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ORIGINAL CONTRIBUTION

Differential regulation of protein phosphatase 1 (PP1) isoforms in human heart failure and atrial fibrillation

Stefanie Meyer-Roxlau[1] · Simon Lämmle[1] · Annett Opitz[1] · Stephan Künzel[1] · Julius P. Joos[1] · Stefan Neef[2] · Karolina Sekeres[1] · Samuel Sossalla[2] · Friedrich Schöndube[3] · Konstantin Alexiou[4] · Lars S. Maier[2] · Dobromir Dobrev[5] · Kaomei Guan[1] · Silvio Weber[1] · Ali El-Armouche[1]

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Abstract Protein phosphatase 1 (PP1) is a key regulator of important cardiac signaling pathways. Dysregulation of PP1 has been heavily implicated in cardiac dysfunctions. Accordingly, pharmacological targeting of PP1 activity is considered for therapeutic intervention in human cardiomyopathies. Recent evidence from animal models implicated previously unrecognized, isoform-specific activities of PP1 in the healthy and diseased heart. Therefore, this study examined the expression of the distinct PP1 isoforms PP1α, β, and γ in human heart failure (HF) and atrial fibrillation (AF) and addressed the consequences of β-adrenoceptor blocker (beta-blocker) therapy for HF patients with reduced ejection fraction on PP1 isoform expression. Using western blot analysis, we found greater abundance of PP1 isoforms α and γ but unaltered PP1β levels in left ventricular myocardial tissues from HF patients as compared to non-failing controls. However, expression of all three PP1 isoforms was higher in atrial appendages from patients with AF compared to patients with sinus rhythm. Moreover, we found that in human failing ventricles, beta-blocker therapy was associated with lower PP1α abundance and activity, as indicated by higher phosphorylation of the PP1α-specific substrate eIF2α. Greater eIF2α phosphorylation is a known repressor of protein translation, and accordingly, we found lower levels of the endoplasmic reticulum (ER) stress marker Grp78 in the very same samples. We propose that isoform-specific targeting of PP1α activity may be a novel and innovative therapeutic strategy for the treatment of human cardiac diseases by reducing ER stress conditions.

Keywords Protein phosphatase 1 (PP1) isoforms · Human heart failure · Atrial fibrillation · Beta-blocker · Endoplasmic reticulum stress response

The original version of this article was revised: The spelling of the author name Samuel Sossalla was incorrect.

Silvio Weber and Ali El-Armouche share senior authorship.

✉ Silvio Weber
Silvio.Weber@tu-dresden.de

✉ Ali El-Armouche
Ali.El-Armouche@tu-dresden.de

[1] Department of Pharmacology and Toxicology, Medical Faculty, Technische Universität Dresden, Fetscherstraße 74, Dresden 01307, Germany

[2] Department of Internal Medicine II, University Hospital Regensburg, Regensburg, Germany

[3] Department of Thoracic and Cardiovascular Surgery, University Medical Centre Goettingen, Göttingen, Germany

[4] Department of Heart Surgery, Dresden University of Technology, Dresden, Germany

[5] Institute of Pharmacology, West German Heart and Vascular Center, University Duisburg-Essen, Essen, Germany

Introduction

Protein phosphatase 1 (PP1) is the predominant dephosphorylating enzyme in the heart and accounts for up to 70% of the entire serine/threonine phosphatase activity [20]. Numerous studies have shown that PP1 dephosphorylates a huge subset of substrates in all compartments of the cardiomyocyte, including membrane, sarcoplasmic reticulum, and myofilament proteins, which work together in a finely tuned manner to enable sufficient excitation–contraction (EC) coupling [30]. Enhanced PP1 expression and activity along with decreased phosphorylation of several key calcium handling proteins [e.g., phospholamban (PLN), cardiac myosin binding protein-C (cMyBP-C), and L-type

Ca^{2+}-channel ($Ca_V1.2$)] has been shown in many cardiac pathologies including heart failure (HF) and atrial fibrillation (AF) [4, 10, 11, 23]. In mice, threefold heart-specific overexpression of PP1α was sufficient to induce severe cardiomyopathy accompanied by a significant decrease (69%) in the phosphorylation of PLN [3].

It is, therefore, not surprising that targeting of PP1 has been postulated as a potential strategy to improve HF phenotypes. However, the usually measured PP1 expression and activity is actually the sum of three different isoforms (α, β, and γ), which are encoded by distinct genes on different chromosomes [10]. Albeit the three isoforms share more than 90% sequence homology, they contain also divergent N- and C-termini with putatively unique interaction sites of substrate or phosphatase regulatory subunit [13]. A number of cell-based and extra-cardiac in vivo studies have demonstrated the isoform-specific dephosphorylation function of PP1. For example, PP1α or γ1, but not PP1β, interacts with neurabins and plays a key role in regulating synaptic transmission in mammalian neurons [27]. However, in skeletal muscle cells, PP1β interacts with the myosin phosphatase targeting subunit 2 (MYPT2) and enhances the dephosphorylation of skeletal muscle myosin [22].

In this study, we examined PP1 isoform expression in different human HF and AF samples using novel and validated PP1 isoform-specific antibodies. To our best knowledge, we are the first, who report selective abundance changes of different PP1 isoforms in human left ventricular (LV) tissues from patients with failing hearts and in right atrial appendage tissues from patients with AF. Interestingly, HF patients with beta-blocker pre-treatment showed lower PP1α abundance accompanied by greater phosphorylation of the PP1α-specific substrate eIF2α, which indicates an augmentation in the endoplasmic reticulum (ER) stress response in cardiomyocytes of HF patients [16].

Methods

Patients

The study conforms with the principles outlined in the Declaration of Helsinki and was reviewed and approved by the ethics committee of the University Hospital Carl Gustav Carus Dresden (Az.: EK 446122011 and EK 1140 82202 and 28/3/07) and the University Medical Center Goettingen (Az.: 31/9/00, this approval is also applicable for the acquisition of samples at the University Hospital Regensburg). Written informed consent was obtained from all participants. A detailed list of patients with brief clinical characteristics can be found in Tables 1, 2, and 3. Frozen non-failing myocardial samples originated from 16 healthy donor hearts that could not be transplanted for technical reasons.

Antibodies, recombinant proteins, and PP1 overexpression lysates

Antibodies Anti-calsequestrin (1:1000, Thermo Fisher Scientific, #PA1-913), anti-PP1α (1:200, Santa Cruz, C-19, Lot #: H0114), anti-PP1β (1:1000, Abcam, ab16369, Lot #: GR279250-3), anti-PP1γ1 (1:1000, Abcam, ab16387, Lot #: GR54077-10), anti-BIP (1:1000, Cell Signaling, #3183, Lot #: 3), anti-eIF2α (1:1000, Cell Signaling, #9722, Lot #: 15), anti-phospho-eIF2α (Ser51) (1:1000, Cell Signaling, #9721, Lot #: 15), and anti-IRE1α (1:1000, Cell Signaling, #3294, Lot #: 9). *Recombinant proteins* PP1α with N-terminal HIS tag (Origene, Cat.-Nr.: TP760285), PP1β with C-terminal Myc-DDK tag (Origene, Cat.-Nr.: TP301142), and PP1γ with N-terminal HIS tag (Origene, Cat.-Nr.: TP720571). *PP1 overexpression lysates* lysate of HEK293 cells transfected with Myc-DDK-tagged human PP1α (Origene, Cat.-Nr.: LY400954), PP1β (Origene, Cat.-Nr.: LY404146), and PP1γ (Origene, Cat.-Nr.: LY419153).

Cardiac tissue preparation, SDS-Gel electrophoresis and immunoblotting

Human cardiac tissues were rapidly frozen by nitrogen, pulverized and lysed in lysis buffer (30 mmol/L Tris/HCl pH 8.8, 5 mmol/L EDTA, 30 mmol/L NaF, and 3% SDS) supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche Diagnostics). The protein samples were separated by weight using SDS-polyacrylamide gel electrophoreses and subsequently transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk (AppliChem) for 1 h and afterwards incubated with the respective primary antibodies overnight at 4 °C. After repeated washing steps and incubation with appropriate secondary antibodies at room temperature for 1 h, chemiluminescence was detected using a Fusion FX imaging system (Vilber Lourmat) and quantified with the Fusion-Capt Advance (Vilber Lourmat) software.

Statistics

Results are presented as mean ± standard error of the mean (SEM). Data sets were compared by unpaired two-tailed Student's *t* test to assess differences between two groups. *P* values of <0.05 were considered as statistically significant.

Results and discussion

Due to the strong sequence homology of PP1 isoforms and multiple reports showing unspecific and isoform-independent binding of several PP1 isoform-specific antibodies, we

Table 1 Clinical characteristics of patients with HF (ICM, DCM) and non-failing controls (NF)

Patient ID #	Age	Gender	Diagnosis		Drugs	LVEF (%)
132	63	F	NF		n.d.	n.a.
90	57	F	NF		n.d.	n.a.
80	78	F	NF		n.d.	n.a.
120	78	M	NF		n.d.	n.a.
43	77	M	NF		n.d.	n.a.
67	77	M	NF		n.d.	n.a.
II/33	52	M	NF		n.d.	n.a.
2/8	36	M	NF		n.d.	n.a.
3/2	58	F	NF		n.d.	n.a.
5/2	25	F	NF		n.d.	n.a.
6/2	39	F	NF		n.d.	n.a.
11/2	42	M	NF		n.d.	n.a.
VI/2	42	F	NF		n.d.	n.a.
III/19	49	F	NF		n.d.	n.a.
IV/19	38	M	NF		n.d.	n.a.
1/22	38	M	NF		n.d.	n.a.
1974	60	M	HF	ICM	A, D, L, O, R, X	15
2023	57	M	HF	ICM	A, X	25
579	60	M	HF	DCM	A, D, G, L, X	15
682	45	M	HF	DCM	A, D, G, R, X	30
792	49	M	HF	DCM	A, D, G, X	25
815	48	M	HF	DCM	A, D, G, X	15
816	49	M	HF	DCM	A, D, G, X	35
1106	49	M	HF	ICM	A, D, G, N, X	15
581	65	F	HF	ICM	A, X, D, L	25
585	58	F	HF	ICM	A, X, D, L, R	20
680	55	F	HF	ICM	A, X, L, D	27
655	29	F	HF	DCM	A, X, G, D	15
611	59	F	HF	DCM	C, R, D	20
628	50	F	HF	DCM	A, X, D	21
671	60	M	HF	DCM	A, X, D, R	20
667	54	M	HF	DCM	C, R, D	14
662	51	M	HF	DCM	R, D	30
669	58	M	HF	ICM	A, X, L, D, G	20
642	52	M	HF	ICM	A, X, D, L	14
612	65	M	HF	ICM	A, X, D, L	20
704	55	F	HF	CM	A, D, R	45

NF non-failing donor, *HF* heart failure, *DCM* dilated cardiomyopathy, *ICM* ischemic cardiomyopathy, *CM* cardiomyopathy (patient with amyloidosis), *A* angiotensin-converting enzyme inhibitors or angiotensin receptor blocker, *C* catecholamines, *D* diuretics (including aldosterone antagonists), *G* cardiac glycosides, *R* antiarrhythmics (except beta-blockers), *V* verapamil, *X* β-blocker, *n.d.* unknown, *n.a.* not applicable

first confirmed the specificity of the PP1 isoform-specific antibodies used in this study. We either applied recombinant proteins of the (human) PP1 isoforms α, β, or γ or protein lysates from HEK293 cells transfected with human PP1 isoforms (Fig. 1a, b). The molecular weight of the different PP1 isoforms detected was slightly higher than expected due to HIS or Myc-DDK tags, which are fused to the respective isoforms. To perform quantitative analysis of western blots, we then made a serial dilution of lysate of a left ventricular sample from an HF patient and carried out western blots using antibodies against PP1α, β, or γ, or calsequestrin. We were able to show that the expression of all three PP1 isoforms and calsequestrin in human samples was within the linear detection range of the

chemiluminescence detection method used in these studies when 20 μg of protein was loaded (Figs. 1c–e, 2e–g) [12]. Based on these data, 20 μg of protein per lane was loaded for western blot analyses throughout the study.

Previous reports showed consistent increase of overall PP1 expression and activity within the most common cardiac pathologies of HF and AF. However, we are not aware of previous studies, which dissected the PP1 isoform expression in the heart. Here, we studied PP1 isoform expression in LV heart tissue samples of patients with (terminal) HF (Table 1) and in right atrial appendages of patients with AF (Table 2) versus non-failing (NF) heart tissues or SR patient samples, respectively. The pattern of PP1 isoform expression was strikingly different within these two human cardiac pathologies compared to NF heart tissues or SR patient samples. In detail, HF patient samples revealed greater abundance of PP1α and γ of 1.6- and 1.5-fold, respectively, when compared to NF samples, whereas the abundance of PP1β was not changed (Fig. 2a–d). In contrast, AF samples showed higher expression of all three PP1 isoforms (2.4-, 3.0-, and 1.8-fold increase of PP1α, β, and γ, respectively), with the most pronounced expression change for PP1β (Fig. 3a–d). These results fit well with the previously reported increase of overall PP1 protein abundance in the human HF and AF settings [5, 6, 21, 24], but highlight a special role of PP1β in different cardiac pathologies. Furthermore, we could show that PP1β is the predominant PP1 isoform in human heart tissue (approximately tenfold higher abundance than PP1 α or γ) (Fig. 2e–g). We propose that the distinct expression pattern of PP1 isoforms is very likely to reflect the different pathogenesis and progression of the underlying cardiac diseases, including significant differences in cardiac protein phosphorylation patterns. It would be of great importance to decipher the specific downstream targets of the different PP1 isoforms, especially PP1β. While cardiomyocyte-restricted overexpression of PP1α in mice induced dilated cardiomyopathy and pump failure strikingly similar to human HF [3], unfortunately, there are no published reports about the outcome of cardiomyocyte-specific overexpression of the other isoforms. It would be interesting to see whether mice with cardiomyocyte-specific PP1β or PP1γ overexpression without concomitant PP1α upregulation would resemble functional phenotypes of AF patients. Furthermore, cardiomyocyte-specific knock-out of the three PP1 isoforms in embryonic and adult mouse shed light into the previously unknown in vivo function and regulation of the different PP1 isoforms in the heart [17].

Table 2 Clinical characteristics of patients with sinus rhythm (SR) and atrial fibrillation (AF)

Patient ID #	Age	Gender	Diagnosis	Drugs	LVEF [%]
3812	69	F	SR	A, X, D	45
3790	77	F	SR	X, C, D, L	65
2891	60	F	SR	A	60
3738	71	M	SR	A, C, S	>55
3741	83	M	SR	A, X, D, L	25–30
2676	68	M	SR	C	40
3751	78	F	AF	A, X	20
2943	58	F	AF	A, X, G	60
3750	81	F	AF	N, X, G, D	55
3676	69	M	AF	X	35
3709	81	M	AF	A, X, C, D	64
3682	65	M	AF	A, X, G, V	>55

SR sinus rhythm, *AF* atrial fibrillation, *A* angiotensin-converting enzyme inhibitors or angiotensin receptor blocker, *C* catecholamines, *D* diuretics (including aldosterone antagonists), *G* cardiac glycosides, *R* antiarrhythmics (except beta-blockers), *V* verapamil, *X* β-blocker

Table 3 Clinical characteristics of HF patients with or without beta-blocker treatment

Patient ID #	Age	Gender	Diagnosis	Pre-existing Medication	LVEF [%]
84	59	M	HF	A, C, D, G, V	20
91	69	M	HF	A, D, R	n.a. (LVAD)
85	65	M	HF	D, C	10–15
101	47	M	HF	A, C, D, G, R	16
102	48	M	HF	C, D	13
103	64	M	HF	D, R	10
90	69	M	HF	A, D, R, X	10–15
111	70	M	HF	D, G, X	15
108	57	M	HF	A, D, X	20
109	59	M	HF	A, D, X	n.a. (LVAD)
119	55	M	HF	A, C, G, X	9

HF heart failure, *A* angiotensin-converting enzyme inhibitors or angiotensin receptor blocker, *C* catecholamines, *D* diuretics (including aldosterone antagonists), *G* cardiac glycosides, *R* antiarrhythmics (except beta-blockers), *V* verapamil, *X* β-blocker, *n.a.* not applicable

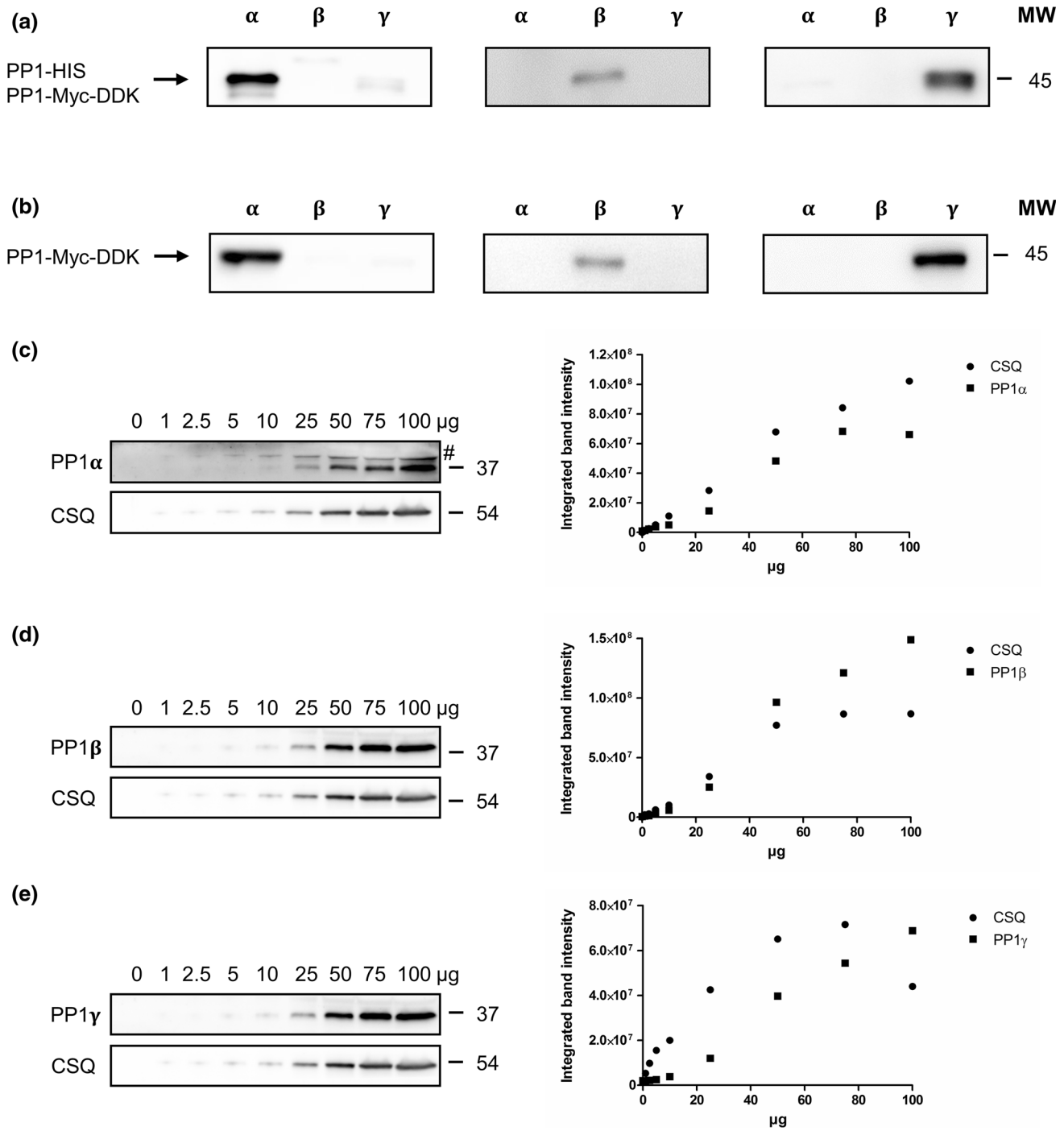
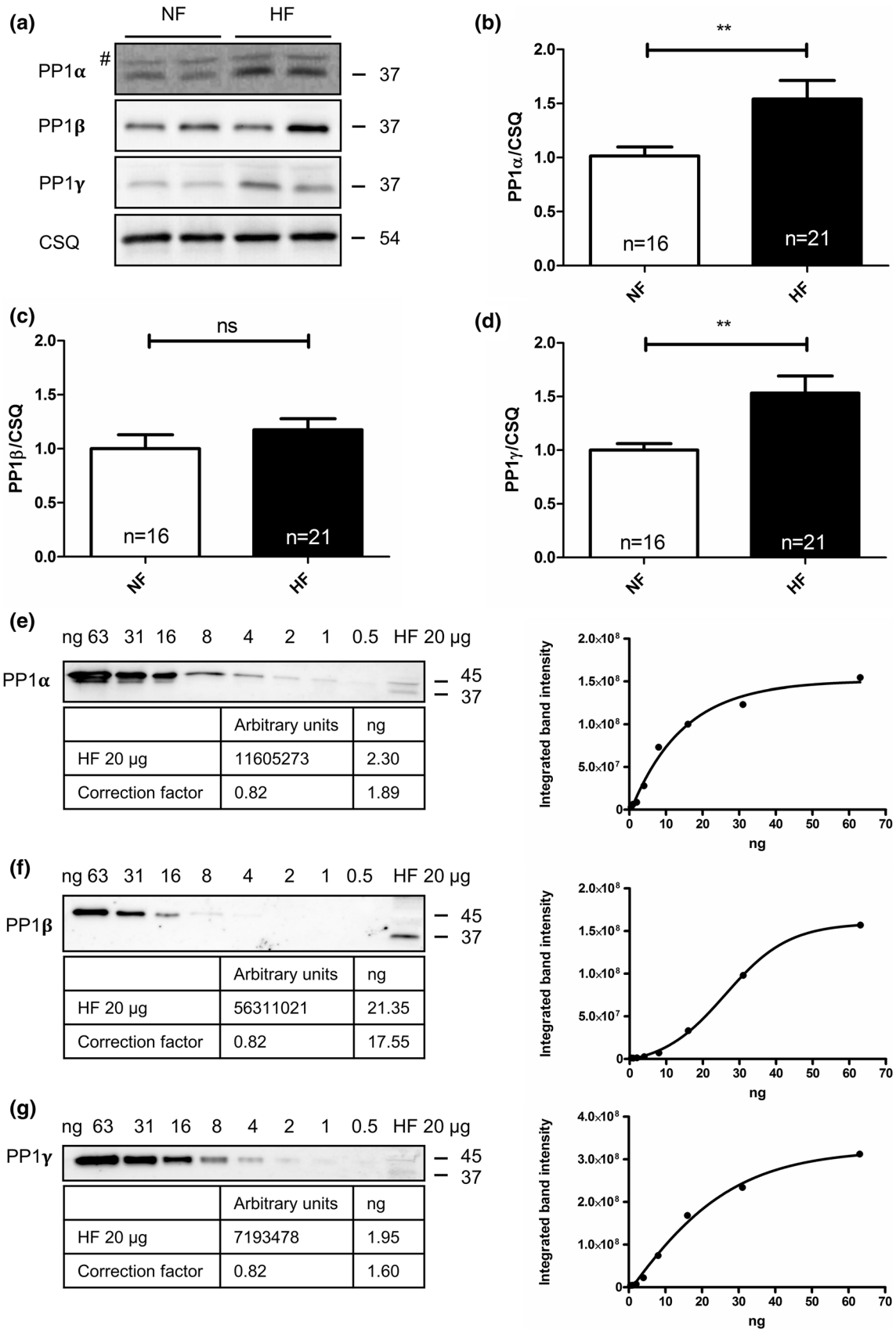


Fig. 1 Validation of protein phosphatase 1 (PP1) isoform-specific antibodies. **a** Western blot analysis of human recombinant PP1 α (with HIS tag), β (with Myc-DDK tag), and γ (with HIS tag) (10 ng each lane) or **b** lysate of HEK293 cells transfected with human PP1α, β, or γ fused with Myc-DDK-tag (10 µg each lane) using isoform-specific antibodies. **c**–**e** Western blot analysis of PP1α, β, γ, and calsequestrin (CSQ) in one experiment with dilution series of human left ventricular heart failure samples. Unspecific bands are marked by a *hash* (#)

While embryonic and adult deletion of PP1α or PP1γ had negligible effects overall, the deletion of PP1β led to concentric remodelling of the heart, interstitial fibrosis, and contractile dysregulation. At the cardiomyocyte level, the deletion of any of the three PP1 isoforms had no effect on phosphorylation of phospholamban; however, PP1β knock-out showed enhanced contractility with concomitant elevated phosphorylation of myosin light chain 2 and cardiac myosin binding protein-C but unchanged Ca^{2+} handling dynamics. Furthermore, the potentially important role of


(a)
NF
HF
PP1α
#
37
PP1β
37
PP1γ
37
CSQ
54
(b)
**
PP1α/CSQ
2.0
1.5
1.0
0.5
0.0
n=16
n=21
NF
HF
(c)
ns
PP1β/CSQ
n=16
n=21
NF
HF
(d)
**
PP1γ/CSQ
n=16
n=21
NF
HF
(e)
ng 63 31 16 8 4 2 1 0.5 HF 20 µg
PP1α
45
37
Arbitrary units
ng
HF 20 µg
11605273
2.30
Correction factor
0.82
1.89
Integrated band intensity
2.0x10^8
1.5x10^8
1.0x10^8
5.0x10^7
0
0 10 20 30 40 50 60 70
ng
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ng 63 31 16 8 4 2 1 0.5 HF 20 µg
PP1β
45
37
Arbitrary units
ng
HF 20 µg
56311021
21.35
Correction factor
0.82
17.55
Integrated band intensity
2.0x10^8
1.5x10^8
1.0x10^8
5.0x10^7
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PP1γ
45
37
Arbitrary units
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HF 20 µg
7193478
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Correction factor
0.82
1.60
Integrated band intensity
4.0x10^8
3.0x10^8
2.0x10^8
1.0x10^8
0
0 10 20 30 40 50 60 70
ng

◀**Fig. 2** Expression of protein phosphatase 1 isoform α (PP1α) and γ (PP1γ) is enhanced in left ventricular tissues of patients with heart failure compared to non-failing patients. **a** Western blot and **b**–**d** densitometric analysis of PP1 isoforms in left ventricular human non-failing (NF; $n = 16$) versus heart failure (HF; $n = 21$) samples. Unspecific bands are marked by a *hash* (#). Results are presented as mean ± SEM. Data sets were compared by unpaired two-tailed Student's *t* test to assess differences between groups. $^{**}p < 0.01$ HF versus NF. All samples were normalized to calsequestrin (CSQ). The patient data are shown in Table 1. **e**–**g** Serial dilution of the human recombinant PP1α, β, and γ protein to calibrate endogenous abundance of PP1α, β, and γ in 20 µg of total protein of a left ventricular human HF sample. The PP1α, β, and γ band intensity was plotted as a function of protein input with a nonlinear regression fit model

PP1β for heart function was confirmed by a study in adult rat cardiomyocytes using adenovirally delivered shRNAs against the different PP1 isoforms. Here, PP1β knockdown not only led to a comparable increase in cardiac force, but also had influence on Ca^{2+} transients and phosphorylation of phospholamban at position serine 16 [1]. Finally, there were very recent findings showing that PP1β de novo missense variants were associated with intellectual disability and congenital heart disease [19].

Beta-blockers such as metoprolol, bisoprolol, and carvedilol are first-line recommended drugs for the treatment of HF patients with reduced ejection fraction, which have been shown to improve survival in HF patients with reduced ejection fraction [25]. Numerous studies have shown beneficial beta-blocker effects by targeting the chronically hyper-activated β-adrenergic signaling pathway. Nevertheless, there are no data about PP1 expression and virtually no data regarding regulation of PP1 isoform expression after chronic beta-blocker treatment available. Here, we observed significantly lower PP1α isoform abundance in the LV tissues from HF patients with beta-blocker treatment (see Table 3), while the expression of the other two PP1 isoforms remained unchanged (Fig. 4a–d). As previous studies showed, PP1α is uniquely interacting with the central ER stress complex Gadd34/eIF2α [2]. We, therefore, postulated that specific PP1α downregulation after chronic beta-blocker treatment may lead to increased or prolonged phosphorylation of eIF2α at position Ser51. Indeed, we observed a higher ratio of phosphorylated (p-Ser51) to unphosphorylated eIF2α in LV tissues from HF patients treated with beta-blocker (HF + BB) versus HF patients without beta-blocker treatment (Fig. 4a, fourth and fifth panels, e, f). Phosphorylation of eIF2α at serine 51 inhibits the translation and subsequent opportunity for the cell to get rid of misfolded proteins or to improve chaperone-mediated protein folding [28]. This process was accompanied by greater expression of ER stress markers, e.g., BIP (also known as Grp78) and IRE1α. Conversely, we observed lower expression of the latter ER stress markers in HF patients, who were on beta-blocker treatment (Fig. 4a, sixth and seventh panels, g, h). These findings fit well with previous studies showing beta-blocker

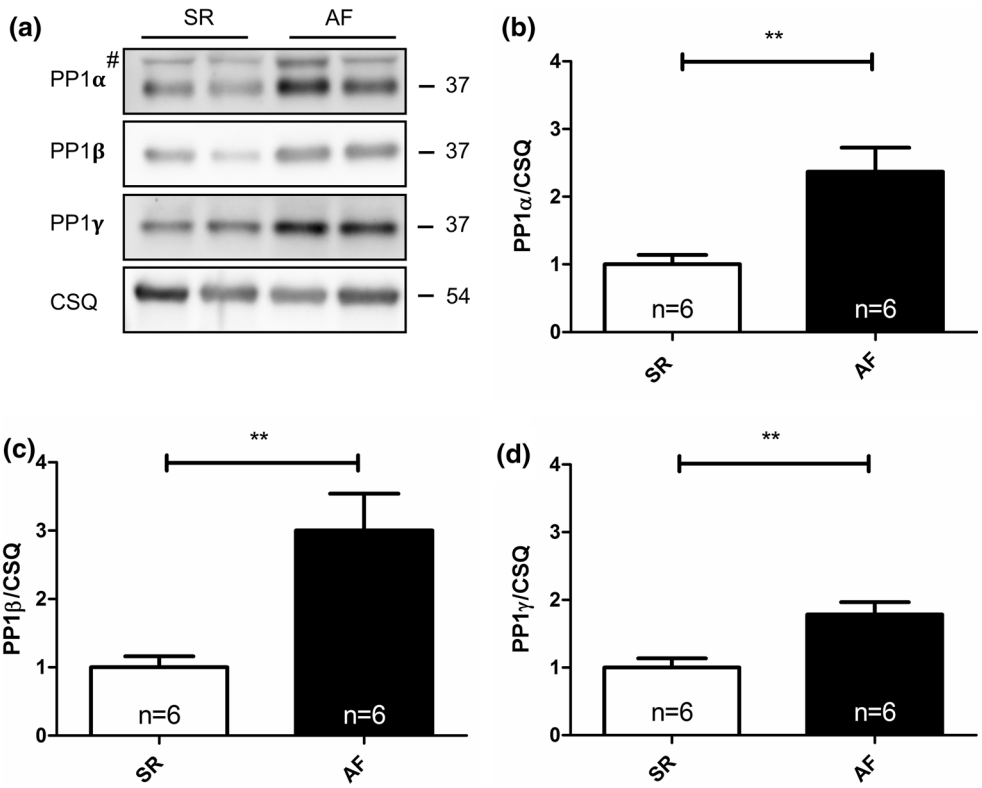

Fig. 3 Comparison of protein phosphatase 1 isoform abundance in right atrial appendages of AF and sinus rhythm (SR) patients. **a** Western blot and **b**–**d** densitometric analysis of PP1 isoforms in human right atrial appendages of patients with sinus rhythm (SR; $n = 6$) versus atrial fibrillation (AF; $n = 6$). Unspecific bands are marked by a *hash* (#). All samples were normalized to calsequestrin (CSQ). The patient data are shown in Table 2. Results are presented as mean ± SEM. Data sets were compared by unpaired two-tailed Student's *t* test to assess differences between groups. $^{**}p < 0.01$ AF versus SR

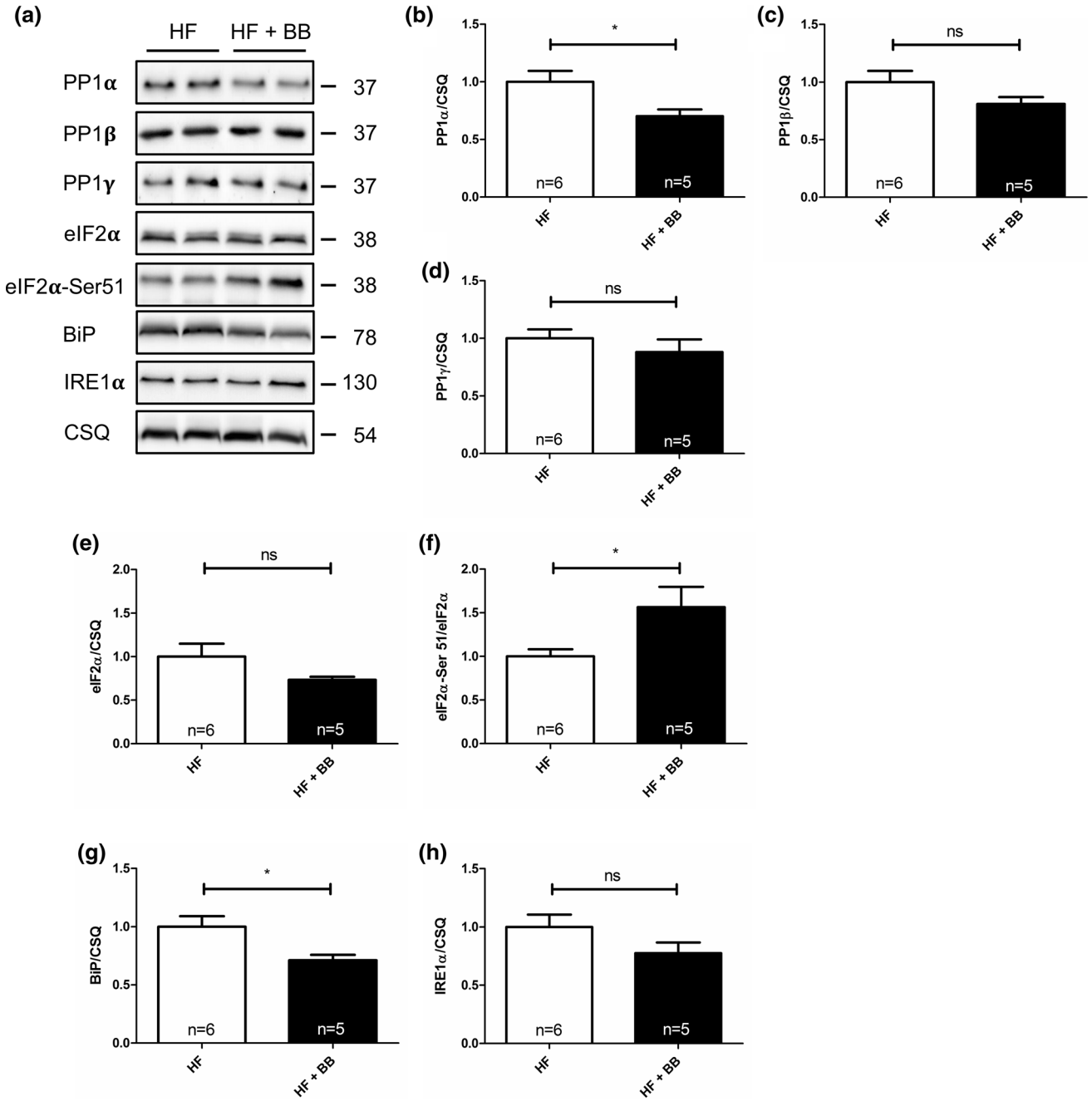

Fig. 4 Expression of protein phosphatase 1 isoform α (PP1α) is decreased in human left ventricular tissues of heart failure patients after beta-blocker treatment. **a** Western blot analysis of PP1 isoforms, eIF2α, phospho-eIF2α (Ser51), and ER stress marker BIP and IRE1α in human left ventricular samples of patients with heart failure (HF) with (HF + BB; $n = 5$) and without (HF; $n = 6$) beta-blocker treatment. **b–d** Densitometric analysis of western blots against PP1 isoforms in human left ventricular samples of HF patients treated with (HF + BB) and without (HF) beta-blocker. **e–f** Densitometric analysis of western blots against PP1α specific substrate eIF2α and the resulting in phospho-eIF2α (Ser51) in human left ventricular samples of HF patients with (HF + BB) and without (HF) beta-blocker treatment. **g–h** Densitometric analysis of western blots against ER stress marker BIP and IRE1α in human left ventricular samples of HF patients with (HF + BB) and without (HF) beta-blocker treatment. Results are presented as mean ± SEM. Data sets were compared by unpaired two-tailed Student's t test to assess differences between groups. *$p < 0.05$ HF + BB versus HF. **$p < 0.01$ HF + BB versus HF. All samples were normalized to calsequestrin (CSQ). The patient data are shown in Table 3

mediated protection against HF-induced ER stress in the heart and cardiomyocytes [9]. Notably, a very recent publication convincingly showed Nox4-dependent inactivation of a similar PP1/p-eIF2α/Grp78 signaling axis in a murine HF model of ischemia–reperfusion [26]. However, it should be noted that counteracting p-Ser51-

eIF2α kinases, e.g., PERK, PKR, and GCN2, were supposed to be regulated under HF conditions as well. It would be interesting to study the expression dynamics of these kinases in response to beta-blocker treatment as well as to receive a more comprehensive picture of this signaling pathway in the future [15, 18, 29]. This further underlines our results, which propose a previously unknown role of the PP1 isoform α in the idea that PP1α-specific downregulation in HF augments beneficial outcome in patients by decreasing ER stress [26].

Potential limitations of the study

While we tried to avoid sex- and age bias as best as possible, the limited number of available human samples did not allow for perfect matching of all potentially confounding factors. This holds especially true when interpreting the results, which we obtained after evaluating PP1α isoform expression and activity in HF patients with or without beta-blocker treatment. It would be of great interest to study, e.g., the expression of PP1α isoform in LV tissues of HF patients before and after beta-blocker treatment to decide if differences in the abundance of PP1α are really a direct response towards beta-blocker treatment. Finally, we did not study the impact of beta-blocker treatment on the abundance of other cardiac phosphatases under heart failure conditions, e.g., protein phosphatase 2A (PP2A), calcineurin, or dual-specific phosphatase 14 (Dusp14), although studies have shown that the abundance and activity of these phosphatases might be changed as well [7, 8, 14].

In conclusion, we believe that differential expression of PP1 isoforms is an obvious issue of human cardiomyopathies and that our novel findings may pave the way to innovative strategies to tackle cardiovascular diseases in the future.

Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflicts of interest with the contents of this article.

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