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Reduced inhibitor 1 and 2 activity is associated with increased protein phosphatase type 1 activity in left ventricular myocardium of one-kidney, one-clip hypertensive rats

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Abstract

In failing hearts, although protein phosphatase type 1 (PP1) activity has increased, information about the regulation and status of PP1 inhibitor-1 (INH-1) and inhibitor-2 (INH-2) is limited. In this study, we examined activity and protein expression of PP1, INH-1 and INH-2 and phosphorylation of sarcoplasmic reticulum (SR) phospholamban (PLB), a substrate of PP1 and modulator of SR Ca^{2+}-ATPase activity, in failing and non-failing hearts. These studies were performed in LV myocardium of seven rats with chronic renal hypertension produced by Goldblatt's one-kidney, one-clip procedure and seven age-matched sham-operated normal controls (CTR). Eight weeks after surgery, LV ejection fraction, LV hypertrophy, and pulmonary congestion were determined in all rats. PP1 activity (nmol ^{32}P/min/mg non-collagen protein) was assessed in LV homogenates using ^{32}P-labeled phosphorylase a as substrate. INH-1 and INH-2 activity was determined in the immunoprecipitate of LV homogenates and expressed as percentage inhibitory activity. Using a specific antibody, LV tissue levels of PP1C and calsequestrin (CSQ), a SR calcium binding protein, which is not altered in failing hearts, were also determined. Further, total and phosphorylated PLB, INH-1 and INH-2 protein levels were determined in the LV homogenate and phosphoprotein-enriched fraction, respectively. The band density of each protein was quantified in densitometric units and normalized to CSQ. *Results*: rats with chronic renal hypertension exhibited significantly reduced LV ejection fraction and increased LV hypertrophy and pulmonary congestion, characteristics of chronic heart failure (CHF). We found that compared to CTR, (1) both INH-1 (10.2 ± 2 versus 57.5 ± 1; $p < 0.05$) and INH-2 activity (3.8 ± 0.4 versus 36.2 ± 4; $p < 0.05$) were reduced, (2) total and phosphorylated PLB amount reduced, (3) protein level of phosphorylated INH-1 was reduced (2.32 ± 0.1 versus 0.73 ± 0.04; $p < 0.05$) whereas that of phosphorylated INH-2 increased (3.05 ± 0.3 versus 1.42 ± 0.1; $p < 0.05$), and (4) PP1 activity was increased approximately 2.6-fold in rats with CHF (1.59 ± 0.05 versus 0.61 ± 0.01; $p < 0.05$) while protein level of the catalytic subunit of PP1 (PP1C) increased 3.85-fold (0.77 ± 0.05 versus 0.20 ± 0.02; $p < 0.05$). These results suggest that reduced inhibitory INH-1 and INH-2 activity, increased PP1C protein level, and reduced PLB phosphorylation are associated with increased PP1 activity in failing hearts. (Mol Cell Biochem **269:** 49–57, 2005)

Key words: heart failure, protein phosphatases, inhibitor-1, inhibitor-2, phospholamban, protein phosphorylation

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Introduction

Chronic heart failure (CHF) is associated with various signaling pathways leading to multiple abnormalities, including abnormal intracellular Ca^{2+} homeostasis, cardiac hypertrophy, apoptosis, fibrosis, and contractile dysfunction [1, 2]. Some of these cellular maladaptations might be due to activation of serine/threonine protein phosphatase (Ser/Thr PP) [3, 4]. In the normal heart, four different subtypes of Ser/Thr PP are present: type 1 (PP1), type 2A (PP2A), type 2B (also known as calcineurin or calcium /calmodulin-dependent PP), and type 2C (PP2C) [3]. Though all four known phosphatase subtypes have been extracted from cardiac tissue, purified to homogeneity and characterized [3], the physiological role of all but calcineurin [5–7] is poorly known in normal or failing hearts. Numerous studies have suggested that PP1 activity is inhibited *in vitro* by isolated heat-stable small molecular weight proteins called inhibitor 1 (INH-1) and inhibitor 2 (INH-2) [3]. These inhibitors were not formerly recognized to be present in cardiac tissue; indeed one study suggested that INH-1 is not present in cardiac tissue [8]. We were the first to demonstrate that INH-1 is not only present in cardiac tissue but also hormonally regulated [9–11]. Although earlier biochemical characterization studies suggested that potency of the isolated INH-1 (but not INH-2) to inhibit PP1 activity increases when it has been phosphorylated by cAMP-dependent protein kinase [3], their roles on PP1 activity *in vivo* is poorly understood. Recently, we demonstrated that acute treatment of the heart or cardiomyocytes with isoproterenol, a β-adrenoceptor agonist, led to inhibition of PP1 activity and this inhibition correlated with phosphorylation of INH-1 [12]. It has been known for some time that phosphorylation of INH-2 by glycogen synthase kinase-3α and 3β leads to activation of PP1 activity [13]. Though several investigators (including us) have reported increased PP1 activity and expression in experimental and human failing hearts [14–17], knowledge of the status and regulation of the PP1 inhibitors INH-1 and INH-2 is limited. Few studies have reported reduced phosphorylation of INH-1 in failing hearts [18, 19], whereas one study reported increased phosphorylation of INH-1 in transgenic mouse heart overexpressing calcium/phospholipid-dependent protein kinase Cα (PKCα) [20]. Recently we reported reduced INH-1 (but not INH-2) associated with the sarcoplasmic reticulum (SR) in LV myocardium of dogs with chronic HF produced by intracoronary microembolizations [21].

Using Goldblatt's one-kidney, one-clip (1K1C) procedure, we observed reduced SR Ca^{2+} uptake and decreased SERCA 2a affinity for calcium in LV myocardium of rats with CHF [22]. Reduced LV SR Ca^{2+} uptake in this rat model could be due to reduced phosphorylation of phospholamban (PLB); if so, that would suggest increased PP1 activity, because activity of cAMP-dependent protein kinase or Ca^{2+}/calmodulin-dependent protein kinase, which phosphorylates PLB, is either not changed or controversial in failing hearts [16, 17, 23]. Using the 1K1C procedure, we assessed *phosphorylation of PLB (PLB-P)*, activity and expression of PP1, INH-1 and INH-2 in LV myocardium of rats with CHF and age-matched sham-operated controls (CTR). Our results suggest that *reduced PLB-P*, INH-1 and INH-2 activity, and increased PP1C protein are associated with increased PP1 activity in rats with CHF due to chronic renal hypertension.

Materials and methods

Materials

Antibodies specific for PP1C and INH-2 were purchased from Transduction Laboratories, and an antibody specific for CSQ was obtained from Affinity Bioreagents. Chemicals and supplies for electrophoresis and electrotransfer were purchased from Bio-Rad. Biochemical supplies were obtained from Sigma. A phosphoprotein enrichment kit was purchased from BD Bioscience.

Methods

Preparation of animals

Goldblatt's 1K1C rat model of systemic hypertension was used to produce CHF [22, 24]. The Institutional Animal Care and Use Committee of Henry Ford Hospital (IACUC) approved the study. Lewis rats ($n = 7$) weighing 180–220 g were anesthetized with pentobarbital (30–35 mg/kg i.p.). Under sterile conditions, the abdominal cavity was opened via a midline incision and the left and right renal arteries and veins exposed. One kidney, selected at random, was removed after arteriovenous ligation. A metal clip was placed on the opposite renal artery to produce 70–75% luminal stenosis. The abdominal cavity was closed with 3-O silk suture and the rat allowed to recover. Age-matched sham-operated controls ($n = 7$) underwent the same operation but without removing one kidney and clipping the other renal artery. Eight weeks after surgery and before killing the rats, LV end-systolic (LVESV) and LV end-diastolic volume (LVEDV) were determined using the area-length method as described previously [24]. LV ejection fraction (LVEF) was calculated as LVEF = (LVEDV-LVSV)/LVDV. A final body weight (kg) was taken; and with the rat still under general anesthesia, the chest was opened and the heart and lung rapidly removed and placed in ice-cold saline solution. The LV and lung were separated, weighed in grams, quickly frozen in liquid nitrogen, pulverized and stored at $-70\,^{\circ}$C until use. LV hypertrophy was determined by calculating the ratio of LV (g) to body weight (kg), and pulmonary congestion was determined by calculating the ratio of lung (g) to body weight (kg).

Preparation of homogenate and assay of PP1 activity

About 100 mg of frozen powdered LV tissue from each rat was used to prepare the LV homogenate at 4 °C. Specimens were thawed in 5-ml homogenization buffer consisting of 50 mM Tris-HCl (pH 7.4), 0.5 mM sodium ethylene diamine tetraacetic acid (EDTA, pH 7.0), 0.3 M sucrose, and protease inhibitors (0.8 mM benzamidine, 0.8 mg/l aprotinin, 0.8 mg/l leupeptin, and 0.4 μg/l antipain). Thawed tissue was homogenized and the resulting homogenate filtered through four layers of cheesecloth. The homogenate was divided into 200-μl aliquots, immediately frozen in liquid nitrogen, and stored at −70 °C until used. Using ^{32}P-labeled phosphorylase a as substrate, PP activity in the homogenate was determined in the absence and presence of 600-ng purified rabbit skeletal muscle INH-2, which is known to inhibit PP1 activity *in vitro* [3] although its role in cardiomyocytes or cardiac tissue *in vivo* in response to external stimuli is not clear. The assay was performed in a 50-μl aliquot consisting of 50 mM Tris-HCl (pH 7.4), 5 mM caffeine, 0.5 mM EGTA, 0.5 mM EDTA, 50 μM β-mercaptoethanol, 100 ng aprotinin (protease inhibitor), with or without 600 ng rabbit skeletal muscle purified INH-2, 2 μg LV homogenate (thawed from frozen), and 550 pmol ^{32}P-labeled phosphorylase a. The assay was initiated by adding the homogenate, and phosphatase activity was determined as described previously [21]. PP1 activity was calculated from INH-2-sensitive PP activity, deducting phosphatase activity with INH-2 from activity without INH-2. Activity was expressed as nmol ^{32}P released/min/mg noncollagen protein in each fraction as determined by Lowry's method [25].

Measurements of INH-1 and INH-2 activity

The assay was performed with the immunoprecipitate of the LV homogenate using a specific antibody. For immunoprecipitation, 200 μl aliquots of LV homogenate corresponding to 200 μg protein were mixed with 100 μl TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) and the resulting mixture was mixed by rotation for 1 h at 4 °C, then centrifuged at 12,000 × *g* for 10 min. The supernatant (350 μl) was mixed overnight with either 2.5 μl anti-INH-1, anti-INH-2, or nonimmune serum, after which 50 μl of 10% (wt/vol) formalin-fixed *Staphylococcus aureus* cells (SAC) containing protein A was added for 60 min at 4 °C. SAC was removed and the pellet resuspended in 100 μl TBS, then centrifuged. This procedure was repeated twice. The final pellet was resuspended in 100-μl 50 mM Tris-HCl buffer (pH 7.4) containing 0.25 mM EGTA and incubated in boiling water for 10 min followed by rapid cooling in ice water. After 10 min, the mixture was centrifuged at 12,000 × *g* for 10 min, the clear supernatant was collected and protein quantified by Bradford method [26]. An aliquot of the heat-treated supernatant was added to the protein phosphatase reaction mixture as described above, except that 0.2 μg of the rabbit skeletal muscle purified catalytic subunit of PP1 was added in place of the LV homogenate. The amount of the purified phosphatase used in the assay should be enough to hydrolyze approximately 30% of the total ^{32}P-labelled phosphorylase a present in the reaction. INH-1 or INH-2 activity was expressed as percentage inhibitory activity of PP1.

Western blot

PP1C, total INH-1 (INH-1), phosphorylated INH-1 (INH-1OP), INH-2 (INH-2), phosphorylated INH-2 (INH-2OP), *total PLB, or phosphorylated PLB protein level* was determined in LV homogenates by Western blots. For *phosphorylated PLB*, INH-1OP and INH-2OP, phosphoprotein-enriched fraction (PPE) from LV homogenate was separated using a BD Bioscience phosphoprotein enrichment kit according to the supplier's instructions. SDS extracts were prepared from the LV homogenate and isolated PPE. Using the SDS-extract, Western blots were performed as described previously [21]. Calsequestrin (CSQ), a calcium-binding protein located in the SR and reportedly unchanged in failing hearts [27, 28], was also quantitated in each sample to normalise protein loading on the gel. Equal volumes of the SDS extract and sample buffer [62.5 mM Tris-HCl (pH 6.8); 20% glycerol, 40 mM DTT, and 0.001% bromophenol blue] were combined and the resulting mixture incubated in boiling water for 10 min. An aliquot of the boiled mixture was subjected to electrophoresis on 4–20% SDS-polyacrylamide gel (Bio-Rad) and the separated proteins transferred to a nitrocellulose membrane [21]. The accuracy of the electrotransfer was confirmed by staining the membrane with 0.1% amido black. For the immunoreaction, the nitrocellulose blot was incubated with diluted primary antibody (monoclonal or polyclonal) based on the supplier's instructions. Antibody-binding protein(s) was visualized by autoradiography after treating the blot with horseradish peroxidase-conjugated secondary antibody (anti-rabbit) and ECL color developing reagents according to the supplier (Amersham). Band intensity was quantified using a Bio-Rad GS-670 imaging densitometer and expressed as densitometric units x mm^2. In all cases we made sure the antibody was present in excess over the antigen and the density of each protein band was in the linear scale.

Miscellaneous methods

The catalytic subunits of PP2A and PP1, INH-1, and INH-2 were isolated from rabbit skeletal muscle, and INH-1 and INH-2 were also purified from dog hearts [21]. The catalytic subunit of cAMP-dependent protein kinase was purified from bovine hearts [9]. A polyclonal antibody was raised against the rabbit skeletal muscle purified INH-1 according to the standard protocol [21]. The antibody interacted with INH-1 but not INH-2 (data not shown).

Statistical analysis

Data are mean ± SEM. Comparisons between sham-operated controls and rats with CHF were based on Student's *t*-test for two means (unpaired *t*-test), taking $p < 0.05$ as significant. The sample size used in this study of seven rats with CHF and seven CTR was based on 80% power to detect a large difference at $\alpha = 0.05$.

Results

Characterization of rats with CHF

Figure 1 shows LV hypertrophy, LVEF, and pulmonary congestion in rats with CHF and CTR. Increased ratio of LV weight (LVW) to body weight (BW) was regarded as an index for LV hypertrophy. LVW/BW increased significantly in rats with CHF by 8 weeks after surgery compared to CTR (3.84 ± 0.08 versus 2.02 ± 0.02; $p < 0.05$) (Fig 1, top). LVEF (%) was significantly reduced in rats with CHF (47 ± 2 versus 74 ± 2; $p < 0.05$) (Fig. 1, center), associated with significant decreases in LVDV (0.36 ± 0.02 versus 0.50 ± 0.03; $p < 0.05$) and LVSV (0.20 ± 0.02 versus 0.13 ± 0.01; $p < 0.05$). Increased ratio of lung weight (LW) to body weight (BW) was regarded as an index for pulmonary congestion. LW/BW increased about 27% by 8 weeks post-surgery (7.03 ± 0.15 versus 5.84 ± 0.09; $p < 0.05$) (Fig. 1, bottom), consistent with LV failure and pulmonary congestion in rats with CHF. We have used the same rat model of systemic hypertension for assessment of SR Ca^{2+} uptake during the development of LV hypertrophy, and the results have always been reproducible [22].

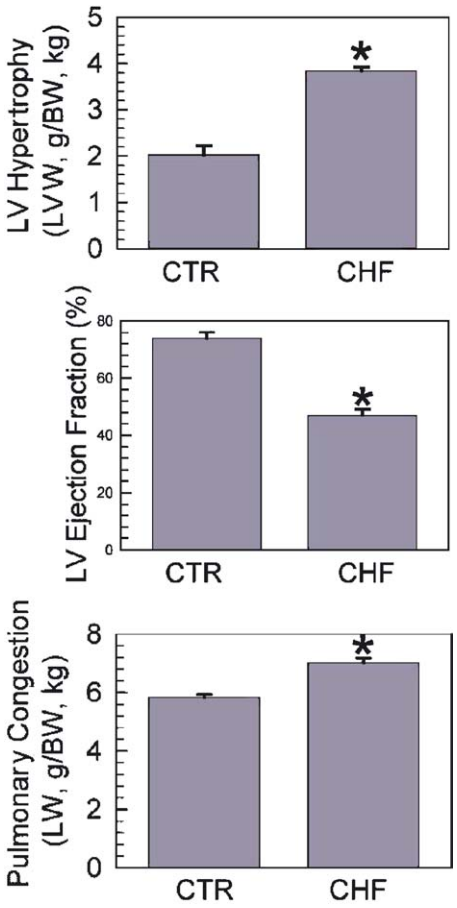

Fig. 1. Left ventricular (LV) hypertrophy (top), LV ejection fraction (center), and pulmonary congestion (bottom) in normal sham-operated controls (CTR) and rats with congestive heart failure (CHF). Values are mean ± SEM from seven different animals in each group; * $p < 0.05$ vs. CTR.

PP1 activity and PP1C protein level

Rat cardiac tissue contains several subtypes of Ser/Thr PP, including PP1 and PP2A [3]. To determine PP1 activity, we measured rabbit skeletal muscle purified INH-2 (a potent inhibitor of PP1) and found that approximately 600 ng was needed to completely inhibit the PP1 activity associated with the LV homogenate (data not shown). Figure 2 shows that PP1 activity in the homogenate (nmol ^{32}P/min/mg non-collagen protein) increased significantly by about 2.6-fold (1.59 ± 0.05 versus 0.61 ± 0.01; $p < 0.05$) in rats with CHF. To examine whether increased phosphatase activity in LV homogenates of rats with CHF is associated with increased expression of the PP1C, protein expression was measured by Western blot (Fig. 3). CSQ expression was also determined to normalise protein loading, since CSQ is not altered in failing hearts [22]. Both antibodies detected a single band in varying LV homogenate concentrations measuring 38 kDa for PP1C and 55 kDa for CSQ (Fig. 3, top). When PP1C and CSQ were quantitated in approximately 5 μg of LV homogenate from rats with CHF, PP1C increased while CSQ remained unchanged (Fig. 3, center). Densitometric and

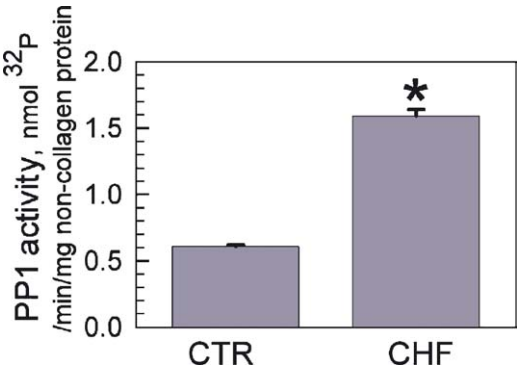

Fig. 2. Protein phosphatase type 1 (PP1) activity in LV homogenates from CTR and rats with CHF. Values are mean ± SEM from seven different animals in each group; * $p < 0.05$ vs. CTR.

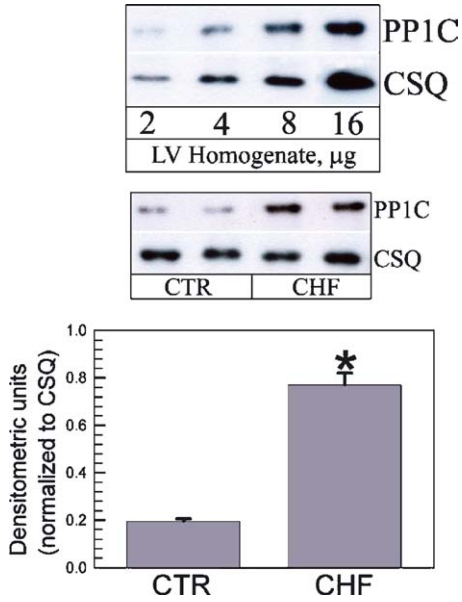

Fig. 3. Top: Western blot of CTR shows immunodetectable levels of the catalytic subunit of protein phosphatase type 1 (PP1C) and calsequestrin (CSQ) in varying amounts of the LV homogenate (2–16 μg). Center: Western blot shows immunodetectable levels of PP1C and CSQ in LV homogenates from two CTR and two rats with CHF. Bottom: densitometric analysis of PP1C normalized to CSQ in LV tissue of seven CTR and seven rats with CHF; $^{*}p < 0.05$ vs. CTR.

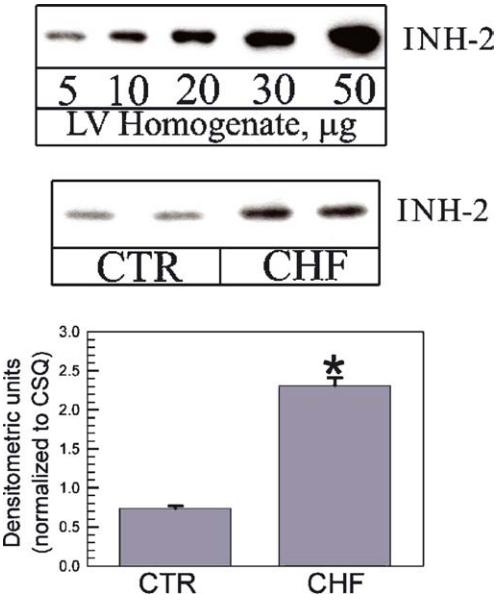

Fig. 4. Top: Western blot shows immunodetectable levels of total inhibitor-2 (INH-2) in varying amounts of LV homogenates (5–50 μg) from CTR. Center: Western blot shows immunodetectable levels of INH-2 in LV homogenates from two CTR and two rats with CHF. Bottom: densitometric analysis of INH-2 normalized to CSQ in LV tissue of seven CTR and seven CHF rats; $^{*}p < 0.05$ vs. CTR.

statistical analysis of PP1C normalized to CSQ showed about a 3.85-fold increase in rats with CHF (0.77 ± 0.05 versus 0.20 ± 0.02; $p < 0.05$) (Fig. 3, bottom).

Activity and total and phosphorylated INH-1 and INH-2 protein levels

Using an INH-2 specific antibody, a predominant 32-kDa protein band was recognized in the SDS extract of the LV homogenate and PPE fraction (Figs. 4 and 5). Band density increased with increasing LV homogenate (Fig. 4, top) or PPE fraction (data not shown) amount. Quantification of protein levels in 10 μg of LV homogenate and 20 μg of PPE fraction, respectively, showed that both INH-2 (Fig. 4, center) and INH-2OP (Fig. 5, center) were increased in the rats with CHF. Densitometric and statistical analysis of the INH-2 (2.31 ± 0.10 versus 0.73 ± 0.04; $p < 0.05$) and INH-2OP bands normalized to CSQ (1.16 ± 0.10 versus 0.60 ± 0.05; $p < 0.05$) was increased about 3.16- and 1.93-fold, respectively, in rats with CHF. Using an INH-1 specific antibody, a predominant 28-kDa protein band was recognized in the SDS extract of the LV homogenate and PPE fraction (Figs. 6 and 7). INH-1 band density increased with increasing LV homogenate (Fig. 6, top) or PPE fraction (Fig. 7, top) amount. When INH-1 and INH-1OP levels were quantified in 10 μg of LV homogenate and 20 μg phosphoprotein-enriched

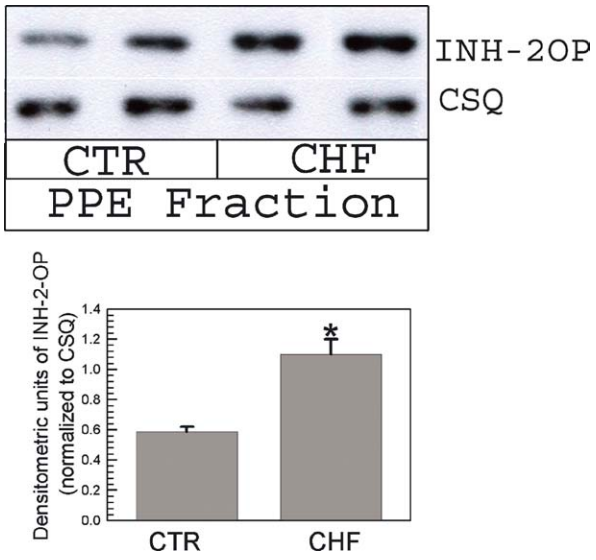

Fig. 5. Top: Western blot shows immunodetectable levels of phosphorylated inhibitor-2 (INH-2OP) and calsequestrin (CSQ) in 20 μg of the phosphoprotein-enriched (PPE) fraction from two CTR and two rats with CHF. Bottom: densitometric analysis of INH-2OP normalized to CSQ in LV tissue from seven CTR and seven rats with CHF; $^{*}p < 0.05$ vs. CTR.

fraction, respectively, INH-1 protein levels increased in the rats with CHF (Fig. 6, center) whereas INH-1OP protein levels was decreased (Fig. 7, center). Densitometric and statistical analysis of INH-1 normalised to CSQ showed

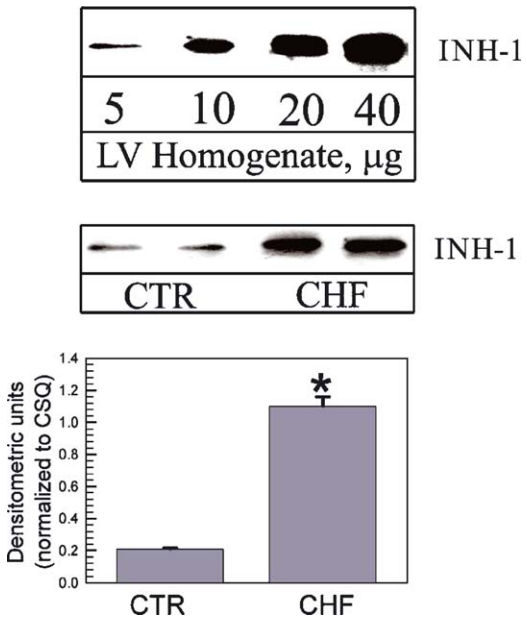

Fig. 6. Top: Western blot of CTR shows immunodetectable levels of total inhibitor-1 (INH-1) in varying amounts of the LV homogenate (5–40 μg). Center: Western blot shows immunodetectable levels of INH-1 in LV homogenates of two CTR and two rats with CHF. Bottom: densitometric analysis of INH-1 normalized to CSQ in LV tissue of seven CTR and seven rats with CHF; $^*p < 0.05$ vs. CTR.

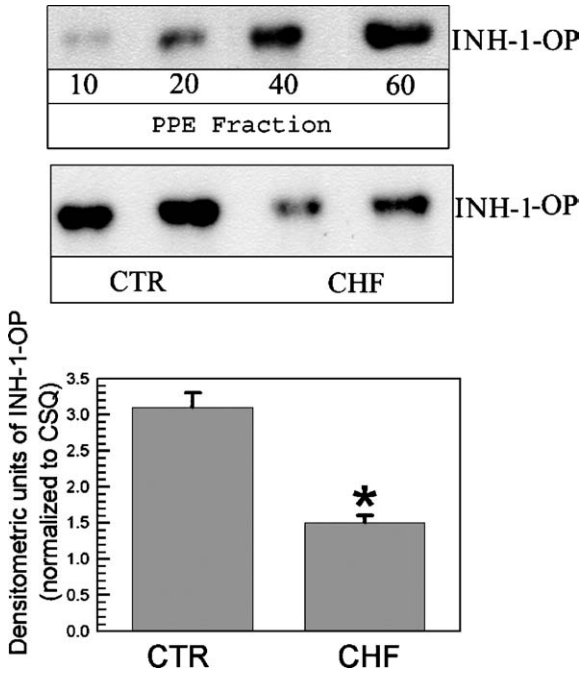

Fig. 7. Top: Western blot shows immunodetectable levels of phosphorylated inhibitor-1 (INH-1-OP) in varying amounts of the phosphoprotein-enriched (PPE) fraction (10–60 μg) from CTR. Center: Western blot shows immunodetectable levels of INH-1-OP in 20 μg of PPE fraction from two CTR and two rats with CHF. Densitometric analysis of INH-1-OP normalized to CSQ in LV tissue from seven CTR and seven CHF rats; $^*p < 0.05$ vs. CTR.

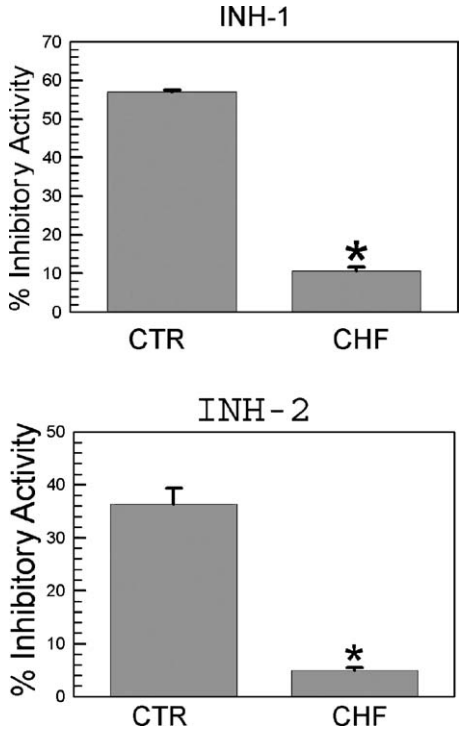

Fig. 8. Activity of Inhibitor-1 (INH-1, top) and inhibitor 2 (INH-2, bottom) in LV homogenates from CTR and rats with CHF. $^*p < 0.05$ vs. CTR ($n = 7$) was considered significant.

a significant increase in rats with CHF (1.1 ± 0.06 versus 0.21 ± 0.01; $p < 0.05$) whereas INH-1OP decreased (3.05 ± 0.3 versus 1.42 ± 0.1; $p < 0.05$). Using a specific antibody, INH-1 and INH-2 activity was determined in the immunoprecipitate of each LV homogenate. Control INH-1 (Fig. 8, top) and INH-2 (Fig. 8, bottom) inhibited PP1 activity by 57.5 ± 1 and $36.2 \pm 4\%$, respectively, in contrast to 10.2 ± 2 and $3.8 \pm 0.4\%$, respectively, in rats with CHF. These results suggest that potency of both inhibitors of PP1 activity is reduced in rats with CHF.

Total and phosphorylated PLB protein levels

Using PLB specific antibody, total PLB protein was quantitated in 5 μg of LV homogenate and phosphorylated PLB protein determined in 10 μg of PPE fraction. Total and phosphorylated PLB protein levels both reduced in LV tissue of rats with CHF compared to age-matched sham operated control; however, reduction was more pronounced in phosphorylated PLB (Fig. 9), suggesting PLB is dephosphorylated in failing heart.

Discussion

Our results demonstrate that PP1 activity is increased in the LV myocardium of rats with CHF. Regardless of the

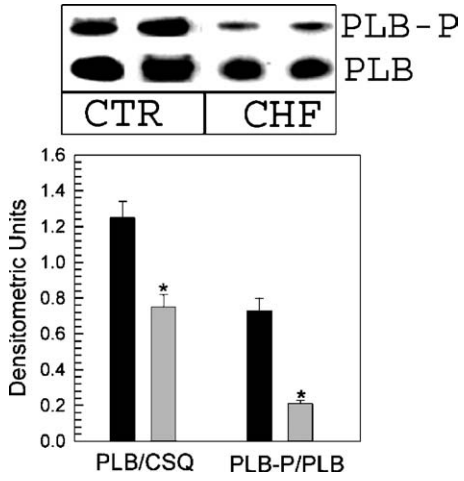

Fig. 9. Top: Western blot of immunodetectable levels of phosphorylated (PLB-P) and total PLB in LV of two rats with two CHF and control (CTR) rats. Using a specific antibody, PLB was determined in 5 μg of LV homogenate and PLB-P determined in 10 μg of PPE fraction prepared from LV homogenate from CTR and rats with CHF. Bottom: densitometric analysis of total PLB normalized to CSQ and PLB-P normalized to total PLB in LV tissue from seven CTR and seven CHF rats; $^{*}p < 0.05$ vs. CTR.

model of HF, recruitment of the sympathetic nervous system, renin-angiotensin-aldosterone system, endothelin, and induction of intracardiac growth factors represent early events associated with the progression of this disease, although they are initially adaptive. We chose Goldblatt's one-kidney, one-clip rat model of systemic hypertension because cardiomyopathy in this model is due to increased systolic wall stress that promotes a concentric pattern of hypertrophy [22, 29, 30]. After 8 weeks, these rats developed LV hypertrophy, reduced LVEF, and increased pulmonary congestion. Using LV tissue from this CHF model, we further demonstrated that increased PP1 activity is associated with increased tissue PP1C protein expression, *reduced PLB-P*, and reduced potency of INH-1 and INH-2 to inhibit PP1 activity. The latter abnormalities are due to reduced phosphorylation of INH-1 but increased phosphorylation of INH-2. Thus increased PP1 activity in failing hearts may cause dephosphorylation of several physiological regulatory proteins, *including PLB*, which control cardiac contractility, apoptosis, fibrosis, and contractile dysfunction, features characteristic of HF.

Reversible protein phosphorylation is an important mechanism that leads to cardiac hypertrophy and contractile dysfunction, salient features of CHF [1, 2, 31]. Several cardiac regulatory proteins have been found to undergo dephosphorylation, including (a) nuclear factor of activated T cells 3 and eukaryotic elongation factor-2 for hypertrophy, (b) slow L-type calcium channels, sodium-calcium exchanger, ryanodine-sensitive calcium release channels, and phospholamban for cardiac contractility, and (c) Bcl-2 and Bcl-2-associated death promoters for apoptosis [32–37]. The phosphorylation state of the cardiac contractility regulatory proteins that control in failing hearts has been studied to some extent. Since the phosphorylation state of any protein is determined by a balance between protein kinase and phosphatase activity, and since protein kinases that phosphorylate these regulatory proteins are not altered in failing hearts [23], the changes in phosphatase activity implicate altered phosphorylation of these proteins. In experimental and explanted human failed hearts, several studies (including ours) reported increased PP1 activity [14–17, 21]. In the present study, we clearly demonstrated that PP1 activity was increased significantly in LV tissue of rats with CHF. Using the same rat model, we have earlier reported reduced SR Ca^{2+} uptake and reduced SERCA 2a affinity for calcium, suggesting that these abnormalities may be partly due to enhanced dephosphorylation of PLB [22]. *In the present study, we observed also reduced PLB-P in failing hearts. Similar results have also been reported using other experimental HF model* [15, 21]. Recently, we reported that PP1 activity associated with the SR (consisting mostly of the longitudinal region of the membrane) is elevated in the LV myocardium of dogs with HF, and this abnormality is associated with hypophosphorylation of PLB in failing hearts [21, 38]. As far as we know, such studies have not been reported in the case of other cardiac contractility regulatory proteins, except for the ryanodine-sensitive calcium release channel, which is hyperphosphorylated in failing hearts [36].

We have only limited data on the molecular mechanisms for increase of PP1 activity in the failing heart. Increased enzyme activity could be partly due to increased protein expression of PP1C and/or altered protein level of INH-1 and INH-2, two potent inhibitors of PP1 activity, which are also regarded as regulatory subunits of this enzyme [3]. We reported in dogs with HF that INH-2 was associated with the LV SR but remained unchanged in failing hearts [21]. In the present study, we observed increased PP1C protein expression in rats with CHF. These findings are very similar to studies of LV tissue from failing hearts of experimental animals or patients with terminal HF [14–17, 21]. INH-1 protein normalised to CSQ in LV tissue was increased in rats with CHF, and this abnormality was associated with its reduced phosphorylation, suggesting that the capacity of INH-1 to inhibit PP1 activity is markedly reduced in failing hearts. Activity of the immunoprecipitated INH-1 was drastically impaired in rats with CHF. Earlier reports have suggested that INH-1 is capable of inhibiting PP1 activity as long as it remains in its phosphorylated state, but is unable to do so once it is dephosphorylated [3]. Our present findings on INH-1 phosphorylation state are very similar to recent reports of explanted failed human hearts [18, 19]. However, the present findings on INH-1 protein level contrast with previous reports in which INH-1 protein was reduced [18, 21]. The difference in INH-1 levels between rats versus human failing hearts could be partly due

to the fact that patients receive drug therapy, which may have a pronounced effect on protein expression in failing hearts.

A recent study concluded that ablation of INH-1 or overexpression of PP1Cα leading to increased PP1C activity caused the development of HF in transgenic mouse hearts, while adenoviral overexpression of constitutively active INH-1 was associated with salvage of β-adrenergic response in failing human cardiomyocytes [19]. Further, adenoviral INH-1 overexpression under CMV control in engineered heart tissue from neonatal rats enhanced both cell shortening and PLB phosphorylation [39], suggesting that a constitutively active form of INH-1 is capable of improving global LV function of failing hearts. In addition to its inhibitory effect on PP1 activity, recent study reported that INH-1 phosphorylation by a catalytic subunit of calcium/phospholipid-dependent protein kinase (PKCα) has a stimulatory effect [20]. In the same study, transgenic mouse hearts overexpressing PP1Cα exhibited reduced ventricular performance and PLB phosphorylation, but increased INH-1 phosphorylation. These studies suggest that INH-1 phosphorylation has a stimulatory or inhibitory effect depending upon as which amino acid in this protein is phosphorylated.

In contrast to INH-1, studies on the status of INH-2 in failing hearts are limited. In dogs with CHF produced by multiple intracoronary microembolizations, we reported that the amount of INH-2 associated with the SR of the LV myocardium was not different from controls [21]. Here, not only total amount of INH-2 but its phosphorylation and activity were determined. To our surprise, total amount and phosphorylation of INH-2 were increased in LV tissue of rats with CHF, but its activity, like that of INH-1, to inhibit PP1 activity was reduced. It has long been known that INH-2 isolated from normal rabbit skeletal muscle and dog and human failing hearts inhibits PP1 activity [3]. However, though its status in diseased hearts has not been reported to our knowledge, there are studies suggesting stimulation or attenuation of its inhibitory effects following phosphorylation by glycogen synthase kinase 3α or 3β, casein kinase II, and activated extracellular signal-regulated protein kinase 1 (ERK1) [13, 40]. Thus, it is quite possible that the observed reduced potency of INH-2 to inhibit PP1 activity is due to its increased phosphorylation by ERK1, which is reportedly increased in failing hearts [41].

In summary, we believe this is the first study to demonstrate that reduced potency of INH-1 and INH-2 activity *to inhibit PP1 activity, reduced PLB-P*, and increased PP1C protein level are associated with increased PP1 activity in failing rat hearts with cardiomyopathy induced by Goldblatt's 1K1C hypertension. Thus increased PP1 activity in failing hearts may be responsible for contractile dysfunction by dephosphorylating various regulatory proteins that control not only cardiac contractility but also apoptosis, hypertrophy, and fibrosis. Thus inhibition of PP1 activity provides a therapeutic target for developing drugs that can treat or at least slow the progression of CHF.

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