Enhancement of Cardiac Function and Suppression of Heart Failure Progression By Inhibition of Protein Phosphatase 1


Abstract—Abnormal calcium cycling, characteristic of experimental and human heart failure, is associated with impaired sarcoplasmic reticulum calcium uptake activity. This reflects decreases in the cAMP-pathway signaling and increases in type 1 phosphatase activity. The increased protein phosphatase 1 activity is partially due to dephosphorylation and inactivation of its inhibitor-1, promoting dephosphorylation of phospholamban and inhibition of the sarcoplasmic reticulum calcium-pump. Indeed, cardiac-specific expression of a constitutively active inhibitor-1 results in selective enhancement of phospholamban phosphorylation and augmented cardiac contractility at the cellular and intact animal levels. Furthermore, the β-adrenergic response is enhanced in the transgenic hearts compared with wild types. On aortic constriction, the hypercontractile cardiac function is maintained, hypertrophy is attenuated and there is no decompensation in the transgensics compared with wild-type controls. Notably, acute adenoviral gene delivery of the active inhibitor-1, completely restores function and partially reverses remodeling, including normalization of the hyperactivated p38, in the setting of pre-existing heart failure. Thus, the inhibitor 1 of the type 1 phosphatase may represent an attractive new therapeutic target. (Circ Res. 2005;96:756-766.)

Key Words: protein phosphatase 1 ■ protein phosphatase 1 inhibitor 1 ■ heart failure ■ hypertrophy ■ phospholamban ■ gene therapy

Reversible protein phosphorylation represents the cellular basis for integration of key signaling pathways, mediating a fine crosstalk between external effector molecules and intracellular events. In the heart, Ca\(^{2+}\) cycling and contractility are controlled by a fine balance of protein kinase and phosphatase activities in response to various second messenger signals. Demands on the heart’s pumping action, during fight-or-flight situations, can increase human cardiac output by nearly 5-fold. This is linked to β-adrenergic activation of the cAMP dependent protein kinase (PKA). PKA then phosphorylates a set of key regulatory Ca\(^{2+}\) handling proteins that control excitation-contraction coupling cycle, such as phospholamban, the ryanodine receptor, the L-type Ca\(^{2+}\) channel, and troponin I.1

The protein kinases and their phosphoprotein substrates underlying augmentation of the heart’s pumping action have been well characterized. However, similar studies on the protein phosphatasases, reversing the increased cardiac contractility, are less well developed. The major Ser/Thr phosphatases [type 1, type 2A, and type 2B (calcineurin)] stem from a common gene family and are highly homologous proteins (40% to 50%) that play critical roles in the control of cardiac contractility and hypertrophy.

Overexpression of the catalytic subunit of the protein phosphatase 1 at similar levels observed in human heart failure was associated with dephosphorylation of phospholamban, depressed cardiac function, dilated cardiomyopathy, and premature mortality.2 Furthermore, PP2A and PP2B (calcineurin) overexpression have been shown to result in decreased function and pathological hypertrophy.3,4

In human and experimental heart failure, the activity of the type 1 phosphatase, associated with the sarcoplasmic reticulum (SR) is significantly increased.5,6 It has been suggested that this increase may be a contributing factor to depressed function, dilated cardiomyopathy, and premature death.2 Some studies indicate that the increased SR-protein phosphatase 1 activity5 may be due to dephosphorylation of its inhibitor protein, inhibitor-1.2,7 Accordingly, expression of
the constitutively active inhibitor-1 in human failing cardiomyocytes increased contractility, indicating that this may represent a viable approach for enhancing the depressed function in heart failure. However, even though such inhibition of protein phosphatase 1, leading to enhanced PKA phosphorylation of calcium-handling proteins, may be initially beneficial, it may become detrimental in the long term. Specifically, phosphorylation of the ryanodine receptor may alter its function by changing its sensitivity to calcium. This would result in leaky channels, which may cause diastolic calcium increases and exacerbate contractile dysfunction. To address the potential benefits and limitations of chronic protein phosphatase 1 suppression, we used transgenenesis and gene-transfer of the constitutively active inhibitor-1. Our findings indicate that inhibition of the type 1 phosphatase is associated with enhanced cardiac function in the long term and may confer protection against heart failure propensity. Furthermore, acute gene transfer of active inhibitor-1 in failing hearts restores cardiac function and rescues left-ventricular contractility. The beneficial effects of inhibitor-1 are mediated, at least partially, through increased phospholamban phosphorylation. Thus, the inhibitor-1 of protein phosphatase 1 may hold promise as a therapeutic agent in heart failure.

Materials and Methods

Generation of Mice

A 5.6-kb transgene, consisting of the α-MHC promoter, the mouse I-T35D (AA1–65) cDNA, and the simian virus 40 polyadenylation site was used for pronuclear microinjection. The transgenic (TG) mice were generated at the University of Cincinnati and were handled as approved by the Institutional Animal Care and Use Committee.

Cardiac Function

In vivo cardiac function was assessed by noninvasive echocardiography, whereas in vitro contractility was examined using the Langendorff perfusion system. Cardiac catheterization and pressure-volume loop measurements in the murine heart were performed, as previously reported.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results

Expression of the Active Inhibitor-1 In Vivo Enhances Cardiac Function

To determine the long-term in vivo effects of decreased protein phosphatase 1 activity, we expressed a constitutively active, truncated inhibitor-1 (I1c) in a cardiomyocyte restricted manner. This form of inhibitor-1 was chosen because...
it specifically inhibits protein phosphatase 1, albeit at higher concentration than the native phosphorylated inhibitor.  

Three transgenic lines were obtained with similar levels of active inhibitor-1 expression. Echocardiographic assessment revealed enhanced basal contractility (see supplemental Table I, available in the online data supplement). Moreover, cardiac function was similarly increased at 6 months of age and longevity studies [19 wild type (WT) and 19 TG] indicated no evidence of sudden death, whereas Kaplan-Meier survival analysis up to 2 years of age revealed no significant differences in mortality rates. Subsequent studies were performed with mice from transgenic line C. Transgenic hearts exhibited a significant decrease (15%) in cardiac protein phosphatase 1 activity (Figure 1A) without changes in its protein level or protein phosphatase 2A activity. The apparent modest decrease in in vitro enzymatic activity may not be reflective of the in vivo activity associated with phospholamban.

Langendorff perfused hearts also indicated enhanced cardiac contractility. In active inhibitor-1–expressing hearts, the maximal left ventricular pressure was increased (23%) and the dP/dt and −dP/dt were augmented by 39% and 36%, relative to wild-type cohorts (Figure 1B). Accordingly, isolated calcium-tolerant cardiomyocytes exhibited increases (56%) in fractional shortening. Rates of myocyte shortening (−dL/dt) and relengthening (+dL/dt) were also enhanced over 2-fold by active inhibitor-1 expression (Figure 1C). The times to 50% peak and relaxation were significantly abbreviated. Furthermore, on maximal stimulation with isoproterenol (100 nmol/L) both dL/dt continued to be enhanced (Figure 1C). The alterations in mechanical parameters reflected similar enhancement in calcium cycling. The amplitude of calcium transients was increased, and the time to 50% decay of the Ca2+ signal (T50) was reduced by 37% (Figure 1D), suggesting enhanced SERCA2 function. Remarkably, even under isoproterenol stimulation, the active inhibitor-1 cardiomyocytes continued to exhibit an abbreviated T50 (Figure 1D), consistent with the enhanced phosphorylation of phospholamban compared with wild-type stimulated cells (supplemental Figure I). However, the amplitude of the calcium transient was not different from WTs (Figure 1D).

**Effect of Active Inhibitor-1 on Ca2+ Handling Proteins**

As described, β-adrenergic receptor–dependent protein phosphorylation of key regulatory phosphoproteins, such as phospholamban, the ryanodine receptor, troponin I, and the L-type calcium channel, constitutes a critical regulatory mechanism that governs Ca2+ cycling and cardiac contractility. Thus, we investigated the expression and phosphorylation levels of these key substrates in our transgenic model. There was no

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Calcium regulatory proteins and phosphoproteins in inhibitor-1 (I1c) hearts. A, Immunoblots (left) revealed that SERCA2, phospholamban (PLN), calsequestrin (CSQ), dihydropyridine receptor (DHPR), and troponin I (TnI) were not altered, although the ryanodine receptor (RYR2) level was decreased (*P<0.05, n=5 each). Phosphorylation of phospholamban (*P<0.05, n=10 each) was increased, whereas ryanodine receptor and troponin I phosphorylation was not altered (*P<0.05, n=4 each). B, Left, Cardiomyocyte current-voltage relationship was not different between WTs and transgenics; Right, Calcium-dependent inactivation kinetics of the L-type Ca2+ channel were accelerated in inhibitor-1 myocytes. *P<0.05, n=5 hearts per group and at least 25 cardiomyocytes per group.
significant difference in β-adrenergic receptor density (data not shown). However, phosphorylation of phospholamban at both its cAMP-dependent (Ser16) and Ca\(^{2+}/\)calmodulin-dependent (Thr17) protein kinase sites was increased significantly compared with wild types (Figure 2A), consistent with previous reports that protein phosphatase 1 is the predominant phospholamban phosphatase.\(^{11}\) Interestingly, the cardiac ryanodine receptor protein levels were decreased by 26%, whereas the relative (mol Pi/mol RyR2) phosphorylation of this channel was not altered (Figure 2A). In addition, the phosphorylation level of troponin I was not different between WT and transgenics (Figure 2A).

There were no alterations in L-type Ca\(^{2+}\) channel protein levels, and examination of the channel activity revealed that the mean peak Ca\(^{2+}\) current (\(I_{\text{Ca}}\)) and the steady-state inactivation of the current-voltage relationship (\(I-V\)) were similar between active inhibitor-1 and WT myocytes. However, inactivation of \(I_{\text{Ca}}\) was faster in the transgenics (Figure 2B), similar to previous observations in the hyperdynamic phospholamban knock-out myocytes.\(^{12}\)

Importantly, we investigated glycogen metabolism and observed no significant difference in glycogen synthase and glycogen phosphorylase activities, between the WTs and transgenics.

### Active Inhibitor-1 Attenuates Functional and Morphological Deterioration on Chronic Pressure-Overload

To examine the hypothesis that the active inhibitor-1 expression, associated with enhanced Ca\(^{2+}\) cycling, may be protective against cardiac remodeling induced by hemodynamic stress, we subjected the transgenic and isogenic wild-type mice to banding of the transverse aorta. Function was evaluated by echocardiography and cardiac catheterization. Echocardiography revealed that banded transgenic mice did not exhibit the pathological increases in left-ventricular end-diastolic and end-systolic dimensions, observed in banded wild-types (Figure 3A; supplemental Table I). The h/r ratio was also markedly increased in the banded inhibitor-1 mice (Figure 3B), consistent with a more compensated hypertrophy and reduced wall-stress, as determined by La Place’s law.

---

**Figure 3.** Inhibitor-1 expression attenuates functional and morphological deterioration on chronic pressure overload. A, Echocardiography at 6 weeks after banding indicated that inhibitor-1 hearts (11c) had less left ventricular luminal dilatation (reduced LV end-diastolic, LVEDD, and end-systolic dimensions, LVESD) than WT cohorts (\(P < 0.05, n > 12\) mice per group). B, Inhibitor-1 mice also had an increased h/r ratio and a reduced HW:BW-ratio relative to WTs (\(P < 0.05\) vs sham, \(\ast P < 0.05\) between WT and 11Cc banded hearts; \(n > 10\) per group). C, HW:BW and \(+dP/dt\) ratio plotted vs the transaortic gradients reveal that inhibitor-1 transgenic mice exhibit less hyper trophy and higher contractility at a given gradient than wild-type mice (\(\ast 11c\ TAC\) and \(\square\) WT TAC). D, Left, Banded WT exhibited substantially increased lung-weight (congestion) than banded 11 mice (\(n = 12\)). \(\ast P < 0.05\) vs WT-Sham (\(n = 9\)) and \(\ast P < 0.05\) between WT-TAC (\(n = 10\)) and 11c TAC (\(n = 12\)). Right, Lung weight plotted against the transaortic gradient reveals that overall banded WT mice progressed to a greater frequency and severity of lung congestion under similar hemodynamic loads.
Although transaortic gradients were similar between the two groups (WT: 55.3 ± 3.68; TG: 53.9 ± 4.64, mm Hg), banded transgenic hearts exhibited less hypertrophy than wild types (Figure 3B), even under similar pressure gradients (Figure 3C). Furthermore, the rate of pressure development of banded inhibitor-1 mice was superior to that of banded wild types at similar pressure gradients (Figure 3C). Examination of lung weights also revealed significant increases in banded wild types, whereas banded transgenics were not different from sham-operated animals. Importantly, the alterations in lung weight were different at similar pressure gradients (Figure 3D).

Figure 4. In vivo physiological assessment of cardiac function and pressure-volume relationships. A, Cardiac catheterization was performed and functional capacity was calculated under baseline conditions and dobutamine stimulation (16 ng/g BW per min). Inhibitor-1 transgenic mice exhibited enhanced contractility (+dP/dt) under basal conditions and under dobutamine stimulation (*P < 0.05 vs WT-Sham). On coarctation, WT mice exhibited a decrease in both +dP/dt and –dP/dt (*P < 0.05) and a reduced dobutamine response (+dP/dt), relative to WT-Shams. However, banded I1c transgenics displayed enhanced function relative banded WTs (†P < 0.05), under both baseline and dobutamine treatment. B, Examination of the end-diastolic volumes also indicated that the WT-banded mice underwent left ventricular lumenal dilatation, on chronic aortic constriction, whereas I1c transgenic mice did not (WT-Sham, n = 8; I1c-Sham, n = 9; WT-TAC, n = 4; I1c-TAC, n = 6; †P < 0.05 vs WT-Sham; ††P < 0.05 vs WT-TAC). C, Representative examples of left ventricular pressure vs left ventricular volume loops.
Aortic constriction was also associated with a functional decline in banded wild types, whereas function was fully preserved in the inhibitor-1 mice, compared with their sham-operated cohorts (Figure 4A). In addition, the dobutamine response of the contraction rate ($\text{dP/dt}$) was blunted in banded WTs, whereas this response was preserved in the banded inhibitor-1 group. Similarly, the end-diastolic volume (EDV) was only increased in the banded WTs, whereas it was not altered in the banded transgenics (Figure 4B). Representative occlusion analysis pressure-volume loops from these groups are shown in Figure 4C. The slope of the end-systolic pressure-volume relationship (ESPVR) was similar between WTs and transgenics under baseline conditions. However, on pressure-overload, this variable was significantly greater only in the banded transgenics (WT sham: $4.1 \pm 1.1$; WT banded: $7.4 \pm 2.0$, mm Hg/µL; TG sham: $4.5 \pm 0.5$; TG banded: $12.2 \pm 3.6$, mm Hg/µL). Similar results were observed for preload-recruitable stroke work and time-varying maximal elastance ($E_{\text{max}}$). Interestingly, peripheral perfusion pressure was well-preserved in the inhibitor-1 banded mice (I1 sham: $108.6 \pm 6.9$; I1 banded: $98.5 \pm 21.9$, mm Hg; $P=0.30$), whereas it significantly declined in banded WTs (WT sham: $82.5 \pm 6.9$; WT banded: $31.4 \pm 2.0$, mm Hg), further supporting the well compensated stage of hypertrophy in the transgenics.

Further examination of the hearts revealed morphological enlargement and increased interstitial fibrosis in banded WTs relative to their transgenic counterparts (Figure 5A). Wheat

Aortic constriction was also associated with a functional decline in banded wild types, whereas function was fully preserved in the inhibitor-1 mice, compared with their sham-operated cohorts (Figure 4A). In addition, the dobutamine response of the contraction rate ($\text{dP/dt}$) was blunted in banded WTs, whereas this response was preserved in the banded inhibitor-1 group. Similarly, the end-diastolic volume (EDV) was only increased in the banded WTs, whereas it was not altered in the banded transgenics (Figure 4B). Representative occlusion analysis pressure-volume loops from these groups are shown in Figure 4C. The slope of the end-systolic pressure-volume relationship (ESPVR) was similar between WTs and transgenics under baseline conditions. However, on pressure-overload, this variable was significantly greater only in the banded transgenics (WT sham: $4.1 \pm 1.1$; WT banded: $7.4 \pm 2.0$, mm Hg/µL; TG sham: $4.5 \pm 0.5$; TG banded: $12.2 \pm 3.6$, mm Hg/µL). Similar results were observed for preload-recruitable stroke work and time-varying maximal elastance ($E_{\text{max}}$). Interestingly, peripheral perfusion pressure was well-preserved in the inhibitor-1 banded mice (I1 sham: $108.6 \pm 6.9$; I1 banded: $98.5 \pm 21.9$, mm Hg; $P=0.30$), whereas it significantly declined in banded WTs (WT sham: $82.5 \pm 6.9$; WT banded: $31.4 \pm 2.0$, mm Hg), further supporting the well compensated stage of hypertrophy in the transgenics.

Further examination of the hearts revealed morphological enlargement and increased interstitial fibrosis in banded WTs relative to their transgenic counterparts (Figure 5A). Wheat
germ agglutinin staining indicated that the cardiomyocyte cross sectional area in banded WTs was substantially increased, relative to banded transgenics (Figure 5B). Given the antihypertrophic effects of inhibitor-1, we examined the MAP-kinase hypertrophic pathways. There was a significant decrease in ERK1/2 activation in the banded transgenics compared with their WT cohorts, but the activation of p38 was not different (Figure 5C). Further examination of other pathways (PKC-α/H9251 and CREB) revealed no alterations. However, expression levels of ANF and β-MHC were significantly increased in the banded WTs, although these genes were not elevated in banded inhibitor-1 hearts.

The protective effects of inhibitor-1 were not associated with any alterations in the levels of phospholamban, SERCA, and calsequestrin but the phosphorylation of phospholamban at Ser16 was markedly increased (Figure 5D), under baseline control conditions as well as posttransverse aortic constriction. There were no differences in the phosphorylation of phospholamban at Thr17 or the ryanodine receptor at Ser2809.

**Active Inhibitor-1 Expression Rescues a Rat Model of Cardiac Pressure-Overload Hypertrophy in Transition to Failure**

Because the studies in our transgenic model suggested that chronic expression of active inhibitor-1 may attenuate hypertrophy and functional deterioration, we investigated whether short-term expression of this inhibitor-1 could improve he-
modynamics in the setting of preexisting heart failure. Thus, we used a rat model of pressure overload, which exhibits characteristics of heart failure by 22 weeks after banding. When decreases of more than 25% in left ventricular fractional shortening were observed, gene transfer was performed. Delivery of active inhibitor-1 or the reporter gene (GFP) induced an expression pattern that was grossly homogeneous throughout the ventricles in failing and nonfailing hearts at one week after gene transfer. The expression level of the active inhibitor-1 was also confirmed by immunoblotting (Figure 6A) and biochemical assays indicated a decrease in type 1 phosphatase activity with no effect on type 2A activity (Figure 6B).

Remarkably, gene transfer of the active inhibitor-1 restored the rate of pressure rise (+dP/dt) to non-failing levels (Figure 6C), whereas left ventricular function was decreased in the failing control group (Figure 6C). Diastolic parameters were also normalized, as evidenced by restoration of the maximal rate of decline of left ventricular systolic pressure (−dP/dt) and the time course for pressure decline, measured by τ (τ), the isovolumic relaxation constant (Figure 6D).

To further define ventricular function in a load-independent fashion, pressure-volume analysis was performed (Figure 6E). The maximal slope of the end-systolic pressure dimension relationship (Emax, or Maximal Elastance) was lower in failing hearts, infected with control virus (Ad.GFP), compared with nonfailing hearts, indicating a diminished state of intrinsic myocardial contractility. Importantly, the expression of the active inhibitor-1 completely restored the maximal elastance to nonfailing levels (Figure 6F).

Biochemical characterization revealed that the SERCA2a levels were significantly decreased in the failing hearts, consistent with previous reports, and they remained depressed on control or active inhibitor-1 gene transfer. The levels of phospholamban or the ryanodine receptor were not different (Figure 7A). Phosphorylation of phospholamban at serine 16 was significantly depressed in failing hearts, but adenoviral gene transfer of the active inhibitor-1 significantly increased it (Figure 7B). Interestingly, both failing groups infected with either control or active inhibitor-1 virus, exhibited increases in Thr-17 phosphorylation of phospholamban (Figure 7B), consistent with increased CAM-kinase activity (see online data supplement). The phosphorylation level of the ryanodine receptor at serine 2809 was also increased in the failing groups, and infection with the active inhibitor-1 had no effect (Figure 7B).

Examination of the effects of active inhibitor-1 on MAP-kinase activation indicated a complete reversal of the overactivated p38-MAP kinase, with no alteration in ERK or JNK activation (Figure 7C). Thus, the enhanced contractility and relaxation, associated with active inhibitor-1 gene transfer, may decrease wall tension leading to normalization of the p38 MAP-kinase.

Discussion

In the present study, we found that chronic increases in the activation of the protein phosphatase 1 inhibitor-1 are associated with enhanced cardiac contractility and may be protective against the development of cardiac hypertrophy and deterioration of function in the face of increased hemody-
namic load. Importantly, acute increases in active inhibitor-1 protein also results in improved function and attenuated remodeling in the setting of preexisting heart failure. To our knowledge, this is the first report demonstrating the efficacy of selective therapeutic protein phosphatase 1 targeting by its inhibitor-1 in the settings of hypertrophy, heart failure, and the transition to heart failure. The beneficial effects of inhibitor-1 may be associated with increases in PLN phosphorylation, because the phosphorylation status of other key phosphoproteins, RyR and troponin I, were not affected. Such unique preference of inhibitor-1 may be important from a therapeutic point of view because inhibition of PLN, and the subsequent enhancement of SR Ca\(^{2+}\) ATPase activity, has been suggested to be beneficial in experimental and genetic heart failure.\(^{14,15}\)

In failing hearts, the downregulation of \(\beta\)-adrenergic receptors and decreased cAMP-dependent protein kinase activity results in decreased phosphorylation (Threonine 35) and inactivation of inhibitor-1, leading to increased protein phosphatase 1 activity\(^{2,3}\) and dephosphorylation of key phosphoproteins, including phospholamban.\(^{16,17}\) Interestingly, hypophosphorylation of inhibitor-1 in heart failure may also reflect enhanced activity of calcineurin (PP2B).\(^{18}\) This fine cross talk between cAMP and Ca\(^{2+}\) at the level of inhibitor 1 has been recently shown to be complemented by PKC-\(\alpha\), a calcium-dependent PKC isoform, which phosphorylates the inhibitor-1 at a different site, Ser67, and reduces its activity.\(^{19}\) Indeed, ablation of PKC-\(\alpha\) is associated with decreased phosphatase activity and enhanced function.\(^{19}\) Thus, inhibitor 1 appears to be an integrator of multiple neurohormonal pathways, associated with regulation of cardiac function and hypertrophy.

**Active Inhibitor-1 Enhances Basal Cardiac Contractility**

Inhibitor-1 has been shown to regulate synaptic mechanisms involved in learning and memory,\(^{20,21}\) as well as the adrenergic and cholinergic pathways in the heart.\(^{22}\) Although this phosphoprotein has long been known to play a major regulatory role in neuronal tissue,\(^{20,23}\) its significance in the heart has only recently been emerging. Previous studies indicated that genetic ablation of inhibitor-1 resulted in depressed cardiac contractility,\(^2\) whereas adenosine infection of rat cardiomyocytes with inhibitor-1 enhanced cardiac contractility.\(^{24}\)

Expression of the active inhibitor-1 in the transgenic mouse heart appeared to specifically increase phosphorylation of phospholamban at Ser16 and Thr17, without altering the phosphorylation levels of the ryanodine receptor and troponin I. This finding was unexpected because both the ryanodine receptor and troponin I are substrates for protein kinase A, similar to phospholamban. Increases in troponin I phosphorylation would be expected to decrease myofilament sensitivity to Ca\(^{2+}\), whereas increases in ryanodine receptor phosphorylation would cause SR Ca\(^{2+}\) leakage and arrhythmias,\(^8\) both counteractive to the beneficial effects of the active inhibitor observed in this study. Importantly, there were no sudden deaths linked to arrhythmias in the inhibitor-1 mice. However, the protein levels of the ryanodine receptor were decreased in the transgenic hearts with enhanced contractility, similar to our previous findings in the phospholamban-knockout hyperdynamic hearts.\(^{25}\) Thus, downregulation of the ryanodine receptor may represent an important compensatory response to maintain calcium homeostasis in models where SR Ca\(^{2+}\)-cycling and SR Ca\(^{2+}\)-load are increased.

It has been suggested that besides protein phosphatase 1, other phosphatases, such as protein phosphatase 2A, also regulate cardiac function.\(^9\) However, the activity of this phosphatase does not appear to change in vivo on isoproterenol stimulation.\(^{22}\) Importantly, the type 2A phosphatase activity was unaffected in the inhibitor-1 transgenic hearts. The apparent specificity of the active inhibitor-1 for the phospholamban-phosphatase may involve its specific anchoring subunit and the relative affinity between the catalytic and binding subunits of this enzyme in the various macromolecular complexes.\(^{26}\) Future phosphoproteomic studies may reveal novel, yet unknown, substrates for protein phosphatase 1/inhibitor-1, which also participate in the augmentation of cardiac contractility in this model.\(^{25}\)

**Inhibitor-1 Prevents Cardiac Dysfunction and Delays Hypertrophy**

To assess whether chronic expression of active inhibitor-1 and associated depressed protein phosphatase 1 activity may be protective against pathological progression of cardiac hypertrophy, the transgenic mice underwent transverse aortic constriction, along with their isogenic wild-type cohorts. Active inhibitor-1 expression was protective against functional deterioration and the geometric alterations leading to cardiac dilatation. The augmentation of phospholamban phosphorylation at Ser16 persisted in the active inhibitor-1 expressing hearts subjected to long-term aortic constriction, indicating enhanced SR Ca\(^{2+}\)-cycling. Interestingly, the banded transgenic mice also exhibited decreased cardiac hypertrophy and decreased activation of the ERK1,2 MAP-kinase pathway. Regardless, our findings indicate a pivotal role of inhibitor-1 in protection against the stress of pressure overload on the heart.

**Acute Gene Transfer of Active Inhibitor-1 Restores Function and Remodeling in the Failing Heart**

The intricate balance of protein kinase and phosphatase activities is shifted in favor of the phosphatases in heart failure due to decreased PKA activation and increased protein phosphatase activity.\(^{5,27}\) To assess whether inhibition of this phosphatase by the active inhibitor-1 may have therapeutic effects in established heart-failure, we used a catheter-based adenoviral delivery technique to transfer this molecule into pressure overload–induced failing rat hearts. Gene transfer resulted in relatively low expression levels of the active inhibitor-1 protein. However, because protein phosphatase 1 activity is chronically increased in heart failure, expression of the active inhibitor-1 resulted in substantial inhibition of this enzyme. This translated into increases in systolic pressure and the maximal rates of pressure development, as well as a
decrease in the time constant of isovolumic relaxation, which indicates enhanced active relaxation. Furthermore, the maximal elastance of the failing rat hearts, treated with the constitutively active inhibitor-1, was restored to normal, indicating complete rescue of intrinsic contractility and contractile reserve.

At the SR level, SERCA2 protein decreased significantly in failing-hearts, consistent with previous studies. There were no differences in phospholamban or ryanodine receptor levels, but phospholamban was hypophosphorylated at Ser16 and hyperphosphorylated at Thr17, consistent with the increased CaM-Kinase activity in these hearts. Importantly, the phosphorylation of the ryanodine receptor was increased in the failing hearts, further confirming that there may be compartmentalization of protein kinase/phosphatase signaling in the SR.

Gene transfer of the active inhibitor-1 was associated with improved function and enhanced phosphorylation of phospholamban. There were no differences in ryanodine receptor phosphorylation levels. These paradoxical findings further exemplify the complex interplay between kinases and phosphatases in the microenvironment of each phosphoprotein. The enhanced phospholamban phosphorylation, leading to enhanced SR calcium-transport and reduced diastolic calcium levels, was probably the predominant mechanism underlying improved contractility in failing hearts in the absence of reversal of other biochemical changes characteristic of heart failure. Similar findings were observed by direct phospholamban inhibition in various experimental models of heart failure. These studies are also consistent with our previous work, showing that cardiac overexpression of protein phosphatase 1 decreased phospholamban phosphorylation and resulted in dilated cardiomyopathy and heart failure. Thus, the active inhibitor-1 may offer a novel approach to interfere with the phospholamban/SERCA2a interaction, independent of the yet unsuccessful strategy of targeting the highly hydrophobic phospholamban by small molecules or antibodies.

Importantly, the active inhibitor-1 gene transfer resulted in partial rescue of the cardiac geometric alterations, associated with heart failure, and complete normalization of the hypertrophic phenotype. However, future studies should address the long-term effects of active inhibitor-1 on cardiac function, molecular pathways, and survival.

Limitations of This Study

The present study along with a number of other recent ex vivo and in vivo reports indicate that SR Ca\textsuperscript{2+}-transport may represent a nodal point in the progression of compensatory hypertrophy and heart failure. Accordingly, restoring the depressed SR Ca\textsuperscript{2+}-cycling through increased SERCA2a expression or decreased activity of PLN has been shown to benefit experimental and genetic models of heart failure. However, concerns have been raised by recent genetic complementation studies with the phospholamban-null mouse. Although some genetic models of heart failure were rescued, others were not affected by phospholamban deficiency. Given that each genetic model carries a large number of secondary alterations, as compensatory responses to the insult by the genetic manipulation, the results from such complementation studies must be viewed with caution. Furthermore, phospholamban ablation did not appear to benefit cardiac function on sustained aortic constriction. However, the transaortic gradients were markedly greater in the knockout relative to WT mice, making direct comparisons difficult.

Nevertheless, the recent findings on human phospholamban mutants, associated with phospholamban ablation, raised additional concerns and challenges on the role of phospholamban and augmented SR Ca\textsuperscript{2+}-cycling in the human heart. While the mechanism associated with the apparently null human phospholamban mutant is currently being explored, parallel studies in higher mammalian species may provide a better understanding of the potential benefit of restoring SR Ca\textsuperscript{2+}-transport in the human failing heart.

Conclusion

The elegant balance between protein kinase and protein phosphatase activities, regulating cardiac function, shifts in favor of the protein phosphatases in heart failure. The present findings demonstrate that partial restoration of this balance through inhibitor-1 may represent a potential therapeutic intervention. Furthermore, chronic increases in this inhibitor-1 activity may enhance basal cardiac contractility and β-adrenergic responses, as well as protect the heart against pressure overload–induced hypertrophy. Unlike agents that increase cAMP, thereby increasing intracellular Ca\textsuperscript{2+}, expression of the active inhibitor-1 improves cardiac function by targeting downstream substrates and by specifically enhancing SR Ca\textsuperscript{2+} cycling.

Acknowledgments

This work was supported in part by grants from the National Institutes of Health: HL26057, HL64018, and HL52318 (E.G.K.), HL 57623 (R.I.H.), DK36569 (A.A.D.-R.), HL07382–27 (A.P.), and the American Heart Association Predoctoral Fellowship 0215138B (A.P.).

References

elements define the specificity of recombinant human inhibitor-1 as a
associated with cardiac sarcoplasmic reticulum. J Biol Chem. 1986;261:
10029–10032.
deficiency alters inactivation kinetics of L-type Ca2+ channels in mouse
7. del Monte F, Williams E, Lebecche D, Schmidt U, Rosenzweig A, 
Gwathmey JK, Lewandowski ED, Hajjar RJ. Improvement in survival and
cardiac metabolism after gene transfer of sarcoplasmic reticulum Ca2+-ATPase in a rat model of heart failure. Circulation. 2000;104:
1424–1429.
6. della Monte F, Harding SE, Schmidt U, Matsui T, Kang ZB, Dec GW,
Gwathmey JK, Rosenzweig A, Hajjar RJ. Restoration of contractile
function in isolated cardiomyocytes from failing human hearts by gene
5. Minamisawa S, Hoshijima M, Chu G, Ferguson DG, Edes I, Kiss E, Sato Y, 
Kranias EG, Yatani A. Phospholamban-sarcoplasmic reticulum calcium ATPase interaction is the critical calcium cycling defect in dilated cardiomyopathy. Cell. 1999;
99:313–322.
4. Schwinger RH, Bolck B, Munch G, Brixius K, Muller-Ehmsen J, 
Erdmann E. cAMP-dependent protein kinase A-stimulated sarcoplasmic
reticulum type 1 protein phosphatase in cardiac muscle. J Biol Chem.
2002;277:25596–25604.
3. Dash R, Frank KF, Carr AN, Moravec CS, Kranias EG. Gender
TF, Lorenz JN, Nairn AC, Liggett SB, Bodi I, Wang S, Schwartz A, 
Lakatta EG, DePaoli-Roach AA, Robbins J, Hewett TE, Bibb JA, 
Westfall MV, Kranias EG, Moltenkorn JD. PKC-alpha regulates cardiac
contractility and propensity toward heart failure. Nat Med. 2004;10:
248–254.
0. Genoux D, Haditsch U, Knobloch M, Michalson A, Storm D, Mansuy JM. 
Protein phosphatase 1 is a molecular constraint on learning and memory.
-1. Protein phosphatase-1 regulation in the induction of long-term poten-
tiation: heterogeneous molecular mechanisms. J Neurosci. 2000;20:
3537–3543.
-2. Ahmad Z, Green FJ, Subahi HS, Watanabe AM. Autonomic regulation of
phosphatase in cardiace muscle. J Biol Chem. 1989;264:
3859–3863.
-3. Nairn AC, Palfrey HC. Identification of the major Mr 100,000 substrate
for calmodulin-dependent protein kinase III in mammalian cells as elon-
-4. El-Armouche A, Rau T, Zolk O, Dietz D, Pammeninger T, Zimmermann
WH, Jackel E, Harding SE, Boknik P, Neumann J, Eschenhagen T. 
Evidence for protein phosphatase inhibitor-1 playing an amplifier role in
beta-adrenergic signaling in cardiac myocytes. FASEB J. 2003;17:
437–439.
-5. Chu G, Ferguson DG, Edes I, Kiss E, Sato Y, Kranias EG. Phospho-
lamban ablation and compensatory responses in the mammalian heart.
-6. DePaoli-Roach AA. Protein Phosphatase 1 Binding Proteins. In: 
Lurie K, Billingham ME, Harrison DC, Sinson EB. Decreased catechol-
-8. Hoshijima M, Ikeda Y, Iwanaga Y, Minamisawa S, Date MO, Gu Y, 
Chronic suppression of heart-failure progression by a pseudophosphory-
lated mutant of phospholamban via in vivo cardiac rAAV gene delivery. 
Grossman W, Morgan JP. Abnormal intracellular calcium handling in
myocardium from patients with end-stage heart failure. Circ Res. 1987;
61:70–76.
-10. Gwathmey JK, Morgan JP. Altered calcium handling in experimental
-11. della Monte F, Harding SE, Dec GW, Gwathmey JK, Hajjar RJ. Targeting
-12. Song Q, Schmidt AG, Hahn HS, Carr AN, Frank B, Pater L, Gerst M, 
Young K, Hoth BD, McConnell BK, Haghigi K, Seidman CE, Seidman 
JG, Dorn GW II, Kranias EG. Rescue of cardiomyocyte dysfunction by
phospholamban ablation does not prevent ventricular failure in genetic
Kranias EG. Hypertrophy and functional alterations in hyperdynamic
phospholamban-knockout mouse hearts under chronic aortic stenosis. 
Fan GC, Tsiapras D, Hahn HS, Adamopoulos S, Liggett SB, Dorn GW II, 
MacLennan DH, Kremastinos DT, Kranias EG. Human phospholamban
null results in lethal dilated cardiomyopathy revealing a critical difference
Enhancement of Cardiac Function and Suppression of Heart Failure Progression By Inhibition of Protein Phosphatase 1


Circ Res. 2005;96:756-766; originally published online March 3, 2005;
doi: 10.1161/01.RES.0000161256.85833.fa

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/96/7/756

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2005/06/06/01.RES.0000161256.85833.fa.DC1
http://circres.ahajournals.org/content/suppl/2005/03/20/01.RES.0000161256.85833.fav1.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research 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 Research is online at:
http://circres.ahajournals.org//subscriptions/
A. SUPPLEMENTAL MATERIALS

Protein Phosphatase Activity

Protein phosphatase 1 activity was examined using $^{32}$P-labeled glycogen phosphorylase $a$ as a substrate in the presence of 4 nM okadaic acid, a concentrations that selectively inhibits phosphatase type 2A, and EDTA (0.5 mM), an inhibitor of phosphatase 2B. The assays were conducted under conditions where no more than 15% of the substrate was utilized to assure linearity of the reaction. Total phosphatase, type 1 and type 2A phosphatase activities were also measured, using myelin basic protein (NEB-P07080S), as the substrate in the presence and absence of 4 nM okadaic acid.

Quantitative Immunoblotting

Quantitative immunoblotting was performed on cardiac homogenates, as previously described. Inhibitor-1 expression was determined using heat-treated extracts and an antibody (#186), kindly provided by Dr. A. Nairn (Yale University).

In Vivo Gene Transfer

Adenoviral vectors were generated to encode for the truncated, constitutively active inhibitor-1 (I-1 T35D, AA 1->65) and green fluorescent protein (GFP), and delivered in a rat model of pressure-overload hypertrophy, transitioned to heart failure. One week following gene transfer, pressure measurements and biochemical assays (upon termination) were performed.

Statistics

The student’s t-test and ANOVA, followed by the Neuman-Keuls t-test, were used to determine the statistical differences between groups. Data are presented as mean±standard error. Statistical analysis was carried out on Prism 3.0 and significance was at $P$ value < 0.05.

Quantitative Immunoblotting and Analysis of the Fetal Gene Program
Quantitative immunobloting was performed on cardiac homogenates, as previously described.\textsuperscript{1,5} Immunoblotting of the heat-stable inhibitor-1 utilized heat-treated extracts. Antibodies to inhibitor-1 were kindly provided by Dr. Angus Nairn (Yale University). The fetal gene program was analyzed by dot-blotting, using probes previously described.\textsuperscript{6}

**Histopathology**

Histopathological studies with H&E, trichrome, PAS and TRITC-labeled wheat-germ agglutinin (Sigma Chemical Co., St. Louis, Missouri, USA) for cardiomyocyte cross-sectional area were performed as previously described.\textsuperscript{7} Specifically, for wheat germ agglutinin labeling of the cell wall, 40 or more cell cross-sectional areas (from multiple sections) were determined for each heart.

**Electrophysiology**

Calcium tolerant cardiomyocytes were isolated and cells with clear cross striations and without spontaneous contractions were used for the measurement of L-type Ca\textsuperscript{2+} current. Current recordings were obtained at constant voltage, and cell capacitance and Ca\textsuperscript{2+} channel inactivation was determined.\textsuperscript{8}

**Transverse Aortic Constriction of Mice**

Transverse aortic constriction on mice was performed, as previously described.\textsuperscript{7} Briefly, 10 week old FVBN male wild-type and transgenic mice, underwent banding of the transverse aorta, using a 27-gauge needle. Echocardiography was performed prior to banding and at various time points post-banding. At the termination point, trans-aortic gradients as well as lung, liver, heart and body weight were measured, and cardiac tissue was stored for subsequent histopathological analysis and biochemical studies.

**Cardiac Catheterization and Pressure Volume Loop Analysis**

Cardiac catheterization and pressure-volume loop analysis were performed 6 weeks after transverse aortic constriction. Mice were anesthetized with intra-peritoneal injections of 50 µg/g body weight (BW) ketamine
and 100 µg/gBW thiobutabarbital (Inactin, Research Biochemicals International, Natick, MA), and placed on a thermally controlled surgical table. Following tracheostomy, the left carotid artery was cannulated with polyethylene tubing connected to a COBE CDXIII fixed-dome pressure transducer (COBE Cardiovascular, Arvada, CO) for measurement of blood pressure distal to the aortic band. The right jugular vein was cannulated for the injection of drugs and saline. The left carotid artery was cannulated with a Millar 1.4F pressure-volume catheter (Model SPR-839), which was advanced across the aortic valve and into the left ventricle. The Millar catheter was interfaced with an Aria 1 conditioner/amplifier (Millar Instruments), and output signals were recorded at 1000 Hz using a PowerLab data acquisition unit (ADInstruments, Colorado Springs, CO). A transverse abdominal incision was made just below the xyphoid process to expose the inferior vena for occlusion analysis. The tracheostomy tube was connected to a rodent respirator (MiniVent Model 845, Harvard Apparatus, Holliston MA) and the animal ventilated at 120 breaths/minute with a stroke volume of 200 µl. For all measurements, ventilation was interrupted momentarily by opening the exhaust port of the respirator. Steady-state and occlusion measurements were made under baseline conditions and during infusion of dobutamine at 16ng/gBW/min. At the end of each experiment, parallel volume was determined by injection of 3 µl of 30% saline into the right jugular vein. The volume signal was calibrated by the cuvette method using warmed heparinized blood in 3-5 mm diameter wells. Each pressure-volume measurement was performed, at least in triplicate. Pressure-volume data were analyzed using Millar PVAN analysis software.

**Aortic Banding of Rats**

Four-week old Wistar rats (70-80 g) were obtained from Charles River Laboratories (Wilmington, MA) and aortic constriction was performed as previously described. The animals were initially randomized in two groups: one group of 30 animals with aortic banding and a second group of 32 animals, which were sham-operated. All animals survived the initial operation.
Serial Echocardiographic Assessment of Rat Heart Failure

At intervals of 6 and 18 weeks post-banding of the rats and then on a weekly basis between 20 and 24 weeks, serial echocardiograms were performed as previously described.⁴

Adenoviral Delivery Protocol in Rat Heart Failure

The group of 30 animals with aortic banding was subdivided in two groups of fifteen with each group receiving either Ad.I-1c or Ad.GFP. The group of 24 sham-operated animals did not receive any gene transfer and were studied in an age-matched fashion. One animal in the I-1c group and one animal in the GFP group died during the gene transfer surgery. The adenoviral delivery system has been described previously.³ In the sham-operated rats, no gene delivery was performed. Previous studies have shown that the sham-operated rats injected with Ad.GFP behaved in a similar way as non-infected sham operated rats.

Left Ventricular Pressure & Dimension Measurements in Rat Heart Failure

We also placed 4 crystals around the heart including one in the which allowed us to estimate ventricular volumes using the Cardiosoft from Sonometrics Corporation with SonoXYZ program (Sonometrics, Canada). The 3-D software allowed us to convert the matrix of distance measurements into the appropriate x,y,z coordinates for each crystal. Data in this format can be used to reconstruct the 3-dimensional image of any structure and show how this is changing over time. The 3-dimensional analysis software uses a triangulation algorithm to convert distance measurements into x,y,z coordinates. Typically, one transducer is taken as the origin of the coordinate system, another defines the x-axis, a third helps to define the x-y plane, and a fourth is used to set the positive z direction. We obtained data from the three different groups: Sham, Failing + Ad.βgal.GFP and failing +Ad.I-1c.

Pressure measurements were performed as previously described.⁴ The time course of isovolumic relaxation (τ) was calculated using the equation: \( P = P_0e^{-\frac{t}{\tau}} + P_b \), where \( P \) is the left ventricular isovolumic
pressure, \( P_o \) is pressure at the time of peak \(-dP/dt\), and \( P_B \) is residual pressure. For the pacing studies, an epicardial lead was placed at an atrial appendage connected to a stimulator (Grass Instruments, MA). In a subset of animals, multiple 0.7 mm piezoelectric crystals (Sonometrics Co., Canada) were placed over the surface of the left ventricle along the short axis of the ventricle at the level of the mitral valve to measure the inter-crystal distance. Left ventricular pressure-dimension loops were generated under different loading conditions by clamping the inferior vena cava. The end-systolic pressure-dimension relationship was obtained by producing a series of pressure dimension loops over a range of loading conditions and connecting the upper left hand corners of the individual pressure-dimension loops to generate the maximal slope.
B. SUPPLEMENTAL FIGURES

Supplemental Figure 1. The phosphorylation of phospholamban at Serine-16 was measured in WT and I1c overexpressing mice, post perfusion with 32 ng/gramBW/min dobutamine. The phosphorylation of phospholamban was increased significantly in the I1c overexpressing mice, compared to the WT controls (*P<0.05, n=4-5 hearts per group).

Supplemental Figure 2. The activity of CaM-Kinase is increased in failing (F) rat hearts and in failing rat hearts infected with Ad.I1c or Ad.GFP, compared to the non-failing (NF) control group (*P<0.05 vs NF, n=3-6 hearts per group).
### Table 1: Echocardiographic Measurements in WT and I1c Transgenic Mice

<table>
<thead>
<tr>
<th></th>
<th>LVEDD (mm)</th>
<th>LVESD (mm)</th>
<th>AWALL (mm)</th>
<th>PWALL (mm)</th>
<th>HR (beats/min)</th>
<th>ETIME (ms)</th>
<th>H/R</th>
<th>LVM (mg)</th>
<th>FS%</th>
<th>Vcf (circ/s)</th>
<th>Vcfc (circ/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong> (N=15)</td>
<td>3.58 ± 0.09</td>
<td>2.13 ± 0.11</td>
<td>0.69 ± 0.04</td>
<td>0.72 ± 0.04</td>
<td>511.18 ± 22.76</td>
<td>50.13 ± 2.82</td>
<td>0.40 ± 0.02</td>
<td>83.97 ± 6.71</td>
<td>40.95 ± 1.62</td>
<td>8.58 ± 0.63</td>
<td>10.01 ± 0.60</td>
</tr>
<tr>
<td><strong>I-1</strong> (N=12)</td>
<td>3.65 ± 0.07</td>
<td>2.00 ± 0.05</td>
<td>0.68 ± 0.03</td>
<td>0.71 ± 0.03</td>
<td>603.87 ± 22.75</td>
<td>44.08 ± 1.90</td>
<td>0.38 ± 0.02</td>
<td>84.30 ± 4.86</td>
<td>45.19 ± 1.12</td>
<td>10.44 ± 0.49</td>
<td>10.95 ± 0.34</td>
</tr>
<tr>
<td><strong>WT-TAC</strong> (N=12)</td>
<td>3.98 ± 0.08</td>
<td>2.42 ± 0.11</td>
<td>0.90 ± 0.02</td>
<td>0.91 ± 0.02</td>
<td>568.18 ± 23.03</td>
<td>49.82 ± 1.80</td>
<td>0.46 ± 0.01</td>
<td>138.43 ± 6.40</td>
<td>39.82 ± 1.77</td>
<td>8.02 ± 0.47</td>
<td>8.25 ± 0.45</td>
</tr>
<tr>
<td><strong>I-1 TAC</strong> (N=12)</td>
<td>3.48 ± 0.14</td>
<td>2.14 ± 0.16</td>
<td>0.99 ± 0.04</td>
<td>1.02 ± 0.04</td>
<td>587.67 ± 15.57</td>
<td>44.25 ± 2.18</td>
<td>0.59 ± 0.03</td>
<td>132.44 ± 8.40</td>
<td>39.49 ± 2.18</td>
<td>8.94 ± 0.47</td>
<td>9.04 ± 0.45</td>
</tr>
</tbody>
</table>

† P < 0.05 vs WT
& P < 0.05 between I-1 TAC and WT-TAC

†Baseline echocardiography was performed on male mice at 3 months of age.

LVEDD: Left Ventricular End Diastolic Dimension
LVESD: Left Ventricular End Systolic Dimension
A Wall: Anterior Wall
P Wall: Posterior Wall
HR: Heart rate
E Time: Ejection Time
H/R: Wall Thickness to Left Ventricular Lumenal Ratio
LVM: Echocardiography Calculated Left Ventricular Mass
FS%: Percent Fractional Shortening
Vcf: Velocity of Circumferential Shortening
Vcfc: Velocity of Circumferential Shortening Corrected for Heart Rate
Table 2: Echocardiographic Measurements in Rats after Sham Surgery or Aortic Banding

<table>
<thead>
<tr>
<th></th>
<th>PW (mm)</th>
<th>LVDD (mm)</th>
<th>LVSD (mm)</th>
<th>FS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sham</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 weeks</td>
<td>1.9±0.1</td>
<td>8.3±0.3</td>
<td>4.9±0.2</td>
<td>46±5</td>
</tr>
<tr>
<td>24 weeks</td>
<td>1.9±0.1</td>
<td>8.7±0.3</td>
<td>5.0±0.2</td>
<td>43±2</td>
</tr>
<tr>
<td><strong>Banded</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 weeks</td>
<td>2.7±0.2*</td>
<td>8.6±0.2</td>
<td>4.3±0.2</td>
<td>48±5*</td>
</tr>
<tr>
<td>20-24 weeks</td>
<td>2.8±0.20*</td>
<td>10±0.3*</td>
<td>6.1±0.1*</td>
<td>31±2*†</td>
</tr>
</tbody>
</table>

* p<0.05 compared to sham at similar time period
† p<0.05 compared to values at 12 weeks

PW: posterior wall thickness during diastole
LVDD: Left ventricular Diameter during diastole
LVSD: Left ventricular Systolic Diameter during Systole
FS: Fractional shortening


Supplemental Figure 1.

WT1  WT2  WT3  WT4  I1c1  I1c2  I1c3  I1c4  I1c5

pSER$_{16}$-PLN
PLN

WT  I1c

pSER$_{16}$ PLN/PLN

WT  I1c

*
Supplemental Figure 2.

Cam-Kinase Activity is Elevated in Failing Rat Hearts

- NF
- F
- F+GFP
- F+I1c

Percent Autonomous Cam-Kinase Activity

* *
A. SUPPLEMENTAL MATERIALS

Protein Phosphatase Activity

Protein phosphatase 1 activity was examined using $^{32}$P-labeled glycogen phosphorylase $a$ as a substrate in the presence of 4 nM okadaic acid, a concentration that selectively inhibits phosphatase type 2A, and EDTA (0.5 mM), an inhibitor of phosphatase 2B. The assays were conducted under conditions where no more than 15% of the substrate was utilized to assure linearity of the reaction. Total phosphatase, type 1 and type 2A phosphatase activities were also measured, using myelin basic protein (NEB-P07080S), as the substrate in the presence and absence of 4 nM okadaic acid.

Quantitative Immunoblotting

Quantitative immunoblotting was performed on cardiac homogenates, as previously described. Inhibitor-1 expression was determined using heat-treated extracts and an antibody (#186), kindly provided by Dr. A. Nairn (Yale University).

In Vivo Gene Transfer

Adenoviral vectors were generated to encode for the truncated, constitutively active inhibitor-1 (I-1 T35D, AA 1->65) and green fluorescent protein (GFP), and delivered in a rat model of pressure-overload hypertrophy, transitioned to heart failure. One week following gene transfer, pressure measurements and biochemical assays (upon termination) were performed.

Statistics

The student’s t-test and ANOVA, followed by the Neuman-Keuls t-test, were used to determine the statistical differences between groups. Data are presented as mean±standard error. Statistical analysis was carried out on Prism 3.0 and significance was at $P$ value < 0.05.

Quantitative Immunoblotting and Analysis of the Fetal Gene Program
Quantitative immunoblotting was performed on cardiac homogenates, as previously described.\textsuperscript{1,5} Immunoblotting of the heat-stable inhibitor-1 utilized heat-treated extracts. Antibodies to inhibitor-1 were kindly provided by Dr. Angus Nairn (Yale University). The fetal gene program was analyzed by dot-blotting, using probes previously described.\textsuperscript{6}

**Histopathology**

Histopathological studies with H&E, trichrome, PAS and TRITC-labeled wheat-germ agglutinin (Sigma Chemical Co., St. Louis, Missouri, USA) for cardiomyocyte cross-sectional area were performed as previously described.\textsuperscript{7} Specifically, for wheat germ agglutinin labeling of the cell wall, 40 or more cell cross-sectional areas (from multiple sections) were determined for each heart.

**Electrophysiology**

Calcium tolerant cardiomyocytes were isolated and cells with clear cross striations and without spontaneous contractions were used for the measurement of L-type Ca\textsuperscript{2+} current. Current recordings were obtained at constant voltage, and cell capacitance and Ca\textsuperscript{2+} channel inactivation was determined.\textsuperscript{8}

**Transverse Aortic Constriction of Mice**

Transverse aortic constriction on mice was performed, as previously described.\textsuperscript{7} Briefly, 10 week old FVBN male wild-type and transgenic mice, underwent banding of the transverse aorta, using a 27-gauge needle. Echocardiography was performed prior to banding and at various time points post-banding. At the termination point, trans-aortic gradients as well as lung, liver, heart and body weight were measured, and cardiac tissue was stored for subsequent histopathological analysis and biochemical studies.

**Cardiac Catheterization and Pressure Volume Loop Analysis**

Cardiac catheterization and pressure-volume loop analysis were performed 6 weeks after transverse aortic constriction. Mice were anesthetized with intra-peritoneal injections of 50 \( \mu \)g/g body weight (BW) ketamine
and 100 µg/gBW thiobutabarbital (Inactin, Research Biochemicals International, Natick, MA), and placed on a thermally controlled surgical table. Following tracheostomy, the left carotid artery was cannulated with polyethylene tubing connected to a COBE CDXIII fixed-dome pressure transducer (COBE Cardiovascular, Arvada, CO) for measurement of blood pressure distal to the aortic band. The right jugular vein was cannulated for the injection of drugs and saline. The left carotid artery was cannulated with a Millar 1.4F pressure-volume catheter (Model SPR-839), which was advanced across the aortic valve and into the left ventricle. The Millar catheter was interfaced with an Aria 1 conditioner/amplifier (Millar Instruments), and output signals were recorded at 1000 Hz using a PowerLab data acquisition unit (ADInstruments, Colorado Springs, CO). A transverse abdominal incision was made just below the xyphoid process to expose the inferior vena for occlusion analysis. The tracheostomy tube was connected to a rodent respirator (MiniVent Model 845, Harvard Apparatus, Holliston MA) and the animal ventilated at 120 breaths/minute with a stroke volume of 200 µl. For all measurements, ventilation was interrupted momentarily by opening the exhaust port of the respirator. Steady-state and occlusion measurements were made under baseline conditions and during infusion of dobutamine at 16ng/gBW/min. At the end of each experiment, parallel volume was determined by injection of 3 µl of 30% saline into the right jugular vein. The volume signal was calibrated by the cuvette method using warmed heparinized blood in 3-5 mm diameter wells. Each pressure-volume measurement was performed, at least in triplicate. Pressure-volume data were analyzed using Millar PVAN analysis software.

Aortic Banding of Rats

Four-week old Wistar rats (70-80 g) were obtained from Charles River Laboratories (Wilmington, MA) and aortic constriction was performed as previously described. The animals were initially randomized in two groups: one group of 30 animals with aortic banding and a second group of 32 animals, which were sham-operated. All animals survived the initial operation.
Serial Echocardiographic Assessment of Rat Heart Failure

At intervals of 6 and 18 weeks post-banding of the rats and then on a weekly basis between 20 and 24 weeks, serial echocardiograms were performed as previously described.⁴

Adenoviral Delivery Protocol in Rat Heart Failure

The group of 30 animals with aortic banding was subdivided in two groups of fifteen with each group receiving either Ad.I-1c or Ad.GFP. The group of 24 sham-operated animals did not receive any gene transfer and were studied in an age-matched fashion. One animal in the I-1c group and one animal in the GFP group died during the gene transfer surgery. The adenoviral delivery system has been described previously.³ In the sham-operated rats, no gene delivery was performed. Previous studies have shown that the sham-operated rats injected with Ad.GFP behaved in a similar way as non-infected sham operated rats.

Left Ventricular Pressure & Dimension Measurements in Rat Heart Failure

We also placed 4 crystals around the heart including one in the which allowed us to estimate ventricular volumes using the Cardiosoft from Sonometrics Corporation with SonoXYZ program (Sonometrics, Canada). The 3-D software allowed us to convert the matrix of distance measurements into the appropriate x,y,z coordinates for each crystal. Data in this format can be used to reconstruct the 3-dimensional image of any structure and show how this is changing over time. The 3-dimensional analysis software uses a triangulation algorithm to convert distance measurements into x,y,z coordinates. Typically, one transducer is taken as the origin of the coordinate system, another defines the x-axis, a third helps to define the x-y plane, and a fourth is used to set the positive z direction. We obtained data from the three different groups: Sham, Failing + Ad.βgal.GFP and failing +Ad.I-1c.

Pressure measurements were performed as previously described.⁴ The time course of isovolumic relaxation (τ) was calculated using the equation: P=P₀e⁻τt + P_b, where P is the left ventricular isovolumic...
pressure, $P_o$ is pressure at the time of peak $-dP/dt$, and $P_b$ is residual pressure. For the pacing studies, an epicardial lead was placed at an atrial appendage connected to a stimulator (Grass Instruments, MA). In a subset of animals, multiple 0.7 mm piezoelectric crystals (Sonometrics Co., Canada) were placed over the surface of the left ventricle along the short axis of the ventricle at the level of the mitral valve to measure the inter-crystal distance. Left ventricular pressure-dimension loops were generated under different loading conditions by clamping the inferior vena cava. The end-systolic pressure-dimension relationship was obtained by producing a series of pressure dimension loops over a range of loading conditions and connecting the upper left hand corners of the individual pressure-dimension loops to generate the maximal slope.
B. SUPPLEMENTAL FIGURES

Supplemental Figure 1. The phosphorylation of phospholamban at Serine-16 was measured in WT and I1c overexpressing mice, post perfusion with 32 ng/gramBW/min dobutamine. The phosphorylation of phospholamban was increased significantly in the I1c overexpressing mice, compared to the WT controls (*P<0.05, n=4-5 hearts per group).

Supplemental Figure 2. The activity of CaM-Kinase is increased in failing (F) rat hearts and in failing rat hearts infected with Ad.I1c or Ad.GFP, compared to the non-failing (NF) control group (*P<0.05 vs NF, n=3-6 hearts per group).
### C. SUPPLEMENTAL TABLES

Table 1: Echocardiographic Measurements in WT and I1c Transgenic Mice

<table>
<thead>
<tr>
<th></th>
<th>LVEDD (mm)</th>
<th>LVESD (mm)</th>
<th>AWALL (mm)</th>
<th>PWALL (mm)</th>
<th>HR (beats/min)</th>
<th>ETIME (ms)</th>
<th>H/R</th>
<th>LVM (mg)</th>
<th>FS%</th>
<th>Vcf (circ/s)</th>
<th>Vcfc (circ/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td>3.58 ±</td>
<td>2.13 ±</td>
<td>0.69 ±</td>
<td>0.72 ±</td>
<td>511.18 ±</td>
<td>50.13 ±</td>
<td>0.40 ±</td>
<td>83.97 ±</td>
<td>40.95 ±</td>
<td>8.58 ±</td>
<td>10.01 ±</td>
</tr>
<tr>
<td>(N=15)</td>
<td>0.09</td>
<td>0.11</td>
<td>0.04</td>
<td>0.04</td>
<td>22.76</td>
<td>2.82</td>
<td>0.02</td>
<td>6.71</td>
<td>1.62</td>
<td>0.63</td>
<td>0.60</td>
</tr>
<tr>
<td><strong>I-1</strong></td>
<td>3.65 ±</td>
<td>2.00 ±</td>
<td>0.68 ±</td>
<td>0.71 ±</td>
<td>603.87 ±</td>
<td>44.08 ±</td>
<td>0.38 ±</td>
<td>84.30 ±</td>
<td>45.19 ±</td>
<td>10.44 ±</td>
<td>10.95 ±</td>
</tr>
<tr>
<td>(N=12)</td>
<td>0.07</td>
<td>0.05</td>
<td>0.03</td>
<td>0.03</td>
<td>22.75 †</td>
<td>1.90 †</td>
<td>0.02</td>
<td>4.86</td>
<td>1.12 †</td>
<td>0.49 † ± 0.34</td>
<td></td>
</tr>
<tr>
<td><strong>WT-</strong></td>
<td>3.98 ±</td>
<td>2.42 ±</td>
<td>0.90 ±</td>
<td>0.91 ±</td>
<td>568.18 ±</td>
<td>49.82 ±</td>
<td>0.46 ±</td>
<td>138.43 ±</td>
<td>39.38 ±</td>
<td>8.02 ±</td>
<td>8.25 ±</td>
</tr>
<tr>
<td>TAC</td>
<td>0.08 †</td>
<td>0.11 †</td>
<td>0.02 †</td>
<td>0.02 †</td>
<td>23.03</td>
<td>1.80</td>
<td>0.01 †</td>
<td>6.40</td>
<td>1.77</td>
<td>0.47</td>
<td>0.45 †</td>
</tr>
<tr>
<td>(N=12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>I-1</strong></td>
<td>3.48 ±</td>
<td>2.14 ±</td>
<td>0.99 ±</td>
<td>1.02 ±</td>
<td>587.67 ±</td>
<td>44.25 ±</td>
<td>0.59 ±</td>
<td>132.44 ±</td>
<td>39.49 ±</td>
<td>8.94 ±</td>
<td>9.04 ±</td>
</tr>
<tr>
<td>TAC</td>
<td>0.14 &amp;</td>
<td>0.16 &amp;</td>
<td>0.04 † &amp;</td>
<td>0.04 † &amp;</td>
<td>15.57 †</td>
<td>1.27 † &amp;</td>
<td>0.03 †</td>
<td>8.40 †</td>
<td>2.18</td>
<td>0.47</td>
<td>0.45</td>
</tr>
<tr>
<td>(N=12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

† P < 0.05 vs WT
& P < 0.05 between I-1 TAC and WT-TAC

‡Baseline echocardiography was performed on male mice at 3 months of age.

- LVEDD: Left Ventricular End Diastolic Dimension
- LVESD: Left Ventricular End Systolic Dimension
- AWALL: Anterior Wall
- PWALL: Posterior Wall
- HR: Heart rate
- ETIME: Ejection Time
- H/R: Wall Thickness to Left Ventricular Lumenal Ratio
- LVM: Echocardiography Calculated Left Ventricular Mass
- FS%: Percent Fractional Shortening
- Vcf: Velocity of Circumferential Shortening
- Vcfc: Velocity of Circumferential Shortening Corrected for Heart Rate
**Table 2: Echocardiographic Measurements in Rats after Sham Surgery or Aortic Banding**

<table>
<thead>
<tr>
<th></th>
<th>PW (mm)</th>
<th>LVDD (mm)</th>
<th>LVSD (mm)</th>
<th>FS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 weeks</td>
<td>1.9±0.1</td>
<td>8.3±0.3</td>
<td>4.9±0.2</td>
<td>46±5</td>
</tr>
<tr>
<td>24 weeks</td>
<td>1.9±0.1</td>
<td>8.7±0.3</td>
<td>5.0±0.2</td>
<td>43±2</td>
</tr>
<tr>
<td>Banded</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 weeks</td>
<td>2.7±0.2*</td>
<td>8.6±0.2</td>
<td>4.3±0.2</td>
<td>48±5*</td>
</tr>
<tr>
<td>20-24 weeks</td>
<td>2.8±0.20*</td>
<td>10±0.3*</td>
<td>6.1±0.1*</td>
<td>31±2*†</td>
</tr>
</tbody>
</table>

* * p<0.05 compared to sham at similar time period
† † p<0.05 compared to values at 12 weeks

PW: posterior wall thickness during diastole
LVDD: Left ventricular Diameter during diastole
LVSD: Left ventricular Systolic Diameter during Systole
FS: Fractional shortening


Supplemental Figure 1.

WT₁  WT₂  WT₃  WT₄  I₁c₁  I₁c₂  I₁c₃  I₁c₄  I₁c₅

pSER₁₆-PLN

PLN

WT  I₁c

*pSER₁₆/PLN

WT  I₁c

*
Supplemental Figure 2.

Cam-Kinase Activity is Elevated in Failing Rat Hearts

![Bar chart showing Cam-Kinase Activity comparison across different conditions: NF, F, F+GFP, F+I1c. Statistical significance indicated by asterisks (*) where applicable.](chart.png)