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Increased level of phosphorylated desmin and its degradation products in heart failure

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ABSTRACT

Although several risk factors such as infarct size have been identified, the progression/severity of heart failure (HF) remains difficult to predict in clinical practice. Using an experimental rat model of ischemic HF and phosphoproteomic technology, we found an increased level of phosphorylated desmin in the left ventricle (LV) of HF-rats. The purpose of the present work is to assess whether desmin is a circulating or only a tissue biomarker of HF. We used several antibodies in order to detect desmin, its proteolytic fragments and its phosphorylated form in LV and plasma by western blot, phosphate affinity electrophoresis, mass spectrometry and immunofluorescence. Plasma was treated with combinatorial peptide ligand library or depleted for albumin and immunoglobulins to increase the sensitivity of detection. We found a 2-fold increased serine-desmin phosphorylation in the LV of HF-rats, mainly in the insoluble fraction, suggesting the formation of desmin aggregates. Desmin cleavage products were also detected in the LV of HF rats, indicating that the increased phosphorylation of desmin results in more susceptibility to proteolytic activity, likely mediated by calpain activity. The native desmin and its degradation products were undetectable in the plasma of rat, mouse or human. These data suggest the potential of serine-phosphorylated form of desmin and its degradation products, but not of desmin itself, as tissue but not circulating biomarkers of HF.

1. Introduction

Chronic heart failure (HF) remains a major cause of illness and death and its prevalence is increasing with a high rate of morbidity and mortality [1]. Despite major significant advances, HF remains a therapeutic challenge, and several adverse consequences of HF are still poorly controlled. New prognostic or diagnostic biomarkers of HF are still important to find and proteomic approaches may be useful [2].

Novel determinants of post-myocardial infarction (MI)-HF were already revealed by previous proteomic [3] and phosphoproteomic [4] approaches. We recently discovered that HF is associated with decreased levels of myocardial and plasma serine^{208}-phosphorylated troponin T (TnT) in an experimental rat model of HF and confirmed the same decrease in plasma of patients with high LV remodeling post-MI, suggesting that the level of circulating phosphorylated TnT could be new biomarker of LV remodeling and may help to predict the development of HF after MI [5]. More recently, seven proteins were found differentially expressed in cardiac tissues in a mouse model of HF (calcineurin transgenic mice) and their presence in plasma of HF mouse models but also in HF patients have been investigated [6]. From these candidate biomarkers highlighted, desmin was found to be detected in plasma from mouse (~45 kDa) and human (~22 kDa) species, suggesting the potential of circulating desmin as biomarker of HF. Desmin is one of the five major groups of intermediate filament (IFs) that packs myofibrils and mitochondria together inside the cardiomyocyte and establishes a crucial link with the sarcolemma and nuclear membranes. Desmin is a 53 kDa protein that forms a cytoskeletal network across the muscle fiber bordering at the plasma and nuclear membrane and is particularly localized to the Z-band [7]. Desmin has been described as a major integrator of IF

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network raising the question of how desmin can be secreted into the blood.

Our experimental rat model of ischemic HF offers an easy access to heart tissue and plasma from the same animal and we have identified increased phosphorylation of desmin in LV of HF-rats compared to the sham-rats (Supplemental Fig.1 and Supplemental Table 1).

It was recently reviewed that during the development of HF, heart homeostasis should be maintained by the balance between protein synthesis and degradation [8]. Three major degradation systems have been identified, namely the calpain systems, autophagy and the ubiquitin proteasome. Interestingly, desmin is the target of proteolytic degradation systems and of post-translation modifications (PTMs), such as phosphorylation and ubiquitinylation [9].

Desmin as other cytoskeletal proteins has been hypothesized to reproducibly degenerate into the same degradation products, upon the activity of the Ca^{2+} dependent calpains [10] that have been localized in heart mitochondria [11]. These recent data suggest that calpains in the heart could be involved in degradation of desmin that could be secreted into the blood.

The purpose of this work performed in a rat model of HF following MI was to characterize (1) whether cardiac desmin is a target of the same or other sites of phosphorylation than those previously described; (2) its susceptibility to degradation proteolytic systems in this experimental model of HF post-MI and, (3) its potential as being a tissue or circulating biomarker of ischemic HF.

2. Materials and methods

2.1. Animal models

All animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication NO1-OD-4-2-139, revised in 2011). Animals were used and experimental protocols performed under the supervision of a person authorized to perform experiments on live animals (F. Pinet: 59-350126, exp. date: 22 June 2016). Approval was granted by the institutional ethics review board (CEEA Nord Pas-de-Calais N°242011, January 2012).

Before surgery, rats were anaesthetized (sodium methohexital, 50 mg/kg intraperitoneally (IP)), while analgesia was administered before (xylazine 5 mg/kg IP) and 1 h after surgery (xylazine 50 mg/kg subcutaneously) as described [12]. Anesthesia and sedation were controlled by monitoring heart rate. MI was induced in 10-week-old male Wistar rats (n=11) (Janvier, Le Genest *St* isle, France) by ligation of the left anterior descending coronary artery [12,13]. Haemodynamic and echocardiographic measurements were taken 2 months after surgery, followed by heart excision, as previously described [4,14].

Hearts and blood from 14-week-old C57/BL6 mices (n=3) were collected after euthanasia by an overdose of sodium pentobarbital (50 mg/kg IP).

2.2. Primary cultures of neonate rat cardiomyocytes

Primary cultures of rat neonatal contractile cardiac myocytes (NCMs) were prepared from heart ventricles of 1- or 2-day-old rats, killed by decapitation, minced in a balanced salt solution containing 20 mmol/L HEPES, 120 mmol/L NaCl, 1 mmol/L NaH_2PO_4, 5.5 mmol/L glucose, 5.4 mmol/L KCl, and 0.8 mmol/L $MgSO_4$ [pH 7.4] as previously described [15]. NCMs were seeded at a density 8×10^5 cells/well in 6-well plates coated with 0.01% of collagen (Sigma-Aldrich) and cultured in a medium containing 4 parts of DMEM and 1 part of Medium199, 10% horse serum (Life Technologies), 5% foetal bovine serum (ATCC), 1% penicillin and streptomycin (10,000 U/mL, Life Technologies) for 7 days at 37 °C under 5% CO_2 atmosphere.

2.3. Plasma preparation

Blood samples from systolic HF-human male patients of ischemic origin (LVEF < 45%) with NYHA class 2 (INCA, CP 98/94 of 5 November 1998, CHRU Lille-FRANCE) [16], HF-rat and from control mouse were collected in EDTA-treated tubes and then centrifuged for 15 min at 1600 g to remove cells and platelets. After centrifugation, the plasma (resulting supernatant) was collected, aliquoted and stored at –80 °C. Plasmas were treated either by albumin and IgG (Alb/IgG) depletion or combinatorial peptide ligand library (CPPL) as previously described [17].

2.4. Tissue fractionation

LV proteins were extracted from 40 mg of frozen tissue (after removing the infarcted area) with Dounce-Potter homogenization into ice-cold RIPA buffer (50 mmol/L Tris [pH7.4], 150 mmol/L NaCl, 1% Igepal CA-630, 50 mmol/L deoxycholate, and 0.1% SDS) containing *anti*-proteases (Complete™ EDTA-free, Roche Diagnostics), serine/threonine and tyrosine protein phosphatase inhibitors (Phosphatase inhibitor Cocktail 2 and 3, Sigma-Aldrich) and 1 mmol/L Na_3VO_4, as described previously [4]. H9c2 and NCM extracts were collected by scraping cells in the same RIPA buffer described for LV proteins above.

Protein concentrations were determined with a Bradford-based method protein assay (Biorad, Marnes-la-Coquette, France) and samples were kept at −80 °C.

2.5. Phosphoproteomic screening

Two-dimensional (2D) gel electrophoresis was performed as previously described [3]. LV proteins (500 μg) from control (n=4) and HF (n=4) rats at 2 months after surgery were analysed on a dry 24-cm strip with a pH linear gradient of 3–10 (Immobilin DryStrip, GE Healthcare). Fluorescent staining of 2-D gel was performed with Pro-Q®Diamond Phosphoprotein Gel Stain (Molecular Probes™) for 90 min for detecting the phosphorylated proteins by image acquisation with an Ettan Dige Imager (GE Healthcare) at an excitation wavelength of 540 nm and an emission wavelength of 595 nm. Gels were then stained for total proteins with Sypro®Ruby Protein Gel Stain (Molecular Probes™) overnight and images of Sypro®Ruby stained gels were acquired with the Ettan Dige Imager at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. Identification of spots of interest (spots 5 and 45) was performed by MALDI-TOF. Detailed methods have already been described [4].

2.6. Antibodies

Three primary antibodies against desmin were used, clone Y66 (ab32362, Abcam Paris, France), clone DE-U-10 (D1033, Sigma-Aldrich, Lyon, France) and clone D33 (M0760, Dako, Les Ulis, France) and one primary antibody against GAPDH (sc-365062, Santa Cruz) was used to confirm equal total proteins loads. The secondary antibody, ECL™ anti-mouse and rabbit IgG horseradish peroxidase-linked whole antibodies from sheep and donkey, respectively (GE Healthcare, Velizy-Villacoublay, France) was used for western blot. Alexa Fluor® 488 coupled anti-mouse and rabbit secondary antibodies (Invitrogen, Life Technologies) was used for immunofluorescence studies.