Long-term levosimendan treatment improves systolic function and myocardial relaxation in mice with cardiomyocyte-specific disruption of the Serca2 gene

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Submitted 24 August 2012; accepted in final form 20 September 2013

In human and experimental HF in animals, abnormalities in myocardial contractility and relaxation are thought to result, in part, from reduced abundance and/or activity of the cardiac sarcoplasmic reticulum ATPase (SERCA2) (28). Reduced SERCA2 function is thought to slow removal of calcium from the cytosol into the sarcoplasmic reticulum (SR) during diastole. This may subsequently reduce the releasable SR calcium store content, thereby also contributing to systolic dysfunction (5, 14). Although decreased levels of SERCA have been shown to occur in patients with HF (28), no treatment has been shown to target this mechanism, except SERCA gene transfer.

Since calcium transients are reduced in patients with reduced SERCA levels, the calcium-sensitizer effect of levosimendan may be particularly suited to target this pathophysiological mechanism. On the other hand, since failing hearts have slowed uptake of calcium and often increased diastolic calcium levels (14), levosimendan may cause worsening of myofilament relaxation and diastolic dysfunction. However, such effects of levosimendan on reduced relaxation caused by diminished SERCA2 activity have not yet been studied in detail. Moreover, the long-term effect of the drug on myocardial relaxation in chronic HF with disturbed calcium transients is not known.

The purpose of this study was to examine the effects of levosimendan in mice with impaired calcium handling due to cardiomyocyte-specific disruption of the Serca2 gene. These mice have reduced relaxation already at 6 days after excision of the Serca2 gene is induced (31). At 4 wk and 7 wk after Serca2 gene disruption, the mice develop a marked diastolic dysfunction as well as impaired contractility (1). These mice were treated with levosimendan for 7 wk.

METHODS

Animal model. Mice with inducible cardiac-specific disruption of the Serca2 gene (Serca2 knockout [Serca2KO]) and control Serca2floxflox (Serca2FF) mice have been described previously (1, 2, 22). Adult SERCA2KO animals are unremarkable until disruption of the Serca2 gene is induced (31). At 4 wk and 7 wk after Serca2 gene disruption, the mice develop a marked diastolic dysfunction as well as impaired contractility (1). These mice were treated with levosimendan for 7 wk.

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METHODS

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Simdax; Orion, Espoo, Finland; diluted in H2O to 0.05 mg/ml), twice daily (total dose 1 mg · kg\(^{-1}\) · day\(^{-1}\); 10 ml/kg; \(n = 9\)) for 7 wk. The animals in the various groups were examined and killed randomly. All investigations conform with the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1985). All experiments were approved by the independent local authority Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Germany, and by the Norwegian Animal Research Authority (project ID 443).

**Cardiac function by echocardiography and left-ventricular pressure hemodynamics.** Seven weeks after tamoxifen injection, the animals were ventilated with 2% isoflurane (Forane; Abbott Laboratories, IL) in ambient air and placed in a supine position. Transthoracic echocardiography was performed using a Vevo 770 High-Resolution in Vivo Micro-Imaging System with a 30-MHz probe (VisualSonics, Toronto, Ontario, Canada). Images were collected in the parasternal long-axis view. Left-ventricular inner-diameter (LVID) dimensions were measured using both M-mode and B-mode. Volumes were obtained by manually tracing the endocardial border in the parasternal long-axis views. Ejection fraction (EF) was calculated using the following formula: $\text{EF} (%) = \frac{\text{LV volume diastole} - \text{LV volume systole}}{\text{LV volume diastole}} \times 100$.

Intraventricular pressure measurements were performed with a Mikro-Tip 1.4 Fr catheter (Millar Instruments, Houston, TX) inserted into the LV through the right carotid artery. Data from 10 consecutive beats were recorded using The Power Lab system and analyzed using Chart Pro 6.1 software. Data for heart rate (HR), LV peak systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), and LV maximal-positive (dP/dt\(_{\text{max}}\)) and minimum (dP/dt\(_{\text{min}}\)) derivatives of the pressure curve were collected. The time constant (\(\tau\)) of isovolumetric relaxation was calculated by fitting the pressure curve of the isovolumetric relaxation phase to the monoexponential curve, described by the following function: $P = P_0 e^{-t/\tau} + P_h$, where P is LV diastolic pressure.

Table 1. Probes used for detection

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Alias</th>
<th>Gene Symbol</th>
<th>Forward Primer, 5′–3′</th>
<th>Reverse Primer, 5′–3′</th>
<th>Probe, 5′-FAM, 3′-TAMRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrial natriuretic peptide</td>
<td>Anp</td>
<td>Nppa</td>
<td>CTTCTTCTCTCAAGGCTGC</td>
<td>CACGATCTGCATGGATTTCA</td>
<td>CACGATCTGCATGGATTTCA</td>
</tr>
<tr>
<td>Brain natriuretic peptide</td>
<td>Bnp</td>
<td>Nppb</td>
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<td>TGAGAAGCTGCTGAGATAGA</td>
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<td>alpha-Myosin heavy chain</td>
<td>Mhyb</td>
<td>Myh7</td>
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<td>Myh7</td>
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<td>TGCAAGCTGCAATGTCAAAG</td>
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<tr>
<td>Collagen type 2, alpha 2</td>
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<td>CACATCGCGGACCTCAGAGAT</td>
<td>CACATCGCGGACCTCAGAGAT</td>
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</tbody>
</table>

FAM, 5′-6-carboxyfluorescein; TAMRA, tetramethyl-6-carboxyrhodamine.
pressure, $P_0$ is pressure at $dP/dt_{\text{min}}$, $e$ is the exponential function, $t$ is time, and $P_B$ is the pressure asymptote, as described previously (16). To verify if the monoexponential model was applicable, the linearity of the relationship between log (pressure) and time was examined. The relationship was found to be close to linear, with a correlation coefficient ($r$) $\approx 0.98$ in all animals. Since Mirsky and Papisov in (27) suggest to assume zero asymptote only when $r > 0.99$, the three-parametric model was preferred. The start and end point of the isovolumetric relaxation phase of the $\tau$ calculations was defined by the commercially available software and was also calculated with the use of a hybrid logistic method, as previously described (25, 30).

Quantification of mRNA abundance by quantitative real-time RT-PCR. LV myocardium was pulverized by grinding with liquid nitrogen and total RNA extracted using the Trizol reagent, according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). Total RNA was digested with DNase I to remove residual genomic DNA. Total RNA integrity was estimated by the absorbance at a 260:280-nm ratio. Total RNA (1 µg) from each sample was reverse transcribed using random hexamer primers and the ImProm-II Reverse Transcription System, according to the manufacturer’s protocol (Promega, Madison, WI). Quantitative real-time PCR (RT-qPCR) analysis was performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) with cycling parameters: 2 min at 50°C; 10 min at 95°C; 40 cycles of 15 s at 95°C, and 1 min at 60°C. Results were normalized to $Rpl32$ controls, and the relative expression of specific transcripts was calculated as relative expression $= 2^{\Delta \Delta CT} = 2^{[(C_{\text{probe}}) - (C_{Rpl32})]}$, where $C_T$ is defined as the cycle number at which the amplification plot for all samples passes a set threshold above baseline. Probes used for detection are shown in Table 1.

Assessment of diastolic pressure-volume relationships. Diastolic pressure-volume relationships were investigated, as described by Zile et al. (35). Briefly, pressure and instantaneous volumes were assessed at three time points during diastole. We corrected for slow relaxation, and the corrected passive-stiffness constant was calculated by per-

Fig. 2. Dimensions and volumes assessed by echocardiography. A: representative images for assessment of left-ventricular (LV) dimensions in each group, whereas B and C show mean values of systolic and diastolic inner-diameter dimensions (LVIDs and LVIDd, respectively). LV volumes were calculated from LV dimensions (D and E). F: LV mass. For all panels, values are shown for control SERCA2FF mice (FF; $n = 9$) and for SERCA2KO mice treated with vehicle (KO/vehicle; $n = 7$) or with levosimendan (KO/levo; $n = 9$). Data are presented as mean ± SE. *$P < 0.05$ vs. FF.
forming a curve fit to the equation: $P = Ae^{\beta V}$, where $A$ is the curve-fitting constant, $\beta$ is the stiffness constant, and $V$ is volume.

**Statistics.** The study was initiated with nine animals in each group. However, some animals died during investigations, mainly due to severe cardiac dysfunction or to bleeding, resulting in unequal group sizes. Thus comparison between groups was made using one-way ANOVA with Newman-Keuls post hoc testing (SigmaStat version 3.5; Systat Software GmbH, Erkrath, Germany). All data are presented as mean ± SE; $P < 0.05$ was considered statistically significant.

**RESULTS**

**Reduced systolic function and slowed myocardial relaxation in SERCA2KO mice.** Seven weeks after tamoxifen injection, SERCA2 mRNA abundance in the myocardium of SERCA2KO mice was strongly reduced to 0.7% of control SERCA2FF values, as assessed by RT-qPCR (data not shown), verifying efficient disruption of the Serca2 gene in the cardiomyocytes.

Cardiac function was reduced significantly in vehicle-treated SERCA2KO mice compared with SERCA2FF control animals, as shown by decreased EF (39% vs. 63%), reduced stroke volume (11.6 µl vs. 26.7 µl), and reduced cardiac output (4.8 vs. 11.79 ml/min; all $P < 0.05$; Fig. 1, A–C). Intraventricular pressure measurements showed that LV dP/dt max, LVSP, and HR were all decreased in SERCA2KO mice compared with control SERCA2FF values (to 32%, 77%, and 84% of control values, respectively; $P < 0.001$; see Fig. 4). LVEDP was increased significantly in SERCA2KO (16.0 ± 1.2 mmHg) compared with SERCA2FF controls (8.4 ± 0.9 mmHg; see Fig. 4), confirming diastolic dysfunction. Both the diastolic LVID and the LV end-diastolic volume were slightly, but significantly, reduced in SERCA2KO compared with SERCA2FF (Fig. 2, C and E). Moreover, dP/dt min was reduced to 33%, and the isovolumetric pressure decay $\tau$ was increased fourfold of SERCA2FF control values, as shown by pressure curves in Fig. 3 and in Fig. 4, E and F. The isovolumetric relaxation time (IVRT) was prolonged significantly, from 10.0 ± 0.4 ms in SERCA2FF to 22.0 ± 1.7 ms in SERCA2KO (Fig. 4G), and the duration of the diastole:$\tau$ ratio was only 1.5 ± 0.3, indicating incomplete relaxation in SERCA2KO, as described by Blaustein and Gasch (6). Overall, both systolic and diastolic dysfunction was evident in vehicle-treated SERCA2KO mice, in keeping with previous results (1, 22).

**Levosimendan treatment improves cardiac systolic function and myocardial relaxation in SERCA2KO.** Strikingly, levosimendan treatment normalized LV EF in SERCA2KO to near-SERCA2FF control values (62% vs. 63%; Fig. 1A). Stroke volume and cardiac output were also improved significantly (19.3 µl and 8.1 ml/min vs. 11.62 µl and 4.8 ml/min in SERCA2KO vehicle, respectively; Fig. 1, B and C). A nonsignificant trend toward decreased end-systolic diameter (LVIDs) and decreased end-systolic volume in the SERCA2KO animals treated with levosimendan also suggests improved contractility ($P = 0.08$ for LVIDs, and $P = 0.19$ for LV end-systolic volume; Fig. 2, B and D). In contrast, there was no significant improvement in LVSP, LVEDP, maximal rates of pressure development and decay, or HR (Fig. 4, A–E). However, the isovolumetric relaxation $\tau$ value was improved significantly in levosimendan-treated compared with vehicle-treated SERCA2KO (three- and fourfold of SERCA2FF control values, respectively; Fig. 4F). Moreover, IVRT was shortened significantly during levosimendan treatment (16.3 ± 1.8 ms) compared with vehicle-treated SERCA2KO (22.0 ± 1.7 ms; Fig. 4G). A ratio of diastole duration:$\tau$ of 2.1 ± 0.3 indicated that relaxation was improved, although still incomplete (6). Additionally, $\tau$ was calculated using a hybrid logistic method, which confirmed the differences found between SERCA2KO vehicle and SERCA2FF (14.2 ± 1.6 ms vs. 4.8 ± 0.2 ms; $P < 0.001$) and between the levosimendan group and the nontreated group (10.2 ± 0.8 ms vs. 14.2 ± 1.6 ms; $P < 0.01$). Taken together, these findings demonstrate that levosimendan markedly improved systolic function and myocardial relaxation.
Markers of cardiac dysfunction and hypertrophy in levosimendan-treated SERCA2KO. We next examined whether levosimendan treatment would attenuate the expression of known markers of cardiac dysfunction and hypertrophy in SERCA2KO. In vehicle-treated SERCA2KO, the abundance of atrial natriuretic peptide and β-myosin heavy chain (β-MHC) mRNA transcripts was increased significantly at 7 wk compared with SERCA2FF control values (Fig. 5, A and D), whereas no significant changes in brain natriuretic peptide or α-MHC mRNA abundance (Fig. 5, B and C) were found. Levosimendan treatment did not affect the expression of these transcripts in SERCA2KO.

Increased expression of ECM markers despite improved cardiac function in levosimendan-treated SERCA2KO. To investigate whether levosimendan treatment had effects on the ECM in SERCA2KO, we analyzed the expression of key gene

Fig. 4. Cardiac function assessed by LV pressure measurements. LVSP (A) and LVEDP (D), dP/dt max (B) and dP/dt min (E) of the pressure curve, heart rate (C), isovolumetric relaxation (F), and isovolumetric relaxation time (IVRT; G) in control SERCA2FF mice (FF; n = 8) and in SERCA2KO mice treated with vehicle (KO/vehicle; n = 4) or with levosimendan (KO/levo; n = 9). Data are presented as mean ± SE. *P < 0.05 vs. FF; †P < 0.05 vs. KO/vehicle.
transcripts important in fibrosis and ECM remodeling (Fig. 6). In SERCA2KO, expression of collagen type 1, \(\alpha_2\) (collagen1a2), and collagen type 3, \(\beta_2\) (collagen3a2), was increased twofold compared with SERCA2FF control values (Fig. 6, B and C). In contrast, expression of collagen type 1, \(\alpha_1\) (collagen1a1), and fibronectin 1 was not altered significantly (Fig. 6, A and D). Thus the cardiac dysfunction in SERCA2KO hearts selectively induced specific transcripts central in ECM remodeling. Levosimendan treatment significantly increased further the expression levels of collagen1a2 and collagen3a2 in SERCA2KO (threefold; Fig. 6, B and C). Levosimendan also significantly increased the expression of collagen1a1 in SERCA2KO (Fig. 6 A). Taken together, levosimendan did not attenuate the altered expression of ECM markers in SERCA2KO but rather, further increased the ECM remodeling response.

Uregulated expression of paracrine factors involved in ECM remodeling in levosimendan-treated SERCA2KO. Several paracrine factors have been shown to be involved in ECM remodeling. We found that transforming growth factor \(\beta_2\) (TGF-\(\beta_2\)) was upregulated fivefold in SERCA2KO compared with SERCA2FF controls (Fig. 7). No significant difference was found between SERCA2KO and SERCA2FF in the expression levels of connective tissue growth factor (CTGF), latent TGF-\(\beta\)-binding protein 2 (LTBP2), or TGF-\(\beta_1\). Levosimendan treatment in SERCA2KO significantly augmented the expression of TGF-\(\beta_2\) (eightfold) and increased the expression of CTGF (sevenfold) and LTBP2 (twofold) relative to control SERCA2FF (Fig. 7). In contrast, there was no effect on TGF-\(\beta_1\) expression. These findings suggest involvement of paracrine-signaling molecules in the observed increase in expression of genes encoding ECM constituents.

Assessment of passive stiffness. Since the SERCA2KO animals had diastolic dysfunction and increased markers of fibrosis, we investigated diastolic pressure-volume relationships and corrected for slow relaxation to create relaxation-adjusted, end-diastolic, pressure-volume relationships, as described by Zile et al. (35). We found no significant alterations in passive stiffness, neither as a result of SERCA2KO nor as a result of levosimendan treatment. Results are shown in Fig. 8.

DISCUSSION

In this study, we show that long-term levosimendan treatment improved cardiac systolic function and myocardial relaxation in an experimental murine HF model with marked reduced myocardial relaxation, primarily due to impaired calcium handling in cardiomyocytes. We show that 7 wk of levosimendan treatment improves cardiac systolic function and myocardial relaxation in this model. Gene-expression analysis showed that long-term levosimendan treatment failed to attenuate the increased expression of several markers of cardiac...
dysfunction and ECM remodeling but instead, augmented these further.

Levosimendan has calcium-sensitizer properties and was introduced on the market 15 years ago. Although the drug is used clinically in many countries, the exact indications still remain to be determined. This may be partly due to a lack of detailed knowledge of the in vivo effects on pathophysiological alterations in failing hearts. Moreover, most studies have investigated the effects of levosimendan in an acute setting using a single intravenous injection of the drug (10). Hence, the long-term effect of oral treatment with levosimendan is not well known, although a few studies indicate positive effects in patients (17) and in experimental models (23, 24). In this study, we investigated the effects of levosimendan in a model with reduced SR calcium reuptake and smaller calcium transients due to cardiomyocyte-specific excision of the SERCA2 gene. The SERCA pump plays a crucial role in calcium homeostasis, as it removes calcium from cytosol and transports it into the SR during early diastole. SERCA2KO animals develop a pronounced reduction in myocardial relaxation and diastolic dysfunction, which is already apparent a short time after induction of gene excision. After 7 wk, an impaired systolic function is also found, as shown by a reduction in EF, stroke volume, and cardiac output.

We treated the SERCA2KO animals with oral levosimendan for 7 wk and observed a marked improvement in cardiac function, as shown by a complete normalization of EF to the levels found in the control mice (SERCA2FF), as well as improved stroke volume and cardiac output. This illustrates that the effects of levosimendan are present when the primary etiology is diminished SR uptake and smaller calcium transients. The assessments of EF were based on measurements of LV volumes. Although the systolic volumes were not significantly different between the SERCA2KO group treated with vehicle and the SERCA2KO group treated with levosimendan, the difference between systolic and diastolic volumes were greater in the levosimendan group, resulting in improved EF. Interestingly, levosimendan treatment also significantly improved relaxation, as shown by a reduction in the isovolumetric pressure decay τ and significant shortening of the IVRT. Although the positive effect of levosimendan on relaxation has been shown to occur in the acute setting (7, 18), we are, to our knowledge, the first to show a sustained effect on relaxation with long-term treatment. For calculation of τ values, we used both a three-parametric model and a hybrid logistic method. It has been discussed whether a two- or a three-parametric model is preferable when calculating τ; however, several studies recommend the latter, as it gives a more accurate estimate in the case of a parallel shift of the pressure curve, which might occur, for example, during respiration (9, 21). The hybrid logistic method has, by some authors, been claimed to give more reliable results (25). We found the same significant differences between the experimental groups using that method. Hence, our study illustrates the potent action of levosimendan on myocardial relaxation, as we show improved relaxation even in the presence of marked reduction in SR relaxation.

![Graph showing relaxation-adjusted, end-diastolic, pressure-volume relationships.](image)

**Fig. 8.** Relaxation-adjusted, end-diastolic, pressure-volume relationships. Correction for slow relaxation was performed, and corrected passive-stiffness constant (β) was calculated by performing a curve fit to the curve described by P = AeβV, where P is LV diastolic pressure, A is the curve-fitting constant, e is the exponential function and V is volume. SERCA2FF control animals are shown in a black, continuous line; SERCA2KO treated with vehicle in gray, triangular symbols and a continuous line; and SERCA2KO treated with levosimendan in a gray, dotted line.
removal of calcium from the cytosol. We think that these findings are clinically relevant, as reduced calcium reuptake by SERCA and smaller calcium transients have been shown to occur in patients with HF (4, 11).

There are several possible explanations for the beneficial effect of levosimendan in this study. By taking a prominent feature of this model into account—the reduced calcium transients—the improved systolic function may be due to the association of levosimendan with troponin C, causing a calcium-sensitizing effect. The observed improvement in relaxation, on the other hand, may be a consequence of several mechanisms. Traditionally, one of the problems with inotropic agents in general and also with the calcium sensitizers has been the aggravated diastolic dysfunction (13). Levosimendan, however, has been shown to have a positive, lusitropic effect, because it binds to troponin C in a calcium concentration-dependent manner and therefore, dissociates from troponin C in diastole (12, 15). We know from previous studies that the SERCA2KO animals have a markedly decreased function of the SR. Levosimendan is known to have a phosphodiesterase III-inhibitory effect, leading to increased SERCA activity through phospholamban phosphorylation and decreased myofibrillar calcium sensitivity through phosphorylation of troponin I (19). Although SERCA mRNA levels are reduced to 0.7% of control levels, we have shown previously that SERCA activity can be enhanced by stimulation of β-adrenergic receptors (1). Hence, phosphorylation of phospholamban and increased activity of the small amount of SERCA that is left might be responsible for the observed improvement in relaxation. Recently, Swift et al. (32) showed that the cardiomyocytes from the SERCA2KO animals display morphological changes, including a marked loss of SR volume but also, T-tubule remodeling with a greater abundance of longitudinal T-tubules. These morphological changes likely lead to enhanced efficiency of calcium release, compensating for the decreased SR volume and function (32). Since levosimendan increases the affinity for calcium to troponin C, levosimendan might contribute to concentrating calcium around the remaining SERCA, facilitating reuptake of calcium and improving relaxation. Hence, even though calcium transients are markedly reduced at an overall level, levosimendan might be particularly beneficial for relaxation in the morphologically changed cardiomyocytes of SERCA2KO animals. Finally, one could not rule out that the vasodilatory effect of levosimendan contributes to the observed effects on contractility and relaxation (26). More favorable working conditions for the heart, as a result of decreased preload and afterload, could be of greater importance in a chronic setting than in an acute setting. Although these positive effects on relaxation are described in literature, most studies have been carried out in an acute setting, using a single dose of the drug. Hence, one could imagine that chronic treatment would aggravate diastolic function. Altogether, we show here for the first time that the beneficial effects on diastolic function are still present after 7 wk of oral levosimendan treatment.

Besides impairment of active relaxation, increased myocardial stiffness is also an important feature of diastolic dysfunction. We investigated expression of genes involved in structural changes of ECM and in particular, collagens and paracrine factors influencing collagen expression. We found no signs of improved fibrosis. On the contrary, the animals treated with levosimendan showed significantly increased expression of several collagen isoforms, as well as paracrine factors known to be involved in fibrosis and tissue repair. TGF-β2 is known to induce CTGF (8), and CTGF may contribute to myocardial fibrosis (20). Therefore, theoretically, levosimendan could influence expression of TGF-β2 and trigger this sequence of events, leading to increased collagen expression and cardiac fibrosis. Our results are inconsistent with previous studies showing that levosimendan improves cardiac remodeling after myocardial infarction in a diabetic rat model (23, 33). However, very few studies have investigated the effect of levosimendan on ECM remodeling. The discrepancy between our results and previous results underlines the need for further investigation of levosimendan’s long-term effect on fibrosis and ECM remodeling in HF of various etiologies.

To investigate whether animals treated with levosimendan had increased passive stiffness, we estimated the relaxation-adjusted, end-diastolic, pressure-volume relationship as suggested by Zile et al. (35). In our study, we did not observe increased passive stiffness in the group treated with levosimendan. However, it cannot be excluded that longer treatment with levosimendan would lead to a significant increase in myocardial stiffness.

In summary, we have shown that 7 wk of oral treatment with levosimendan improves cardiac systolic function and myocardial relaxation in hearts with impaired calcium handling. However, increased expression of several collagen isoforms and profibrotic paracrine factors indicates that long-term use of levosimendan may lead to adverse remodeling of the ECM. Hence, this study suggests that levosimendan has favorable effects on myocardial relaxation in animals with reduced SERCA2, but our data also indicate unfavorable effects on development of fibrosis.

ACKNOWLEDGMENTS

We are grateful to Andreas Geerts, Gabriele Imberge, and Manuela Weldert for technical assistance.

GRANTS

Support for this work was provided by the Norwegian Research Council, Anders Jahre’s Fund for the Promotion of Science, South-Eastern Norway Regional Health Authority, the University of Oslo, and Bayer Pharma AG, Germany.

DISCLOSURES

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS


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