Altered Calcium Handling Is Critically Involved in the Cardiotoxic Effects of Chronic \(\beta\)-Adrenergic Stimulation

Stefan Engelhardt, MD, PhD; Lutz Hein, MD; Vitaly Dyachenkov; Evangelia G. Kranias, PhD; Gerrit Isenberg, MD; Martin J. Lohse, MD

Background—Chronic adrenergic stimulation leads to cardiac hypertrophy and heart failure in experimental models and contributes to the progression of heart failure in humans. The pathways mediating the detrimental effects of chronic \(\beta\)-adrenergic stimulation are only partly understood. We investigated whether genetic modification of calcium handling through deletion of phospholamban in mice would affect the development of heart failure in mice with transgenic overexpression of the \(\beta_1\)-adrenergic receptor.

Methods and Results—We crossed \(\beta_1\)-adrenergic receptor transgenic (\(\beta_1\)TG) mice with mice homozygous for a targeted deletion of the phospholamban gene (\(\text{PLB}^{-/-}\)). Phospholamban ablation dramatically enhanced survival of \(\beta_1\)TG mice. The decrease of left ventricular contractility typically observed in \(\beta_1\)TG mice was reverted back to normal by phospholamban ablation. Cardiac hypertrophy and fibrosis were significantly inhibited in \(\beta_1\)TG/\(\text{PLB}^{-/-}\) mice compared with \(\beta_1\)TG mice, and the heart failure–specific gene expression pattern was normalized. Analysis of intracellular calcium transients revealed increased diastolic calcium levels and decreased rate constants of diastolic calcium decline in \(\beta_1\)TG mice. In \(\beta_1\)TG/\(\text{PLB}^{-/-}\) mice, diastolic calcium concentration was normal and rate constants of diastolic calcium decline were greater than in wild-type mice.

Conclusions—We conclude that modification of abnormal calcium handling in \(\beta_1\)TG mice through ablation of phospholamban resulted in a rescue of functional, morphological, and molecular characteristics of heart failure in \(\beta_1\)-adrenergic receptor–transgenic mice. These results imply altered calcium handling as critical for the detrimental effects of \(\beta_1\)-adrenergic signaling. (Circulation. 2004;109:1154-1160.)

Key Words: calcium ■ heart failure ■ sarcoplasmic reticulum ■ hypertrophy ■ heart failure

The incidence of heart failure has been constantly increasing during the past decades, and heart failure is among the most frequent causes of morbidity and mortality in the Western world. Most patients with heart failure show chronic activation of the sympathetic nervous system, and the resulting elevation of plasma norepinephrine concentration correlates with the severity of the disease.1 Mimicking chronic adrenergic stimulation in transgenic animal models results in progressive hypertrophy and heart failure.2 The importance of this dysregulation of the sympathetic nervous system has been additionally underlined by several large clinical trials demonstrating beneficial effects of \(\beta\)-adrenergic receptor antagonists.3 Thus, whereas chronic adrenergic stimulation initially serves to compensate for the functional impairment of cardiac function, it ultimately results in deterioration of cardiac structure and function. The mechanisms by which \(\beta\)-adrenergic receptor stimulation contributes to the progression of heart failure are still not clear. Several signaling pathways have been shown to be involved in adrenergically induced hypertrophy and heart failure. Many of these pathways are linked to cardiac sarcoplasmic reticulum (SR) \(\text{Ca}^{2+}\) cycling directly or indirectly.

In the present study, we tested whether alterations in SR \(\text{Ca}^{2+}\) handling are a critical step involved in the detrimental effects of chronic \(\beta\)-adrenergic stimulation. To investigate this hypothesis, we used a genetic strategy to enhance SR \(\text{Ca}^{2+}\) uptake and deleted the principal inhibitor of SR \(\text{Ca}^{2+}\) uptake, phospholamban, in \(\beta_1\)-adrenergic receptor (\(\beta_1\)-AR) transgenic (\(\beta_1\)TG) mice.

Methods

Transgenic Animals

The generation of transgenic mice overexpressing human \(\beta_1\)-AR under the control of the \(\alpha\)-myosin heavy chain promoter has been described previously.4 For this study, we crossed heterozygous \(\beta_1\)-TMice (line TG4) with mice carrying a homozygous deletion of the phospholamban gene (both maintained on the same genetic background, FVB/N). Heterozygous intercrosses for the phospholamban deletion and the \(\beta_1\)-AR transgene were then mated to...
heterozygous phospholamban knockout mice. From the resultant F2 generation, we followed a total of 17 β, TG+/−/PLB−/− (termed β1TG), 28 β, TG+/−/PLB−/− (termed β1TG/PLB−/−), 8 β, TG−/−/ PLB−/− (termed β1TG/PLB−/−), and 20 β, TG+/−/PLB−/− β1TG (wild-type controls [WT]) for survival analysis. Four to 7 mice per genotype (aged 12 months) were used for experiments determining cardiac function, gene expression, and morphology. All animals were housed under specified pathogen-free conditions. All animal experiments were approved by the responsible authorities (protocol No. 621-2531.01-10/98 and 28/01).

**Morphological Analysis**

Sections (4 μm) were stained with H&E and picric acid/Sirius red, essentially as described. For the determination of myocyte cross-sectional area, 50 individual cells per genotype from at least 3 different animals were analyzed morphometrically. Only nucleated cardiac myocytes from areas of transversely cut muscle fibers were included in the analysis. Quantification of left ventricular fibrosis was achieved by Sirius red staining followed by semiautomated image analysis, as described.

**Left Ventricular Catheterization**

Left ventricular catheterization was carried out via the right carotid artery, as previously described. Data analysis was carried out with Chart software (Chart 4.2, AD Instruments).

**Cells and Solutions**

After the aorta was cannulated in situ, the heart was retrogradely perfused and cells were enzymatically dissociated as described previously. Isolated ventricular myocytes were loaded with 7 μmol/L Indo-1 AM (Molecular Probes) at 37°C for 30 minutes. During the experiment, cells were continuously superfused by a physiological salt solution containing (in mmol/L) NaCl 150, KCl 5,4, CaCl2 1,8, MgCl2 1,2, glucose 10, and HEPES/NaOH 10 (pH 7,4, 37°C).

**[Ca2+] Measurements**

Cells were washed in Indo-1 free physiological salt solution for at least 20 minutes. Fluorescence (F) of Indo-1 was excited at 350 nm. Emitted F was collected through 20-nm bandpass filters at 400 and 490 nm with a pair of photomultipliers. Time-dependent F400 and F490 were filtered at 100 Hz, sampled at 3 kHz (CED1401 interface), and stored in a personal computer (customer written software). Cells were voltage clamped with 40-ns pulses from −45 to −15 mV at a rate of 1 Hz. After a 4-minute equilibration period, measurements started. Offline, 10 traces of stimulation were averaged, and [Ca2+] was calculated from the ratio of F400 to F490. Diastolic and peak systolic [Ca2+] were identified by computer algorithms. The ratio constant of the diastolic decay of [Ca2+] was obtained by nonlinear fit of [Ca2+] after the end of the ms pulse. The number of animals was ≥4 for each group, and the number of cells was ≥10 for each animal.

**RNase Protection Analysis of Atrial Natriuretic Factor and SERCA**

Rnase protection analysis was carried out as described. The 18S band intensities of total RNA isolated from WT and transgenic animals determined after denaturing agarose gel electrophoresis were essentially identical and were used to normalize the specific RNA levels. The size of the unprotected fragments was 60 nucleotides longer than that of the protected fragments, thus excluding the contribution of undigested probe to the signal.

**Statistical Analysis**

Average data are presented as mean±SEM. Statistical analysis was carried out using the Prism software package (GraphPad). ANOVA followed by Bonferroni’s test was used for comparisons unless indicated otherwise. Differences were considered significant when <0.05.

**Results**

**PLB Deletion Enhances Survival of β, AR Transgenic Mice**

To determine the role of phospholamban in the development of heart failure in mice with cardiac-specific overexpression of the β, AR, we generated 4 different transgenic lines by crossing of β, TG with mice homozygous for deletion of the phospholamban gene (PLB−/−). We followed a total of 73 mice for 12 months and monitored survival by Kaplan-Meier analysis (Figure 1). No deaths occurred in the WT group (n=20) and in PLB−/− mice (n=8). Survival of β, TG mice was significantly reduced, with 10 deaths during the study period. In contrast, none of the 28 β, TG/PLB−/− animals died during the study period (P<0.01 β, TG vs WT and β, TG/PLB−/− vs β1TG).

**Inhibition of Cardiac Hypertrophy, Fibrosis, and Pulmonary Congestion**

Morphological analysis indicated marked hypertrophy of left ventricular myocardium in β1TG mice compared with WT mice (Figure 2A). This increase in ventricular weight was completely abolished in β1TG/PLB−/− mice. Likewise, the ratio of heart weight to body weight of PLB−/− mice showed no signs of cardiac hypertrophy. During pathological examination, severe pulmonary congestion paired with pleural effusions became apparent in β1TG mice (Figure 2B, inset). In accordance with these findings, the lung weights of β1TG mice were more than doubled compared with WT mice (Figure 2B). However, on PLB ablation, the morphology and
lung weights of β1TG/PLB−/− mice were in the normal range and did not differ from WT animals.

The histological analysis of left ventricular myocardium revealed severely hypertrophic cardiac myocytes throughout the whole left ventricular myocardium of β1TG mice (Figure 2C). In addition, the cellular arrangement of cardiac myocytes was disturbed with large areas of cellular disarray. Nuclear morphology was highly abnormal, with numerous pleomorphic cardiomyocyte nuclei. Quantitative morphometrical analysis revealed marked cardiomyocyte hypertrophy in β1TG mice compared with WT mice (Figure 2E). In sharp contrast, cardiomyocyte hypertrophy was absent when phospholamban was deleted in β1TG/PLB−/− mice.

A hallmark of heart failure in β1TG mice is the development of profound interstitial fibrosis (Figure 2D). We stained left ventricular sections with picric acid/Sirius red and quantified the percentage of left ventricular fibrosis by semiautomated image analysis. β1TG mice showed an increase of left ventricular fibrosis to 19±0.5% of the myocardium compared with 3±0.5% in WT animals (Figure 2F). β1TG/PLB−/− mice showed significantly decreased left ventricular fibrosis (5±1%) compared with β1TG mice. Phospholamban ablation alone had no detectable impact on the formation of left ventricular fibrosis.

Rescue of Left Ventricular Function in β1TG Mice
Left ventricular function was determined in vivo by ventricular catheterization of anesthetized animals. Deletion of phospholamban in β1TG mice prevented the decrease of left ventricular systolic pressure observed in the surviving β1TG mice (Figure 3A). Phospholamban deletion also markedly improved left ventricular congestion, as evidenced by a normalization of left ventricular end-diastolic pressure (LVEDP) (Figure 3B). Furthermore, the decrease of left ventricular contractility observed in β1TG mice (Figure 3C) was completely inhibited by phospholamban ablation. This beneficial effect of phospholamban deletion was also observed for diastolic dysfunction in these mice (Figure 3D).

Figure 2. Inhibition of cardiac hypertrophy, fibrosis, and pulmonary congestion in β1TG mice through phospholamban ablation. A, β1TG mice displayed marked cardiac hypertrophy. Concomitant deletion of phospholamban (β1TG/PLB−/−) greatly reduced cardiac hypertrophy (P<0.05, n=7 to 11). Mice homozygous for the deletion of phospholamban did not develop cardiac hypertrophy (n=4). B, Inhibition of pulmonary congestion through phospholamban ablation. Inset, Thickening of the alveolar septa and rarification of free alveolar space were evident throughout the lungs of β1TG mice. In sharp contrast, the morphological structure remained normal in β1TG/PLB−/− mice. Bar=100 μm. C, Midventricular sections (4 μm) were stained with H&E. The myocardium of β1TG mice shows cardiomyocyte hypertrophy and cellular disarray. Bar=100 μm. D, Determination of left ventricular fibrosis by staining with Sirius red. E, Morphometric quantification of cardiomyocyte cross-sectional areas from left ventricular myocardium. Fifty cardiac myocytes per genotype from at least 3 different animals were analyzed. F, Quantification of left ventricular fibrosis by Sirius red staining and subsequent semiautomated image analysis. Data are from 2 representative left ventricular sections per animal from 3 different animals per group.
Again, β TG/PLB−/− mice did not show impairment of left ventricular relaxation compared with wild-type animals.

**Reversal of Heart Failure–Specific Changes in Cardiac Gene Expression**

We then assessed the influence of phospholamban ablation on cardiac gene expression by RNase protection analysis of mRNAs for atrial natriuretic factor (ANF) and the SR Ca2+-ATPase (SERCA). We found expression of ANF to be significantly enhanced in β TG mice (Figures 4A and 4B). The principal effector molecule of phospholamban, SERCA2A, was downregulated in β TG mice (Figures 4A and 4C), a finding observed in several models of heart failure and also in human heart failure. Deletion of phospholamban reversed this pathological gene expression pattern back to normal (Figure 4C).

**Rescue of Calcium Handling Defects**

To determine the potential mechanisms underlying the detrimental effects of chronic β-adrenergic stimulation on cardiac myocytes, we assessed intracellular calcium signaling. We isolated cardiac myocytes from mice at a very young age (8 weeks) to detect crucial defects occurring early in the development of the disease. At this age, β TG mice do not show any signs of cardiac impairment. Cells were loaded with the fluorescent Ca2+-indicator Indo-1, and we detected 2 major alterations of the intracellular calcium transient (Figure 5). First, β TG mice displayed significantly prolonged calcium transients compared with WT mice (Figure 5A). Analysis of the rate constant of diastolic calcium decline demonstrated a significant impairment of diastolic calcium decline (Figure 5B). Second, β TG mice displayed enhanced diastolic calcium levels, whereas peak systolic calcium was unaltered (Figures 5A, 5C, and 5D). Concomitant deletion of phospholamban resulted in a complete reversal of both abnormalities (Figures 5A through 5C), resulting in intracellular calcium transients decaying even faster than calcium transients from wild-type controls (Figures 5A and 5B). Diastolic calcium levels from β TG/PLB−/− mice were significantly decreased both versus β TG mice and versus WT mice (Figure 5C). As a result, the Ca2+ load, ie, the time average of systolic and diastolic [Ca2+]i, was higher in β TG than in WT mice. Again, ablation of phospholamban in β TG/PLB−/− mice

---

**Figure 3.** Rescue of the impairment of left ventricular function in β AR-transgenic mice. A 1.4F high-fidelity micromanometer was advanced through the right carotid artery into the left ventricle, and left ventricular pressure was determined (n=5 to 8 for all determinations, except n=4 for PLB−/− mice). A, A significant decrease of left ventricular systolic pressure was detectable in β TG mice. This decrease was completely abolished in β TG/PLB−/− mice. B, As a measure for backward failure, we assessed LVEDP. LVEDP was strongly enhanced in β TG mice. Again, this was rescued almost completely by phospholamban ablation. C, Maximum of the first derivative of the left ventricular pressure curve (dp/dt max) served as a measure for left ventricular systolic contractility. Depression of dp/dt max in β TG mice was reverted back to the normal range by phospholamban ablation. D, The minimum of the first derivative of the left ventricular pressure curve (dp/dt min) was used to assess diastolic function in these animals. The determination of dp/dt min revealed marked diastolic dysfunction in β AR transgenic mice, which was rescued in β TG/PLB−/− mice.

**Figure 4.** Reversal of heart failure–specific changes in cardiac gene expression. RNase protection analysis was used to assess the levels of the mRNAs for ANF and SR Ca2+-ATPase (SERCA2A). Total RNA 4 μg from left ventricular myocardium was used for hybridization with the respective radioactively labeled antisense probes. A, Representative autoradiograph of left ventricular RNA from 12-month-old mice. B, Quantitative analysis of mRNA levels for ANF and SERCA. n=4 to 5 for both experiments.
progressive myocyte hypertrophy, myocyte damage, and fibrosis and eventually heart failure. Inhibition of phospholamban via phosphorylation and thereby enhancement of SERCA function is one of the key effectors of this signaling pathway, and it might therefore be expected to be an integral part of the detrimental signaling cascade. We have previously shown phospholamban phosphorylation to be significantly enhanced in this mouse model. Accordingly, complete phospholamban inhibition through ablation of the gene might be expected to aggravate the phenotype of β1-AR transgenic mice. Surprisingly, histological analysis revealed that hypertrophy and interstitial fibrosis, indicators of tissue damage, were significantly decreased in β1-TG/PLB−/− mice compared with β1-TG animals. Isoproterenol-stimulated adenyl cyclase activity (data not shown) was preserved in β1-TG/PLB−/− mice compared with β1-TG mice, indicating that phospholamban ablation did not interfere with the β1-AR-mediated cAMP signal. Thus, neither the enhancement of cardiac contractility nor phosphorylation of phospholamban can be regarded as part of the toxic signal transduction cascade originating from the cardiac β1-AR. It appears more likely that additional signal transduction pathways are involved, possibly via enhanced Ca2+ entry into cardiac myocytes or enhanced release from internal stores, eg, through the ryanodine Ca2+ release channel. In accordance with the latter hypothesis, leakiness of the ryanodine Ca2+ release channel has been described in heart failure through PKA-mediated hyperphosphorylation.

**Calcium Signaling in Heart Failure**

Similar alterations in cardiomyocyte SR Ca2+ handling with slow rate constants and enhanced diastolic calcium concentrations have been identified in a variety of animal models as well as in human heart failure. Specifically, in the human disease, prolonged intracellular Ca2+ transients have been described and have been attributed to a decreased reuptake of diastolic calcium into the SR. The impairment of Ca2+ reuptake has been attributed to alterations in the function of several SR proteins, including a decreased ratio of the SERCA function through gene knockout of phospholamban has led to less clear results. Whereas phospholamban ablation markedly improved the phenotype of some heart failure models, it did not attenuate the phenotype of tropomodulin transgenic mice and mice with transgenic overexpression of Gqα and a mutant myosin-binding protein C. Thus, some forms of cardiomyopathy seem to be resistant to the therapeutic inhibition of phospholamban function, whereas others benefit from phospholamban inhibition. Additional complexity is suggested by 2 recent reports studying phospholamban mutations in humans. Although a premature stop-codon is described to be associated with cardiomyopathy in the homozygous state, Schmitt et al report that a heterozygous R9C mutation in phospholamban leads to local trapping of PKA, thereby minimizing PKA-

**Discussion**

The main result of the present study is that abnormal intracellular calcium handling underlies the detrimental effects of chronic β1-adrenergic signaling in the heart. Modification of the abnormal calcium transients through ablation of phospholamban protects from the detrimental functional, morphological, and molecular consequences of chronically enhanced β1-AR signaling in the heart.

**Enhancement of Cardiac Contractility Through Inhibition of Phospholamban Is Beneficial Rather Than Detrimental**

Acute enhancement of myocardial performance is regarded as the main effect of the cardiac β-adrenergic signaling pathway. However, chronic activation of the β adrenergic receptor–G–protein kinase A (PKA) axis in the heart leads to reduced the calcium load significantly (Figure 5E). Analysis of voltage-clamp data indicated no significant differences in the amplitude, rate of inactivation, and time of the L-type calcium currents (not shown), suggesting that the influx of extracellular Ca2+ was comparable between the 3 types of cells.

![Figure 5. Calcium signaling in isolated cardiac myocytes. A, Representative Ca2+ transients of adult ventricular myocytes. Each of the 3 panels indicates Ca2+ transients recorded with Indo-1 fluorescence. B, Rate constants of intracellular calcium decline (s−1). C, End-diastolic calcium concentrations. D, Average peak systolic calcium concentration and calcium load (E) (nmol/L, time integral over 10 seconds, divided by the sampling period of 10 seconds). Data are derived from ≥10 cells per animal with ≥4 animals per group.

![Figure 5.](image)
mediated inhibition of the remaining phospholamban allele. The disinfected WT allele becomes functionally dominant, and thereby SERCA function is decreased. Thus, the 2 studies found either complete absence or hyperactivity of phospholamban to be associated with the development of heart failure. At present it is not clear what might explain these differences and the divergent effects of phospholamban inhibition in different transgenic mouse models of heart failure. We observed downregulation of SERCA in βAR TG mice and a significant upregulation of SERCA in βAR TG/PLB–/– mice, which may contribute to the rescue of the detrimental effects of β-adrenergic stimulation. However, our results cannot prove a causal role for SERCA expression changes in this model, because phospholamban ablation may also improve cardiac function independent of changes in expression and also reverse remodeling indirectly through other pathways that may be altered in PLB-deficient hearts. Thus, in most heart failure models tested to date and in cardiac myocytes from failing human hearts, enhancement of SERCA function and inhibition of phospholamban exerted markedly beneficial effects. A direct comparison of these alternative concepts performed in a model of chronic heart failure is needed to decide which represents the most promising therapeutic principle to follow.

Thus, a paradigm evolves, with signaling pathways enhancing diastolic calcium being detrimental and strategies preventing calcium entry into the cell either directly (L-type Ca$^{2+}$ channel blockade$^{25,27}$) or indirectly (Na$^+$/H$^+$ exchange inhibition$^{29}$) being beneficial for the heart. Importantly, it is now clear that this occurs independently of hemodynamic unloading or negative inotropy. What might be the downstream mechanisms exerting the detrimental action of cytosolic calcium? During the past few years, a multitude of experimental results has refined our picture of how enhanced calcium levels might chronically harm cardiac myocytes.$^{28}$ Specifically, activation of calcineurin$^{29}$ and the calmodulin/calmodulin kinase pathway seem to be essentially involved in Ca$^{2+}$-mediated cardiomyocyte hypertrophy. Recently, the signaling cascade downstream of calmodulin kinase has been additionally explored in cardiac myocytes and has been shown to involve regulation of histone deacetylases,$^{30}$ potent inhibitors of cardiac gene transcription.

**Phospholamban Inhibition as Therapeutic Strategy to Remove the Cardiotoxic Effects of βAR Stimulation**

Heart failure is a complex clinical syndrome originating from a wide variety of different causes, including longstanding pressure overload, ischemic damage, and less common familial forms of cardiomyopathies. Because of this diversity of disease origins, effective therapeutic strategies focus on common pathophysiological mechanisms crucial for the progression of the disease. Among these mechanisms, chronic stimulation of the β-adrenergic receptor system is common in most patients with heart failure,$^3$ and blockade of this detrimental mechanism represents the most effective therapeutic principle to date.$^3$ However, treatment of hemodynamically compromised patients with heart failure is clinically challenging, because the negative inotropic effect of this therapy acutely impairs cardiac function and as a direct result excludes a significant number of patients from the application of this therapy. Our data suggest an alternative therapeutic strategy by inhibition of phospholamban, which rescues the detrimental effects of βAR signaling without the negative inotropic effects of β-blockade.

**Acknowledgments**

These studies were supported by grants from the Deutsche Forschungsgemeinschaft (SFB 355, SFB 598, and Leibniz award), the Fonds der Chemischen Industrie, and NIH HL26057, HL64018, and HL52318 (to D Kranias). The authors would like to thank Lydia Vlaskin for help with cross-breeding and genotyping of the animals. The excellent technical assistance of Ursula Keller is gratefully acknowledged.

**References**


Altered Calcium Handling Is Critically Involved in the Cardiotoxic Effects of Chronic β-
-Adrenergic Stimulation
Stefan Engelhardt, Lutz Hein, Vitaly Dyachenkow, Evangelia G. Kranias, Gerrit Isenberg and
Martin J. Lohse

Circulation. 2004;109:1154-1160; originally published online February 16, 2004;
doi: 10.1161/01.CIR.0000117254.68497.39
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circ.ahajournals.org/content/109/9/1154

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published
in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial
Office. Once the online version of the published article for which permission is being requested is located,
click Request Permissions in the middle column of the Web page under Services. Further information about
this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/