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, Ca2+ 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 Ca2+ handling proteins that control excitation-contraction coupling cycle, such as phospholamban, the ryanodine receptor, the L-type Ca2+ channel, and troponin I.

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 phosphatases, 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. Furthermore, PP2A and PP2B (calcineurin) overexpression have been shown to result in decreased function and pathological hypertrophy.

In human and experimental heart failure, the activity of the type 1 phosphatase, associated with the sarcoplasmic reticulum (SR) is significantly increased. It has been suggested that this increase may be a contributing factor to depressed function, dilated cardiomyopathy, and premature death. Some studies indicate that the increased SR-protein phosphatase 1 activity may be due to dephosphorylation of its inhibitor protein, inhibitor-1. 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 transgenesis 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 catheritization 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

![Image](http://circres.ahajournals.org/)

Figure 1. Mice with cardiac-specific expression of the active inhibitor-1 (I1c) exhibit a decrease in cardiac type 1 phosphatase activity and an increase in cardiac contractility. A, Active inhibitor-1 protein was expressed 25-fold of endogenous inhibitor-1, which resulted in 15% decreases in type 1 phosphatase activity (P<0.05, n=6 per group). B, Langendorff analysis indicated that inhibitor-1 hearts exhibited significantly enhanced rates of pressure development (dP/dt) and maximal LVP. Inhibitor-1 expressing cardiomyocytes also exhibited enhanced (C) rates of shortening (dL/dt) and relengthening (−dL/dt) and percent fractional shortening (%FS), as well as (D) increased amplitude and time to 50% decay (T50) of the calcium transient. Isoproterenol (100 nmol/L) stimulation further enhanced dL/dt and T50, compared with WTs. *P<0.05 vs WT and #P<0.05 vs WT+ISO, n=30 cardiomyocytes from 6 to 8 hearts per group.
it specifically inhibits protein phosphatase 1, albeit at higher concentration than the native phosphorylated inhibitor.\textsuperscript{10}

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 \( \frac{dP}{dt} \) and \( -\frac{dP}{dt} \) 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 (\( -\frac{dL}{dt} \)) and relengthening (\( +\frac{dL}{dt} \)) 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 \( +\frac{dL}{dt} \) and \( -\frac{dL}{dt} \) 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 Ca\textsuperscript{2+} signal (T\textsubscript{Ca}) 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 T\textsubscript{Ca} (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 Ca\textsuperscript{2+} Handling Proteins**

As described, \( \beta \)-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 Ca\textsuperscript{2+} cycling and cardiac contractility. Thus, we investigated the expression and phosphorylation levels of these key substrates in our transgenic model. There was no

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**Figure 2.** Calcium regulatory proteins and phosphoproteins in inhibitor-1 (I\textsubscript{1c}) hearts. A, Immunoblot (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 \( *P<0.05, \text{n}=5\text{ each} \). Phosphorylation of phospholamban \( *P<0.05, \text{n}=10\text{ each} \) was increased, whereas ryanodine receptor and troponin I phosphorylation was not altered \( *P<0.05, \text{n}=4\text{ each} \). B, Left, Cardiomyocyte current-voltage relationship was not different between WTs and transgenics; Right, Calcium-dependent inactivation kinetics of the L-type Ca\textsuperscript{2+} channel were accelerated in inhibitor-1 myocytes. \( \text{*P}<0.05, \text{n}=5\text{ hearts per group and at least 25 cardiomyocytes per group.} \)
significant difference in β-adrenergic receptor density (data not shown). However, phosphorylation of phospholamban at both its cAMP-dependent (Ser16) and Ca²⁺/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. 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 Ca²⁺ channel protein levels, and examination of the channel activity revealed that the mean peak Ca²⁺ current (IₙCa) and the steady-state inactivation of the current-voltage relationship (I-V) were similar between active inhibitor-1 and WT myocytes. However, inactivation of IₙCa was faster in the transgenics (Figure 2B), similar to previous observations in the hyperdynamic phospholamban knock-out myocytes. 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 Ca²⁺ 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.](http://circres.ahajournals.org/)
Although transaortic gradients were similar between the two groups (WT: 55.3 ± 3.6; TG: 53.9 ± 4.6, 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).

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 luminal 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 \((\frac{dP}{dt})\) 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±1.1; WT banded 7.4±2.0, mm Hg/µL; TG sham 4.5±0.5; TG banded 12.2±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±6.9; I1 banded: 98.5±21.9 mm Hg; \(P=0.30\)), whereas it significantly declined in banded WTs (WT sham: 82.5±6.9; WT banded: 31.4±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 of WT and I1c banded hearts (1000× and 400×); Right, Average cross-sectional area of WT-shams and banded WT and I1c cardiomyocytes \((^{*}P<0.001 \text{ vs } \text{WT-Sham}; ^{†}P<0.05 \text{ between WT and I1c-banded hearts}; n=120 \text{ myocytes each from 3 hearts each})\). Analysis of: C, MAP-kinase activation; and D, P-Ser16-phospholamban in banded mice (WT-Sham, n=5; I1c-Sham, n=5; WT-TAC, n=10; I1c-TAC, n=10; \(^{*}P<0.05 \text{ vs WT-Sham}; ^{†}P<0.05 \text{ between WT-TAC and I1c-TAC}\).
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 \(\tau\), 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 \((E_{\text{max}}\) 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.\textsuperscript{14,15}

In failing hearts, the downregulation of β-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\textsuperscript{2,3} and dephosphorylation of key phosphoproteins, including phospholamban.\textsuperscript{16,17} Interestingly, hypophosphorylation of inhibitor-1 in heart failure may also reflect enhanced activity of calcineurin (PP2B).\textsuperscript{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-α, a calcium-dependent PKC isoform, which phosphorylates the inhibitor-1 at a different site, Ser67, and reduces its activity.\textsuperscript{19} Indeed, ablation of PKC-α is associated with decreased phosphatase activity and enhanced function.\textsuperscript{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,\textsuperscript{20,21} as well as the adrenergic and cholinergic pathways in the heart.\textsuperscript{22} Although this phosphoprotein has long been known to play a major regulatory role in neuronal tissue,\textsuperscript{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,\textsuperscript{2} whereas adenoviral infection of rat cardiomyocytes with inhibitor-1 enhanced cardiac contractility.\textsuperscript{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,\textsuperscript{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.\textsuperscript{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.\textsuperscript{2} However, the activity of this phosphatase does not appear to change in vivo on isoproterenol stimulation.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 hyperactive p38 MAP-kinase. 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\(^{2+}\)-transport may represent a nodal point in the progression of compensatory hypertrophy and heart failure. Accordingly, restoring the depressed SR Ca\(^{2+}\)-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 knock-out 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\(^{2+}\)-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\(^{2+}\)-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\(^{2+}\), expression of the active inhibitor-1 improves cardiac function by targeting downstream substrates and by specifically enhancing SR Ca\(^{2+}\) cycling.

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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.\(^1,5\)

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.\(^6\)

**Histopathology**

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Supplemental Figure 1.

The figure shows a Western blot analysis with lanes labeled WT1 to WT4 and I1c1 to I1c5. The bands are labeled as pSER16-PLN and PLN.

A bar graph below the blot shows the ratio of pSER16 PLN/PLN for WT and I1c. The I1c group shows a significantly higher ratio indicated by an asterisk.
Supplemental Figure 2.

Cam-Kinase Activity is Elevated in Failing Rat Hearts

Percent Autonomous Cam-Kinase Activity

NF  F  F+GFP  F+I1c
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.

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