PROTEOMIC REMODELLING OF PROTEASOME IN RIGHT HEART FAILURE

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Abstract

The development of Right Heart Failure (RHF) is characterized by alterations of Right Ventricle (RV) structure and function, but the mechanisms of RHF remain still unknown. Thus, understanding the RHF is essential for improved therapies. Therefore, identification by quantitative proteomics of targets specific to RHF may have therapeutic benefits to identify novel potential therapeutic targets. The objective of this study was to analyze the molecular mechanisms changing RV function in the diseased RHF and thus, to identify novel potential therapeutic targets. For this, we have performed differential proteomic analysis of whole RV proteins using two experimental rat models of RHF. Differential protein expression was observed for hundred twenty six RV proteins including proteins involved in structural constituent of cytoskeleton, motor activity, structural molecule activity, cytoskeleton protein binding and microtubule binding. Interestingly, further analysis of down-regulated proteins, reveals that both protein and gene expression of proteasome subunits were drastically decreased in RHF, which was accompanied by an increase of ubiquitinated proteins. Interestingly, the proteasomal activities chymotrypsin and caspase-like were decreased whereas trypsin-like activity was maintained. In conclusion, this study revealed the involvement of ubiquitin-proteasome system (UPS) in RHF. Three deregulated mechanisms were discovered: (1) decreased gene and protein expression of proteasome subunits, (2) decreased specific activity of proteasome; and (3) a specific accumulation of ubiquitinated proteins. This modulation of UPS of RV may provide a novel therapeutic avenue for restoration of cardiac function in the diseased RHF.

Keywords: Quantitative proteomic**,** Right heart failure, Proteasome, Ubiquitin, Proteasomal activities.

1.1 Introduction

Left heart disease, including Left Heart Failure (LHF) associated with ischemia and endotoxemia, has been widely studied. Many advances have been made in the understanding of left heart disease, including characterization of left ventricular anatomy and its response to acute cardiac events. Furthermore, functional studies of the left heart have provided a vast array of data for further understanding left heart disease. In spite of its importance, right heart disease, which is distinctly different from LHF, has not been studied with the same degree as other aspects of left heart disease and remains relatively poorly understood. The primary function of the Right Ventricle (RV) is to deliver deoxygenated blood to the lungs for gas exchange. The RV effectively serves as a reservoir for blood returning to the heart via the right atrium, thereby optimizing venous return and providing sustained low-pressure perfusion through the lungs. To this end, the RV ejects blood somewhat continuously from the right atria to the lungs. On the contrary, the Left Ventricle (LV) generates high pressure pulsatile flow through arterial vessels with low compliance. In LHF it is well accepted that the process of cardiac remodeling itself, regardless of the initial cardiac event, is, although compensatory at first, detrimental in the long run [1]. There is now convincing evidence that intervening in the process of remodeling importantly reduces morbidity and mortality in patients with LHF [2][3]. Thus, initially, LV dilatation may be considered as a protective mechanism to maintain cardiac "pump" function. However, this phenomenon ultimately leads to alterations of global function of the LV and ultimately aggravates LHF. RHF is now being increasingly recognized as distinctly different from LHF [4], and an important mediator of overall cardiovascular collapse. However, very little is known about i) the structural and functional evolution of RV dysfunction in RHF, ii) the determining molecular and cellular mechanisms, iii) the direct (not afterload reducing) interventions that could preserve RV function.

We postulate that the study of RV is very interesting and important to understand the cardiac remodeling mechanisms observed in RHF. Since there is still unexploited potential for therapies that directly target the RV, a better understanding of the complex molecular events that initiate and perpetuate the process of RV dilation would facilitate the development of more selective and efficient therapies. Studying RV dilation leading to RHF is therefore fundamental, and requires the evaluation of RV proteome as well as the integration of these findings into the overall disease context.

Proteomic technology allows the examination of global alterations pattern in protein expression in the diseased heart, and can provide new insights into the cellular mechanisms involved in cardiac dysfunction [5]. It is believed that the use of proteomic analysis to investigate heart disease should result in the generation of new diagnostic and therapeutic markers [6]. To search for cardiac specific biomarkers, the analysis of cardiac tissue remains necessary [7] and experimental animal models allow to study serial changes in cardiac tissue proteins over time, and relate these changes to RHF and cardiac dysfunction. Our objective was thus to find changes in protein expression, which are specifically linked to right ventricular remodeling in RHF. For this purpose, we performed differential proteomic analysis of RV in rats in which RHF was induced either by monocrotaline (MCT) [8] or chronic hypoxia [9] as compared to RV from normal rats. The chronically hypoxic animal model, one of the most commonly used animal models, is obtained by exposure of rats to chronic–hypoxic conditions (four weeks) under hypobaric conditions (keeping animals in a chamber with barometric pressure adjusted to 0.5 atmosphere)[10, 11]. These conditions induce a progressive increase in both the mean pulmonary artery pressure (pap) and the Fulton index (the ratio of right ventricle to left ventricle plus septum weight) from 12–15 to 30–35 mm Hg and 0.25% to 0.5%, respectively. An increase in the pap and right ventricle

hypertrophy are maximal at three to four weeks of hypoxia[10, 11]. The other model is the MCT-treated rat. A single administration of MCT (generally a subcutaneous injection at the concentration of 60 mg/kg) is sufficient to induce PAH [12-14]. The initial injuries result in endothelial degeneration or hyperplasia, hypertrophy of medial smooth muscle, and adventitial edema [15]. These changes result in the augmentation of vascular resistance and the pressure overload of the right ventricle. MCT induced a higher increase in the pap (50–80 mm Hg) than hypoxia [16]. MCT-induced pulmonary hypertension (MCT-PAH) and cardiac dystrophy are irreversible [16]. MCT-PAH is also accompanied by a massive mononuclear infiltration into the perivascular regions of arterioles and muscular arteries. From this point of view (i.e., the inflammatory process), the MCT model is considered as a standard model for idiopathic PAH (IPAH)[17]. All the experiments through the paper were un-pooled experiments to make sure that we avoid any bias effect due to the use of two models. We observed that the RHF is associated with the modulation of numerous gene and proteins in the RV. Importantly, our data provide strong evidence of an ubiquitin-proteasome system (UPS) dysfunction in RHF. Our study offers new insight into the complex integration of UPS expression and activities within metabolic network that are linked to changes in pathophysiological state of RHF.

1.2 Materials and Methods

1.2.1 Heart Models

All animal experiments, euthanasia and tissue collection were performed according to protocols approved by the Institutional Animal Care and Use Committee of Bordeaux University. To induce Right Heart Failure (RHF) in rats, six week-old male Wistar rats received a single subcutaneous injection of 60mg/kg (body weight) monocrotaline (MCT), while control animals were treated with an equal volume of vehicle. In the other group, for induction of RHF by chronic hypoxia, six week-old male Wistar rats were placed in a ventilated chamber system with an inspired O2 fraction (FiO2) of 0.10, while control animals were maintained under normoxia. All animals were euthanized on day 28, when they showed accelerated breathing, lethargy, and ruffled fur.

1.2.2 Tissue Sample

Rats were euthanized after intraperitoneal injection with 50 mg/kg of ketamine and 10 mg/kg of xylazine. Hearts were rapidly excised and removed of any blood cells. The right ventricle (RV) and left ventricle (LV) plus septum (LV+S) were dissected and snap-frozen in liquid nitrogen. Samples were stored at -80°C until further use. Right ventricular hypertrophy was confirmed by the ratio of the right ventricular weight to body weight and compared to the left ventricular weight to body weight ratio.

1.2.3 Measurement of Fulton Index

The Fulton index was measured as the ratio of the RV divided by LV+S weights.

1.2.4 Hemodynamic Studies

Right Ventricular Pressure (RVP) was measured using the technique as previously described by van Suylen RJ *et al.* [18]. Mean RVP was calculated by digital integration.

1.2.5 Right ventricle protein identification by LC-MS/MS

RV proteins were extracted from each tissue sample, six animals per condition, by homogenization and solubilization in 30 mmol/L Tris-HCl pH 8.0, 150 mmol/L NaCl, 1.5% CHAPS and protease inhibitor cocktail (Sigma). The lysates were clarified by centrifugation at 435000 X g_{max} for 30 min at 4°C. Protein concentrations were determined according to the Bradford assay. Isolated RV proteins were solubilized in Laemmli buffer, and subjected to SDS-PAGE gel to evaluate concentration and for cleaning purpose. After entering the resolving gel, separation was stopped. Following a colloidal blue staining, bands were cut out from the SDS-PAGE gel and subsequently cut in 1 mm x 1 mm gel pieces. Gel pieces were destained in 25 mmol/L ammonium bicarbonate 50%, rinsed twice in ultrapure water and shrunk in acetonitrile (ACN) for 10 min. After ACN removal, gel pieces were dried at room temperature, covered with the trypsin solution (10 ng/µl in 40 mmol/L NH4HCO3 and 10% ACN), rehydrated at 4 °C for 10 min, and finally incubated overnight at 37 °C. Gel pieces were then incubated for 15 min into 40 mmol/L NH_4HCO_3 and 10% ACN at room temperature on a rotary shaker. The supernatant was collected, and an extraction solution (H2O/ACN/HCOOH (47.5:47.5:5)) was added onto gel slices for 15 min. The extraction step was repeated twice. Supernatants were pooled and concentrated with a vacuum centrifuge to a final volume of 25 µL. Digests were finally acidified by addition of 1.5 µL of formic acid (5%, v/v) and stored at -20 °C. Peptide mixture was analyzed on a Ultimate 3000 nanoLC system (Dionex) coupled to a nanospray LTQ-Orbitrap XL mass spectrometer

(ThermoFinnigan, San Jose, CA). Ten microliters of peptide digests were loaded onto a 300-µm-inner diameter x 5-mm C18 PepMapTM trap column (LC Packings) at a flow rate of 30 µL/min. The peptides were eluted from the trap column onto an analytical 75-mm id x 15-cm C18 Pep-Map column (LC Packings) with a 5–40% linear gradient of solvent B in 105 min (solvent A was 0.1% formic acid in 5% ACN, and solvent B was 0.1% formic acid in 80% ACN). The separation flow rate was set at 200 nL/min. The mass spectrometer operated in positive ion mode at a 2-kV needle voltage and a 24-V capillary voltage. Data were acquired in a data-dependent mode alternating an FTMS scan survey over the range m/z 300–1700 and six ion trap MS/MS scans with Collision Induced Dissociation (CID) as activation mode. MS/MS spectra were acquired using a 3-m/z unit ion isolation window and normalized collision energy of 35. Mono-charged ions and unassigned charge-state ions were rejected from fragmentation. Dynamic exclusion duration was set to 30s.

1.2.6 Database search and results processing

Data were searched by SEQUEST through Proteome Discoverer 1.3 (Thermo Fisher Scientific Inc.) against a subset of the 2012.01 version of UniProt database restricted to Rattus norvegicus Reference Proteome Set (35,598 entries). Spectra from peptides higher than 5000 Da or lower than 350 Da were rejected. The search parameters were as follows: mass accuracy of the mono-isotopic peptide precursor and peptide fragments was set to 10 ppm and 0.8 Da respectively. Only b- and y-ions were considered for mass calculation. Oxidation of methionines (+16 Da) was considered as variable modification. Two missed trypsin cleavages were allowed. Peptide validation was performed using Percolator algorithm [19] and only “high confidence” peptides were retained corresponding to a 1% False Positive Rate at peptide level.

1.2.7 Label-Free Quantitative Data Analysis

Raw LC-MS/MS data were imported in Progenesis LC-MS 4.0 (Non Linear Dynamics). Data processing includes the following steps: (i) Features detection, (ii) Features alignment across the 12 LC-MS/MS runs, (iii) Volume integration for 2-6 charge-state ions, (iv) Normalization on total protein abundance (volume sum in each sample), (v) Import of sequence information, (vi) ANOVA test at peptide level and filtering for features $p < 0.05$, (vii) Calculation of protein abundance (sum of the volume of corresponding peptides), (viii) ANOVA test at protein level and filtering for features $p < 0.05$. Proteins are grouped according to the parsimony principle so as to establish the minimal protein list covering all detected peptides. Noticeably, only non-conflicting features and unique peptides were considered for calculation at protein level. Quantitative data were considered for proteins quantified by a minimum of 2 peptides.

1.2.8 Protein isolation and western blotting from ventricular tissue

Right and left ventricle protein extracts were extracted as described above (Right ventricle protein identification by LC-MS/MS), and then processed for immunoblot analysis as previously described [20]. Quantification was performed using ImageJ software. Results were normalized to the anti-Gapdh protein and are presented as fold change compared with control rat, which was set to one.

1.2.9 Analysis of mRNAs

Total mRNA was extracted from right and left rat ventricles using Trizol (Invitrogen) extraction procedure as previously described [20]. RNA concentration, purity and quality were determined using the Nanodrop (Thermo Fisher scientific). For cDNA synthesis, reagents and incubation steps were applied according to the manufacture's protocol using Superscript III (Invitrogen). Real-time PCR was used for relative quantification of each gene using Gapdh as the reference gene. Quantification was performed by using ImageJ software. Results were normalized to the invariant transcript Gapdh protein and are presented as fold change compared with control rat, which was set to one. Primers sequences are shown in Supplemental Table S1.

1.2.10 Determination of overall proteosomal activities

Experiments were performed as we previously described [21]. Briefly, heart tissues were kept on ice and homogenized in extraction buffer (20 mmol/L Tris-HCl, pH 7.8, 1 mmol/L dithiothreitol, 1 mmol/L ATP, 10% Glycerol, 0.5 mmol/L EDTA, 0,5% Igepal and 5 mmol/L MgCl2). The lysates were centrifuged at 14000 g at 4°C for 15 min. The supernatants were placed on ice and assayed for protein concentrations by the Lowry method (Bio-Rad DC Protein Assay, Marnes-la-Coquette, France). The three activities of the proteasomes (chymotrypsin-like, trypsin-like, and caspase-like activities) were analyzed using fluorogenic substrates: Suc-LLVY-MCA, Boc-LSTR-MCA, and Z-LLE-β-naphthylamide (Sigma). Based on the linearity curves presented on Supplemental figure 1A-C, 50µg of protein were used for trypsin and chymotrypsin, and 200µg of protein was used for caspase-like activity. Fifty or two hundred µg of protein (depended on fluorogenic substrates, see above) and the

fluorogenic substrate (final concentration, 50 µmol/L) were incubated together in a buffer containing 20 mmol/L Tris-HCl (pH 7.8), 5 mmol/L MgCl2, 1 mmol/L DTT and 1 mmol/L ATP. Since the activity of the 19S regulatory subunit and the assembly of the 20S catalytic subunit require energy [22], the assay was therefore performed in presence of ATP to measure the overall proteasome activity which requires ATP [23, 24]. Therefore we have used 1mM ATP as previously described [23, 24]. All assays were performed in triplicate. The incubation took place at 37°C for 60 min in a 96-well plate. Fluorescence was determined at 355 nm excitation / 460 nm emission in a Microplate Spectrofluorometer (DTX 880, Beckman Coulter). Proteasomal activity was expressed as arbitrary units. The specificity of the proteasomal assay was confirmed by the ability of 50 µmol/L MG132 (Enzo Life Sciences,Villeurbanne,France), an inhibitor of proteasomal function, to nearly totally inhibit fluorescence change. Chymotrypsin and caspase activities were also conducted in the absence and presence of the specific proteasomal inhibitor 20 µmol/L epoxomicin.

1.2.11 Statistical analysis

Data are presented as standard error of the mean ± SEM for all studies. Statistical differences between groups were analyzed using a Student's *t*-test with GraphPad Prism 4 software. A *P* value of 0.05 or lower was considered significant. All experiments were designed with matched control conditions to enable statistical comparison.

1.2.12 Antibodies and reagents

The sources of the primary antibodies used in these studies were as follows: Anti-Proteasome Subunit alpha-5 (PSMA5) (ThermoScientific), anti- Proteasome Subunit beta-5 (PSMB5)

(Enzo, Life Sciences), anti- ubiquitin (Sigma-Aldrich) and anti-Gapdh (Chemicon).

1.3 Results

1.3.1 Animal and heart analysis

To assess the global protein alteration pattern during Right Heart Failure (RHF), two rats model of RHF were generated: rats under hypoxia conditions and rats injected with monocrotaline (MCT). Representative pictures of the heart from the different animals (control as compared to hypoxia or MCT conditions) displayed an increase of heart morphological size. No statistical differences were observed between MCT and chronic hypoxia controls animals (Figure Supp. 3A-C).Both conditions either MCT or chronic hypoxia induced RHF therefore from now and latter on, we will group both conditions under RHF animals (Figure 1A). We next evaluate the heart to body weight ratio (Figure 1B, E and H). The right ventricular weight/body ratio was significantly increased in RHF animals as compared to control animals (control 0.55 ± 0.031mg/g *vs* RHF 1.16 ± 0.074mg/g, Figure 1H), while the left ventricular weight/body weight ratio was unaltered (control 2.02 ± 0.134mg/g *vs* heart failure 2.11 ± 0.117mg/g, Figure 1I), indicating that conditions with MCT or chronic hypoxia resulted in pronounced myocardial hypertrophy, which was restricted to the Right Ventricle (RV). As previously reported [8, 9], RHF animals present a significant increased of the Fulton index (control 0.28 ± 0.007mg/g *vs* heart failure 0.54 ± 0.004mg/g, Figure 1J) as compared to control rats.

1.3.2 Quantitative analysis of RV proteins associated with RHF

To specifically screen for expressional changes in RV from RHF rats, we isolated RV and performed a LC-MS/MS analysis with RV from six un-pooled RHF animals (three MCT and three chronic hypoxia) and six rat controls (see Materials and Methods).

By identifying features across all LC-MS/MS runs, analysis in Progenesis LC-MS 4.0 allowed relative quantification of all peptides identified. The label-free quantitation of all proteins identified is presented in Supplemental Table 4. We have thus conducted an exhaustive study where we have carefully compared un-pooled control animals to un-pooled MCT and un-pooled control normoxia to un-pooled hypoxia animals (Supplemental Table S4) to finally conserve the common protein changes that could explain right heart failure. Per proteins, a minimum of 2 peptides, were quantified. Proteins displaying changes in intensity, which were significantly associated with RHF, were detected by ANOVA test based on the protein level, and p value under <0.05 were considered as biological relevant. Our quantitative proteomics resulted in the confident detection of 126 proteins differentially expressed with either an increased or decreased intensity in RHF rats as compared to normal rats. A listing of these 126 proteins was provided in Supplemental Table 2 and 3. Fifty six RV proteins were up-regulated (Supplemental Table S2) and seventy RV proteins were down-regulated (Supplemental Table S3) in RHF-rats.

1.3.3 Classification of the RV proteins displaying RHF-related changes

A graphical presentation of the cellular components, biological processes and molecular functions affected in RHF as predicted by analysis with the PANTHER program (http://www.pantherdb.org/) is displayed in Figure 2.

The majority of changes were associated with cytoskeleton proteins (Figure 2A – left panel). According to the observed alteration of ventricular function from RHF rats, modifications in the levels of cytoskeleton proteins were expected. Other groups of altered proteins were represented by proteins of the intracellular proteins and ribonucleoprotein

complex (Figure 2A – left panel). The majority of the proteins involved in these cellular components displayed higher levels in RHF rats compared to control group (Figure 2A – right panel).

Morphogenesis, muscle contraction and cellular organization processes constituted the most prominent categories in biological processes (Figure 2B – left panel). The majority of the proteins involved in these biological processes displayed higher levels in RHF rats compared to the control group (Figure 2B – right panel).

As expected, the majority of changes in molecular functions were associated with structural constituent of cytoskeleton, motor activity, structural molecule activity, cytoskeleton protein binding and microtubule binding (Figure 2C – left panel), which are typical sign of hypertrophy. The majority of the proteins involved in these molecular functions displayed higher levels in RHF rats compared to the control group (Figure 2C – right panel). Surprisingly, the second group of altered molecular functions was represented by proteins of the peptidase activity (Figure 2C – left panel). Thirteen out of fourteen proteins involved in the peptidase activity displayed lower levels in RHF rats compared to the control group (Figure 2C – right panel). Moreover, another group of altered proteins was represented, the proteins involved in peptidase inhibitor activity. Interestingly, a deep analysis of the proteins belonging to peptidase molecular function showed eight proteins. These eight proteins Psma1, Psma2, Psma4, Psma5, Psma7, Psmb4, Psmb5 and Psmb6, belong to the proteasome complex and were dramatically decreased (Table 1). In contrast, within the group of five peptidase inhibitors, three are up-regulated and two of them have for function to be protease inhibitors. These two proteins, Cast and Serpina3n, were expressed at very high levels in RHF animals (Table 1). In addition, all the proteasome proteins identified in this study, displayed drastically low levels in RHF animals (Supplemental Table S3, and Table 1).

Since the proteasome complex seems to be differentially regulated in RHF, we therefore focus on the proteasome for further analysis.

1.3.4 Validation of proteasome proteomic data by protein expression profiles

With the use of Western blot [20], the down-regulation of two representative proteasome proteins, Psma5 and Psmb5, were assessed. RV and LV proteins were extracted from six RHF animals (three MCT and three chronic hypoxia) as compared to six rat controls (see Materials and Methods). The mean of six different control and six different RHF rats, as well as the standard deviation and *p*-values, was determined for both the RV and Left Ventricle (LV). These data were summarized in histogram format in Figure 3. The images of the samples used for histogram quantification are presented in Supplemental Figure 2. As compared to Gapdh, a statistically significant decrease for Psma5 (Figure 3A) and Psmb5 (Figure 3B) was detected specifically in the RV from RHF rats. Thus, the trend towards down-regulation for theses two proteasome proteins confirmed and validated the directionality revealed by our proteomic quantitative analysis (Table 1 and Supplemental Table S3).

1.3.5 Validation of proteasome proteomic data by gene expression profiles.

The proteasome consists of a 20S proteasome core and two 19S regulatory subunits [25]. The 20S proteasome core is composed of 28 subunits that are arranged in four stacked rings, resulting in a barrel-shaped structure. The two end rings are each formed by seven α-subunits, and the two central rings are each formed by seven β-subunits [25]. Our results obtained above on the proteasome proteins show proteins from α- and β-subunits, therefore

the subsequent analyses were only focused on α- and β-subunits of proteasome differentially regulated (Table 1). To validate the proteomic dataset but also to evaluate the effect of inducing RHF on both ventricles (RV and LV), we investigated first to measure the genomic expression level of these proteins in the RV (Figure 4A) and LV (Figure 4B) from rats with RHF as compared to control rats. We extracted total mRNA of RV and LV from six RHF animals (three MCT and three chronic hypoxia) as compared to six rat controls (see Materials and Methods). RV and LV mRNA expressions of α- and β-subunits of proteasome normalized to Gapdh are summarized in Figure 4. The level of GAPDH was unaffected during RHF (Figure 4 E-F). Psma1, Psma2, Psma4, Psma5 and Psma7 (α-subunits) transcript levels were all statistically decreased in the RV of RHF compared to control rats (Figure 4A, black boxes *vs* white boxes). In contrast, these α-subunits were not statistically regulated in LV in RHF rats as compared to control rats (Figure 4B, black boxes *vs* white boxes). We also investigated the expressions of β-subunits genes: Psmb4, Psmb5 and Psmb6. In the RV, these β-subunits mRNAs were significantly decreased in RHF rats compared to control rats (Figure 4A, black boxes *vs* white boxes), whereas in the LV the mRNA levels did not differ significantly between RHF and control rats (Figure 4B, black boxes *vs* white boxes).

Interestingly, Calpastatin (Cast) and Serine protease inhibitor A3N (Serpina3n), which are two protease inhibitors were significantly up-regulated in RHF rats (Table 1). Messenger RNA content of Cast and Serpina3n were strongly increased in RV of RHF rats compared to control rats (Figure 4C, black boxes *vs* white boxes), whereas their LV transcript levels were unchanged in RHF and control rats (Figure 4D, black boxes *vs* white boxes).

1.3.6 Comparison of poly-ubiquitinated proteins profiles

A direct consequence of the down-regulation of both α- and β-subunits of proteasome mRNA (Figure 4A and B) and proteins (Table 1 and Figure 3) could be a modification of poly-ubiquitinated proteins patterns. Therefore, we next evaluated the amount of poly-ubiquitinated proteins in the LV and RV homogenates of normal and RHF rats. We isolated RV and LV proteins from six RHF animals (three MCT and three chronic hypoxia) as compared to six rat controls (see Materials and Methods). We found that the intensity of high-molecular weight bands was increased in the RV of the RHF rats compared to control rats (Figure 5A and B), whereas no major differences in the ubiquitin signals was observed in the LV between RHF and control rat (Figure 5A and C). These results confirmed that a specific modification of the ubiquitination pattern occurs in the RV of RHF animals.

1.3.7 Proteasome activities in RHF

Proteasome inhibition is known to lead to the accumulation of ubiquitinated proteins. Thus, based on the specific accumulation of ubiquitinated proteins shown in Figure 5, and the clear down-regulation of both α- and β-subunits of proteasome at the mRNA (Figure 4) and proteins levels (Table 1 and Figure 3) in the RV, we hypothesize that the proteasome activities must be altered in RHF. To determine whether this drastic decreased in protein abundance results in decreased proteasome catalytic activity, we used a fluorescent assay to measure the three activities. Figure Supplemental 1A-C shows the linearity of this assay in a ventricle extract from control animals based on the amount of protein added.

Thus, we have next measured the three different proteasome activities, caspase-like, trypsin-like and chymotrypsin-like activities of RV in RHF and control rats (Figure 6). We

isolated RV and LV tissues from six RHF animals (three MCT and three chronic hypoxia) as compared to six rat controls (see Materials and Methods). All proteasome activities were distinguished from background proteolitic activities with a proteasome inhibitor as detailed in Materials and Methods. The trypsin activity was decreased by 75% after addition to the assay buffer of the inhibitor (Figure Supp. 1D). The chymotrypsin activity was decreased by 92% after addition to the assay buffer of the inhibitor (Figure Supp. 1E) and caspase activity was decreased by 89% (Figure Supp. 1F). To further assess the contribution of nonproteasomal enzymes, we have used a highly specific inhibitor of proteasomes, epoxomicin. The most widely used proteasome inhibitor MG132 is not suitable for these purposes because it can also inhibits lysosomal enzymes [24, 26, 27]. Using epoxomicin, we found that the chymotrypsin activity was decreased by 76% (bar graph dark grey) after addition of the inhibitor whereas caspase-like activity was not significantly affected (bar graph light grey) (Figure Supp. 1G). Interestingly, proteasome activities were differentially regulated (Figure 6), trypsin-like activity was unaltered (Figure 6A, black boxes *vs* white boxes), whereas chymotrypsin and caspase-like activities, were significatively decreased around 60% in RHF compared to the activities in control rats (Figure 6B and C, black boxes *vs* white boxes).

Recently, Kim *et al.*, have shown that a daily administration of Bortezomib after induction of RHF suppressed substantially RHF [28]. To investigate how a proteasome inhibitor could alleviate RHF after RHF induction, we have followed the proteasome activity during the development of RHF every week until 4 weeks of RHF-induced by hypoxia. The morphological parameters are presented in Supplemental Table S5. Animals under hypoxia exhibited significant right ventricular hypertrophy, defined as an increase of right ventricular weight to body weight ratio (Figure 7A-B and Supp. Table S5) as compared to control animals. The right ventricle to body weight ratio increase significantly after one week of

hypoxia (0.98+/- 0.08, $p<0.001$) reaching a plateau at 2 weeks of hypoxia (1.33+/- 0.18, $p<0.001$) until 4 weeks (Figure 7A) whereas the left ventricle to body weight ratio present no significant change overtime (Figure 7B). The fulton index, representative of myocardial hypertrophy, shows the same trends, an increase starting after one week of hypoxia exposure (0.52+/- 0.09, $p<0.001$), reaching a plateau at two weeks (0.61+/- 0.09, $p<0.001$) and remaining constant until four weeks (Figure 7C). During the development of RHF, the right ventricular pressure increased with time of hypoxia exposure (Supp. Table S5 and Figure 7D). Since the proteasome inhibitor (Bortezomib) after induction of RHF has been shown to be beneficial to treat RHF, we hypothesize that during the development of RHF, the proteasome activity increase to reach a maximum activation, and then the activity decreased below the basal level to adapt the heart to its morphological changes. Thus, we have next measured the changes in proteasome activity in response to pressure overload induced by hypoxia following four weeks. We observed that after one week both activities chymotrypsin and caspase increased (Figure 7E-F) to then drop down under basal levels.

1.4 Discussion

Until recently, right heart disease has been relatively understudied and underappreciated. A recent report using in-depth quantitative proteomics have investigated the differential proteome of isolated mouse right and left ventricle [29]. Interestingly they found very subtle change between LV and RV, suggesting that changes in protein expression due to HF could be specifically linked to remodeling of the ventricle. In order to dissect differences particular to RHF hypertrophy, the aim of this study was to search for protein that were differentially expressed. Proteomic expression profiling of RV in RHF had identified 126 differential protein expressions (Supplemental Tables S2 and S3). Among the proteins that are up-regulated in RHF, we found several proteins already reported at the RNA level in RHF mice model [30] such as tropomyosin 1, myosin heavy polypeptide 9, Synpo protein, Reticulon-4, Neural cell adhesion molecule 1. However few reports in the literature have look at down-regulated proteins rather than up-regulated proteins. Interestingly, many proteasome subunit α- and β- proteins quantified in our study were found highly depressed in the RV of RHF rats (Table 1). The cardiac proteasome contains a variable proteasome complex consisting of different proportions of β-subunits [31]. Alternations in proteasome subunit composition affect overall proteasome proteolytic activity, which, in turn, may alter the specificity and selectivity of the proteasome for various substrates under certain conditions. The proteasome subunit α- and β- proteins, Psma1, Psma2, Psma4, Psma5, Psma7, Psmb4, Psmb5 and Psmb6 show low expression levels in RHF as compared to the control group (Table 1). Interestingly, the increased expression of protease inhibitors (Figure 4) in RHF let us to suggest that the balance between proteasome components and protease inhibitors is specifically altered in the RV of RHF. Indeed, Serpina3n belongs to the Serpin family of protease inhibitors. Some of them such as Plasminogen activator inhibitor type-1 (PAI-1) [32]

and type-2 (PAI-2) [33] have been shown to interact with proteasome and affect its activity, suggesting that an increase in the expression of protease inhibitors could reflect and confirm a decrease in proteasome activity. By Western blot, the down-regulation of two representative proteins, Psma5 and Psmb5, was confirmed (Figure 3). Thus, the trend towards down-regulation for theses two proteasome proteins confirmed the directionality revealed by our proteomic quantitative analysis. Psma1, Psma2, Psma4, Psma5 and Psma7 (α-subunits) transcript levels were all statistically decreased specifically in the RV of RHF compared to control rats (Figure 4A). In contrast, as expected, these α-subunits were not statistically regulated in LV in RHF rats as compared to control rats (Figure 4B). As well, in the RV, the β-subunits mRNAs (Psmb4, Psmb5 and Psmb6) were also significantly decreased in RHF rats compared to control rats (Figure 4A), whereas in the LV the mRNA levels did not differ significantly between RHF and control rats (Figure 4B). In LHF, this type of alteration has also been reported that the transcript levels of some of the α- and β-subunits of the 20S proteasome were downregulated in failing human hearts compared with non-failing hearts [34, 35], however the pattern of alteration such as the type of subunits seems to be slightly different in the RVH as compared to the LHF. This is consistent with the physiological requirement of the RV as compared to the LV. In a non-disease state, the LV encounters the relatively high pressures of systemic circulation, which requires the formation of a thick myocardium, while RV has only to cope with the low pressure of pulmonary circulation and develops a thin wall. Due to their anatomic differences, any increase in pressure will have a much more severe impact on the RV than on the LV. Comparative microarray based transcriptome analysis of RV and LV remodeling identified distinct responses to pressure-induced hypertrophy [30].

The alterated proteasome proteins identified belong to the ubiquitin–proteasome system (UPS). The UPS is the major non-lysosomal pathway for intracellular degradation of proteins and plays a major role in regulating many cellular processes. The key components of the UPS are the 26S proteasome and ubiquitin [36].The role of ubiquitin in the UPS is to act as a tag for the proteasome to identify how the protein targeted for proteolytic degradation is destroyed. It has been reported, that during the transition of hypertrophy to LHF in patients, there was an increased level of ubiquitination during hypertrophy followed by increase upon the onset of heart failure [37] [38]. In our model RHF, we found that the intensity of several bands of poly-ubiquitinated proteins was only increased in the RV of the RHF rats compared with control rats (Figure 5A and B), whereas no major differences in the ubiquitin signals was observed in the LV between RHF and control rats (Figure 5A and C).

Here, we report by immunoblot analysis an accumulation of ubiquitinated proteins (Figure 5) and also a down-regulation of both α- and β-subunits of proteasome at the mRNA (Figure 4) and proteins levels (Table 1 and Figure 3) specifically in RV from RHF rats. Altogether, these results suggest impairment of RV proteasomal activity in RHF rats. The vast majority of proteins in mammalian cells is degraded by 26S proteasomes [39]. The 26S enzyme consists of the 20S proteasome and one or two 19S regulatory complexes [40]. The 20 S proteasome is composed of two outer α- and two inner β-rings [40]. Each ring contains seven different subunits, and each β-ring contains three proteolytic sites, which differ in their substrate specificities. The “chymotrypsin-like” site cleaves peptide bonds preferentially after hydrophobic residues; the “trypsin-like” site cuts mainly after basic residues, and the third site “caspase-like” cuts preferentially after acidic residues [41]. It has been reported that the association of 20 S proteasomes with the 19 S regulatory complexes to form 26 S proteasomes leads to much higher rates of peptide hydrolysis [42] and confers the ability to degrade

ubiquitinated proteins as well as certain non-ubiquitinated polypeptides [43]. Thus the decrease of β subunits proteins (Figure 4) and the increased in ubiquitinated proteins (Figure 5) correlates with the effect on 26S proteasome activities (Figure 6). Recently, a growing body of evidence suggests that alteration of proteasome-mediated protein degradation also contributes to the initiation and/or progression of cardiac diseases [44-46]. Inhibition of the proteasome has been shown to impair the heart function, as indicated by the use of bortezomib, a FDA-approved chemotherapeutic anti-cancer medication, which increases the occurrences of heart failure [47]. It has been also demonstrated that bortezomib induced complications in the stressed mouse heart [48]. Kim *et al.*, have shown that a daily administration of Bortezomib after induction of RHF suppressed substantially RHF [28]. Indeed, in both models hypoxia- and MCT-induced animals Bortezomib inhibits RV hypertrophy and vascular remodeling. This type of inhibitors already used clinically for the treatment of multiple myelanoma, could therefore be beneficial in the development of RHF. However, other studies in rat, murine, and porcine models indicate that proteasome inhibition could be cardioprotective [49]. The effects of proteasome inhibition during myocardial ischaemia on cardiac function have been controversial, as both beneficial and deleterious effects have been reported. In heart failure with proteasome dysfunction, bortezomib has a detrimental effect. In contrast, during early remodeling and left ventricular hypertrophy, Hedhli *et al.* [49] as well as Drews *et al.* [50] observed increased proteasome activities, showing that under these circumstances, Bortezomib was cardioprotective. Thus, the proteasome activities in cardiac disease remain a subject of debate and the specific molecular components, function, and regulation of the cardiac proteasome still remains largely unknown. Analyses of human biopsies of failing hearts support the concept that defective protein degradation contributes to heart failure. However, although most studies are congruent in showing high total levels of ubiquitinated proteins in failing hearts [51], it remains unclear

whether this is attributable to impaired proteasome function. Some reports show that the levels and activity of the proteasome are unchanged in failing hearts [52]. In contrast, other reports show that proteasome activity is impaired in failing hearts, possibly as a result of oxidative modifications on proteasome subunits [53].

Here, we correlate the time-frame of RHF development to proteasome activity and found that proteasome activity increase and then drop down below basal level, these results might explained the differences between studies based on the day for which proteasome activity was measured. Based on the time-course of proteasome activity during the development of RHF, we believe that caution should be taken before comparing studies since the activity has two phases, first an increase followed by a drastic decrease at the end-stage of RHF. This model is consistent with Tsukamoto *et al.* paper in mouse model [54]. Thus, the proteasome activities in cardiac disease remain a subject of debate and the specific molecular components, function, and regulation of the cardiac proteasome still remain largely unknown. Several experimental studies have demonstrated the contribution of UPS dysfunction to the pathogenesis of LHF disease, however, there is no report investigating the role of UPS in the pathogenesis of RHF, until recently. In parallel to our study, Rajagopalan *et al.* have reported that proteasome activity is decreased in another model of RHF also suggesting that UPS dysfunction contributes to RHF [55]. It has been demonstrated previously that ubiquitinated proteins are accumulated when proteasome is deactivated and also that the inhibition of proteasome chymotrypsin-like activity was linked to an accumulation of ubiquitinated proteins [56]. Thus, depression of these two proteasome activities found in our study (Figure 6 and 7) correlates with the accumulation of ubiquitinated proteins observed in Figure 5.

1.5 Conclusion

In summary, our study demonstrates an impairment of UPS in the RHF disease with a decrease of transcript and protein levels of some α- and β-subunits of proteasome, an accumulation of ubiquitinated proteins and a specific decrease of the caspase-like and chymotrysin-like proteolitic activities in the RV. In conclusion, we demonstrate a potential role of impairment of ubiquitin–proteasome system in the development of RHF. However, it remains unclear whether the UPS is impaired as a byproduct of the pathogenesis or in fact contributes to the development of the pathogenesis. Future work in human tissue is necessary to define the precise mechanisms for proteasome dysfunction and to establish a causal link to RHF.

1.6 Appendices

A - Table

Table A.1. Differential expression of protease proteins based on their common function.

B - Figure legends

Figure B.1. Right ventricular hypertrophy in rat experimental models**. A.** Representative images of heart in rats treated with monocrotaline or in rats under chronic hypoxia as compared to control rats. **B,E,F.** Right ventricular (RV) weights normalized for body weight (mg/g) in rats under chronic hypoxia (**B**), in rats treated with monocrotaline (**E**), (**F**) in right heart failure animals (RHF). (n = 6, mean ± SEM); ***p < 0.001. **C,F,I.** Left ventricular plus septum (LV + S) weights normalized for body weight (mg/g) in rats under chronic hypoxia (**C**), in rats treated with monocrotaline (**F**), (**I**) in right heart failure animals (RHF). (n = 6, mean ± SEM). **D,G,J.** The mean Fulton index is measured as described in the *Methods*, in rats under chronic hypoxia (**D**), in rats treated with monocrotaline (**G**), (**J**) in right heart failure animals (RHF). (n = 6, mean ± SEM); ***p < 0.001.

Figure B.2. Cellular components, biological processes and molecular functions mainly influenced by RHF. Most pronounced cellular components (**A**), biological processes (**B**) and molecular functions (**C**) revealed by PANTHER analysis (http://www.pantherdb.org/) of proteins displaying differences in intensity. On the left panel horizontal axis indicates the significance (-log *p* value) of the functional association, which is dependent on the number of proteins in the class. On the right panel, changes in the protein cellular components pattern are

displayed as the number of proteins with increased (gray) or decreased (black) levels (horizontal axis).

Figure B.3. Validation of abundance protein changes by western blot. **A.** Lower panels: Immunoblot analysis of Psma5 and Gapdh in the right and left ventricles of RHF and control rats. Upper panels: histogram representation of Psma5 normalized to Gapdh (n = 6, mean ± SEM); **p < 0.01. **B.** Lower panels: Immunoblot analysis of Psmb5 and Gapdh in the right and left ventricles of RHF and control rats. Upper panels: histogram representation of Psmb5 normalized to Gapdh (n = 6, mean ± SEM); **p < 0.01.

Figure B.4. Reduced proteasome subunits mRNA levels in ventricles from right heart failure animals. **A.** RT-PCR analysis of mRNA proteasome subunits levels in RV from RHF as compared to control rats (black bars and white bars, respectively) (n=6, mean ± SEM). **B.** RT-PCR analysis of mRNA proteasome subunits levels in LV from RHF animals as compared to control (black bars and white bars, respectively) (n=6, mean ± SEM). **C.** RT-PCR analysis of mRNA levels of Calpastatin (*Cast*) and Serine protease inhibitor A3N (*Serpina3n*) in RV from RHF as compared to control rats (black bars and white bars, respectively) (n=6, mean ± SEM). **D.** RT-PCR analysis of mRNA levels of Calpastatin (*Cast*) and Serine protease inhibitor A3N (*Serpina3n*) in LV from RHF as compared to control animals (black bars and white bars, respectively) (n=6, mean ± SEM). Means and standard errors are shown, and statistical significance by Student's t test is indicated by one ($p < 0.05$), two ($p < 0.01$) or three ($p<0.001$) asterisks. **E-F**, RT-PCR of RNA expression of Gapdh from right ventricle (RV,

panel **E**) or left ventricle (LV, panel **F**) for each condition normal *vs.* RHF animals induced by hypoxia or MCT.

Figure B.5. Significant changes in ubiquinated proteins in the RHF. **A.** Representative immunoblot analysis of ubiquitinated proteins in the right and left ventricles of RHF rats as compared to control rats using α-ubiquitin and Gapdh antibodies are shown. Gapdh served as a loading control. **B.** Histogram representation of ubiquitinated proteins in the right ventricles of RHF rats (black box) and control rats (white box) normalized to Gapdh (n = 6, mean ± SEM); ***p < 0.001. **C.** Histogram representation of ubiquitinated proteins in the left ventricles of RHF (black box) and control rats (white box) normalized to Gapdh (n = 6, mean ± SEM).

Figure B.6. Divergent regulation of proteasome activities in the RHF. Proteasomal trypsin-like (**A**), chymotrypsin-like (**B**), caspase-like (**C**) activities were evaluated using fluorogenic substrates as described in *Material and methods*. Reaction rates are standardized by comparison to normal animal values and expressed as arbitrary units (n = 6, mean ± SEM); ***p < 0.001.

Figure B.7. Time course of development of RHF-induced by hypoxia **A.** Right ventricular (RV) weights following weeks of hypoxia normalized for body weight (mg/g) (n = 6, mean ± SEM); ***p < 0.001. **B.** Left ventricular plus septum (LV + S) weights normalized for body weight (mg/g) following weeks of chronic hypoxia. (n = 6, mean ± SEM). **C.** The mean Fulton index is measured as described in the *Methods,* in rats under chronic hypoxia following

weeks (n = 6, mean ± SEM); ***p < 0.001. **D.** Right ventricular (RV) pressure following weeks of hypoxia (mmHg) (n = 6, mean ± SEM); ***p < 0.001. **E-F.** Proteasomal chymotrypsin-like (**E**), caspase-like (**F**) activities were evaluated using fluorogenic substrates as described in *Material and methods*. Reaction rates are standardized by comparison to normal animal values and expressed as arbitrary units (n = 6, mean ± SEM);***p < 0.001.

C - Supplemental data

Supplemental Figure 1. Linearity and specificity of proteasome activities. **A-C.** Linearity of the trypsin assay (**A**), chymotryptic assay (**B**) and caspase assay (**C**) (n=3) per group in hearts from control animals, as function of the protein amount. **D-F**. Proteasome activity for trypsin (D), chymotrypsin (**E**) and caspase (**F**) in control *vs.* RHF animals with or without MG132 (n=6/group). **G.** Proteasome activity for chymotrypsin and caspase in control animals with or without Epoxomicin (n=6/group).

Supplemental Figure 2. A-B, Protein expression of PSMA5 from right ventricle for RHF-induced by MCT (**A**) or by hypoxia (**B**) for each samples tested. **C-D**, Protein expression of PSMB5 from right ventricle for RHF-induced by MCT (**C**) or by hypoxia (**D**) for each samples tested. Data are normalized per expression of Gapdh and used for graph quantification (See Figure 3).

Supplemental Figure 3. A-C, Statistical comparison between controls for RHF-induced by MCT or by hypoxia. . **A.** Right ventricular (RV) weights normalized for body weight (mg/g) in rats controls for hypoxia animals (**light grey**),or in rats treated with monocrotaline (**dark grey**), **B.** Left ventricular plus septum (LV + S) weights normalized for body weight (mg/g) in rats under chronic hypoxia , and in rats treated with monocrotaline. (n = 3, mean ± SEM).

C. The mean Fulton index is measured as described in the *Methods*, in rats under chronic hypoxia and, in rats treated with monocrotaline. (n = 3, mean ± SEM).

Supplemental Table S1. Primers used for PCR verification (5' - 3').

Supplemental Table S2. Summary of proteins showing enrichment levels in RHF.

Supplemental Table S3. Summary of proteins showing loss levels in RHF.

Supplemental Table S4. Full reports for peptide counts, peptide coverage, unique peptides and unique spectra for each animal: normoxia *vs.* hypoxia and control *vs*. MCT (un-pooled data).

Supplemental Table S5. Parameters of rat controls compared to rats under hypoxia over time

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