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Original Article

Posttranslational modifications of calcium/calmodulin-dependent protein kinase IIδ and its downstream signaling in human failing hearts

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Abstract: Background: In human failing hearts (HF) of different origin (coronary artery disease-CAD, dilated-DCM, restrictive and hypertrophic cardiomyopathy-OTHER), we investigated the active forms of Ca^{2+}/calmodulin-dependent protein kinase IIδ (p-Thr^{287}-CaMKIIδ, oxMet$^{281/282}$-CaMKIIδ) and their role in phenotypes of the disease. Methods and results: Although basic diagnostic and clinical markers indicating the attenuated cardiac contractility and remodeling were comparable in HF groups, CaMKIIδ-mediated axis was different. P-Thr^{287}-CaMKIIδ was unaltered in CAD group, whereas it was upregulated in non-ischemic cardiomyopathic groups. No correlation between the upregulated p-Thr^{287}-CaMKIIδ and QT interval prolongation was detected. Unlike in DCM, oxMet$^{281/282}$-CaMKIIδ did not differ among HF groups. Independently of CaMKIIδ phosphorylation/oxidation, activation of its downstreams-phospholamban and cardiac myosin binding protein-C was significantly downregulated supporting both diminished cardiac lusitropy and inotropy in all hearts. Content of sarcoplasmic reticulum Ca^{2+}-ATPase 2a in all HF was unchanged. Protein phosphatase1β was upregulated in CAD and DCM only, while 2A did not differ among groups. Conclusion: This is the first demonstration that the posttranslational activation of CaMKIIδ differs in HF depending on etiology. Lower levels of downstream molecular targets of CaMKIIδ do not correlate with either activation of CaMKIIδ or the expression of major protein phosphatases in the HF. Thus, it is unlikely that these mechanisms exclusively underlie failing of the heart.

Keywords: Human heart failure, Ca^{2+}/calmodulin-dependent protein kinase II, sarcoplasmic reticulum calcium handling, cardiac myosin binding protein-C

Introduction

Heart failure (HF) is a progressive cardiac disease ranging from stages which impair the patient's quality of life and finally progress to a stage that is characterized by symptoms resistant to treatment. HF can occur as a result of coronary artery disease (CAD), dilated cardiomyopathy (DCM) and other hereditary and idiopathic causes [1]. In contrast to the most prevalent HF due to CAD, the age-adjusted prevalence of other forms of HF is lower (1:500 to 1:2500) [1-3]. Regardless of etiology, all types of systolic HF are characterized by seriously impaired cardiac contractility leading into inadequate perfusion of organs. Great effort has been undertaken to understand the pathomechanisms of systolic myocardial dysfunction. The abnormal Ca^{2+} cycling and defects in the sarcomeric proteins are proposed to be one of the crucial players in the adverse remodeling and dysfunction of the heart [4].

Ca^{2+}/calmodulin-dependent protein kinase IIδ (CaMKIIδ) has emerged as an important Ca^{2+} handling protein regulating excitation-contraction coupling. Alterations in its activity have been proposed to exaggerate mishandling in Ca^{2+} homeostasis, and thereby underlie the depressed cardiac contractile function and arrhythmogenesis [5, 6]. Overactivation of CaMKIIδ can arise from its capability to undergo the vari-

ous posttranslational modifications, including autophosphorylation (pThr^{287}-CaMKIIδ) and oxidation (oxMet$^{281/282}$-CaMKIIδ) due to oxidative stress [7]. As CaMKIIδ phosphorylates a wide variety of proteins involved in the regulation of both Ca^{2+} handling (phospholamban-PLN), and contractile proteins (cardiac myosin binding protein-C-cMyBP-C), this protein kinase might be an interesting target in the management of HF treatment [8]. In fact, it has been previously reported that in failing hearts, sarcoplasmic reticulum (SR) Ca^{2+} uptake is significantly reduced due to the inhibitory effects of PLN on SERCA2a [9, 10]. This may occur as a result of a decreased activity/expression of SERCA2a [11] or a higher expression of PLN [12, 13]. In addition to Ca^{2+} dysregulation, attenuation of cMyBP-C function, which serves as a regulatory and structural protein of the sarcomere, due to its dephosphorylation and subsequent degradation of cMyBP-C is associated with the diminished cardiac contractile function and poor prognosis of patients with HF [14]. However, it is not known if the above-mentioned link involving SR Ca^{2+} proteins, cMyBP-C and CaMKIIδ, as a central signal-transducing element, is altered depending on the certain type of systolic ventricular dysfunction. Therefore, the objective of this study was to provide a comprehensive analysis of the posttranslational activation of CaMKIIδ and if it is associated with the alterations of downstream target proteins in the SR and sarcomere in human failing heart samples. We also investigated a potential role of protein phosphatases PP1 and PP2A counterbalancing the effects of CaMKIIδ and its downstream proteins [15]. As CaMKIIδ has been suggested as a pro-arrhythmogenic marker [16, 17], and arrhythmias are a common complication of HF [18], therefore we also analyzed a correlation between the levels of the active forms of CaMKIIδ and QT interval duration.

Methods

Study design

All procedures were in accordance with ethical standards for human experiments based on Heslinki declaration. All experiments were approved by national and institutional ethical commissions (statement no. IK-NP-0021-24/1426/14 and NUSCH EK 126/180509). Samples of left ventricles were obtained from explanted hearts of patients with diagnosed terminal stadium of HF (NYHA III-IV) and reduced left ventricle ejection fraction (LVEF<25%). We employed the failing hearts due to coronary artery disease (CAD, n=6), dilated cardiomyopathy (DCM, n=10), and other cardiomyopathies being either restrictive or hypertrophied cardiomyopathy (OTHER, n=6). All patients underwent echocardiographical, hemodynamical and biochemical examination before transplantation. Additionally, the HF pharmacotherapy was analyzed from the last medical records. Samples of left ventricles from healthy donor patients (C, n=4), whose hearts cannot have been used for transplantation from various medical reasons (CMV infection, size/donor recipient mismatch, major damage during procedure), served as a control group. Tissues from the walls of left ventricles have been harvested in the time of explanation avoiding the scared, fibrotic and adipose tissues. They were afterwards rinsed, dried and snap-frozen in liquid nitrogen until further processing.

Immunoblotting

The samples of left ventricles were processed for the immunoblotting analysis based on our standard laboratory protocol for SDS-PAGE and Western Blotting [19]. Proteins were transferred on PVDF membranes (Immobilon-P, EMD Millipore, USA) and incubated with primary antibodies against p-Thr^{287}-CaMKII (Cell Signaling, USA), oxMet$^{281/282}$-CaMKII (EMD Millipore, USA), total CaMKIIδ (Santa Cruz, USA), p-Thr^{17}-PLN (Badrilla, UK), p-Ser^{16}-PLN (Badrilla, UK), total PLN (Badrilla, UK), p-Ser$^{282(284)}$-cMyBP-C (Enzo, USA), total cMyBP-C (Santa Cruz, USA), total SERCA2a (Badrilla, UK), PP1β (Abcam, USA) and PP2A (Sigma-Aldrich, USA). Afterwards, the incubation with HRP-conjugated secondary antibodies, donkey anti-rabbit IgG (GE Healthcare Life Sciences, UK) and rabbit anti-goat IgG (Sigma-Aldrich, USA) was performed. Signals were detected by enhanced chemiluminiscence (Crescendo Luminata, EMD Millipore, USA) and chemiluminiscence imaging system (myECL imager, Thermo Scientific, USA). Total protein staining with Coomasive Brilliant Blue R-250 or Reactive Brown 10B evaluated by scanning densitometry was used as the loading control as a substitution to the housekeeping protein immunodetection [20-22]. Intensity of a particular protein band was compared to a whole lane.

Table 1. Echocardiographic, hemodynamic and biochemical parameters of probands

	Controls	CAD	DCM	OTHER	
				RCM	HCM
Age (years)	30 ± 5	52 ± 4	47 ± 3	47 ± 6	60 ± 4
LVEF (%)	n.a.	21 ± 1	18 ± 1	34 ± 8	30 ± 5
LVEDD (mm)	n.a.	75 ± 4	72 ± 3	60 ± 3	59 ± 1
RVEDD (mm)	n.a.	34 ± 3	35 ± 1	33 ± 1	n.a.
QT (s)	n.a.	0.42 ± 0.01	0.41 ± 0.02	0.44 ± 0.02	n.a.
sPAP (mm Hg)	n.a.	44 ± 7	53 ± 5	52 ± 5	45 ± 6
dPAP (mm Hg)	n.a.	21 ± 4	27 ± 3	28 ± 3	21 ± 2
CI ($dm^{-3} \cdot min^{-1} \cdot m^{-2}$)	n.a.	2.2 ± 0.1	1.8 ± 0.1	1.8 ± 0.2	n.a.
NT-proBNP ($ng \cdot dm^{-3}$)	n.a.	3541 ± 1035	6571 ± 1682	3394 ± 1007	10124 ± 4152
Creatine kinase ($\mu kat \cdot dm^{-3}$)	3.09 ± 0.92	1.18 ± 0.26	1.17 ± 0.21	1.08 ± 0.47	0.97 ± 0.21

End-stage HF groups of ischemic and non-ischemic origin did not significantly differ in neither echocardiographic nor hemodynamic parameters. CAD-coronary artery disease; DCM-dilated cardiomyopathy; RCM-restrictive cardiomyopathy; HCM-hypertrophied cardiomyopathy. LVEF-left ventricular ejection fraction; LVEDD-left ventricular end-diastolic diameter; RVEDD-right ventricular end-diastolic diameter; QT-QT interval; sPAP-systolic pulmonary artery pressure; dPAP-diastolic pulmonary artery pressure; CI-cardiac index; NT-proBNP-N-terminal pro-B type natriuretic peptide. Data are expressed as the means ± SEM.

Statistical analysis

The results are expressed as means ± standard error of means (S.E.M.), unless stated otherwise. One-way ANOVA analysis with Newman-Keuls and Tukey's post-hoc tests and two-tailed unpaired Student's *t*-test were used for evaluation of group differences in variables with normal distribution. In case of the non-normal distribution, a Man-Whitney's analysis was used. Correlation between biochemical parameters, QT duration or N-terminal pro-B type natriuretic peptide (NT-proBNP) and the levels of certain proteins was analyzed by Pearson's test. All analyses were performed with GraphPad Prism 6.00 for Windows (GraphPad Software, USA). Differences between groups were considered significant when $P<0.05$.

Results

Characteristics of study subjects

A summary of the main pre-transplant data of the study subjects are presented in **Table 1**. All patients irrespective of the etiology of HF were in either NYHA III or IV class. Among the HF groups, there were no significant differences in neither of the values of left ventricular ejection fraction (LVEF), parameters of cardiac remodeling (the size of left and right end-diastolic ventricular diameters-LVEDD, RVEDD), markers of pulmonary hypertension (systolic and diastolic pulmonary pressures-sPAP, dPAP) nor NT-proBNP, a common biochemical marker of HF. HF patients were treated at least with ACE inhibitors or AT1R-blockers, beta-blockers and diuretics. The control subjects were treated with the infusion of noradrenaline and dopamine and fluid balance was maintained with desmopressin and hydroxyethyl starch, an intravenous colloid volume expander. They presented preserved systolic/diastolic function. No metabolic disorders were diagnosed in the study subjects.

Posttranslationally modified forms of CaMKIIδ in end-stage human HF show no association with the QT interval duration

Western blot analysis of the posttranslationally modified forms of CaMKIIδ by phosphorylation at Thr287 and oxidation at Met281/282 is shown in **Figures 1**, **2**.

The content of p-Thr^{287}-CaMKIIδ in the group of CAD did not differ from the values of the control subjects. On the other hand, in all cardiomyopathic groups of HF, the expression of p-Thr^{287}-CaMKIIδ was significantly increased as compared with the control hearts and CAD (**Figures 1**, **2A**). Of note, the levels of oxMet$^{281/282}$-CaMKIIδ did not differ among HF group. However, by using Student's *t*-test, significantly lower expression of oxMet$^{281/282}$-CaMKIIδ was observed in the DCM as compared to control group (**Figures 1**, **2B**). This is the first analysis of the oxidized form of this protein kinase in human end-stage HF.

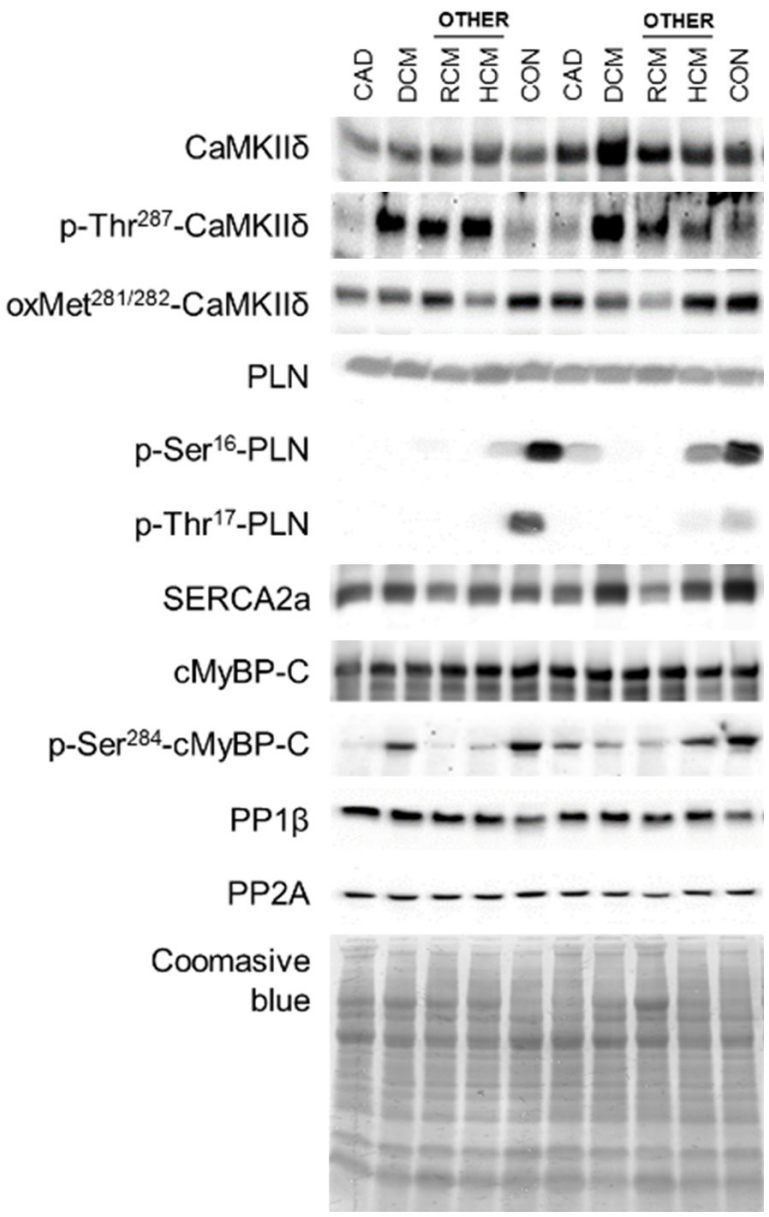

Figure 1. Representative blots of evaluated proteins in control and human failing hearts. Total CaMKIIδ-total Ca^{2+}/calmodulin-dependent protein kinase 2δ; p-Thr^{287}-CaMKIIδ-phospho-Thr^{287}-Ca^{2+}/calmodulin-dependent protein kinase 2δ; ox-$Met^{281/282}$-CaMKIIδ-oxidized-$Met^{281/282}$-Ca^{2+}/calmodulin-dependent protein kinase 2δ; total PLN-total phospholamban; p-Thr^{17}-PLN-phospho-Thr^{17}-phospholamban; p-Ser^{16}-PLN-phospho-Ser^{16}-phospholamban; SERCA2a-sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; total cMyBP-C-total cardiac myosin-binding protein C; p-Ser^{284}-cMyBP-C-phospho-Ser^{284}-cardiac myosin-binding protein C; PP1β-protein phosphatase 1β; PP2A-protein phosphatase 2A.

The total CaMKIIδ was comparable in all HF groups and did not differ from the values of controls (**Figures 1**, **2C**). Thus, the ratio of both p-Thr^{287}/total CaMKIIδ and ox$Met^{281/282}$-CaMKIIδ/total CaMKIIδ mimicked the pattern of changes of the particular posttranslational modifications (**Figures 1**, **2D**, **2E**).

QT interval prolongation is known as a predisposing factor for increased ventricular arrhythmia risk and a higher activity and/or expression of CaMKIIδ has been associated with arrhythmia triggering [23, 24]. Therefore in the groups of HF with the altered expression of posttranslationally modified form of CaMKIIδ (DCM, OTHER), a potential correlation between the content of phosphorylated form and prolongation of QT interval was analyzed. However, we did not identify any link between p-Thr^{287}-CaMKIIδ and the prolongation of QT interval (**Figures 1**, **2F**).

Regulation of SR Ca^{2+} uptake in end-stage human HF

As SR Ca^{2+} cycling is known to be diminished in HF [25] and CaMKII activates certain SR proteins [26] we further analyzed p-Thr^{17}-PLN, a downstream of this protein kinase, and SERCA2a. In spite of the different state of the phosphorylation of CaMKIIδ in the particular HF types (ischemic vs. non-ischemic) (**Figures 1**, **2A**), the levels of p-Thr^{17}-PLN were greatly downregulated in all HF groups in comparison to non-failing hearts (**Figures 1**, **3A**). Likewise, PKA-dependent phosphorylation of PLN at Ser16 residue was decreased and there was no difference among the diseased groups indicating that PKA does not substitute a role of CaMKIIδ to maintain the function of PLN (**Figures 1**, **3B**). The expression of the total PLN (**Figures 1**, **3C**) was unchanged in the HF groups, thereby the ratio of both

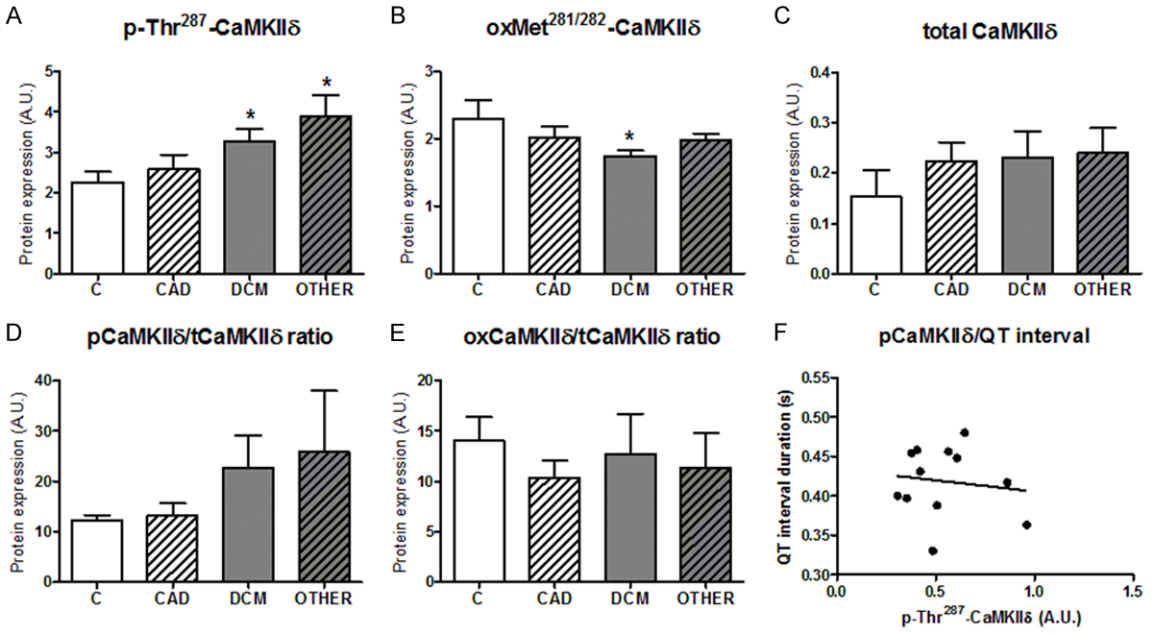

Figure 2. Phosphorylation (p-Thr^{287}-CaMKIIδ) and oxidation (oxMet$^{281/282}$-CaMKIIδ) of CaMKIIδ in different types of terminal human HF. (A) Levels of p-Thr^{287}-CaMKIIδ; (B) Content of oxMet$^{281/282}$-CaMKIIδ; (C) Expression of total CaMKIIδ; (D) Ratio of phosphorylated to total CaMKIIδ; (E) Ratio of oxidized to total CaMKIIδ; (F) Comparison between QT interval duration and phosphorylation of CaMKIIδ (n=12). Data for (A-E) are expressed as the means ± S.E.M. n=4-10 hearts per group. **P<0.05* vs. control hearts.

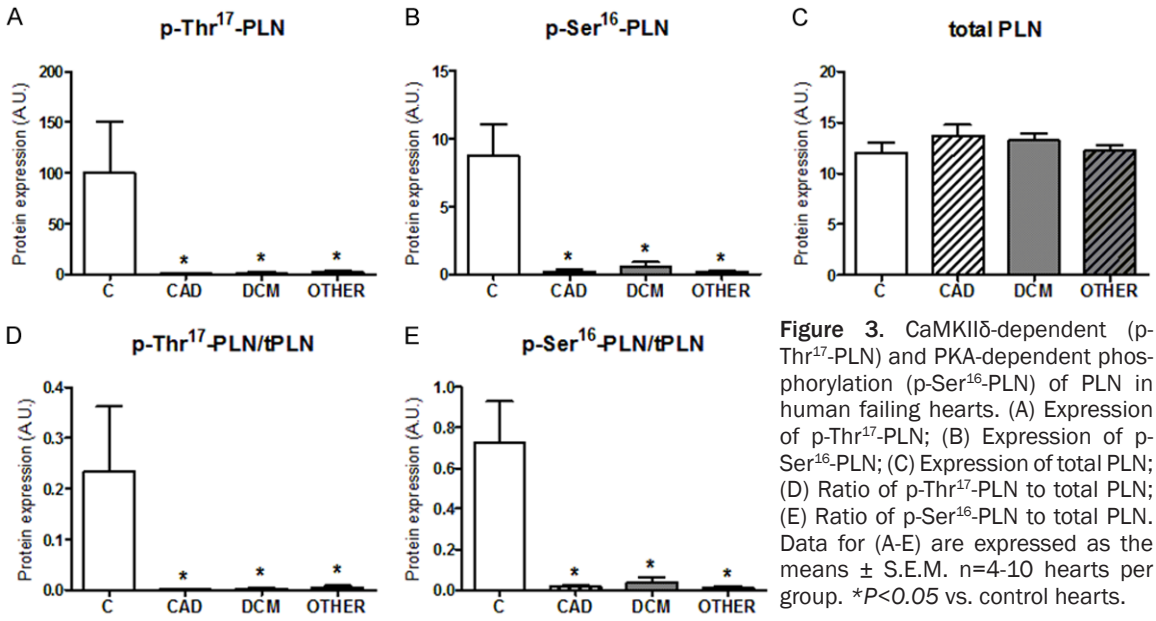

Figure 3. CaMKIIδ-dependent (p-Thr^{17}-PLN) and PKA-dependent phosphorylation (p-Ser^{16}-PLN) of PLN in human failing hearts. (A) Expression of p-Thr^{17}-PLN; (B) Expression of p-Ser^{16}-PLN; (C) Expression of total PLN; (D) Ratio of p-Thr^{17}-PLN to total PLN; (E) Ratio of p-Ser^{16}-PLN to total PLN. Data for (A-E) are expressed as the means ± S.E.M. n=4-10 hearts per group. **P<0.05* vs. control hearts.

p-Thr^{17}-PLN/total PLN and p-Ser^{16}-PLN/total PLN showed the same pattern as the respective active forms of this protein (**Figures 1**, **3D**, **3E**). These data, as general characteristics of diastolic dysfunction, indicates no important role of the expression of either of active form of the upstream kinase in terminal HF.

Non-phosphorylated form of PLN produces inhibitory effects on SERCA2a activity and restricts Ca^{2+} filling back into the SR [27, 28]. Therefore, we estimated a ratio between the total PLN and SERCA2a. Similarly to the total PLN, the levels of total SERCA2a were unchanged in all HF groups when compared to control

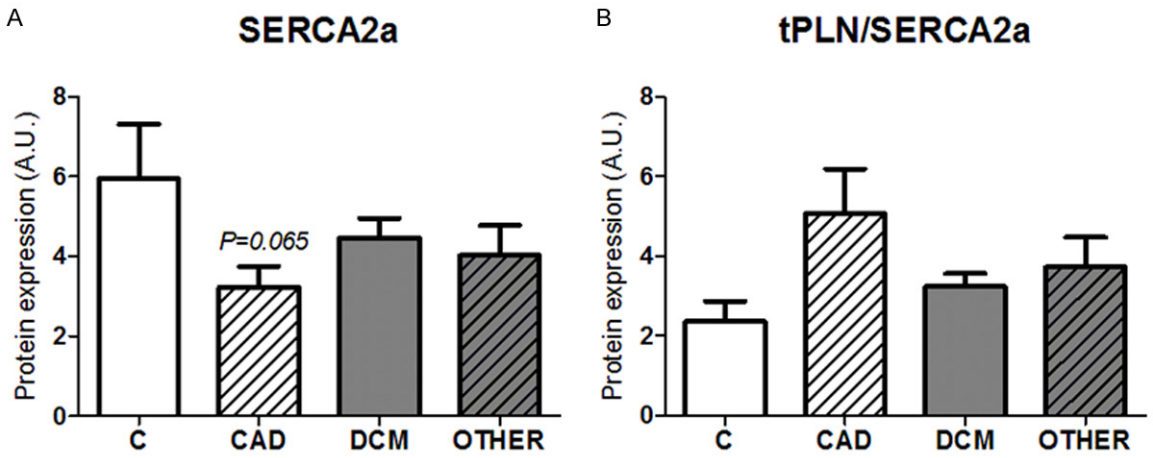

Figure 4. Content of SERCA2a in human failing hearts and ratio of total PLN to SERCA2a. (A) Expression of SERCA2a. (B) Ratio of total PLN to SERCA2a. Values for (A) and (B) are expressed as the means ± S.E.M. n=4-10 hearts per group.

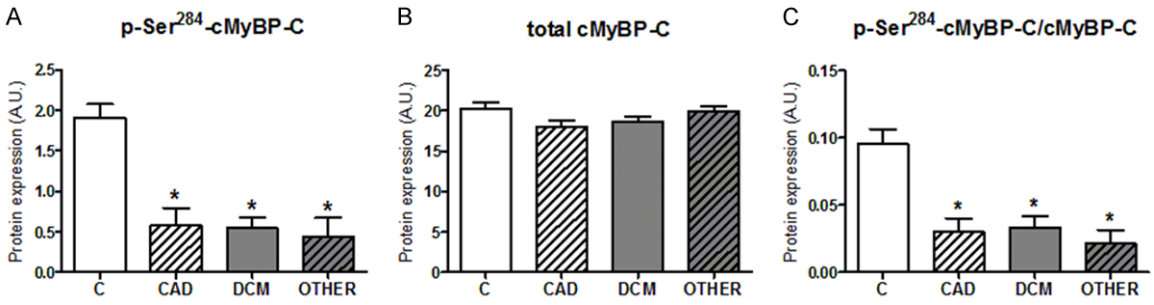

Figure 5. Expression and phosphorylation of cMyBP-C in human failing hearts. (A) Expression of p-Ser^{284}-cMyBP-C; (B) Content of total cMyBP-C; (C) Ratio of p-Ser^{284}-cMyBP-C to total protein. Values for (A-C) are expressed as the means ± S.E.M. n=4-10 hearts per group. **P<0.05* vs. control hearts.

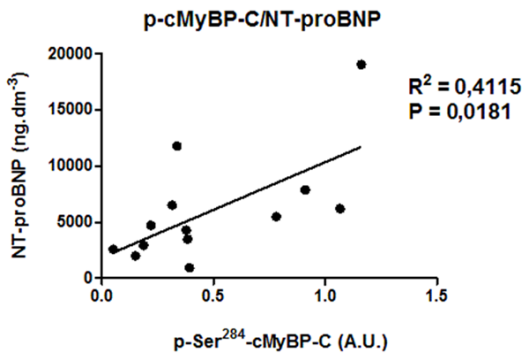

Figure 6. Correlation between the expression of p-Ser^{284}-cMyBP-C and marker of volume overload and myocyte stretch NT-proBNP in failing hearts. n=13 hearts. Correlation was considered as positively significant (*P=0.0181)*.

group (**Figures 1**, **4A**). Accordingly, no significant changes were found in the PLN/SERCA2a ratio in HF groups as compared with non-failing hearts (**Figures 1**, **4B**).

Cardiac MyBP-C in human HF

In addition to the altered Ca^{2+} cycling, the response of the contractile proteins is diminished in HF [29]. Therefore, we evaluated expression of total cMyBP-C and its phosphorylated form at Ser284. Unlike the expression of the total protein (**Figure 5B**), the levels of p-Ser^{284}-cMyBP-C were significantly downregulated in all HF groups regardless the expression of particular posttranslationally modified forms of CaMKIIδ (**Figures 1**, **5A**, **5C**). These results indicate serious systolic dysfunction in HF and similarly to the status of p-Thr^{17}-PLN propose no role of the levels of the active forms of CaMKIIδ in this context.

To further expand the knowledge about this active form of cMyBP-C in end-stage human HF, a correlation between the serum NT-proBNP and p-Ser^{284}-cMyBP-C was evaluated. Interestingly, the levels of NT-proBNP positively corre-

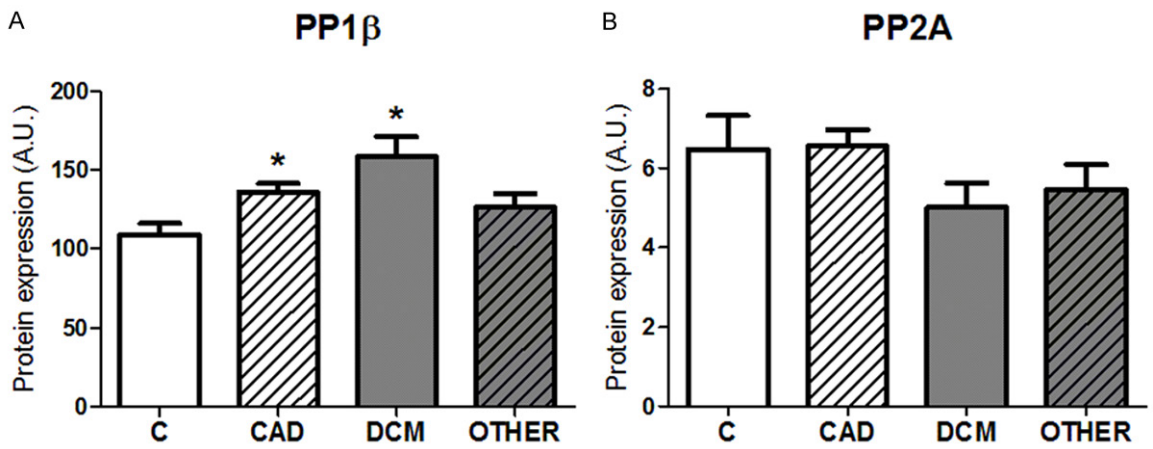

Figure 7. Expression of protein phosphatases PP1β and PP2A in left ventricles of various types of human heart failure. (A) Expression of PP1β; (B) Expression of PP2A. Values for (A) and (B) are expressed as the means ± S.E.M. n=4-10 hearts per group. **P<0.05* vs. control hearts.

Table 2. Expression of evaluated proteins in left ventricles of human failing hearts of different origin

Proteins	CAD	DCM	OTHER
Total CaMKIIδ	≈	≈	≈
p-Thr^{287}-CaMKIIδ	≈	↑	↑
ox-Met$^{281/282}$-CaMKIIδ	≈	↓	≈
Total PLN	≈	≈	≈
p-Thr^{17}-PLN	↓	↓	↓
p-Ser^{16}-PLN	↓	↓	↓
SERCA2a	≈	≈	≈
Total cMyBP-C	≈	≈	≈
p-Ser^{284}-cMyBP-C	↓	↓	↓
PP1β	↑	↑	≈
PP2A	≈	≈	≈

Table summarizes protein expression detected by immunoblot analysis between particular HF groups: CAD-coronary artery disease; DCM-dilated cardiomyopathy; RCM-restrictive cardiomyopathy; HCM-hypertrophied cardiomyopathy and control group. Total CaMKIIδ, p-Thr^{286}-CaMKIIδ, ox-Met$^{281/282}$-CaMKIIδ-total, phospho-Thr^{286} and oxidized-Met$^{281/282}$-Ca^{2+}/calmodulin-dependent protein kinase IIδ; total PLN, p-Thr^{17}-PLN, p-Ser^{16}-PLN-total, phospho-Thr^{17} and phospho-Ser^{16}-phospholamban; SERCA2a-sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; total cMyBP-C, p-Ser^{284}-cMyBP-C-total and phospho-Ser^{284}-cardiac myosin-binding protein C; PP1β-protein phosphatase 1β; PP2A-protein phosphatase 2A. (≈)-no difference in expression; (↑)-significantly increased expression in comparison to control group; (↓)-significantly decreased expression in comparison to controls. Differences between the groups were evaluated by ANOVA test and considered significant when *P<0.05*.

lated with the levels of p-Ser^{284}-cMyBP-C in the HF groups (**Figures 1**, **6**). This may indicate a compensatory increase in cMyBP-C phosphorylation in response to increased myocyte stretch documented by the higher levels of NT-proBNP.

Modulation of protein phosphorylation status by protein phosphatases in failing hearts

We also analyzed certain protein phosphatases which counterbalance the phosphorylation of PLN, cMyBP-C and CaMKIIδ itself. Indeed, two major isoforms of protein phosphatases in human myocardium, PP1 subunit β (PP1β) and PP2A were investigated. PP1β, proposed to dephosphorylate the most of PLN as well as cMyBP-C [30], was increased in the CAD and DCM group but not in other cardiomypathic groups (**Figures 1**, **7A**). PP2A sharing similar catalytic sites with PP1 [31, 32] proposed also to dephosphorylate cMyBP-C [33, 34] and most of CaMKII in cytosolic and membrane fraction [35] did not differ among the diseased groups nor in comparison with control healthy hearts (**Figures 1**, **7B**).

Discussion

In the present comprehensive study we have shown that the levels of both active, posttranslationally modified forms of CaMKIIδ, p-Thr^{287}-CaMKIIδ and oxMet$^{281/282}$-CaMKIIδ in failing hearts due to CAD were comparable to those of healthy hearts. On the other hand, in HF of non-ischemic origin (DCM, RCM and HCM), p-Thr^{287}-CaMKIIδ was upregulated whereas expression of oxMet$^{281/282}$-CaMKIIδ was unchanged. Regardless the levels of these active forms of the kinase, the downstream targets of CaMKIIδ, such as p-Thr^{17}-PLN or p-Ser^{284}-cMyBP-C were

significantly downregulated in all HF types. Evaluation of protein phosphatases revealed the upregulated content of PP1β in CAD and DCM but not in other types of HF. Expression of PP2A in failing hearts did not differ of the levels of controls. Thus, this study employing the samples of various types of HF (both of a high and low incidence) provides the first demonstration that i) the oxidative activation of CaMKIIδ does not go hand in hand with its activation through phosphorylation, ii) CaMKIIδ activation through phosphorylation and oxidation differs depending on the etiology of HF, iii) the lower phosphorylation of the downstream proteins indicating diminished inotropy and lusitropy in these diseased hearts is not paralleled with either active forms of CaMKIIδ or with the expression of the main cardiac protein phosphatases (**Table 2**).

Under physiological conditions, the activation of CaMKII in heart is mainly regulated by autophosphorylation in the presence of Ca^{2+}/CaM [36]. This mechanism allows the kinase to activate various Ca^{2+}-sensitive proteins to ensure proper Ca^{2+} handling, including proteins regulating the function of the sarcoplasmic reticulum and sarcomere [37-39]. Therefore, the observed higher activation state of CaMKIIδ and Ca^{2+} mishandling may contribute to the disturbances in contraction and relaxation in HF [40]. Considering these deleterious effects of the overactivated protein kinase, increased p-Thr^{287}-CaMKIIδ could be expected in the failing hearts. However, in spite of this logical hypothesis, data shown in this study as well as in others [41, 42] are unequivocal. In fact, all HF hearts characterized by depressed cardiac contractility and remodeling have shown the different pattern of oxidation and phosphorylation of CaMKIIδ. Moreover, the pattern of changes in the oxidative activation of the kinase did not mimic the changes in phosphorylation and vice versa. Interestingly, in spite of this observation the markers of HF such as NT-proBNP, pulmonary arterial pressures, EF or cardiac indices did not differ among the types of HF.

So far published papers have mainly been dealing with CaMKII in human HF of a higher incidence. In study of Miyamoto et al. [43], in DCM, the increased phosphorylation of CaMKII has been reported what is in line with our data. On the other hand, similarly to Fisher et al [42] the ratio of the phosphorylated form/total protein kinase has not indicated the changes among the groups. In another study, the activity of CaMKII measured by enzymatic assay was found to be increased in DCM while unchanged in CAD [41]. By predicting a linear link between phosphorylation and catalytic activity of the kinase this observation is in line with results reported in this study. In the present study, we have extended the current knowledge on the activation of CaMKIIδ and reported for the first time the content of p-Thr^{287}-CaMKIIδ being also upregulated in HF types of a lower incidence. It has been suggested that the overactivated/hyperphosphorylated CaMKII is associated with electrical instability along with contractile dysfunction [19, 42, 44]. However, we have been unable to found any correlation between p-Thr^{287}-CaMKIIδ expression and the duration of QT interval in the HF groups of non-ischemic origin which exerted the higher phosphorylation of this protein kinase.

In addition to phosphorylation, CaMKIIδ can undergo other posttraslational modifications [7]. In the presence of oxidative stress, which is commonly observed in HF [45], the activation of CaMKIIδ through oxidation might be of a great relevance thereby suggesting the levels of oxMet$^{281/282}$-CaMKIIδ being increased in our samples of human HF. Of note, the increased levels of oxMet$^{281/282}$-CaMKIIδ have been shown to underlie electrical remodeling and hypertrophy in cardiac myocytes and isoprenaline-treated rats [46, 47]. Surprisingly, no changes in the expression of oxMet$^{281/282}$-CaMKIIδ were found among HF groups. However, while comparing DCM and control group, oxMet$^{281/282}$-CaMKIIδ was found to be decreased in the diseased hearts. It has been proposed that methionine residues at 281/282 can undergo oxidation by superoxide produced by NADPH oxidases what leads into the kinase activation [48, 49]. Another enzyme, methionine sulfoxide reductase A is able to reverse this effect and thereby decrease pro-oxidant potential of the kinase [48]. It would be interesting to measure the activity of this enzyme; however, at the time of performing the study it was impossible due to the unavailability of assay and antibody. Thus, the aforementioned data indicate that a role of CaMKIIδ including its posttranslational forms should be carefully investigated and the particular etiology of HF should be taken into account while assessing

its involvement in the pathologic mechanisms of the disease. Of note, in our recent study, we have shown that both p-Thr^{287}-CaMKIIδ and oxMet$^{281/282}$-CaMKIIδ are not elevated, rather markedly decreased in the late phase of reperfusion of previously ischemic hearts which experienced contractile dysfunction and higher oxidative stress [19].

CaMKIIδ is one of critical proteins regulating proper Ca^{2+} cycling. For instance, by phosphorylation of PLN at Thr^{17} it relieves the inhibitory effects on SERCA2a and thereby mediates lusitropic effects [50]. Since HF is characterized by significantly abolished cardiac contractility and relaxation we have hypothesized CaMKIIδ-dependent phosphorylation of PLN being decreased. In agreement with this hypothesis, the expression of CaMKIIδ-phosphorylated form of p-Thr^{17}-PLN was decreased to almost non-detectable levels in all HF groups. Similarly to our study, Dash et al. [12] and Miyamoto et al. [43] have also reported CaMKII-mediated phosphorylation of PLN being decreased in DCM. The content of p-Thr^{17}-PLN was parallel with the levels of p-Ser^{16}-PLN regulated by PKA. Additionally, we have shown that SERCA2a levels did not differ among the groups what is in agreement with some previous studies [11, 51]. On the other hand, downregulation of SERCA2a in heart failure resulting from DCM has also been reported [12, 52]. The expression of the ratio of total PLN to SERCA2a was unaltered in all diseased groups. The levels of non-phosphorylated PLN have been suggested to act as a marker of decreased SERCA2a pumping activity [53]. However, our data and of others [39, 43, 54] do support the role of phosphorylated PLN rather than the non-phosphorylated protein in diminished Ca^{2+} SR handling in HF.

CaMKIIδ is also known to directly phosphorylate some proteins of contractile apparatus and thereby regulates cardiac contractility independently of Ca^{2+} cycling modulation. In fact, it phosphorylates cMyBP-C, regulates actin-myosin cross-bridging, and controls force generation within the sarcomere [29]. Recently, cMyBP-C has been proposed to serve as a novel biomarker of cardiac injury [14, 55]. Dephosphorylation and subsequent degradation of this protein may indicate cardiac dysfunction and HF [56, 57]. Likewise, several gene mutations of this protein have been reported in CAD, DCM and HCM [58, 59]. 17 phosphorylation sites of cMyBP-C being phosphorylated by four various kinases have been identified so far; however, Ser282 residue (Ser284 in humans), which can be phosphorylated by CaMKII [60], is the most frequent target of phosphorylation *in vivo* [61]. Here, we have shown that phosphorylation of Ser284 residue was significantly downregulated while the total protein was unaltered in all HF groups. In support, other studies dealing with genetic mutations promoting cardiac dysfunction have reported total cMyBP-C being downregulated [59]. In context with p-Thr^{287}-CaMKIIδ, these observations open several questions. Similarly to p-Thr^{17}-PLN, p-Ser^{284}-cMyBP-C was decreased independently of the phosphorylation/oxidation of the upstream protein kinase CaMKIIδ. However, as indicated above, it should be mentioned that the Ser284 residue of cMyBP-C can also be phosphorylated by some other protein kinases such as PKA, PKC or ribosomal S6 kinase [29]. Nevertheless, the phosphorylation of Ser284 mediated by CaMKII seems to play an important role because its inhibition resulted in the decreased phosphorylation of cMyBP-C [62, 63].

As the ability of the kinase to phosphorylate the proteins is counterbalanced by phosphatases we investigated the expression levels of main cardiac protein phosphatases PP1 and PP2A. The PP1 has been proposed to serve as a negative regulator of cardiac function. It is able to dephosphorylate substrates of CaMKIIδ and thus may play an important role in gradual blunting of heart function during the development of HF [15, 64]. In this and in previously published studies [30, 43, 65], the expression of β subunit of PP1 was increased only in CAD and DCM failing hearts. Interestingly, the most abundant PP, PP2A did not differ among the HF groups and was comparable to the value of controls. The data about PP2A are controversial. The increased mRNA levels have been reported in DCM [43], while the protein content of this phosphatase was unchanged [66] supporting the findings of our current study.

Although this study has reported several novel findings and raised some new questions, there are few limitations. Firstly, the conclusions are based on proteomic data only. Nevertheless, we aimed to investigate an association between certain posttranslational modifications of Ca-

MKIIδ and its downstream proteins, with cardiac function of all known types of HF. Secondly, phosphorylation status of proteins can be affected by certain drugs, however, by assuming that all HF patients were given the standard, almost the same therapy, this is unlikely to influence our present data.

Conclusion

In failing hearts of ischemic and non-ischemic origin, not differing in any main diagnostic characteristics, we have detected a different phosphorylation and oxidation status of CaMKIIδ. The changes in posttranslational status of the kinase have not reflected the phosphorylation of its downstream targets neither in p-Ser^{284}-cMyBP-C nor p-Thr^{17}-PLN which, however were significantly downregulated and thereby could underlie the diminished cardiac contractile and relaxation function in HF. By investigating a link between the duration of the QT interval and the posttranslationally modified levels of CaMKIIδ we have been unable to confirm that the altered activation of CaMKIIδ promotes pro-arrhythmogenic environment. Thus, the assessment of a role of CaMKIIδ, its active forms and potential consequences on cardiac function in particular forms of HF needs more detailed investigations.

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Disclosure of conflict of interest

None.

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