The Presence of Lys27 Instead of Asn27 in Human Phospholamban Promotes Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase Superinhibition and Cardiac Remodeling

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Background—Phospholamban (PLN) is an inhibitor of the Ca\(^{2+}\) affinity of sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase (SERCA2). The amino acid sequence of PLN is highly conserved, and although all species contain asparagine (Asn), human PLN is unique in containing lysine (Lys) at amino acid 27.

Methods and Results—Human PLN was introduced in the null background. Expression of human PLN, at similar levels to mouse wild-type PLN, resulted in significant decreases in the affinity of SERCA2 for Ca\(^{2+}\), attributed to unique spatial conformation of this PLN form and increases in its monomeric active unit compared with mouse PLN. The increased inhibition by human PLN was associated with attenuated cardiac contractility in the intact-animal, organ, and cardiomyocyte levels and with depressed calcium kinetics. These inhibitory effects could not be fully reversed even on maximal isoproterenol stimulation. There were no alterations in the expression levels of SERCA2, calsequestrin, ryanodine receptor, and FKBP12, although the sodium/calcium exchanger and the L-type Ca\(^{2+}\) channel expression levels were upregulated. The depressed function resulted in increased heart/body weight ratios and phosphorylation levels of Akt, p38, and Erk1/2.

Conclusions—Human PLN may play a more inhibitory role than that of other species in Ca\(^{2+}\) cycling. Expression of human PLN in the mouse is compensated by alterations in Ca\(^{2+}\)-handling proteins and cardiac remodeling in an effort to normalize cardiac contractility. Thus, the unique amino acid sequence of human PLN may be critical in maintaining a high cardiac reserve, which is of paramount importance in the regulation of human cardiac function. (Circulation. 2006;113:995-1004.)

Key Words: contractility • myocytes • phospholamban • sarcoplasmic reticulum • hypertrophy

Heart failure, the leading cause of human morbidity and mortality, represents a final common pathway in response to a variety of different pathological stimuli and reflects the complex interaction of multiple intracellular cascades. A nearly universal characteristic of human and experimental heart failure is the attenuated sarcoplasmic reticulum (SR) Ca\(^{2+}\) cycling, which is associated with decreased expression of the cardiac SR Ca\(^{2+}\)-ATPase (SERCA2) and relative overabundance of the SERCA2 inhibitory protein phospholamban (PLN).1,2 Accordingly, the restoration of SR Ca\(^{2+}\) cycling either by adenovirus-mediated overexpression of SERCA2 or through manipulation of PLN activity appeared to hold promise in the treatment of heart failure.3-4 The restored SR Ca\(^{2+}\) transport was associated with improved energetics, survival, and cardiac function at the cellular, organ, and intact-animal levels.3 To date, PLN inhibition as a means of improving SR Ca\(^{2+}\) cycling and contractile performance has received wide attention. Studies in experimental systems indicate that PLN levels inversely correlate with cardiac contractile parameters; PLN ablation may rescue several genetic models of heart failure; inhibition of PLN activity may restore function and halt the progression of heart failure.5 Conversely, increased inhibition by PLN mutants impairs heart function, which leads to cardiac remodeling and dilated cardiomyopathy.5

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The importance of PLN in human cardiac Ca\(^{2+}\) homeostasis was recently supported by identification of a PLN mutant (R9C) that had no effect on SERCA2 activity under basal conditions, which is consistent with a lack of inhibitory function.
conditions but trapped protein kinase A, resulting in dominant inheritance of dilated cardiomyopathy and early death. The long-term detrimental effects of such chronic inhibition of SERCA2 activity were also observed in transgenic mice, which indicates that inhibition of PLN phosphorylation is sufficiently deleterious to cause the onset of dilated cardiomyopathy in humans. However, another human PLN mutation (L39stop), which results in PLN deficiency, was also associated with human dilated cardiomyopathy and heart failure in the homozygous state, which indicates that lack of PLN expression may be detrimental to humans. This unexpected finding, given previous reports in mouse models, was suggested to reflect differences in cardiac reserve and myocyte Ca\(^{2+}\) cycling characteristics between the 2 species.

Another overlooked, compounding factor in the interpretation of these studies, however, is the subtle difference in the primary structure of PLN between human and mouse. The amino acid sequence of PLN is highly conserved with Asn27 in almost all mammalian species except humans, where Lys is found at this position. Amino acid 27 in PLN interacts with Leu321 in SERCA2, and any substitution in these amino acids may result in significant alterations in PLN/SERCA2 activity. Indeed, a previous in vitro study showed that the K27-PLN gained inhibitory function relative to N27-PLN in HEK-293 cells. Thus, it was of special interest to investigate whether human (K27) PLN could also inhibit SERCA2 and cardiac function to a greater extent than mouse (N27) PLN in vivo, which may underlie the differences in Ca\(^{2+}\) cycling between human and mouse species. To mimic the human PLN amino acid sequence, the mouse PLN cDNA was mutated (N27K) and expressed in the PLN null mouse heart. Our findings suggest that human PLN plays a more inhibitory role in cardiac Ca\(^{2+}\) handling and contractility, which leads to cardiac remodeling in the mouse. This single amino acid substitution at position 27 of PLN in part may underlie the known differences between mouse and human hearts with respect to Ca\(^{2+}\) cycling characteristics and their degree of SR Ca\(^{2+}\) dependence.

**Methods**

Transgenic mice expressing N27K-PLN (human PLN) in the PLN-deficient mouse background (FVB/N) were generated by standard procedures. Cardiac contractile parameters were determined by in vivo echocardiography, ex vivo Langendorff perfusion, and isolated cardiomyocytes. Animals were handled and maintained according to protocols established by the ethics committee of the University of Cincinnati. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the
National Institutes of Health. A detailed description of the methods used in the study is provided in the online-only Data Supplement.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agreed to the manuscript as written.

Results

Generation and In Vivo Characterization of Transgenic Mice Expressing Human (K27) PLN in the PLN-Deficient Background

The amino acid sequence of PLN is highly conserved across species, and the critical amino acid 27, proposed to mediate part of its functional effects, is asparagine (Asn) in all known mammalian species with the exception of human, which contains Lys27 (Figure 1A). To determine the functional significance of this single amino acid substitution, cardiomyocyte-specific expression of human N27K(AAT→AAG)-PLN with the α-MHC (myosin heavy chain) promoter was directed in the PLN-null background. 10,11 Three transgenic lines (TGL1, TGL2, and TGL3) were obtained, and quantitative immunoblotting indicated that the cardiac PLN levels in TGL2 (92.6±9.5%) were similar to those (100±10.8%) present in wild-type mouse hearts (Figure 1B). Thus, TGL2 was propagated for further characterization studies in 10- to 12-week-old mice, except as indicated. This age was chosen to compare our results with previous studies in transgenic models that expressed either wild-type PLN or other PLN mutants. 10,14

In vivo cardiac function, assessed by echocardiography, revealed significant decreases in fractional shortening (82%) and velocity of circumferential shortening corrected for heart rates (44%), whereas the left ventricular end-diastolic dimension (118%), left ventricular end-systolic dimension (139%), ejection time (134%), and calculated left ventricular mass (156%) were significantly increased in human-PLN compared with mouse-PLN animals (Table 1; Figure 1C). Cardiac remodeling was observed as early as 4 weeks of age in this line, which indicates early compensatory changes in the face of depressed function elicited by human PLN (data not shown). Examination of TGL1, which expressed 40% of human PLN levels, indicated intermediate effects on geometric and functional alterations compared with TGL2, which suggests a gene dosage effect (Table 1). Interestingly, the inhibitory effects of 40% expression levels of human PLN were of similar magnitude as those elicited by 100% mouse PLN in the heart (Table 1), which points to the potent nature of human (K27)-PLN.

Superinhibitory Effects of Human PLN on SR Ca2+ Transport

The subcellular effects of human PLN on SR function were assessed in cardiac homogenates with conditions that restricted Ca2+ uptake to SR. 10,14 The initial rates of SR Ca2+ transport, obtained over a wide range of [Ca2+]o, indicated that human PLN resulted in a significant increase in the EC50 of SERCA2 for calcium (0.49±0.01 μmol/L) compared with mouse PLN (0.32±0.01 μmol/L; Figure 1D). However, the maximal velocity of calcium uptake was not altered (Figure 1D). These data suggest that human PLN interacts with and inhibits SERCA2 to a greater extent than mouse PLN.

Because the interaction between PLN and SERCA2 may be modified by the concentration of the active PLN monomer and the primary structure of PLN, we examined these parameters using quantitative immunoblotting and nuclear magnetic resonance (NMR) analysis.16,17 The PLN monomer content was significantly higher in human PLN than in mouse PLN. In accordance with this, the PLN pentamer content was lower (65% of total) in human PLN hearts than in mouse PLN. Because the interaction between PLN and SERCA2 may be modified by the concentration of the active PLN monomer and the primary structure of PLN, we examined these parameters using quantitative immunoblotting and nuclear magnetic resonance (NMR) analysis.16,17 The PLN monomer content was significantly higher in human PLN than in mouse PLN. In accordance with this, the PLN pentamer content was lower (65% of total) in human PLN hearts than in mouse PLN. These findings indicate that higher relative levels of the active PLN monomeric species in human PLN hearts would lead to increased SERCA2 inhibition.

Furthermore, 2 PLN peptides (AA 1–36) with either mouse or human PLN were synthesized and subjected to NMR studies. The structural statistics (Table 2) confirmed that the number of NOE (nuclear Overhauser effect) cross peaks, which indicate the proximity of 2 nuclear hydrogen atoms in the molecular structure, the quality of the spectra, and the quality of the structural families were comparable for the 2 peptides.16 Forty structures deduced from NMR data with no violations >0.1 Å were obtained for each peptide. In both peptides, the N-terminal residues from AA 4–18 and the C-terminal residues from AA 22–35 displayed α-helical configurations (Figures 2B and 2C). The crossing angle Ω (defined as the torsion angle between the axes of the 2 helices when projected on a contact plane) was not significantly different for the 2 families; however, the distance of closest contact, d, between the 2 helices was 7.0±2.8 Å for mouse PLN and 11.7±4.2 Å for human PLN, which indicates a more loose packing of human PLN. In addition, the axes of the 2 peptides.16 Forty structures deduced from NMR data with no violations >0.1 Å were obtained for each peptide. In both peptides, the N-terminal residues from AA 4–18 and the C-terminal residues from AA 22–35 displayed α-helical configurations (Figures 2B and 2C). The crossing angle Ω (defined as the torsion angle between the axes of the 2 helices when projected on a contact plane) was not significantly different for the 2 families; however, the distance of closest contact, d, between the 2 helices was 7.0±2.8 Å for mouse PLN and 11.7±4.2 Å for human PLN, which indicates a more loose packing of human PLN. In addition, the axes of the 2

| TABLE 1. Echocardiography Parameters in Human PLN (TGL1 and TGL2) and Mouse PLN Mice |
|--------------------------------------|-----------|-----------|-----------|---------|
|                                      | mPLN (n=6) | TGL1 (n=6) | TGL2 (n=7) | P       |
| LVEDD, mm                            | 3.3±0.1   | 3.6±0.1   | 3.9±0.1*  | 0.002   |
| LVESD, mm                            | 1.8±0.1   | 2.1±0.07  | 2.5±0.1*  | <0.001  |
| Anterior wall, mm                    | 0.58±0.03 | 0.79±0.02*| 0.58±0.04 | NS      |
| Posterior wall, mm                   | 0.59±0.03 | 0.63±0.05 | 0.68±0.03 | NS      |
| HR                                   | 57±4:22   | 563±26    | 291±19*   | <0.001  |
| Ejection time, ms                    | 53±2.2    | 49±2.0    | 71±2.4*   | <0.001  |
| H/R                                  | 0.36±0.03 | 0.47±0.02*| 0.32±0.02 | 0.07    |
| LVM                                  | 54±1.6    | 75±7.1*   | 84±5.5*   | 0.001   |
| FS%                                  | 45±2.1    | 40±2.4    | 37±1.2*   | 0.01    |
| Vcf (circ −)                         | 16.3±1.9  | 15.2±0.9  | 7.2±0.4*  | <0.001  |

mPLN indicates mouse PLN; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; HR, heart rate; H/R, ratio of wall thickness to left ventricular radius; LVM, left ventricular mass; FS%, percentage fractional shortening; and Vcf, velocity of circumferential shortening corrected for heart rates.

Values are mean±SE.

TGL1 and TGL2 expressed 40±3.8% and 92.6±9.5% of mouse PLN (100%).
P indicates comparison of 3 groups; *P<0.05, TGL1 or TGL2 vs mPLN (1-way ANOVA).

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α-helices of human PLN lay on planes more distant than in mouse PLN. These data suggest a significant difference between the 2 PLN structures in the space conformation, which may play a key role in PLN interaction with SERCA2.

### Table 2. Characteristics of the Structures of Human PLN and Mouse PLN (AA1–36)*

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*Forty structures were selected from 200 computed for each peptide on the bases of energy and restraints violations. Region 4–16 indicates N-terminal helix of PLN36; region 22–35, C-terminal helix of PLN36; Rmsd, root mean square deviation, calculated on the basis of the analysis by PROCHECK-NMR.16

### Figure 2. PLN pentamer and monomer content, NMR structure of human PLN (hPLN) and mouse PLN (mPLN), and their effects in isolated hearts. A, Quantitative immunoblotting of hPLN (n=8) and mPLN (n=8). Upper panel shows a representative immunoblot. Lower panel shows quantitation of total PLN, PLN pentamer, and PLN monomer. Experiments were performed in triplicate. Values are mean±SE. *P<0.05 vs mPLNs. B and C, Examples of the structures of mouse N27-PLN1–36 (red) and human K27-PLN1–36 (gray or blue). Main chain atoms of the C-terminal helix from residues M20 to S36 of the 2 peptides are superimposed (C). The N-terminal helix of the human K27-PLN1–36 adopts a different course than the mouse N27-PLN1–36. D and E, Ex vivo Langendorff-perfused hearts showed significantly decreased rates of contraction (+dP/dt) and relaxation (−dP/dt), as well as delayed time to 50% relaxation (RT1/2) in hPLN mice (n=7) compared with mPLNs (n=7) under paced (400 bpm, D) and unpaced (E) conditions. Values are mean±SE. *P<0.05 vs mPLNs.

**Effects of Human PLN in Isolated Hearts and Cardiomyocytes**

To further delineate the functional effects of human PLN at the organ level, Langendorff-perfused hearts were paced at 400 bpm, and contractile parameters were assessed. The rates of contraction (+dP/dt) and relaxation (−dP/dt) in human PLN hearts were decreased to 70% and 72% of mouse PLN hearts, respectively. Furthermore, the time to 50% relaxation (RT1/2) was increased to 1.6 times of that observed in mouse PLN hearts (Figure 2D). Similar findings were obtained in unpaced hearts. In human PLN hearts (233 bpm), +dP/dt and −dP/dt were decreased to 79% and 78% of those in mouse PLN hearts (329 bpm), respectively. RT1/2 was prolonged by 1.3 times compared with that in mouse PLN hearts (Figure 2E). These data indicate that human PLN is associated with depressed cardiac contractility in intact hearts.

Similar inhibition of myocyte mechanics and Ca2+ kinetics was also observed in isolated cardiomyocytes (Figure 3). Human PLN cells exhibited decreases in fractional shortening and rates of contraction and relaxation to 77%, 61%, and 65% of mouse PLN cells, respectively (Figures 3A, 3C, 3E, and 3G). After maximal isoproterenol stimulation (100 nmol/L), fractional shortening and rates of contraction and relaxation remained similarly depressed in human PLN cardiomyocytes. Similar findings were obtained when the rates of contraction...
and relaxation were divided by the fractional shortening (data not shown). Accordingly, the peak of the Ca\(^{2+}\) transient was decreased to 74\%, whereas T\(_{80}\) (time to 80\% decay of calcium) and \(\tau\) were prolonged to 148\% and 145\% in human PLN cells compared with mouse PLN cells, respectively (Figures 3D, 3F, and 3H). No difference in diastolic Ca\(^{2+}\) levels were observed between human and mouse PLN myocytes (Figure 3B). After maximal isoproterenol (100 nmol/L) stimulation, the calcium peak remained depressed, and T\(_{80}\) and \(\tau\) remained significantly prolonged in human PLN cells (Figures 3D, 3F, and 3H). However, the relative increases by isoproterenol stimulation appeared similar between mouse PLN and human PLN myocytes (data not shown). Assessment of \(\beta\)-adrenergic receptor expression, as determined by radioligand binding (29±6.9 versus 16±1.4 fmol/mg, n=4, \(P=0.19\)) or coupling to adenyl cyclase (basal: 14±3.3 versus 15±3.0 pmol·min\(^{-1}\)·mg\(^{-1}\); isoproterenol: 36±6.8 versus 38±5.5 pmol·min\(^{-1}\)·mg\(^{-1}\); EC\(_{50}\) 213±37 versus 202±49 nmol/L, respectively, n=4; Figure 4A), indicated no alterations in transgenic hearts. Furthermore, human PLN could be phosphorylated to the same extent as mouse PLN in vitro by either cAMP-dependent or Ca\(^{2+}\)/calmodulin-dependent protein kinase (Figures 4B and 4C), which indicates that the N27K substitution did not alter the ability of PLN to be phosphorylated. Interestingly, in vivo phosphorylation of human PLN in nonstimulated or \(\beta\)-adrenergic–stimulated hearts was depressed on both Ser16 (50\% and 43\%, respectively) and Thr17 (53\% and 53\%, respectively) compared with mouse PLN (Figures 4D and 4E).

Assessment of frequency-dependent increases of myocyte contractile parameters indicated significantly higher stimulation of myocyte fractional shortening and rates of contraction and relaxation (+/−dL/dt) in the human PLN myocytes than in mouse PLN cells (Figures 5A to 5C). However, the actual values of these contractile parameters at maximal stimulation (5 Hz) were similar between human PLN and mouse PLN myocytes (Figures 5D to 5F), which indicates that the maximal Ca\(^{2+}\) capacitance of the SR was similar between these models.
Effects of Human PLN on Major Calcium-Handling Proteins

Quantitative immunoblotting revealed that the levels of SERCA2, calsequestrin, ryanodine receptor, and FKBP12 remained similar between human and mouse PLN hearts (data not shown), which indicates that superinhibition by human PLN did not influence the expression of these SR proteins. However, the relative expression levels of the

Figure 4. Adenylyl cyclase activity, in vitro and in vivo phosphorylation of PLN. A, Adenylyl cyclase activity in mouse PLN (mPLN, n=6) and human PLN (hPLN, n=6) hearts, measured with increasing concentrations of isoproterenol (Iso). B and C, Cardiac homogenates from mPLN (n=6) and hPLN (n=6) mice were phosphorylated by either catalytic subunit of protein kinase A (PKA; B) or endogenous calcium/calmodulin-dependent protein kinase II (CaMKII; C) in vitro and subjected to quantitative immunoblotting to determine phosphorylation of PLN at Ser16 and Thr17. D and E, Hearts from mPLN (n=6) and hPLN (n=6) mice were homogenized after maximal dobutamine treatment (32 ng · g⁻¹ · min⁻¹ for 5 minutes) in vivo and subjected to quantitative immunoblotting, as above. Values are mean±SE. *P<0.05 vs mPLN.

Figure 5. Cardiac frequency-dependent increase of myocyte contraction. A and B, Rates of shortening and relengthening (+/-dL/dt). C, Fractional shortening (FS) from isolated human PLN (hPLN, n=4) and mouse PLN (mPLN, n=4) cardiomyocytes, stimulated at 0.5, 2, and 5 Hz and expressed as percentage value of 0.5 Hz. Rates of shortening and relengthening (D and E; +/-dL/dt, μm/s) and fractional shortening (F) stimulated at 0.5, 2, and 5 Hz, expressed as actual values. Values are mean±SE. At least 6 to 10 myocytes were analyzed per heart; n, represents number of hearts. *P<0.05 vs mPLNs.
sodium/calcium exchanger and the L-type calcium channel or dihydropyridine receptor were increased by 40% and 20%, respectively, compared with mouse PLN hearts (Figures 6A and 6B) based on quantitative immunoblotting (see Data Supplement). To further investigate the properties of the L-type calcium channel, recordings were made in isolated ventricular myocytes with a whole-cell voltage clamp. Figure 6C shows typical \( \text{Ca}^{2+} \) currents in response to a series of depolarizing steps from a holding potential of \(-50 \text{ mV}\). Figure 6D compares the average current-voltage relationships of the L-type Ca\(^{2+}\) currents for mouse and human PLN ventricular cells. The average peak current density for human PLN cells was \(13.0 \pm 0.5 \text{ pA/pF}\), and this was 33% higher than that from mouse PLN cells \((-9.8 \pm 0.4 \text{ pA/pF}, P<0.001)\). Kinetic properties of the Ca\(^{2+}\) currents, including inactivation rates, steady-state inactivation, and recovery from inactivation at \(-60 \text{ mV}\), were similar in the 2 cell types (data not shown). The slow MHC isoform, which predominates in normal human hearts, was also significantly increased in the human PLN transgenic hearts compared with mouse PLN hearts (Figure 6E). Thus, the increases in both the sodium/calcium exchanger and the \(\beta\)-MHC, associated with human PLN expression, suggested a shift toward the “human heart” in the mouse.

**Cardiac Hypertrophy**

Our echocardiography studies above indicated an increase in calculated left ventricular mass (Table 1), and there was a significant increase in heart-to-body-weight ratios (Figure 7A) in human PLN compared with mouse PLN animals. The average myocyte membrane capacitance (Figure 7B) and cardiomyocyte cross-sectional area (123%) were also increased significantly by human PLN expression. Cardiac gross morphology and microscopic analysis by Masson trichrome staining and fluorescein-conjugated wheat germ agglutinin staining showed significant heart and myocyte hypertrophy, as well as fibrosis, in human PLN mice (Figures 7C and 7D). To determine the intracellular signaling pathways involved in cardiac remodeling, the activation status of the Ca\(^{2+}\)-dependent phosphatase, calcineurin (A\(\alpha\) or A\(\beta\)), calcium/calmodulin-dependent protein kinase II, mitogen-activated protein kinases, and Akt were investigated.\(^{18-20}\) There were no alterations in the levels or activity of Ca\(^{2+}\)-dependent phosphatase, calcineurin, and calcium/calmodulin-dependent protein kinase II (data not shown), whereas phospho-Akt was increased significantly without alteration in total Akt levels in human PLN hearts (Figure 7E). The levels of phospho-p38 and phospho-ERK1/2 were also significantly higher, whereas the corresponding total protein levels of p38 and ERK1/2 were not altered (Figures 7E and 7F). These results suggest at
least some of the potential molecular underpinning for the observed hypertrophy associated with human PLN expression.

Discussion

The present study shows that cardiac expression of PLN, which carries the human amino acid sequence (K27), is associated with superinhibition of contractility that leads to remodeling in the mouse heart. It has been suggested that the inhibitory effects of PLN involve amino acids in domain Ib or residues 21 to 30, which may be relatively unstructured. Domain Ib contains a single positive charge in the form of Arg25 in most species. However, Lys27 replaces Asn27 in humans, and this results in 2 positive charges, which may alter the secondary and tertiary structure of PLN, as well as its interaction with SERCA2. Indeed, NMR analysis of AA 1–36 indicated that the spatial conformation of human PLN is significantly different from the PLN of mouse or any other mammalian species. The major difference is the relative position of the N-terminal and C-terminal helices of the 2 peptides and the looser packing of the human peptide, which may influence the interaction between PLN and SERCA2. Indeed, the flexibility of residues 22 to 30 has been suggested to be critical in allowing PLN to adapt its conformation to several different targets (including SERCA2 and protein kinase A) by facilitating PLN recognition. Furthermore, Asn27 in PLN was reported to interact with Leu321 in SERCA, and mutation of any of these residues has significant effects on the PLN-SERCA affinity. Expression of human PLN in the mouse heart was associated with increased inhibition of SERCA2 Ca\textsuperscript{2+} affinity, depressed cardiomyocyte Ca\textsuperscript{2+} cycling, and impaired cardiac contractility in both unpaced and paced hearts. These inhibitory effects may also be attributed to increases in the concentration of the active PLN monomers, similar to previous observations with other superinhibitory monomeric PLN mutants. However, neither N27A-PLN mutant, which is the strongest superinhibitor of SERCA2a, nor another superinhibitory mutant, V49G-PLN, altered the monomer:pentamer ratio. Thus, increases in either monomeric PLN forms or its efficacy for SERCA2 may mediate the PLN inhibitory effects in vivo. Interestingly, even on maximal β-adrenergic receptor stimulation, human PLN remained substantially inhibitory compared with mouse PLN. This may be due to the depressed Ser16 and Thr17 phosphorylation of PLN in vivo, although both sites could be fully phosphorylated in vitro. These findings indicate that the degree and regulation of PLN phosphorylation in vivo are far more complicated than that in vitro.

The depressed contractility elicited by human PLN was associated with significant increases in L-type calcium chan-
nel current density, which may compensate for the depressed calcium cycling in these hearts. Furthermore, sodium/calcium exchanger (NCX) levels were increased, which provides another important attempt to normalize cardiac relaxation in the phase of increased inhibition of SR Ca$^{2+}$ transport function. However, the increased NCX expression may also contribute to the decreased SR calcium content and depressed contractility in human PLN transgenic mice. Interestingly, the degree of NCX upregulation (40%) suggested a shift in mouse cardiac Ca$^{2+}$ handling similar to that in the human heart. In mouse, Ca$^{2+}$ removal during relaxation is ≈90% SR-dependent, whereas it is only ≈70% SR-dependent in humans, with a greater role of the NCX.25 Substitution of mouse PLN with human PLN in the present study and the increased inhibition of SERCA2 could not be accommodated, which resulted in recruitment of compensatory mechanisms. This is because the mouse heart normally operates close to its theoretical maximum, and its SR Ca$^{2+}$ storage is nearly full at normal heart rates. However, humans, with heart rates ranging between 60 and 180 bpm, have a large cardiac reserve, and increased basal inhibition of SERCA2 by K27-PLN may be an important factor in the potential for gain in SR Ca$^{2+}$ content.26 Indeed, increases in frequency of cardiac stimulation resulted in relatively higher enhancement of contractile parameters in human PLN—expressing cardiomyocytes than in mouse PLN—expressing cardiomyocytes, consistent with previous studies, which showed that PLN plays a key role in frequency-dependent increases of myocyte contraction.27,28 Human PLN may be more inhibitory than mouse PLN at basal conditions, but it allows for a relatively greater degree of stimulation of contractile parameters.

The inhibiting effects of human PLN on SR Ca$^{2+}$ transport and cardiac function, associated with increased wall stress, led to hypertrophy as an initial compensatory response. Similarly, previous studies on N27A-, V49G-, L37A- and I40A-PLN mutations10,14,15 showed that severely depressed SR function resulted in cardiac hypertrophy. The molecular pathways linking decreased SR Ca$^{2+}$ handling to reprogramming of gene expression appeared to involve the activation of Akt, p38, and ERK1/2, consistent with recent reports that these pathways may underlie cardiac remodeling, as a response to initial alterations of calcium handling.29 Thus, compensatory mechanisms by Ca$^{2+}$ cycling, MHC isoform switches, and hypertrophic proteins may also contribute to the observed phenotype of the human PLN hearts.

An important limitation of the present study is the expression of K27-PLN, mimicking human PLN, in the mouse heart, which exhibits Ca$^{2+}$ cycling characteristics that differ from humans.25 Furthermore, the interaction of PLN with mouse SERCA2 may differ from that with human SERCA2, although there is 98.7% homology between these 2 enzymes. Nevertheless, the present study, coupled with the finding that a human “null PLN” mutant results in dilated cardiomyopathy and heart failure in the homozygous state, points to the paramount importance of PLN in the human heart. The increased inhibition by K27-PLN allows for a high cardiac reserve in response to “flight or fight” situations, given that heart rate and SR Ca$^{2+}$ cycling need to be increased by 2- to 3-fold in the human heart. Although mouse and human hearts differ in excitation-contraction coupling and the isoforms of contractile proteins, it is very important to pursue future studies in mouse hearts expressing the human PLN, because the present study indicated a significant functional effect by this PLN form and a shift in Ca$^{2+}$-handling characteristics to those seen in human hearts. Furthermore, the availability of a mouse model mimicking the function of human PLN may provide an effective platform in the design of therapeutic modalities aimed at disruption of PLN/SERCA2 interaction to benefit the compromised heart.

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Disclosures

None.

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Calcium cycling is the critical determinant of cardiac contraction/relaxation and is characteristically abnormal in failing hearts from human and experimental models. In fact, these experimental models have unambiguously demonstrated the potential for dysfunctional calcium cycling to cause heart failure and have suggested that the correction of calcium cycling abnormalities has therapeutic potential. Thus, there is intense interest in modulating phospholamban (PLN), the inhibitory protein for the sarcoplasmic reticular calcium pump (SERCA), in heart disease. Interestingly, the amino acid sequence of PLN is highly conserved, and although all other species contain asparagine at amino acid 27, human PLN is unique in containing lysine. To assess the functional significance of this single amino acid substitution, the endogenous form of PLN-containing lysine. To assess the functional significance of this single amino acid substitution, the endogenous form of PLN

**CLINICAL PERSPECTIVE**

Calcium cycling is the critical determinant of cardiac contraction/relaxation and is characteristically abnormal in failing hearts from human and experimental models. In fact, these experimental models have unambiguously demonstrated the potential for dysfunctional calcium cycling to cause heart failure and have suggested that the correction of calcium cycling abnormalities has therapeutic potential. Thus, there is intense interest in modulating phospholamban (PLN), the inhibitory protein for the sarcoplasmic reticular calcium pump (SERCA), in heart disease. Interestingly, the amino acid sequence of PLN is highly conserved, and although all other species contain asparagine at amino acid 27, human PLN is unique in containing lysine. To assess the functional significance of this single amino acid substitution, the endogenous form of PLN in the mouse heart was replaced with the human form. Expression of human PLN resulted in superinhibition of the sarcoplasmic reticulum calcium-transport system, with impairment of cardiomyocyte calcium kinetics and contractility. The chronic depression of basal function in mouse hearts resulted in cardiac remodeling, which led to hypertrophy. Our findings indicate that human PLN plays a greater inhibitory role in basal calcium cycling than the PLN form present in all other species, which may be critical in maintaining a high cardiac reserve in the human heart. However, when the relative levels increase or the activity of PLN increases, as is observed in human failing hearts, then the superinhibitory function of human PLN may become detrimental, resulting in impaired calcium handling. These observations demonstrate the importance of correcting PLN function in heart failure.
The Presence of Lys27 Instead of Asn27 in Human Phospholamban Promotes Sarcoplasmic Reticulum Ca\textsuperscript{2+}-ATPase Superinhibition and Cardiac Remodeling

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