Enhanced cardiac function in mice overexpressing protein phosphatase Inhibitor-2

Uwe Kirchhefer\textsuperscript{a}, Hideo A. Baby\textsuperscript{b}, Peter Boknik\textsuperscript{a}, Kristine M. Breeden\textsuperscript{c}, Nirmala Mavila\textsuperscript{c}, Nicole Brücher\textsuperscript{a}, Isabel Justus\textsuperscript{a}, Marek Matus\textsuperscript{a}, Wilhelm Schmitz\textsuperscript{a}, Anna A. DePaoli-Roach\textsuperscript{c,*}, Joachim Neumann\textsuperscript{d,*}

\textsuperscript{a}Institut für Pharmakologie und Toxikologie, Westfälische Wilhelms-Universität, 48149 Münster, Germany
\textsuperscript{b}Institut für Pathologie, Universität Essen, 45147 Essen, Germany
\textsuperscript{c}Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202, USA
\textsuperscript{d}Institut für Pharmakologie und Toxikologie, Martin-Luther-Universität Halle-Wittenberg, 06112 Halle, Germany

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Abstract

Objective: Protein phosphatase 1 (PP1) has been implicated in the control of cardiac function. Cardiac specific overexpression of the catalytic subunit, PP1c, results in hypertrophy and depressed contractility.

Methods: To further address the role of PP1, transgenic mice (TG) were generated that overexpress in heart a functional COOH-terminally truncated form (amino acids 1-140) of the PP1 inhibitor-2 (I-2\textsuperscript{140}).

Results: The TG hearts show increased levels of I-2\textsuperscript{140} mRNA as well as protein and activity. No increase in absolute or relative heart weight was observed, nor any changes in gross pathology or increase in morbidity or mortality in the TG mice. Immunohistochemical and biochemical analyses revealed that expression of the I-2\textsuperscript{140} protein is confined to cardiomyocytes where it is mainly localized in the cytosol. The total protein phosphatase (PP) activity was reduced by 80% in TG hearts as compared to wild-type littermates (WT). The PP1c mRNA level was the same in TG and WT, while the protein level was increased by \( \approx 7 \)-fold in TG animals. The maximal rates of contraction (+dP/dt) and of relaxation (−dP/dt) were increased by 32% and 40%, respectively, in the intact catheterized TG mice compared to WT. However, the maximal contractile response to β-adrenergic agonists was comparable in hearts from TG and WT mice. In isolated cardiomyocytes of TG mice, Ca\textsuperscript{2+} transient amplitude was increased by 50% under basal conditions and by 60% upon rapid caffeine application. The phospholamban (PLB) protein level was unchanged whereas the basal phosphorylation of PLB at Ser\textsuperscript{16} was significantly increased in TG hearts.

Conclusion: These results indicate that I-2\textsuperscript{140} overexpression results in decreased PP1 activity and enhanced contractility in the heart, underscoring the fundamental role of PP1 in cardiac function.

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Keywords: Protein phosphatase 1; Inhibitor-2; Heart; transgenic mouse; contractility

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1. Introduction

Protein phosphorylation is a dynamic process, in which the action of protein kinases is counterbalanced by protein phosphatases (PP). The main serine/threonine phosphatase activity in the heart is due to PP1 and PP2A. The phosphorylation of phospholamban (PLB) at Ser16 by cAMP-dependent protein kinase (PKA) in heart is reversed by type 1 and type 2A phosphatases [1,2]. PP1 enzymes are heteromeric complexes consisting of a catalytic subunit (PP1c) associated with an ancillary protein. There are some 70 PP1c binding/regulatory proteins [3] that target the phosphatase activity to various cellular compartments or organelles, confer substrate specificity or control enzyme activity. PP1c has been shown to bind the ryanodine receptor (RyR) possibly via an ancillary protein, spinophilin [4]. The glycoprotein targeting proteins, R$_{ex}$/G$_{M1}$, G$_{I}$ and PTG, the myofibrillar-targeting proteins, MYPT1/M110, and the nuclear targeting protein NIPP1 and PNUTS all direct the phosphatase to distinct subcellular locales [3]. PP1 and PP2A can be distinguished by their sensitivity to specific protein inhibitors; for example PP1 but not PP2A, is inhibited by the heat stable proteins inhibitor-1 (I-1) and inhibitor-2 (I-2) [5–8]. These inhibitors are highly conserved and the I-2 primary sequence is over 90% identical in mammals. Whereas unphosphorylated I-2 inhibits PP1 activity, I-1 requires phosphorylation by PKA for inhibitory activity. We have previously shown that β-adrenergic receptor (β-AR) stimulation led to enhanced I-1 phosphorylation and reduced PP1 activity in the intact heart, in isolated heart, and in cardiomyocytes [9–11]. The decreased PP1 activity would amplify the PKA-induced phosphorylation of PLB and other regulatory proteins under sympathetic stimulation [9].

Several lines of evidence indicate that increased PP1 activity might contribute to cardiac hypertrophy and heart failure. Increased PP1c mRNA levels and PP1 activity have been observed in end-stage human failing hearts [12,13]. Consistent with this finding, several laboratories have reported dephosphorylation of cardiac regulatory proteins such as PLB [12,14,15] and troponin I [16] in samples from patients with end-stage heart failure. A causal link between heart failure and PP1 is further strengthened by studies in animal models. Chronic β-AR stimulation by infusion of isoproterenol led to cardiac hypertrophy, increased PP1 activity and PLB dephosphorylation [17]. Cardiac-specific overexpression of PP1c in mice results in depressed basal and β-adrenergic agonist stimulated cardiac functions, dilated cardiomyopathy, and premature mortality consistent with heart failure [2]. Ablation of I-1 led to a 23% increase in PP1 activity and an impaired contractile response to β-AR stimulation [2].

Based on these findings, we speculated that increased expression of I-2 might alter cardiac function in the opposite sense to PP1c overexpression. To address this question we generated mice with cardiac-specific overexpression of a COOH-terminally truncated I-2 form and analyzed the biochemical and physiological consequences in attempts to further understand the role of PP1 in control of heart function. The results strongly suggest that inhibition of PP1 activity improves cardiac contractility and, therefore, performance.

2. Methods

2.1. Generation of mice overexpressing a COOH-terminally truncated form of I-2

Total RNA was isolated from human left ventricle as described by Chomczynski and Sacchi [18]. First strand cDNA was synthesized by standard RT-PCR protocols and amplified using Taq DNA Polymerase (Promega, Madison, WI) and I-2 oligonucleotides as follows. The forward primer (33nt) extended from bp-3 through +21 and contained a 5'-engineered XbaI restriction enzyme site. The reverse primer (33 nt) corresponded to the sequence 511-543 bp downstream of the I-2 translational stop codon. The amplified cDNA fragment was purified using the DNA Purification System (Promega). Sequencing of the PCR product revealed that around nt 410 there were 6 As instead of the expected 7. After digestion with XbaI and Clal the resulting 799 bp fragment was subcloned into pBluescript and then excised with NorI and KpnI. This fragment was inserted into a mouse cardiac α-myosin heavy chain promoter expression cassette [19]. The orientation of the I-2 cDNA was confirmed by sequencing. The one bp deletion caused a frame shift and generation of a stop codon 14 nt downstream. Consequently, the protein produced is a COOH-terminally truncated 140 amino acid I-2 (I-2140), instead of the normal 204 residues, with Lys135 followed by 5 mutated amino acid residues: Ser136-Asp-Lys138-Leu-Lys140 before the stop codon. Generation of transgenic (TG) mice and screening were performed by standard procedures. One I-2140 TG mouse founder was obtained, which was crossed with DBA/C3H mice for propagation and generation of experimental animals. All experiments were performed on mice 16–20 weeks of age. Animals were handled and maintained according to protocols approved by the animal welfare committee of the University of Münster, Germany, which also conform to the NIH Guidelines for the Care and Use of Laboratory Animals.

2.2. Northern blot analysis

Total RNA was prepared from individual mouse ventricles [18]. 20 µg of RNA samples were separated on a 1% formaldehyde-agarose gel and transferred to a nylon membrane. After prehybridization the membrane was hybridized overnight sequentially with a $^{32}$P-labeld 466 bp rat I-2 and a 295 bp rat PP1c [20] cDNA fragment. Membranes were washed and signals were visualized in a PhosphorImager.
2.3. Immunohistochemistry and immunoelectron microscopy

Longitudinal tissue sections of 5 µm thickness were obtained from the left ventricular wall and treated as described [21]. Prior to application to the sections, a monoclonal antibody raised against residues 1-144 of the human I-2 (Transduction Laboratories, Lexington, KY) was reacted with a biotinylated anti-mouse immunoglobulin (DAKO, Carpinteria, CA). After blocking with non-immune mouse serum, the sections were treated either with the biotinylated primary antibody (recognizing only the transgenic human I-2[140]) or an affinity-purified polyclonal antibody raised against recombinant rabbit I-2 (recognizing both the endogenous mouse I-2 and the transgenic human I-2[140]) followed by incubation with streptavidine-peroxidase and reacted using diaminobenzidine/hydrogen peroxidase as a chromogenic-substrate (DAKO) or by incubation with a goat anti-rabbit alkaline phosphatase-conjugated antibody and reacted using diaminobenzidine/hydrogen peroxidase as a chromogenic-substrate (DAKO) or by incubation with a goat anti-rabbit alkaline phosphatase-conjugated antibody (Dianova, Hamburg, Germany), respectively. Sections were counterstained with hematoxylin. Preparation of ultrathin frozen sections (50 nm) from hearts and labeling were performed as described by Tokuyasu [22]. Tissue sections were incubated with the I-2 monoclonal antibody, labeled with immunogold, and examined in a Philips 301 electron microscope.

2.4. Preparation of subcellular fractions

The preparation of cardiac crude homogenates and subcellular fractions (myofilaments, cytosol, and membrane vesicles) was performed as described [23].

2.5. Protein phosphatase and I-2 activity assays

Protein phosphatase activity was assayed using 32P-phosphorylase a as substrate as previously described [24]. Mouse ventricular tissue was homogenized at 4 °C for 1 min in buffer containing 4 mM EDTA (pH 7.4) and 15 mM 2-mercaptoethanol. Homogenates were centrifuged at 14,000 ×g for 20 min at 4 °C and the supernatants were used for determination of phosphorylase phosphatase activity. The reaction mixture contained 20 mM Tris/HCl (pH 7.4), 5 mM caffeine, 0.1 mM EDTA, and 15 mM 2-mercaptoethanol. The dephosphorylation reactions were initiated by adding 32P-phosphorylase a to a final concentration of 0.5 mg/ml (40,000 cpm/nmol) and carried out at 30 °C for 10 min. Where appropriate, okadaic acid was added before the initiation of the reaction. The reaction was terminated by addition of 50% trichloroacetic acid. The precipitated proteins were sedimented at 10,000 ×g for 5 min and an aliquot of the supernatants was counted in a liquid scintillation counter. To test the activity of the overexpressed I-2[140], ventricular tissue was homogenized in the buffer above containing additionally 10 mM HEPES and 1 mM MnCl2 [25]. After centrifugation the supernatants were boiled for 10 min and centrifuged sequentially 3 times at 14,000 ×g for 10 min each. The boiled supernatants (2.5–2500 ng protein) were incubated with 2.5 ng recombinant catalytic subunit of PP1 (Sigma-Aldrich, Taufkirchen, Germany) for 10 min on ice and then the phosphatase activity was determined as described above, except that the reaction was carried out for 30 min. No more than 18% of the substrate was utilized in the assay in order to assure linearity of the reaction.

2.6. Heart perfusion

Hearts were subjected to anterograde perfusion using the work-performing modus with an oxygenated Krebs–Henseleit buffer [26]. After a 30-min perfusion period, hearts were rapidly freeze-clamped.

2.7. Immunoblotting analyses

Hearts were homogenized at 4 °C for 1 min in buffer containing 20 mM Tris/HCl (pH 7.4), 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, and 5 µg/ml leupeptin. Homogenates were centrifuged at 4 °C for 20 min at 10,000 ×g. Supernatants were treated with equal volumes of loading buffer (7.5% SDS, 62.5 mM Tris/HCl, pH 6.8, 20% glycerol, 40 mM dithiothreitol). For immunoblot analysis, supernatant proteins were separated on 8% SDS-PAGE [27], and transferred to nitrocellulose membranes which were probed with affinity-purified polyclonal antibodies to PP1C or PP2Ac (Upstate, Lake Placid, NY). For quantitation of expression of endogenous I-2 and I-2[140], cardiac muscle extracts (13 µg for the WT and 0.65 µg for TG) were separated on an 11% SDS-polyacrylamide gel, transferred to nitrocellulose membrane and the membrane was blotted with affinity-purified polyclonal antibody raised against recombinant rabbit I-2. The polyclonal antibody recognizes both the mouse and the human I-2. Moreover, homogenates and subcellular fractions were separated on 10% SDS-PAGE and transferred to membranes, which were probed for I-2[140], full-length mouse I-2, PP1C, myoglobin (DAKO), troponin inhibitor (TnI), and SERCA2a [26]. For analysis of PLB phosphorylation, perfused hearts were homogenized at 4 °C for 90 s in 10 mM NaHCO3, 50 mM NaF, and 5 mM Na2P2O7 (pH 7.4). Homogenates were centrifuged at 10,000 ×g for 20 min at 4 °C. Aliquots of supernatants (100 µg) were subjected to 10% SDS-PAGE, and blotted onto nitrocellulose membranes which were probed sequentially with antibodies against PLB phosphoserine16 (Upstate) or phosphothreonine17 (Badrilla, Leeds, UK) and the non-phosphorylation-specific A1 monoclonal antibody (Upstate). Antibody binding was detected by alkaline phosphatase-conjugated secondary antibody, by horseradish peroxidase-conjugated secondary antibody, or by 125I-protein A.

2.8. Hemodynamic performance

Left ventricular catheterization was performed in closed-chest mice by modifications of the method described by
Engelhardt et al. [28]. Increasing doses of dobutamine were perfused into the left jugular vein.

2.9. Ca\(^{2+}\) transients, cell shortening and SR Ca\(^{2+}\) load

Cardiomyocytes were enzymatically isolated from hearts as described [26]. Subsequently, cardiomyocytes were loaded with Indo-1/AM and stimulated at 0.5 Hz. Indo-1 was excited at 365 nm. The cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) was estimated by calculating the ratio of emitted Indo-1 fluorescence signals (405/495 nm). The shortening of cardiomyocytes was recorded simultaneously using a video edge detection system, as described [21]. Intracellular free, cytosolic Ca\(^{2+}\) levels (maximum Ca\(^{2+}\) levels = systolic ratio, minimum Ca\(^{2+}\) levels = diastolic ratio and their difference = \(\Delta[Ca]_i\) amplitude) and shortening of the cardiomyocytes were recorded at steady-state conditions. The sarcoplasmic reticulum (SR) Ca\(^{2+}\) content was estimated by measurement of caffeine-induced (10 mM) increases in Ca\(^{2+}\) levels as reported before [21].

2.10. Statistical analysis

Data are reported as means \(\pm\) S.E.M. Statistical significance was assessed by ANOVA analyses followed by Bonferroni’s or Student’s \(t\)-test. \(P < 0.05\) was considered significant.

3. Results

3.1. Generation and characterization of transgenic mice overexpressing I-2

To determine the physiological role of I-2, we generated TG mice with cardiac-specific overexpression of a truncated form of human I-2 (amino acids 1-140) lacking the 64 COOH-terminal residues and containing 5 mutated amino acids at the end of the sequence (I-2\(^{140}\)). The truncated I-2 is equally potent as the full-length and has the advantage that its interaction with the PP1c forms a complex that can no longer be reactivated by phosphorylation at Thr\(^{72}\), effectively resulting in a constitutively active inhibitor [29,30]. No differences in the heart and body weight or their ratio were observed in WT compared to TG (ratio 6.4 \(\pm\) 0.3 vs. 6.1 \(\pm\) 0.3, resp.; \(n = 15\)). TG mice exhibited a normal life span and no apparent morbidity. There was no evidence of cardiac hypertrophy or dilatation as revealed by echocardiography (data not shown). Northern blot analysis revealed the presence of an I-2 transcript of \(\sim 1.7\) kb in WT hearts (Fig. 1A), most likely representing the endogenous I-2, and a second of \(\sim 0.8\) kb in TG hearts, corresponding to the predicted mRNA size of the transgene. A protein band of \(\sim 24\) kDa was visualized in extracts from TG hearts separated on SDS-PAGE and stained with Coomassie Blue (Fig. 1B), indicating a very high level of expression of I-2\(^{140}\). The identity of the polypeptide as I-2\(^{140}\) was confirmed by immunoblotting with a polyclonal antibody that recognizes both the mouse and human I-2 (Fig. 1C). Quantitative analyses indicate that I-2\(^{140}\) is expressed \(\sim 40\)-fold over the endogenous protein (Fig. 1D). Similar levels of expression were recently reported for overexpression of a truncated form of I-1 in heart [31].

3.2. Subcellular localization of the overexpressed I-2\(^{140}\) protein

Immunostaining of heart sections using an antibody that recognizes the I-2\(^{140}\) transgene (Fig. 2B, D) but not the endogenous I-2 (Fig. 2A, C), revealed a homogeneous

Fig. 1. Analysis of I-2 expression. Panel A shows a Northern blot from WT and TG heart. 20 \(\mu\)g of total RNA were subjected to electrophoresis, transferred to a nylon membrane, and then hybridized with a \(^{32}\)P-labeled rat I-2-specific cDNA probe. m, endogenous I-2 mRNA and h, human I-2 transgene transcript. Panel B shows a Coomassie Blue-stained gel of cardiac extracts (13 \(\mu\)g of protein/lane). The truncated I-2\(^{140}\) migrates with an apparent molecular mass of 24 kDa. Panel C shows an autoradiogram from an immunoblot (WT 13 \(\mu\)g protein/lane and TG 0.65 \(\mu\)g/lane) with polyclonal antibodies raised against recombinant rabbit I-2 that recognized both mouse and human I-2 as described under Methods. Note that the full-length I-2 is not detected in the TG samples because of the 20-fold lower protein loading. Panel D shows quantitation of an immunoblot by densitometric scanning of the autoradiogram.
distribution throughout the cardiomyocytes. I-2\textsuperscript{140} was not detectable in vascular smooth muscle cells, endothelial cells or fibroblasts, consistent with the known specificity of the \(\alpha\)MHC promoter. Staining with antibodies that react with both endogenous and the transgenic I-2 showed similar localization, mainly in the cytosol of the cardiomyocytes (Fig. 2E, F). Ultrastructural analysis by immunoelectron microscopy also revealed the presence of I-2\textsuperscript{140} throughout the TG heart but not WT heart (Fig. 3A). Expression of the transgene was confirmed by Western analysis of subcellular fractions by analyzing equivalent amounts of the cytosolic, the membrane vesicle and the myofilament fractions. Analysis of immunoblots showed that I-2\textsuperscript{140}, full-length I-2, and PP1c\(\alpha\) were mainly present in the cytosol indicating co-localization of the transgene and PP1c (Fig. 3B). The efficacy of the fractionation procedure was confirmed by the detection of specific marker proteins like myoglobin, TnI, and SERCA2a in appropriate fractions (Fig. 3B).

3.3. Decreased phosphatase activity in TG hearts

The ability of the overexpressed I-2\textsuperscript{140} to inhibit PP1 activity was determined by measuring phosphorylase phosphatase activity. Total phosphatase activity was re-
duced by ~80% in the soluble fraction of TG as compared to WT hearts (Fig. 4A). Interestingly, although the PP1cα mRNA level was unchanged between TG and WT (Fig. 4B, left panel), the protein level of PP1cα was upregulated ~7-fold (Fig. 4B, right panel and C) with no changes in the level of PP2Ac protein (data not shown), indicating that the overexpressed I-2140 stabilizes the PP1c protein, an effect also observed by overexpression of Rgl/GM, another PP1c regulatory/targeting subunit [32]. I-2 is known to be heat stable and this property was utilized to determine its activity. As shown in Fig. 4D, heat-treated extracts from TG heart inhibited recombinant phosphatase activity (+1 mM MnCl2) more effectively than those from WT (IC50: 13.5 ± 2.7 ng for TG vs 158.3 ± 27.4 ng for WT), consistent with the high level I-2140 expression as detected by immunoblotting (Fig. 1C, D). These results indicate that the truncated I-2140 is functionally active in vitro and most likely also in vivo.

In order to dissect the relative contribution of the activities of PP1 and PP2A, okadaic acid dose responses were performed on extracts from TG and WT (Fig. 5A). Okadaic acid at 0.1 nM concentration inhibited phosphatase activity in both samples by ~25% and complete inhibition was attained at 1 μM. However, at concentrations of the toxin of 10 nM and 100 nM, phosphatase activity was more strongly reduced in the extracts of TG mice (Fig. 5B). Since okadaic acid is a more potent inhibitor of PP2A than of PP1 activity and I-2140 overexpression specifically inhibits PP1 activity, these results indicate that in the extracts from TG, there is a higher contribution of PP2A to total phosphatase activity, which may also reflect a functional predominance of PP2A activity versus PP1 activity in TG mice.

3.4. Enhanced basal cardiac contractility in catheterized TG mice

Evaluation of in vivo contractile parameters using catheterization revealed an enhanced maximal left ventricular pressure in TG (by 19%, Fig. 6A). In addition, under basal conditions, the maximum rate of pressure development (+dP/dt) and the maximum rate of relaxation (−dP/dt) were increased by 32% and 41%, respectively, in TG compared to WT mice (Table 1). Following maximum β-AR stimulation by dobutamine, the cardiac contractile parameters were comparable in the two groups of mice (Fig. 6A and Table 1), consistent with the maximal response to the pharmacological agent. However, at lower doses (~EC50) contractility was significantly higher in the TG as compared to the WT. Importantly, the heart rate in response to dobutamine administration was not different between TG and WT (Fig. 6B). This is noteworthy, as changes in intrinsic heart rate will affect force of contraction, which would complicate the interpretation of the results. Although the total amount of PLB protein was unchanged between TG and WT animals (Fig. 7), expression of I-2140 resulted in ~2.6-fold increased phosphorylation of PLB at Ser16, with no changes in the phosphorylation at Thr17 (Fig. 7).
This result is consistent with overexpression of PP1c in heart, which caused decreased phosphorylation only at Ser\(^{16}\) [2].

3.5. Increased shortening, \(\text{Ca}^{2+}\) transients and SR \(\text{Ca}^{2+}\) load in TG cardiomyocytes

To test whether the enhanced basal contractility in catheterized mice corresponds to similar alterations in the cardiomyocytes, we measured simultaneously cell shortening and \(\text{Ca}^{2+}\) transients in isolated cardiomyocytes. The cell shortening was determined at a stimulation rate of 0.5 Hz as the difference between diastolic (maximum) and systolic (minimum) length of the cardiomyocyte. The shortening was increased by 77% in the TG (Table 2) and was accompanied by an accelerated relengthening. The time to 90% relengthening (i.e., mechanical relaxation) was decreased by 33% in TG (Table 2). The increased contraction of the TG cardiomyocytes was associated with a 50% higher \(\Delta[\text{Ca}]_i\) amplitude (Table 2). Caffeine-triggered \(\Delta[\text{Ca}]_i\) amplitude was enhanced by 61\(\%\)\((n = 10–11, p < 0.05)\) in TG compared to WT, suggesting an enhanced SR \(\text{Ca}^{2+}\) load in TG (Fig. 8). The time constant of exponential decline of caffeine-induced \([\text{Ca}]_i\) was similar in both groups (3.4 ± 0.4 s in TG versus 2.8 ± 0.3 s in WT.

![Graph](image)

**Fig. 5.** Effect of okadaic acid on phosphatase activity. Phosphatase activity was determined in supernatants from WT and TG hearts incubated with increased concentrations of okadaic acid (A, B). Shown are the absolute values (pmol/min/mg) of phosphatase activity measured in supernatants of both groups (A) or phosphatase activity expressed as % of control (Ctr; B) which is the phosphatase activity in the absence of okadaic acid.

![Graph](image)

**Fig. 6.** LV hemodynamic parameters in response to \(\beta\)-AR stimulation. Left ventricular pressure (LVP, A) and heart rate (B) were measured in closed chest-anaesthetized WT and TG mice by cardiac catheterization. Contractile parameters were determined under basal conditions (Ctr) and in response to increased dobutamine administration.

**Table 1**

<table>
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<tr>
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<th>WT</th>
<th>TG</th>
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<tr>
<td>(+dP/dt) (mmHg/s)</td>
<td>5791.7 ± 436.5</td>
<td>7634.2 ± 382.9</td>
</tr>
<tr>
<td>(+dP/dt) (mmHg/s)+dobutamine</td>
<td>15,109.4 ± 1096.9</td>
<td>16,516.2 ± 1071.1</td>
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<tr>
<td>(-dP/dt) (mm Hg/s)</td>
<td>5480.7 ± 371.1</td>
<td>7712.8 ± 688.6</td>
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<tr>
<td>(-dP/dt) (mm Hg/s)+dobutamine</td>
<td>11,590.2 ± 344.4</td>
<td>12,380.6 ± 933.5</td>
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The contractile parameters of closed chest-anaesthetized WT and TG male mice of 4 months of age were studied by cardiac catheterization. Contractile parameters were determined under basal and maximum \(\beta\)-AR stimulation (+dobutamine; 458 ng/g body weight). \(+dP/dt\) indicates the maximum rate of left ventricular pressure development and \(-dP/dt\) is the maximum rate of left ventricular pressure decline. The indicated values are mean ± S.E.M. (\(n = \text{mice}\)). *\(p < 0.05\) vs. WT.
\( n = 10 - 11, \text{n.s.} \), suggesting an unchanged function of the Na\(^+\)–Ca\(^{2+}\) exchanger between the two groups.

### 4. Discussion

The most important finding of the present study is that inhibition of PP1 activity by I-2140 expression can increase contractility in the heart. The transgenic mouse hearts exhibited an 80% reduction in phosphatase activity. These results are consistent with in vitro studies demonstrating that the inhibitory function of I-2 resided in the NH\(_2\)-terminal region of the protein \[29,30\]. Interestingly, although PP1 activity was reduced, the PP1c protein level was upregulated by \( \approx 7\)-fold. Because of the unchanged mRNA level of PP1c in TG, the increased PP1c protein level most likely is due to stabilization by the overexpressed I-2. PP1c is not present in the cell in the free form and, probably, excess free PP1c is rapidly degraded. Therefore, overexpression of regulatory components that associate with the catalytic subunit may protect it from degradation. Indeed, overexpression or gene knockout of the striated muscle-specific PP1-binding RGL/GM \[32–34\] also resulted in increased or decreased levels of PP1c protein, respectively. Importantly, the stabilization of PP1c by I-2140 indicates that the truncated form interacts with PP1c, consistent with in vitro studies \[29,30\]. Since the complex formed with the COOH-terminally truncated I-2 cannot be reactivated by phosphorylation of the I-2 at Thr\(^{72}\).

<table>
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<th>Table 2</th>
<th>Contractile parameters and Ca(^{2+}) transients of isolated cardiomyocytes</th>
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<tr>
<td></td>
<td>WT</td>
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<td>( n )</td>
<td>15</td>
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**Contractile parameters**

- Cell shortening (\( \mu \text{m} \)) \( 1.34 \pm 0.16^* \) \( 2.37 \pm 0.46^* \)
- Time of 90% relaxation (ms) \( 363.5 \pm 39.9 \) \( 245.1 \pm 31.3^* \)

**Ca\(^{2+}\) transients**

- \( \Delta[\text{Ca}] \) amplitude \( 0.08 \pm 0.01 \) \( 0.12 \pm 0.01^* \)

The contractile measurements were performed by edge detection. Isolated myocytes were paced at 0.5 Hz. Cell shortening was calculated as the difference between maximum diastolic and minimum systolic cardiomyocyte length. Ca\(^{2+}\) transients were determined as the Indo-1 fluorescence ratio at 405 and 495 nm in isolated ventricular cardiomyocytes from WT and TG mice. \( \Delta[\text{Ca}] \) amplitude indicates the difference between systolic (maximum) and diastolic (minimum) Indo-1 ratio during electrical stimulation. Values represent the mean \( \pm \) S.E.M. \( (n = \text{cardiomyocytes}) \).

* \( p < 0.05 \) vs. WT.

### Fig. 7. Phosphorylation state of PLB.

Figure shows representative immunoblots (upper panel) and the quantitation of the expression level of PLB and its phosphorylated forms (lower panel). Total PLB (A1), PLB phosphorylated at Ser\(^{16}\) (P-Ser16) or at Thr\(^{17}\) (P-Thr17) were identified with specific antibodies as described in Methods.

\( \ast \ p < 0.05 \) vs. WT

### Fig. 8. Effects of caffeine on Ca\(^{2+}\) transients.

Ca\(^{2+}\) transients were recorded in response to 10 mM caffeine in WT and TG. Cardiomyocytes were stimulated at 0.5 Hz, then stimulation was stopped for 15 s and caffeine was applied for 1 min on quiescent cells. The maximum amplitude of caffeine-induced Indo-1 ratio in WT was set as 100%.
most likely I-2^{140} acts as a constitutively active inhibitor. The decreased phosphatase activity, despite upregulation of the PP1c, can be explained by the higher level of expression of I-2^{140} (40-fold) that may also inhibit other, but not all forms, of PP1 in the cell [3]. Interestingly, very high protein levels were also observed in TG hearts overexpressing a constitutively active I-1 [31], which elicit significant effects on cardiac function. These studies indicate that inhibitors or targeting components may affect distinct subsets of phosphatase holoenzymes directed to different intracellular substrates, and therefore, cellular functions.

The conclusion that contractility was increased is substantiated by the following results. (i) Overexpression of I-2^{140} was accompanied by elevated basal tension and by an augmented rate of tension development, (ii) the rate of mechanical relaxation was elevated, and (iii) enhanced mechanical function was also observed in isolated cardiomyocytes. The basis for the improved contractility is probably due to increased Ca\(^{2+}\) transients. All these alterations can be explained by the decrease in PP1 activity in this model. The decreased PP1 activity resulted in elevated phosphorylation of PLB at Ser\(^{16}\) but not at Thr\(^{17}\). In vitro, PP1 accounts for ~70% of the phosphatase activity towards both PLB Ser\(^{16}\) and Thr\(^{17}\) [35]. However, PP1c overexpression in heart elicited decreased phosphorylation only at Ser\(^{16}\) [2]. The I-2^{140} and the PP1c models suggest that either PP1 preferentially acts on PLB-Ser\(^{16}\) or that I-2^{140} inhibits only the PP1 holoenzyme responsible for dephosphorylation of Ser\(^{16}\). Regardless, Ser\(^{16}\) phosphorylation appears to be sufficient to mediate maximal response to \(\beta\)-agonists [36] by relieving the inhibition of SERCA2a. Thus, the elevated phosphorylation of PLB at Ser\(^{16}\) should promote Ca\(^{2+}\) transport into the SR and hence cardiac relaxation. Indeed, the SR Ca\(^{2+}\) content was increased in TG hearts. Moreover, the rate of relaxation was enhanced in anasthetized TG mice corresponding with a reduced time of relengthening in isolated cardiomyocytes. The apparent discrepancy of the extent of improved relaxation in TG can be explained by the different physiological conditions of the experimental techniques used. The higher SR Ca\(^{2+}\) load led to higher Ca\(^{2+}\) transients, activation of myofilaments and subsequent increased mechanical contraction in intact hearts and isolated cardiomyocytes of TG mice. Hence, in this model, we have described a consistent chain of events in which I-2^{140} expression lowers PP1 activity causing higher PLB phosphorylation at Ser\(^{16}\), which results in enhanced contractility and relaxation.

These results are consistent with the findings in other reported animal models in which PP1 activity was altered. Overexpression of PP1c led to a 3-fold increase in PP1 activity, which was associated with decreased PLB phosphorylation at Ser\(^{16}\) and depressed contractility as determined by the blunted response to \(\beta\)-AR stimulation and by reduced fractional shortening measured by echocardiography [2]. Deletion of I-1 increased PP1 activity, decreased force development and mechanical relaxation, and reduced the response to \(\beta\)-AR stimulation in isolated hearts and in vivo [2]. Likewise, this impairment of contractility can be explained by the observed decrease in the phosphorylation state of PLB [2]. Conversely, overexpression of a constitutively active I-1 resulted in decreased phosphatase activity, increased PLB phosphorylation and enhanced contractility [31]. Moreover, our findings are consistent with a recent study in which PKCo-deficient mice exhibited an enhanced contractility, reduced PP1 activity resulting from decreased I-1 potency, and higher PLB phosphorylation at Ser\(^{16}\) [37]. Interestingly, deletion of PKC rescued the PP1c TG phenotype. Conversely, overexpression of PKCo was associated with a reduced contractility, increased PP1 activity, and lower PLB-Ser\(^{16}\) phosphorylation.

A role of PP1 activity in cardiac contractility is also supported by previous work using cell membrane permeant organic inhibitors of PP activity such as okadaic acid and cantharidin. Treatment of isolated heart preparation with these compounds induced a positive inotropic effect [24]. Moreover, cantharidin was shown to raise the force of contraction in preparations from failing human hearts [38]. This effect was accompanied by accelerated relaxation. Under the same conditions, PLB phosphorylation and free intracellular Ca\(^{2+}\) were enhanced, which could explain the higher contractility [39,40]. Additional evidence for a role of PP1 in the control of cardiac function comes from the observations that PP1 activity is increased [13] and the level and phosphorylation state of I-1 is decreased [41] in human failing hearts. In fact, the increase in PP1 activity could be due either to enhanced expression of PP1c, to decreased expression of I-1 [41,42], to reduced phosphorylation and hence diminished inhibitory action of I-1 [2] or to a combination of all three mechanisms. It has been speculated that, in heart failure, I-1 might be dephosphorylated by PP2B, because PP2B expression and activity is upregulated under this pathological condition [43]. Interestingly, overexpression of PP2B in mouse hearts resulted in enhanced Ca\(^{2+}\) transients and cell shortening [44]. This is consistent with higher SERCA2a protein expression. The increased PLB phosphorylation at Ser\(^{16}\) was unexpected since PP2B should cause activation of PP1 [45]. This finding leads to the suggestion that the SR associated PP1 in myocardium may not be controlled by PP2B, a hypothesis supported by the fact that cardiac-specific overexpression of protein inhibitors of PP2B like CAIN, MCIP or AKAP79 did not alter contractile function [46,47].

Regardless of the underlying mechanism for higher PP1 activity, in isolated electrically-driven cells from failing human hearts, the effect of \(\beta\)-AR stimulation on cell shortening and Ca\(^{2+}\) transients was enhanced after infection of cells with an adenovirus that codes for an active mutant of I-1 [2]. In addition, delivery of I-1 adenovirus also restored cardiac function in a model of heart failure [31]. Hence, inhibition of PP1 activity can restore impaired cardiac contractility, underscoring the importance of PP1 in control
of heart function. The fact that PP1 activity is elevated in the failing human heart and that phosphorylation of proteins, that are involved in regulation of contractile behavior, is reduced raises the possibility that enhanced PP1 activity may be detrimental for cardiac performance. A negative role of PP1 activity is consistent with the observation that PP1c overexpression in the heart leads to impaired contractility, cardiac hypertrophy and increased mortality after 6 months of age [2]. Therefore, pharmacological inhibition of PP1 activity may provide a therapy for the treatment of heart failure. Inotropic drugs that inhibit PP1 activity could have two advantages in the treatment of heart failure as compared to β-AR agonists. First, while the response to β-AR stimuli in failing hearts is blunted, the response to phosphatase inhibitors is not attenuated but somewhat accentuated in preparations from failing human hearts [38]. Second, PP1 inhibitors do not elevate cAMP content in cardiomyocytes, as β-AR agonists do [24]. An increase in cAMP is known to lead to cardiac ventricular arrhythmias, a potentially fatal side effect of β-AR stimulation [48].

In summary, heart specific overexpression of I-2 140 augmented basal cardiac contractility. Hence, this work provides a novel transgenic mouse model that highlights the importance of PP1 activity in the regulation of cardiac contractility and the potential pathophysiological role of the enzyme in human heart failure. Inhibition of PP1 specifically in heart may represent a target for therapeutic interventions.

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