Implications of the cardiomyocyte stress response on protein homeostasis in atrial fibrillation

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2016

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter 2

Proteostasis and Scope of the thesis
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The proteostasis network
Proteostasis is the homeostasis of protein synthesis, folding, assembly, trafficking, function and degradation. The protein quality control (PQC) network represents the complex machinery that maintains this homeostasis and consists of the translational machinery, enzymes (such as folding and deubiquitinating enzymes), molecular chaperones and co-chaperones, the ubiquitin-proteasome system, the autophagic pathway and mitochondria. A healthy proteostasis is crucial for cell survival, therefore, components of the PQC network can be found in all cellular compartments. Physiological, metabolic and environmental changes are able to cause proteotoxic stress, i.e. aberrant, misfolded, accumulated and/or aggregated proteins, and thereby challenge a healthy proteostasis. Proteotoxic stress has been found in several cardiac diseases, including desmin-related, hypertrophic, familial and hypertensive cardiomyopathies, (congestive) heart failure, metabolic syndrome-related cardiac disease and ischemic heart disease. Proteotoxic stress induces the activation of one or more stress-responsive pathways of the PQC network. These pathways can assist either in refolding or directing aberrant proteins towards degradation. Depending on the cellular compartment affected by proteotoxic stress, one or multiple stress-responsive pathways can be activated. The three main stress-responsive pathways of the PQC network are: the heat shock response (HSR), the mitochondrial unfolded protein response (UPR_mito) and the endoplasmic reticulum (ER) unfolded protein response (UPR_{ER}).

The HSR is activated by cytosolic and/or nuclear proteotoxic stress and this pathway is mainly governed by the activation of heat shock factor 1 (HSF1). Under normal conditions, the molecular chaperones heat shock protein 90 (HSP90) and TriC bind to HSF1 in the cytosol, thereby rendering HSF1 inactive. Proteotoxic stress drives the dissociation of HSP90 and TriC from HSF1 and promotes homotrimerization and translocation of the HSF1 trimer to the nucleus. There, HSF1 binds to the promoter region of hsp genes, including hsp70 and hsp90, which induces HSP70 and HSP90 expression (Figure 1, grey lines). The increase in HSP70 and HSP90 in the cytosol not only assists in refolding, but also alleviates the HSR by binding to active HSF1.

The UPR_mito is activated by mitochondrial stress, either proteotoxic or metabolic as observed in heart failure and metabolic syndrome-related cardiac disease, in which obesity serves as one of the cardiac disease-generating substrates and activators of the UPR_mito. The regulation of this stress-responsive pathway is extensively studied in Caenorhabditis elegans, but is not entirely known in mammals. In C.
elegans, activating transcription factor associated with stress 1 (ATFS1) is the main regulator. This transcription factor has a mitochondrial targeting sequence and is, therefore, canonically present in the mitochondria. In the absence of mitochondrial stress, ATFS1 is degraded by mitochondrial proteases. However, mitochondrial stress impairs mitochondrial ATFS1 import, resulting in cytosolic accumulation of ATFS1. This accumulation leads to translocation of ATFS1 to the nucleus, where it binds to the promotor region of genes coding for mitochondrial chaperones, such as

Figure 1 The three stress-responsive pathways of the proteostasis network. Proteotoxic stress activates ER stress (depicted by the green lines), which drives the dissociation of HSPA5 from IRE1, PERK and ATF6, thereby enabling HSPA5 to bind to aberrant proteins, resulting in attenuation of proteotoxic stress. Both IRE1 and PERK homodimerize and autophosphorylate upon activation. IRE1 splices XBP1 mRNA, resulting in an active XBP1 transcription factor. PERK phosphorylates eIF2α, resulting in a general protein translation block, and activates the transcription of genes related to ER stress, including transcription factor ATF4. ATP6 is translocated to the Golgi apparatus, where it is cleaved. XBP1, ATF4 and spliced ATF6 all translocate to the nucleus and bind to the promotor of genes of molecular chaperones involved in resolving ER stress. Cytosolic proteotoxic stress (depicted by the grey lines) promotes the dissociation of HSP90 and TriC from HSF1, thereby activating the latter. Active HSF1 homotrimerizes and translocates to the nucleus as a transcription factor to promote expression of HSPs, which help to restore proteostasis. Mitochondrial stress (depicted by the blue lines) inhibits the import of ATFS1 into the mitochondria, where it is degraded by mitochondrial proteases under normal conditions. The cytosolic accumulation of ATFS1 leads to the translocation of this transcription factor to the nucleus, where it binds to the promotor of genes involved in resolving mitochondrial stress. Activation of these three stress-responsive pathways lead either to refolding or degradation of aberrant proteins.
HSP60 and HSP10, and components of mitochondrial import, fission and respiratory chain complexes (Figure 1, blue lines). The restoration of the mitochondrial import machinery attenuates the UPR\textsubscript{mito} as, consequently, ATFS1 is imported again into the mitochondria and, therefore, the cytosolic amount of ATFS1 reduces. In mammals, several components of the UPR\textsubscript{mito} are found to be conserved from \textit{C. elegans}, such as HSP60 and HSP10 upregulation. Nevertheless, the transcriptional regulation of mammalian UPR\textsubscript{mito} is not yet elucidated.\textsuperscript{15}

The UPR\textsubscript{ER} is activated upon ER stress. ER stress is found in dilated\textsuperscript{16,17} and ischemic\textsuperscript{16} cardiomyopathy and heart failure,\textsuperscript{18,19} and results from the accumulation of unfolded, misfolded and/or aberrant proteins in the ER lumen. As the ER is the site where the major part (at least one-third) of all proteins are folded and synthesized,\textsuperscript{9,20} it is no surprise that the UPR\textsubscript{ER} is the most complex and elaborate stress-responsive pathway of all three. This pathway activates three stress-responsive sensors in parallel: inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6) and protein kinase-like endoplasmic reticulum kinase (PERK). These proteins are inactive under normal conditions by the binding of the ER chaperone HSPA5 (also known as BiP or GRP78) to their luminal domain. During ER stress, HSPA5 binds unfolded proteins by their externalized hydrophobic regions, resulting in the dissociation of HSPA5 from the stress sensors. This dissociation leads to the activation of IRE1, ATF6 and PERK. Activated IRE1, a transmembrane protein, homodimerizes and autophosphorylates, thereby gaining RNase activity. This RNase activity enables activated IRE1 to splice \textit{X-box binding protein 1} (XBP1) mRNA. The spliced XBP1 (XBP1\textsubscript{s}) is an activated transcription factor that translocates to the nucleus and binds to the promoter region of genes of UPR\textsubscript{ER} molecular chaperones and folding catalysts (Figure 1, green lines). In the second branch of the UPR\textsubscript{ER} stress-responsive pathway, the transmembrane protein ATF6 is transported from the ER to the Golgi apparatus upon dissociation of HSPA5. In the Golgi apparatus, ATF6 is cleaved by proteases and the N-terminal fragment translocates to the nucleus and binds to the promoter region of other molecular chaperone genes involved in the UPR\textsubscript{ER} (Figure 1, green lines).\textsuperscript{1,9,20,21} The third branch of the UPR\textsubscript{ER} consists of activation of the transmembrane protein PERK, which, similar to IRE1, homodimerizes and autophosphorylates. Autophosphorylation causes PERK to phosphorylate eukaryotic initiation factor 2 alpha (eIF2α). Phosphorylated eIF2α blocks the entire protein translation and synthesis, except for activating transcription factor 4 (ATF4), which is selectively transcribed during PERK activation (Figure 1, green lines). ATF4 subsequently induces expression of C/EBP homologous protein (CHOP),
which leads to activation of apoptosis during chronic or severe UPR\textsubscript{ER} activation.\textsuperscript{1,9,20,21} All three stress-responsive pathways activate expression of molecular chaperones, which, therefore, make up the major part of the PQC network. These molecular chaperones regulate the folding, refolding, trafficking or degradation of substrates, often aided by co-chaperones. The degradation of proteins (aberrant, misfolded, damaged or aged) can be accomplished by two different degradation pathways: the ubiquitin-proteasome system (UPS) or macro-autophagy (hereafter ‘autophagy’).\textsuperscript{1,9} The UPS is a degradation pathway for single proteins, especially short-lived or misfolded. Molecular chaperones promote the polyubiquitination and relocation of proteins to the cytosolic proteasomes. In order to be degraded by the proteasome, the polyubiquitin chain must be removed from the substrate, which is accomplished by deubiquitinating enzymes. Then, the substrate is unfolded and enters the proteasome, where it is degraded by

![Figure 2](image_url)
proteosomal proteases (Figure 2A).\textsuperscript{1,9,22} The second degradation pathway, autophagy, is a bulk degradation system in which large cytosolic proteins, protein aggregates and/or organelles are sequestered into a double-membrane structure, called an autophagosome. After the fusion of the autophagosome with a lysosome, lysosomal hydrolases degrade the contents into ATP, amino acids and fatty acids, which are being recycled (Figure 2B).\textsuperscript{23} Autophagy is an important degradation pathway in the PQC network, as upon UPS failure, substrates are directed to this pathway and do not need to be unfolded before degradation.\textsuperscript{9,22}

**Central role for autophagy in the proteostasis network**

The importance of the autophagic pathway is exemplified by the notion that each of the stress-responsive pathways of the PQC network by its own is capable of activation of autophagy. HSF1 was shown to not only increase expression of molecular chaperones, but also of autophagy-related protein 7 (ATG7)\textsuperscript{24} and sequestrome 1 (p62/SQSTM1),\textsuperscript{25} which are both involved in the autophagic pathway. Moreover, HSP70 was found to be involved in the formation of the autophagosomes.\textsuperscript{26} The UPR\textsubscript{mito} can activate an organelle-specific autophagic pathway, called mitophagy, which specifically degrades old or damaged mitochondria.\textsuperscript{27,28} Mitophagy is regulated by the expression of PTEN-induced putative kinase 1 (PINK1). PINK1 is transported to the outer mitochondrial membrane and is, under normal conditions, cleaved and degraded by the UPS.\textsuperscript{27,28} However, old or damaged mitochondria do not cleave PINK1, leading to accumulation of PINK1 on the mitochondria. This accumulation triggers the translocation of the E3 ubiquitin ligase Parkin to the mitochondria. Parkin ubiquitinates several proteins on the outer mitochondrial membrane.\textsuperscript{27,28} This ubiquitination recruits LC3\textsuperscript{29} and p62,\textsuperscript{28} an adaptor protein for autophagic cargo that binds to LC3. These proteins bind to PINK1\textsuperscript{29} and parkin-ubiquitinated substrates,\textsuperscript{28} respectively. Finally, ER stress can activate autophagosome formation by action of HSPA5\textsuperscript{30} and phosphorylated eIF2α, which enables the transcription of ATG5,\textsuperscript{31} ATG12,\textsuperscript{32} and LC3.\textsuperscript{31} ATG5 and ATG12 form a complex that is necessary for the formation of the autophagosomes and for the conversion of LC3.\textsuperscript{32}

**Stress-responsive pathways activated in parallel**

As mentioned before, multiple stress-responsive pathways may be activated in parallel, depending on the proteotoxic stress encountered. Mitochondrial stress often leads to increased mitochondrial and cytosolic expression of reactive oxygen species (ROS),
which activate both the UPR_{mito}^{33} and the HSR,^{34} respectively. Moreover, cytoplasmic proteotoxic stress triggers translocation of the mitochondrial SSPB1, involved in the UPR_{mito} to the nucleus where it binds to HSF1 to induce both cytoplasmic and mitochondrial chaperones.^{35} Especially the UPR_{ER} and UPR_{mito} influence each other as the ER and mitochondria are in close contact through the mitochondria-associated membranes (MAMs), which promote the exchange of metabolites, such as lipids and Ca^{2+}, between both organelles. Moreover, due to the close contact between these two organelles, mitochondria are sensitive to develop stress in response to ER stress,^{36} and vice versa.^{37} Notably, PERK activation strongly influences mitochondrial function. PERK is enriched in MAMs^{38} and is therefore able to influence the function of MAMs and mitochondria. The subsequent eIF2α phosphorylation blocks overall transcription,^{1,9,20,21} including transcription of mitochondrial electron transport chain subunits, thereby decreasing ATP production.^{39} This may result in excessive cellular stress as many chaperones involved in the PQC network are ATPases and, therefore, dependent on ATP for their folding properties. In addition, eIF2α phosphorylation drives the selective degradation of the mitochondrial translocase of the inner membrane 17A (TIM17A), which is part of TIM23, the protein responsible for two-third of all mitochondrial protein import, including that of ATFS1.^{40} On the other hand, PERK activation also mobilizes proteins to counteract mitochondrial stress. For instance, ATF4 is able to increase expression of Parkin^{37} and activates the nuclear factor-erythroid-derived 2-related factor 2 (NRF2) transcription factor.^{41} Parkin was shown to protect against apoptosis during the UPR_{ER/mito} by promoting degradation of pro-apoptotic proteins by the UPS.^{42,43} Moreover, in addition to promoting mitophagy, Parkin plays a physiological role in mitochondrial ATP production, as Parkin potentiates the formation of MAMs.^{44} This increases the mitochondrial Ca^{2+}-uptake,^{44} which promotes ATP synthesis.^{45} NRF2 plays an important role in the cellular redox homeostasis, and high intracellular/intraorganellar ROS levels cause this transcription factor to augment transcription of several genes coding for antioxidant proteins.^{46} Moreover, NRF2 was also found to increase ATP production.^{46}

**Derailment of proteostasis**

It has been recognized that proteostasis is crucial for cell survival, and derailment of proteostasis can have detrimental effects on cellular and even organismal homeostasis. This is exemplified by the large number of disorders caused by proteostasis derailment,
due to extensive accumulation of aberrant proteins, deficits of PQC network components, chronic activation of single PQC network components, stress-responsive pathways and/or degradation pathways or mutations in PQC network components. As mentioned earlier, many of the components of the PQC network are ATPases. Therefore, mitochondrial dysfunction will have profound effects on the functioning of the PQC network. This is underscored by the amount of disorders, including cardiovascular diseases, cancer, neurodegenerative diseases and metabolic disorders, that are associated with mitochondrial dysfunction and, subsequently, with proteostasis derailment. For example, mutations in *hspd1*, the gene encoding the mitochondrial chaperone HSP60, are linked to neurodegenerative diseases. HSP60 is an important mitochondrial chaperone as it is necessary for folding of mitochondrial proteins and counteracting mitochondrial proteotoxic stress. Mutations in *hspd1* result in a compromised folding activity of HSP60, leading to chronic UPRmito activation. The neurodegenerative disorder French Canadian Leigh syndrome has been linked to a mutation in *lrpprc*, which reduces the activity of complex IV of the mitochondrial electron transport chain (ETC), resulting in ATP depletion and chronic activation of the UPRmito. Further, UPR-associated protein degradation can be impaired by mitochondrial stress, which could possibly contribute to neurodegenerative diseases by impaired degradation of misfolded proteins. Lastly, a *dynamin-related protein 1* (*drp1*) mutation showed dysregulation of protein assembly, leading to inhibition of mitophagy, ATP depletion, inflammation and, ultimately, dilated cardiomyopathy. The autophagic pathway is aberrantly activated in multiple diseases, including cancer, neurodegenerative, lung, renal, liver, intestinal and cardiovascular disorders. Autophagy activation is not uniform in all disorders, in some autophagic upregulation is detrimental, while in others downregulation has detrimental effects. For example, autophagy is oncosuppressive in healthy cells, but once cancerous transformation has occurred it promotes further proliferation. In addition, dysfunction in lysosomal metabolism, such as in lysosomal storage disorders, is characterized by impaired clearance of autophagosomes and induction of ER and oxidative stress. Chronic UPRER activation has been found to accelerate disease progression in several conditions, including neurodegeneration, sleep apnea, diabetes, cancer, viral infections and cardiovascular diseases. Augmented HSF1 expression levels have been shown to promote tumorigenesis and have been found in several malignancies, including breast cancer, leukemia, myeloma, skin cancer, lung cancer, liver cancer and endometrial cancer. Often,
increased expression of HSF1 is associated with poor patient survival and drug resistance in cancer patients.\textsuperscript{24,83-85} The importance of HSF1 in cancer is emphasized by the finding that HSF1 knockout mice are highly resistant to developing cancer.\textsuperscript{79} Besides HSF1, components of the UPR\textsubscript{ER} and UPR\textsubscript{mito} are also involved in cancer development. IRE1, PERK, ATF6 and HSPA5 were shown to be involved in angiogenesis, cell adhesion and survival, metastasis and/or resistance to chemotherapy.\textsuperscript{70-74} Furthermore, mitochondria were shown to promote cancer development,\textsuperscript{86,87} as HSP60 expression is increased in multiple cancers and correlates with prognosis and tumor progression.\textsuperscript{88-91}

Most diseases that are associated with proteostasis derailment do not feature defects in a single component of the PQC network, such as in the few examples given below. Neurodegenerative diseases, such as Parkinson’s disease, Alzheimer’s disease, Huntington’s disease and amyotrophic lateral sclerosis, are characterized by accumulation of disease-causing protein aggregates and profound derailment of proteostasis has been found to account for disease progression. As the PQC network is not able to degrade the overload of misfolded proteins,\textsuperscript{92} the UPR\textsubscript{ER} is chronically activated in these diseases, leading to autophagy and CHOP activation, induction of apoptosis and, ultimately, neuronal cell death.\textsuperscript{93} However, the accumulation of autophagosomes both in patients and in \textit{in vivo} models show that the autophagic pathway can be impaired.\textsuperscript{94,95} The importance of this pathway is emphasized by the finding that disruption of autophagy in \textit{atg5}- and \textit{atg7}-deficient mice induces severe neurodegeneration.\textsuperscript{96,97} Furthermore, mitochondrial dysfunction\textsuperscript{98} and impaired HSR\textsuperscript{99} (mostly at the end of disease progression) were found in these neurodegenerative diseases.

In diabetes mellitus type 2 (DM2), protein aggregation of IAPP (islet amyloid polypeptide) also leads to CHOP upregulation, apoptosis activation and, ultimately, pancreatic β-cell death.\textsuperscript{69} Moreover, altered autophagy,\textsuperscript{100} increased oxidative stress\textsuperscript{101} and reduced expression of HSPs\textsuperscript{102,103} are present in pancreatic β-cells and skeletal muscle, respectively, of DM2 patients, the latter correlating with insulin resistance in these patients.\textsuperscript{102,103} Furthermore, vascular complications are correlated with single nucleotide polymorphisms in \textit{psmd9}, a gene encoding a proteosomal component,\textsuperscript{104} suggesting UPS involvement in DM2 disease progression.

Hutchinson Gilford progeria, an accelerated aging disorder, is due to a mutation in the laminin A (\textit{lmna}) gene. This mutation results in overproduction of reactive oxygen species and divergent activities of ETC complexes I, IV and V, leading to mitochondrial
dysfunction, enhanced activation of autophagy and increased protein misfolding.\textsuperscript{105} Multiple lung diseases, including cystic fibrosis and COPD, are associated with disease-causing mutations leading to derailment of proteostasis. In cystic fibrosis, mutations in \textit{cftr} lead to misfolded protein products, which are correctly degraded by the PQC system, resulting in loss of the CFTR protein and cystic fibrosis. In addition, chronic activation of the HSR has been reported in cystic fibrosis.\textsuperscript{106} Next to this, several polymorphisms on different genes have been shown to associate with COPD development,\textsuperscript{107-109} a disorder in which loss of ATP,\textsuperscript{110} oxidative stress, mitochondrial dysfunction\textsuperscript{111} and inappropriate activation of the UPS and autophagy\textsuperscript{110} have been reported.

### Derailment of proteostasis in atrial fibrillation

Atrial fibrillation (AF) is characterized by electrical and structural remodeling of the atria.\textsuperscript{112} Structural remodeling of cardiomyocytes includes myolysis (loss of sarcomeres), fibrosis, myocyte hypertrophy and atrial dilatation, mitochondrial morphologic changes and loss of sarcoplasmic reticulum, which are a consequence of AF, but, subsequently, also a trigger for AF.\textsuperscript{112,113} Although electrical remodeling in AF is reversible,\textsuperscript{114,115} structural remodeling is not.\textsuperscript{116} Therefore, a ‘second factor’, contributing to structural remodeling and progression of AF, has been proposed to underlie impaired cardiomyocyte reversibility.\textsuperscript{117} Over the years, evidence has emerged that derailment of proteostasis represents this ‘second factor’.

A key feature of AF is intracellular Ca\textsuperscript{2+} overload. As Ca\textsuperscript{2+} is able to activate several Ca\textsuperscript{2+}-dependent substrates, such as calpain, intracellular Ca\textsuperscript{2+} overload is an early trigger for cardiomyocyte stress.\textsuperscript{118,119} This cardiomyocyte stress induces the activation of three key pathways, namely protein degradation,\textsuperscript{120} post-translational modifications\textsuperscript{121-124} and RhoA GTPase,\textsuperscript{125} which contribute to the derailment in proteostasis in AF initiation and progression.

RhoA GTPase is an enzyme involved in multiple cellular functions, including stabilization of microtubules, cell adhesion and migration, cardiomyocyte contractility, gene expression, cell cycle progression and enzyme activation.\textsuperscript{126} RhoA GTPase activity is increased upon AF initiation in an \textit{in vitro} AF model and this increase drives the formation of F-actin stress fibers. F-actin stress fibers contribute to changes in structural proteins, which, ultimately, lead to contractile dysfunction.\textsuperscript{125} RhoA activity, and the subsequent detrimental changes, are attenuated by overexpression of a specific HSP, HSPB8.\textsuperscript{125} The HSR is exhausted during AF progression, and induction of the HSR showed protective effects.\textsuperscript{125,127-129} Although RhoA activation is involved in AF
disease progression and HSPB8 boosting is protective, the effect of RhoA activation on the HSR in AF, however, is not known (Figure 3).

Changes in protein function, activity and/or stability, due to aberrant post-translational modifications, also cause proteostasis derailment and AF-induced remodeling. Aberrant phosphatase and kinase activities were associated with decreased L-type Ca\(^{2+}\) current,\(^{123,124}\) increased potassium current\(^{122}\) and increased sarcoplasmic reticulum Ca\(^{2+}\) leakage,\(^{121}\) which are all related to AF progression. Interestingly, previous studies in dogs treated with the HSP inducer GGA revealed protective effects against AF, indicating boosting of the HSR to protect against AF. So, by comparing the kinomic profile between dogs with AF and with AF in combination with an HSP-inducing treatment, we may be able to identify kinases involved in AF remodeling (Figure 3).

**Figure 3 AF-induced derailment of cardiomyocyte proteostasis.** AF is characterized by cellular Ca\(^{2+}\) overload, which leads to cardiomyocyte stress and, subsequently, to derailment of proteostasis. Pathways leading to induction of protein degradation, posttranslational modifications and changes in structural proteins and gene expression are implicated in the derailment of proteostasis. Components of these pathways are also able to activate each other. The derailment of proteostasis leads to irreversible structural remodeling, which is preceded by reversible electrical remodeling (creating a substrate for AF) and results consequently in contractile dysfunction. The UPR\(_{\text{ER}}\), UPR\(_{\text{mito}}\), autophagy, kinome changes and HSP induction may represent key modulators of structural remodeling due to their role in proteostasis derailment.
Previous research identified calpain to degrade structural proteins, resulting in myolysis in cardiomyocytes. In these studies, autophagosomes have also been identified in patients with longstanding persistent AF. This suggests that next to calpain activation, autophagy also underlies derailment of proteostasis, structural remodeling and AF progression. Nevertheless, it is not known how autophagy is activated in AF. One possible pathway of autophagy activation is through the UPRER. As many chaperones of this stress-responsive pathway are regulated through Ca\textsuperscript{2+} binding, it is plausible that the UPRER is activated due to the divergent Ca\textsuperscript{2+} homeostasis in AF. Moreover, calpain is also able to activate the UPRER (Figure 3).

As mentioned before, ER and mitochondrial function are closely linked to each other through their connection by MAMs. Therefore, UPRER activation often results in a subsequent UPR\textsubscript{mito} activation, and vice versa (Figure 3). Acute UPRER activation has been shown to increase the amount of MAMs, in order to increase Ca\textsuperscript{2+} influx into the mitochondria. This leads to increased ATP production to promote protein refolding, as many ER chaperones are ATPases, and alleviation of ER stress. However, chronic UPRER activation results in depletion of the ER Ca\textsuperscript{2+} stores into the mitochondria. Although mitochondria are Ca\textsuperscript{2+} buffering organelles, mitochondrial Ca\textsuperscript{2+} overload, such as through ER Ca\textsuperscript{2+} depletion, will lead to increased ROS production, fragmentation and dysfunction of the mitochondria and, therefore, ATP depletion. ATP depletion is detrimental for cardiomyocytes, as contractile function is dependent on ATP. Moreover, ATP depletion, through failing mitochondria, is known to increase autophagic activity, as one of the end products of this degradation pathway is ATP.

Although mitochondria are essential for contractile function, and oxidative stress is associated with AF pathogenesis, the precise role of mitochondrial function in AF pathogenesis is not yet studied (Figure 3).

**SCOPE OF THE THESIS**

The main aim of this thesis is to elucidate the role of key stress-responsive pathways within the proteostasis network in the derailment of cardiomyocyte proteostasis, structural remodeling (including hibernation) and AF initiation and progression. Identified targets were addressed therapeutically and examined in *in vitro* and *in vivo* models for AF progression. Previously, involvement of the HSR in AF was shown, as genetic and/or pharmacological boosting of HSP expression protected *in vitro* tachypaced HL-1 atrial cardiomyocytes and *in vivo* tachypaced *Drosophila melanogaster* and dogs against AF-induced contractile dysfunction. Furthermore, we showed
an increased RhoA stimulation in \textit{in vitro} tachypaced HL-1 atrial cardiomyocytes. This increase was associated with contractile dysfunction, which was attenuated by HSPB8 overexpression.\textsuperscript{125} Since the relation between RhoA and HSP expression was unknown, we examined the connection between RhoA and the HSR in \textbf{chapter 3}. We show that RhoA stimulation in heat-shocked \textit{in vitro} HL-1 atrial cardiomyocytes suppresses HSR activation by impairing the binding of HSF1 to the \textit{hse} promotor sequence within \textit{hsp} genes. Moreover, we show that abrogation of RhoA counteract these effects. As multiple components of the proteostasis network are either regulated by kinases or are kinases themselves, we performed in \textbf{chapter 4} a kinase array in order to examine kinome homeostasis in AF and determine key kinases involved in AF progression. In order to do this, we compared the kinome in atrial tissue of non-paced dogs, atrial tachypaced dogs and atrial tachypaced dogs treated with the cardioprotective HSP-inducer GGA. An altered kinome homeostasis, induced by tachypacing, was found, which could be attenuated by GGA treatment. We verified the findings of 2 key kinases, Akt and CDK4, in our \textit{in vitro} AF model and show that inhibition of these kinases protects against contractile dysfunction. In \textbf{chapter 5}, the stress-responsive pathways of the proteostasis network were examined in the heart of an \textit{in vivo} natural hibernator, the Syrian hamster. Cardiac diseases, including AF, are often characterized by ‘cardiac hibernation’, in which the altered features of cardiomyocytes resemble those found in natural hibernators, such as hypocontractility and subcellular morphological changes.\textsuperscript{138-140} However, myolysis is present in cardiac diseases,\textsuperscript{116,139,140} but not in the heart of natural hibernators.\textsuperscript{138} As structural damage does not occur in natural hibernators, we hypothesized hibernators to have a highly efficient PQC network in order to cope with stress during hibernation, which protect against derailment of proteostasis.\textsuperscript{138} Moreover, natural hibernators, including ground squirrels, woodchucks and hedgehogs, do not develop arrhythmias.\textsuperscript{141-144} Therefore, we examined the HSR, UPR\textsubscript{ER} autophagy and posttranslational modifications in heart of the Syrian hamster in torpor, which is characterized by low body temperature, reduced metabolic rate and gross changes in physiology, and upon arousal, in which these physiological functions are restored to normal levels, and show upregulation of autophagy and protein ubiquitination during arousal. The HSR did not show changes in activation during torpor or arousal phases and the UPR\textsubscript{ER} showed to be slightly upregulated during arousal. Our results suggest that natural hibernators have a mechanism to cope quickly and efficiently with the built-up proteotoxic stress during torpor. The function of a major degradation pathway, autophagy, in AF was examined in \textbf{chapter 6}. We found increased UPR\textsubscript{ER} activation
resulting in an increased autophagic activation, which was associated with myolysis in both \textit{in vitro} tachypaced HL-1 atrial cardiomyocytes and in AF patients. Inhibition of autophagy seems to be protective in HL-1 atrial cardiomyocytes and \textit{in vivo} tachypaced \textit{D. melanogaster}. Moreover, the chemical chaperone 4-phenyl butyrate, an ER stress inhibitor and FDA-approved drug, shows protective effects not only in HL-1 atrial cardiomyocytes and \textit{D. melanogaster}, but also in an \textit{in vivo} tachypaced canine model for AF. As ER and mitochondrial function are closely linked to each other, we examined mitochondrial function in AF in \textit{chapter 7}. In HL-1 atrial cardiomyocytes, mitochondrial function was greatly compromised upon AF, as shown by ATP depletion, upregulation of mitochondrial stress markers and collapse of the mitochondrial membrane potential and network. We found this was due to aberrant mitochondrial Ca$^{2+}$ energetics, and the mitochondrial Ca$^{2+}$ uptake blocker Ru360 showed protective effects both in HL-1 cardiomyocytes and in \textit{D. melanogaster}. Finally, in \textit{chapter 8}, we summarize and discuss the data obtained in our experimental chapters and provide future perspectives. We discuss the role of proteostasis in the aging heart and age-related cardiac diseases, such as AF, and we discuss novel therapeutic targets in respect to proteostasis to prevent and/or treat cardiac disease in the aging population.
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Part II: Experimental chapters