATPase type 2a cDNAs

An expression vector plasmid-encoding mouse SAR was constructed using the polymerase chain reaction (PCR) strategy. The specific regions of the full-length mouse SAR cDNA were amplified by PCR using the following forward and reverse primers, 5'-TCAAGATCCGGCAGTCACCTGA-AGGCCGCTG-3' and 5'-CAGATATCAGTGGGAACCCCAAGAATAGC-3'. The full-length mouse SAR cDNA was inserted at the BanHI and EcoRV sites of pcDNA-3.1(+) (Invitrogen, Tokyo, Japan). A full-length human SERCA2a cDNA was kindly provided by Dr Masahiko Hoshijima at the University of California, San Diego. We confirmed all constructs by direct DNA sequencing analysis.

2.3. Cell culture and DNA transfection

Human embryonic kidney 293T (HEK-293T) cells were incubated in Dulbecco’s modified Eagle’s medium (Sigma, Tokyo, Japan) supplemented with 10% fetal calf serum and 1% antibiotics. HEK-293T cells were co-transfected with SAR and SERCA2a cDNAs by using FuGen6 transfection reagent (Roche Applied Science, Tokyo, Japan) at a ratio of 1:3 of SAR:SERCA2a. A total amount of 6 μg of DNA was used for transfection in a 10 cm dish.

2.4. Immunofluorescent staining

Three days after co-transfection, HEK-293T cells were fixed to the glass coverslips with 3.7% formaldehyde. FITC and TRITC-conjugated goat IgG (MP Biomedicals, Solon, OH, USA) were used as secondary antibodies. Stained cells were analysed with an LSM510 confocal laser scanning microscope (Carl Zeiss, Tokyo, Japan) and Adobe Photoshop software.

2.5. Immunoprecipitation and immunoblot analysis

For immunoprecipitation, we prepared SR proteins from left ventricular (LV) tissues of C57BL/6J mice (20–24 weeks of age) and whole cell lysates from HEK-293T cells co-transfected with SERCA2a and SAR genes. Co-immunoprecipitation and immunoblot analysis were performed as described previously. Antibodies that were used in the present study are shown in online data supplements.

2.6. Northern blot analysis

A northern blot analysis was performed as described previously, with slight modifications by using 10 μg of total RNA.

2.7. $\text{Ca}^{2+}$ uptake assay

ATP-dependent, oxalate-supported $\text{Ca}^{2+}$ uptake was measured, as described previously, with slight modifications. The assays were performed in duplicate by using the microsomes derived from three independent transfections.

2.8. Analysis of sarcoplasmic reticulum $\text{Ca}^{2+}$-ATPase type 2a protein stability

HEK-293T cells were transfected with SERCA2a alone or together with SAR as described earlier. All cells were treated with 5 μg/mL cycloheximide (Sigma, Tokyo, Japan) after 48 h of transfection and harvested at different time points. Whole cell lysates were subjected to SDS-PAGE and immunoblot analysis. Assays were performed in triplicate.

2.9. Statistical analysis

All values are expressed as mean ± SEM. The group data were compared using one-way or two-way ANOVA followed by multiple comparisons testing by Turkey-Kramer’s post hoc test. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Downregulation of sarcalumenin in stressed hearts

Using three independent animal models, (i) the pressure-overloaded LV by TAC, (ii) the activated H-Ras-v gene overexpressed LV, and (iii) the pressure-overloaded right ventricles
(RV) by monocrotaline administration, we attempted to survey relationship between cardiac hypertrophy and SERCA2 and SAR expression. Western blotting demonstrated that SERCA2 protein levels were significantly lower by 38%, and 18% in the H-Ras-v-overexpressed LV (Figure 1B) and the monocrotaline-administered RV (Figure 1C), respectively, when compared with the respective control ventricles. There was no change in SERCA2 protein levels in the TAC-induced LV (Figure 1A). SAR protein levels were significantly lower by 14, 24, and 17% in the TAC-induced LV (Figure 1A), the H-Ras-v-overexpressed LV (Figure 1B), and the monocrotaline-administered RV (Figure 1C), respectively, when compared with the respective control ventricles. Further, the expression levels of SAR mRNA were also decreased in hypertrophic ventricles (data not shown).

These data indicated that the downregulation of SAR protein was a common feature in stressed hearts.

3.2. Pressure overload promoted mortality and cardiac dysfunction in sarcalumenin-knockout mice

Since the earlier-mentioned findings suggest that SAR plays a role in the progression of cardiac hypertrophy, we examined the effects of SAR disruption on cardiac hypertrophy caused by a biomechanical stress such as TAC-induced pressure overload. In our designed experimental conditions, no mice that underwent a sham operation died in the experimental period. As shown in Figure 2, mortality was markedly greater in SAR-KO relative to WT mice; 4 weeks after TAC, 50% of SAR-KO mice died, whereas only ~20% of WT

<table>
<thead>
<tr>
<th>Different animal models of hypertrophy</th>
<th>Groups</th>
<th>Number of animals prepared</th>
<th>Number of samples in western blot analyses</th>
<th>Number of animals in haemodynamic analyses</th>
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<tr>
<td>TAC mice</td>
<td>WT sham operation</td>
<td>6</td>
<td>5 (LV)</td>
<td>6</td>
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<tr>
<td></td>
<td>WT-TAC</td>
<td>11</td>
<td>5 (LV)</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>SAR-KO sham operation</td>
<td>6</td>
<td>5 (LV)</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>SAR-KO TAC</td>
<td>10</td>
<td>4 (LV)</td>
<td>4</td>
</tr>
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<td>H-Ras-v transgenic mice</td>
<td>NTG</td>
<td>4</td>
<td>4 (LV)</td>
<td>ND</td>
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<tr>
<td></td>
<td>TG</td>
<td>4</td>
<td>4 (LV)</td>
<td>ND</td>
</tr>
<tr>
<td>MCT Wistar rats</td>
<td>NS</td>
<td>4</td>
<td>4 (RV)</td>
<td>ND</td>
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<tr>
<td></td>
<td>MCT</td>
<td>4</td>
<td>4 (RV)</td>
<td>ND</td>
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</tbody>
</table>

TAC, transverse aortic constriction; MCT, monocrotaline; WT, wild-type mice; SAR-KO, sarcalumenin-knockout mice; NTG, non-transgenic mice; TG, transgenic mice; NS, saline-administered rat; LV, left ventricles; RV, right ventricles; ND, not done.

Table 1  The experimental design of different animal models of cardiac hypertrophy

Figure 1  Expression of sarcalumenin protein in stress-loaded hearts. (A) Pressure-overloaded left ventricle by transverse aortic constriction (n = 4); (B) Left ventricle overexpressed with H-v-Ras (n = 4); (C) Pressure-overloaded right ventricle by monocrotaline-administered rat administration (n = 4). (Protein expression was normalized by β-actin.) Controls were expressed as 1.0. *P < 0.05. SERCA2, SR Ca\(^{2+}\)-ATPase; SAR, sarcalumenin; Sham, sham operation; TAC, transverse aortic constriction; NTG, non-transgenic mice; Ras-TG, Ras-transgenic mice; NS, saline-administered rat; MCT, monocrotaline-administered rat.
mice died. It is unlikely that SAR-KO mice received severer TAC-induced pressure overload when compared with WT mice, because the pressure gradient between the ascending and descending aortas was similar in survivors of both mutant and control groups (Table 2). The dying mice exhibited symptoms of advanced heart failure, such as accelerated breathing and ruffled fur, and the dead mice exhibited fluid in the pleural cavity and thrombi in the atria at autopsy. It should be noted that in the absence of pressure overload, SAR-KO mice survived over a year at a similar survival rate with WT mice (data not shown).

The degree of cardiac hypertrophy, as measured by LV weights and LV wall thickness, was similar between WT and SAR-KO mice after TAC (Table 2), suggesting that SAR deficiency did not dramatically induce the exacerbation of cardiac hypertrophy in response to pressure overload. Subsequently, the cardiac function of all survivors was evaluated after TAC by echocardiographic and haemodynamic studies (Table 2). TAC deteriorated both systolic and diastolic functions to a greater degree in SAR-KO than in WT mice. Percent fractional shortening (%FS) in SAR-KO mice

![Figure 2](image-url) Effects of pressure overload on survival in sarcalumenin knockout mice. Survival analysis of sarcalumenin knockout mice after transverse aortic constriction. Mortality was markedly higher in sarcalumenin knockout mice relative to wild-type mice. SAR, sarcalumenin; SAR-KO, SAR knockout mice; WT, wild-type mice; TAC, transverse aortic constriction.

### Table 2 Effects of pressure overload on morphology and left ventricle function

<table>
<thead>
<tr>
<th></th>
<th>WT (Sham)</th>
<th>TAC (survivors)</th>
<th>KO (Sham)</th>
<th>TAC (survivors)</th>
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<tr>
<td>n</td>
<td>6</td>
<td>8</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>BW, g</td>
<td>25.5 ± 1.1</td>
<td>28.5 ± 1.9</td>
<td>27.4 ± 1.0</td>
<td>26.4 ± 2.8</td>
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<tr>
<td>TL, mm</td>
<td>19.8 ± 0.1</td>
<td>19.6 ± 0.3</td>
<td>19.8 ± 0.1</td>
<td>19.0 ± 0.3</td>
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<td>RA, mg</td>
<td>3.4 ± 0.4</td>
<td>4.8 ± 0.7</td>
<td>3.6 ± 0.5</td>
<td>5.2 ± 0.4*</td>
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<td>LA, mg</td>
<td>4.4 ± 0.3</td>
<td>10.6 ± 1.7*</td>
<td>4.4 ± 0.4</td>
<td>7.9 ± 1.4*</td>
</tr>
<tr>
<td>RV, mg</td>
<td>24.3 ± 1.0</td>
<td>28.4 ± 2.3</td>
<td>23.1 ± 0.9</td>
<td>26.5 ± 3.6</td>
</tr>
<tr>
<td>LV, mg</td>
<td>100.2 ± 2.8</td>
<td>168.8 ± 11.8*</td>
<td>103.2 ± 2.9</td>
<td>149.0 ± 7.4*</td>
</tr>
<tr>
<td>Lung, mg</td>
<td>151.2 ± 5.9</td>
<td>178.5 ± 11.3</td>
<td>167.2 ± 10.5</td>
<td>193.4 ± 12.5</td>
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<tr>
<td>Liver, g</td>
<td>1.15 ± 0.08</td>
<td>1.30 ± 0.06</td>
<td>1.36 ± 0.06</td>
<td>1.52 ± 0.13</td>
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<tr>
<td>RA/BW, mg/g</td>
<td>1.3 ± 0.1</td>
<td>1.7 ± 0.3</td>
<td>1.3 ± 0.2</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>LA/BW, mg/g</td>
<td>1.7 ± 0.1</td>
<td>3.9 ± 0.7*</td>
<td>1.6 ± 0.1</td>
<td>3.3 ± 1.0</td>
</tr>
<tr>
<td>RV/BW, mg/g</td>
<td>9.6 ± 0.5</td>
<td>10.4 ± 1.1</td>
<td>8.4 ± 0.4</td>
<td>10.1 ± 0.9</td>
</tr>
<tr>
<td>LV/BW, mg/g</td>
<td>4.0 ± 0.2</td>
<td>6.1 ± 0.5*</td>
<td>3.7 ± 0.2</td>
<td>5.9 ± 0.8*</td>
</tr>
<tr>
<td>LV/TL, mg/mm</td>
<td>5.1 ± 0.1</td>
<td>8.6 ± 0.6*</td>
<td>5.2 ± 0.1</td>
<td>7.8 ± 0.4*</td>
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<tr>
<td>LVEDD, mm</td>
<td>3.81 ± 0.04</td>
<td>4.06 ± 0.06*</td>
<td>3.96 ± 0.05*</td>
<td>4.50 ± 0.08*</td>
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<td>LVESD, mm</td>
<td>2.46 ± 0.04</td>
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<td>2.70 ± 0.06*</td>
<td>3.47 ± 0.17*</td>
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<td>LVFS, %</td>
<td>35.5 ± 0.7</td>
<td>31.1 ± 0.5*</td>
<td>31.8 ± 0.6*</td>
<td>22.9 ± 0.9*</td>
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<td>IVSth, mm</td>
<td>0.67 ± 0.03</td>
<td>0.98 ± 0.03*</td>
<td>0.65 ± 0.01</td>
<td>0.92 ± 0.05*</td>
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<td>LVPth, mm</td>
<td>0.69 ± 0.03</td>
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<td>0.67 ± 0.01</td>
<td>0.94 ± 0.07*</td>
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<td>ET, ms</td>
<td>50.0 ± 0.8</td>
<td>52.3 ± 0.5*</td>
<td>56.2 ± 0.7*</td>
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<td>V circ/s</td>
<td>2.57 ± 0.07</td>
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<td>Pressure gradients, mmHg</td>
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<td>–</td>
<td>47.4 ± 4.6</td>
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<td>LVP, mmHg</td>
<td>104.5 ± 8.6</td>
<td>148.2 ± 10.9</td>
<td>86.4 ± 3.5</td>
<td>127.8 ± 4.1</td>
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<tr>
<td>LV dp/dt max, mmHg/s</td>
<td>9391 ± 652</td>
<td>7946 ± 394*</td>
<td>6836 ± 298*</td>
<td>3897 ± 134*</td>
</tr>
<tr>
<td>LV dp/dt min, mmHg/s</td>
<td>–6442 ± 263</td>
<td>–6217 ± 515</td>
<td>–3853 ± 301*</td>
<td>–3563 ± 183*</td>
</tr>
<tr>
<td>EDP, mmHg</td>
<td>2.5 ± 0.3</td>
<td>2.8 ± 0.1</td>
<td>3.1 ± 0.3</td>
<td>4.5 ± 0.1*</td>
</tr>
<tr>
<td>Tau, ms</td>
<td>6.0 ± 0.3</td>
<td>6.3 ± 0.3</td>
<td>7.5 ± 0.1*</td>
<td>9.1 ± 0.3*</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. *P < 0.05 vs. sham, †P < 0.05 vs. WT.

WT, wild-type mice; SAR-KO, sarcalumenin-knockout mice; Sham, sham operation; TAC, transverse aortic constriction; BW, body weight; TL, tibia length; RA, right atria; LA, left atria; RV, right ventricles; LV, left ventricles; HR, heart rate; b.p.m., beats per minute; LVEDD, left ventricular end-diastolic dimension; ESD, end-systolic dimension; FS, per cent fractional shortening; IVSth, interventricular wall thickness; LVPth, left ventricular posterior wall thickness; ET, ejection time; V circ, corrected velocity of circumferential fibre shortening; circ, circumference; LV dp/dt max or LV dp/dt min, maximal or minimal first derive of left ventricular pressure; LVP, maximal left ventricular systolic pressure; EDP, end-diastolic pressure.
was decreased by 30% after TAC, when compared with sham-operated SAR-KO mice (Figure 3A), whereas WT mice showed only 12% decrease. Although the degree of hypertrophy was similar between WT and SAR-KO mice after TAC, TAC-induced LV dilatation estimated by LV end-diastolic dimension (LVEDD) was greater in SAR-KO than in WT mice (Figure 3B). The maximal first derivate of LV pressure (dP/dt\text{max}) was decreased by 50% after TAC (Figure 3C), though it was decreased by only 14% in WT mice. Tau, an indicator of LV diastolic function, was significantly prolonged after TAC in SAR-KO when compared with that in WT mice (Figure 3D). Thus, the deterioration of cardiac function was augmented and, presumably, led to increased mortality in SAR-KO mice in response to pressure overload, indicating that SAR plays an important role in preserving cardiac function under a pathological condition such as pressure overload.

3.3. Pressure overload progressively downregulated sarcoplasmic reticulum Ca$^{2+}$-ATPase type 2a protein and decreased Ca$^{2+}$ uptake in sarcalumenin-knockout mice

Because we have previously demonstrated the downregulation of SERCA1 protein and a decrease in Ca$^{2+}$ uptake in the skeletal muscle of SAR-KO, the disruption of SAR is very likely to be associated with a downregulation of SERCA2 protein and a decrease in Ca$^{2+}$ uptake in cardiac muscle. Our blot experiments demonstrated that SERCA2 protein, but not mRNA, was significantly downregulated in the ventricular muscles of SAR-KO mice at basal level (Figure 4A and B).

The expression level of SERCA2 protein was further decreased in the ventricular muscles of SAR-KO after TAC, but it remained unchanged in those of WT after TAC (Figure 4C and D). The expression level of PLN protein was already lower in ventricular muscles of SAR-KO than in those of WT before TAC (Figure 4C and E), but did not decrease further after TAC in the ventricular muscles of WT and SAR-KO mice. Because the SERCA2/PLN protein ratio is known as an important determinant of cardiac function, our observations suggested that this ratio was also decreased in ventricular muscles of SAR-KO mice in response to pressure overload stress (Figure 4F). However, protein levels of phosphorylated PLN (serine 16), CSQ2, RyR2, and sodium–Ca$^{2+}$ exchanger 1 were similar between the ventricular muscles of WT and SAR-KO mice (data not shown).

Next, we examined Ca$^{2+}$ uptake activity in SR vesicles prepared from hearts. The maximum velocity of Ca$^{2+}$ uptake ($V_{\text{max}}$) at pCa 5.5 (Figure 5) was significantly lower by 46% in ventricular muscles of SAR-KO (14.1 ± 1.4 nmol/mg/min) than that in those of WT before TAC (20.7 ± 1.4 nmol/mg/min). A further significant reduction in $V_{\text{max}}$ occurred in the ventricular muscles of SAR-KO mice after
3.4. Sarcalumenin and sarcoplasmic reticulum Ca\(^{2+}\)-ATPase type 2a were co-localized and co-immunoprecipitated

Since the earlier-mentioned in vivo findings indicate that a disruption of SAR is associated with a progressive downregulation of SERCA2a protein and SR Ca\(^{2+}\) uptake in pressure-overloaded hearts, it can be assumed that SAR directly or functionally interacts with SERCA2a in the SR. Therefore, we next focused on interaction between SAR and SERCA2a to examine the molecular mechanism underlying the deteriorated hypertrophy in SAR-KO mice.

When both SAR and SERCA2a were overexpressed in HEK-293T cells, immunofluorescent staining pointed to possible co-localization of SAR and SERCA2a in the endoplasmic reticulum (Figure 6A). Co-immunoprecipitation using lysates from such HEK-293T cells demonstrated that SAR and SERCA2a were physically bound to each other (Figure 6B). To examine whether SAR also interacts with SERCA2a in vivo, the detergent extracts from mouse LV tissues were subjected to co-immunoprecipitation assays. As shown in Figure 6C, SAR could be co-immunoprecipitated with an anti-SERCA2a antibody, but not with control mouse IgG in LV tissues.

3.5. Sarcalumenin augmented Ca\(^{2+}\) sequestration into the sarcoplasmic reticulum

To examine whether SAR regulated the Ca\(^{2+}\) transport activity of SERCA2a, the ATP-dependent, oxalate-supported Ca\(^{2+}\) uptake rates were measured by using the microsomes from SERCA2a-overexpressed HEK-293T cells in the presence or absence of SAR co-expression. The accumulation of Ca\(^{2+}\) at pCa 6.8 was greater during the first 7 min in SERCA2a and SAR co-expressed HEK-293T cells than those overexpressing SERCA2a alone, suggesting that SAR increased the rates of Ca\(^{2+}\) uptake (Figure 7A).

Determination of the K0.5 value, i.e. the Ca\(^{2+}\) concentration required to attain the half-maximal Ca\(^{2+}\) uptake rates, in the presence of SAR was lower (K0.5 = 0.13 ± 0.01 µmol/L) than that in the absence of SAR (K0.5 = 0.21 ± 0.01 µmol/L), suggesting that SAR increased, slightly

**Figure 5** Effects of the pressure overload on Ca\(^{2+}\) uptake rates in sarcalumenin-knockout mice. The maximum velocity of Ca\(^{2+}\) uptake (V\(_\text{max}\)) by the sarcoplasmic reticulum. Data are normalized to the V\(_\text{max}\) of sham-operated wild-type mice (n = 4 for each group). *P < 0.05, TAC vs. Sham; #P < 0.05, WT vs. SAR-KO. WT, wild-type mice; SAR-KO, sarcalumenin-knockout mice; Sham, sham operation; TAC, transverse aortic constriction; SAR, sarcalumenin.

**Figure 6** Possible co-localization and physical interaction of sarcalumenin and sarcoplasmic reticulum Ca\(^{2+}\)-ATPase type 2a. (A) Possible co-localization of sarcalumenin and sarcoplasmic reticulum Ca\(^{2+}\)-ATPase type 2a in HEK-293T cells. We used two lasers for excitation: FITC (sarcoplasmic reticulum Ca\(^{2+}\)-ATPase type 2a) at 488 nm and TRITC (sarcalumenin) at 543 nm. (a)-(c) Overlay images of differential interference contrast and fluorescence; (d)-(f) fluorescence alone; (c) and (f) overlay images of sarcalumenin and sarcoplasmic reticulum Ca\(^{2+}\)-ATPase type 2a expression. The upper and lower images were obtained from two different fields. Scale bar 10 µm. (B) Co-immunoprecipitation of sarcalumenin and sarcoplasmic reticulum Ca\(^{2+}\)-ATPase type 2a in HEK-293T cells. Sarcalumenin was not expressed endogenously in HEK-293T cells. Anti-sarcalumenin monoclonal antibody shows minimal cross-reactivity to endogenous sarcoplasmic reticulum Ca\(^{2+}\)-ATPase type 2b. When both sarcalumenin and sarcoplasmic reticulum Ca\(^{2+}\)-ATPase type 2a were transfected in HEK-293T cells, strong immunoreaction was detected in the immunoprecipitated pellets. (C) Co-immunoprecipitation by using the detergent extracts from mouse left ventricular tissues. The immune complexes generated with antisarcoplasmic reticulum Ca\(^{2+}\)-ATPase type 2a monoclonal antibody were blotted with anti-sarcalumenin polyclonal antibody (upper panel) or vice versa (lower panel). The gels were loaded with homogenate (Lysate, lane 1), immunoprecipitation pellet (IP-P, lanes 2 and 4), and post-immunoprecipitation supernatant (IP-S, lanes 3 and 5; same volume as lane 1). No binding signal was detectable in control beads prepared with normal mouse IgG or rabbit IgG. SAR, sarcalumenin; SR, sarcoplasmic reticulum; SERCA2a, sarcoplasmic reticulum Ca\(^{2+}\)-ATPase type 2a; IP, immunoprecipitation; IB, immunoblot; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate.
but significantly, the affinity of SERCA2a to Ca^{2+} (Figure 7B).
In contrast, the maximum velocity of Ca^{2+} uptake ($V_{\text{max}}$) remained unchanged by SAR co-expression ($V_{\text{max}} = 45.0 \pm 1.1$ vs. $43.2 \pm 1.2$ nmol/mg/min). In our experimental system, the rates of Ca^{2+} uptake were decreased by 95% in the presence of thapsigargin (10 $\mu$mol/L), a SERCA blocker, and negated in the absence of ATP in the reaction mixture (data not shown). The uptake rate in the absence of SERCA2a overexpression, i.e. uptake rate by endogenous SERCA2b in HEK-293T cells, was ~11% of that of SERCA2a-overexpressed cells. Thus, these results indicate that SAR functionally interacts with SERCA2a and regulates the Ca^{2+} transport activity of SERCA2a.

3.6. Sarcalumenin extended the half-life of sarcoplasmic reticulum Ca^{2+}-ATPase type 2a protein

Because a previous study with knockout mice showed that the disruption of SAR decreased the SERCA2a protein expression\textsuperscript{11}, we were interested in examining whether SAR plays a role in stabilizing SERCA2a protein, as exemplified by calreticulin, another Ca^{2+}-binding glycoprotein that acts as a molecular chaperone in the endoplasmic reticulum.\textsuperscript{18} Thus, we examined the half-life of SERCA2a protein in SERCA2a overexpressed HEK-293T cells in the presence or absence of a co-expression of SAR. Forty-eight hours after transfection, the cells were treated with cycloheximide, a translation inhibitor, to stop de novo SERCA2a protein synthesis. The half-life of SERCA2a protein was $2.8 \pm 0.3$ days in the absence of SAR co-expression, whereas it increased to $4.6 \pm 0.4$ days in the presence of SAR co-expression (Figure 8). The initial amount of SERCA2a protein expression was not altered by SAR co-expression. These findings suggest that SAR increased the stability of SERCA2a protein most likely by preventing the degradation of SERCA2a protein.

4. Discussion

A major finding in the present study is that TAC induced the higher incidence of mortality in SAR-KO mice. This indicates that SAR plays an important role in adapting to biomechanical stresses such as pressure overload. We think that the higher incidence of mortality was due to the progressive
decline in cardiac function in SAR-KO mice. Numerous studies have demonstrated that SERCA2a activity is a critical determinant for adapting to biomechanical stresses.19-21 Because we found that SAR increased the activity and stability of SERCA2a protein in in vitro experiments, decreased SERCA2a activity was most likely responsible for progressive cardiac dysfunction in SAR-KO mice. Therefore, we postulate that the loss of SERA–SERCA2a interaction promotes a subsequent degradation of SERCA2a protein and decreased SERCA2a activity in response to pressure overload, which eventually resulted in advancing cardiac dysfunction in SAR-KO mice.

The expression level of SERCA2a protein is regulated transcriptionally22,23 and post transcriptionally.24,25 Maintaining the expression level of SERCA2a protein is considered important in controlling heart failure.3,26 To date, the mechanism of the post-transcriptional regulation of SERCA2a has remained unclear. Here, we found that SAR prolonged the stability of SERCA2a protein in the heart. It is important that we found the SAR expression downregulated in three independent animal models of cardiac hypertrophy. Lohan and Ohlendieck27 also demonstrated that the SAR was drastically downregulated in dystrophic-deficient cardiac muscle. Thus, the downregulation of SERCA protein may be a common feature in hearts under various pathological conditions and may contribute to a progressive reduction in SERCA2a protein in failing hearts. However, it should be noted that the reduction in SERCA protein does not always accompany the downregulation of SERCA protein. In TAC-induced hypertrophic hearts in WT, the expression of SERCA protein was decreased, whereas that of SERCA2a protein was unchanged. Dowling et al.10 also demonstrated that SAR, but not SERCA1, was downregulated in dystrophic-deficient skeletal muscle. These results suggest that downregulation of SAR precedes downregulation of SERCA in pathological muscles.

Previous studies have demonstrated that the channel activity of RyR is increased when CSQ directly interacts with purified RyRs.7 Similarly, we found that SAR accelerated the rates of Ca2+ uptake when SAR was associated with SERCA2a. These results indicate that functional interaction between SAR and SERCA2a regulates the apparent Ca2+ affinity and the Ca2+ transport activity of SERCA2a. The relationship between SAR and SERCA in the longitudinal SR is reminiscent of that between CSQ and RyR in the terminal cisternae of the SR. Similar to the relation between CSQ and RyR, the affinity for the binding interaction between SAR and SERCA is probably low. No signal sequence responsible for its subcellular localization has been identified in SAR protein so far. We assume that SAR could be anchored to the longitudinal SR by its weak interaction with SERCA. This notable finding raises the possibility that SAR acts as a regulator of SERCA2a activity by sensing the changes in Ca2+ concentration in the SR lumen.

In conclusion, SAR functionally interacts with SERCA2a to stabilize SERCA2a protein and modulates the apparent Ca2+ affinity as well as the Ca2+ transport activity of SERCA2a. Thus, the SERCA2a–SAR interaction plays an important role in preserving cardiac function under biomechanical stresses such as pressure overload.

### Supplementary material

Supplementary material is available at Cardiovascular Research online.

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### Conflict of interest

none declared.

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