Chapter 8

Summary
Discussion
Future perspectives

Adapted from:
Derailed proteostasis as a determinant of cardiac aging

Marit Wiersma¹, Robert H. Henning¹, Bianca J.J.M. Brundel¹²

¹Department of Clinical Pharmacy and Pharmacology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
²Department of Physiology, Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, The Netherlands

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SUMMARY

Atrial fibrillation (AF) is the most common sustained clinical tachyarrhythmia, which is associated with increased mortality and morbidity. The incidence is age-related and expected to rise due to the aging population. AF is characterized by electrical and structural remodeling, especially degradation of sarcomeres (myolysis) in left and right atria, which are not only a consequence of AF, but also create a trigger for this arrhythmia. Although electrical remodeling is reversible, structural remodeling is not, which limits the efficacy of current therapies. Thus, the need for mechanism-related AF therapies directs current research to uncover the underlying mechanisms of AF-induced structural remodeling.

Derailment of proteostasis, i.e. the homeostasis of protein synthesis, folding, assembly, trafficking, function and degradation, plays a key role in AF pathogenesis. Previous research demonstrated activation of proteases and histone deacetylases to contribute to degradation of contractile and structural proteins. Moreover, induction of the heat shock response (HSR) proved protective against AF-induced remodeling and contractile dysfunction. Nevertheless, the precise molecular mechanisms underlying derailment of proteostasis in AF are not yet identified.

In this thesis, we explored the role of stress-responsive and protein degradation pathways of the proteostasis network in AF-induced derailment of protein homeostasis and their implications as druggable target. Previously, genetic and/or pharmacological boosting of heat shock proteins (HSPs) was found to protect against AF pathogenesis in experimental models for AF. Furthermore, AF-induced RhoA activation contributes to contractile dysfunction, which is abrogated by HSPB8 overexpression. Therefore, we elucidated the role of RhoA in HSP expression in chapter 3. We show that RhoA activation suppresses the heat shock response (HSR) by impairing the binding of heat shock factor 1 (HSF1) to the hse promotor sequence within hsp genes. Inhibition of RhoA counteracts this effect and thus maintains proper mounting of a HSR upon stress. In chapter 4 we applied a kinase array to identify key kinases involved in tachypacing-induced cardiac remodeling by comparing non-paced dogs, atrial tachypaced dogs and atrial tachypaced dogs treated with the cardioprotective HSP-inducer geranylgeranyacetone (GGA). While tachypacing changed activity of 50 kinases, GGA treatment precluded such changes in 40 (80%) of these. Verification of two key kinases, CDK4 and Akt, by pharmacological and/or genetic inhibition protected against AF-induced contractile dysfunction in the tachypaced HL-1 cardiomyocyte model. The results of this kinase array analysis signify
that 1) GGA treatment alleviates the majority of changes in cardiac kinase activity in AF and 2) it may be used to uncover druggable targets underlying AF pathogenesis. Next, in chapter 5 we examined the stress-responsive pathways of the proteostasis network in the heart of an in vivo natural hibernator, the Syrian hamster. Cardiomyocytes of cardiac diseases, including AF, are often denoted as ‘hibernating’, as their key features resemble those of cardiomyocytes of natural hibernators.23-25 Nevertheless, myolysis, which is present in AF and other cardiac diseases, cannot be found in cardiomyocytes of natural hibernators.10,23-25 More interesting is the fact that natural hibernators do not develop arrhythmias, although they are under severe stress,26-29 which implies them to have a well-timed regulation of their stress-responsive pathways. We show that the stress-responsive pathways in Syrian hamsters are activated during transition from torpor to arousal. In particular, during the transition from torpor to arousal, the endoplasmic reticulum (ER) unfolded protein response (UPR_{ER}), autophagy and protein ubiquitination are increased and protein sumoylation is decreased. This suggests that cellular stress is successfully cleared in the 1.5 hour transition time, which likely precludes detrimental effects of the huge increase in cardiac function. Understanding how these natural hibernators successfully regulate their stress-responsive pathways in relation to cellular stress might lead to identification of key pathways underlying ‘cardiac hibernation’ encountered in cardiac diseases. The activation of a stress-responsive pathway and, subsequently, a protein degradation pathway of the proteostasis network in AF was shown in chapter 6. Here, we show that tachypacing induces activation of the UPR_{ER}, which subsequently results in autophagy activation. In an experimental model for AF as well as in clinical AF, activation of autophagy correlates with myolysis. Inhibition of the UPR_{ER} and, consequently, autophagy by the chemical chaperone and FDA approved drug 4-phenyl butyrate (4PBA) protected against AF-induced remodeling and contractile dysfunction in in vitro HL-1 cardiomyocytes and in vivo tachypaced Drosophila melanogaster and a canine model for AF. These findings imply that 4PBA comprises a novel therapeutic intervention for treatment of clinical AF. Finally, in chapter 7 we explored mitochondrial function in an experimental AF model, as the ER and mitochondrial function are linked to each other and ER stress often leads to mitochondrial stress.30 We show tachypacing to induce mitochondrial stress and mitochondrial dysfunction, as exemplified by the increased transcription of the mitochondrial stress chaperones HSP60 and HSP10, fragmentation of the mitochondrial network and reduction of cellular ATP levels, mitochondrial membrane potential, mitochondrial calcium transients and maximal respiratory capacity. Features
of mitochondrial dysfunction, including dispersed mitochondrial localization, decreased cellular ATP levels and increased HSP60 expression, were also observed in AF patients. Aberrant Ca\(^{2+}\) influx through the mitochondrial calcium uniporter (MCU) seemingly underlies mitochondrial dysfunction in HL-1 atrial cardiomyocytes, as inhibition of the MCU by Ru360 and downregulation of its expression by siRNA protect against mitochondrial dysfunction during tachypacing. In addition, data from a small group of AF patients indicates the potential of circulating mitochondrial DNA in serum as a biomarker for AF. These findings suggest that maintenance of mitochondrial function by inhibition of the MCU comprises a novel therapeutic option in AF and circulating mitochondrial DNA in serum as a potential biomarker for AF.

Figure 1 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\(_{\text{ER}}\), mitochondrial dysfunction, autophagy, kinome changes and RhoA activation represent key modulators of structural remodeling due to their role in proteostasis derailment. The indicated drugs of these modulators are able to prevent the AF-induced changes and are, therefore, novel therapeutic targets.
In summary, data presented in this thesis indicate that the activation of the stress-responsive and protein degradation pathways of the proteostasis network contributes to derailment of proteostasis and AF pathogenesis. We identified several novel therapeutic targets, which preserved cardiac proteostasis in the models used. Consequently, these targets may be exploited to prevent AF substrate formation and pathogenesis in patients. An overview of the stress-responsive and protein degradation pathways involved in AF-induced derailment of proteostasis and possible novel therapeutic targets is depicted in Figure 1.

DISCUSSION

AF is an age-related disease and due to the aging population its incidence is expected to rise, which will increase hospitalization and medical costs, contributing significantly to the socioeconomic burden.\(^3\,^4\) In 2010, the prevalence of AF was 2.7-6.1 million and 6.5-12.3 million in the USA and the EU, respectively, and this prevalence is expected to rise, due to the aging population, to 5.6-12 million and 13.6-23.7 million in 2050 in the USA and the EU, respectively.\(^3\) Importantly, aging is accompanied by a decline in function of the stress-responsive and protein degradation pathways of the proteostasis network,\(^3\,^1\) making cardiomyocytes susceptible for cellular damage, which aggravates the development of age-related cardiac diseases, such as AF.

A healthy proteostasis is the keystone of a healthy heart

Cardiovascular diseases (CVDs) are the main cause of death worldwide and in the EU CVDs account for 47% of all disease-related deaths yearly. Its incidence is age-related, with 3 per 1000 and 74 per 1000 persons affected in the age groups of 35-44 and 85-94 years, respectively. CVDs are present in 33% of the adult population in the USA; over 50% of this CVD population is older than 60 years of age. Moreover, CVDs account for a high socioeconomic burden; its medical costs outnumber those of any other disease, as CVDs are the most common cause for hospitalizations especially in the elderly. As the population is aging, projections foresee a steep increase in the incidence, prevalence and medical costs of CVDs (from $565 billion in 2015 to $1 trillion in 2030).\(^3\,^3\,^2\) In view of global aging, it is ever more important to develop adequate strategies to counteract aging-related effects to arrest this trend.

Recent findings indicate that a healthy protein homeostasis (proteostasis) plays a key role in safeguarding the proper functioning of the heart by maintaining proper cell function through adequate synthesis, folding, assembly, trafficking, function and
degradation of proteins. A proper proteostasis is especially warranted in post-mitotic cells, such as cardiomyocytes, which have limited regenerative capacity. Similarly to various neurodegenerative diseases, including Parkinson’s, Huntington’s and Alzheimer’s disease, derailed proteostasis also underlies CVDs as shown for cardiomyopathies, heart failure, metabolic syndrome-related cardiac disease and atrial fibrillation. Accordingly, an optimal function of proteostasis is required to maintain a healthy heart.

Proteostasis is maintained by a regulated network of molecular chaperones, stress-responsive pathways and protein degradation pathways. In healthy conditions, this network shows a basal activation that is induced upon cellular stress, including CVD. To respond to cellular stress, cells may deploy three main stress-responsive pathways: the heat shock response (HSR), the mitochondrial unfolded protein response (UPR$_{\text{mito}}$) and the endoplasmic reticulum (ER) unfolded protein response (UPR$_{\text{ER}}$) as well as two main degradation pathways: the ubiquitin-proteasome system and macroautophagy (hereafter ‘autophagy’). As the name implies, each of the stress-responsive pathways is activated upon stress in a specific cellular compartment. Nevertheless, these pathways may also be activated and act in parallel upon severe cellular stress. When these stress-responsive pathways and/or protein degradation pathways are dysregulated, proteostasis becomes derailed, which is observed in multiple cardiac diseases. For example, heart failure features the activation of the UPR$_{\text{ER}}$ and UPR$_{\text{mito}}$ and the reduced activity of the HSR, UPS and autophagy. Similarly, cardiomyopathies are associated with increased UPR$_{\text{ER}}$ activation and reduced UPS and autophagic activity. Furthermore, metabolic syndrome-related cardiac disease shows reduced autophagy and mitochondrial function. In contrast, activation of degradation pathways is increased and the HSR gets exhausted in (longstanding) persistent atrial fibrillation. The common dominator of derailed proteostasis in various – mostly age-related – cardiac diseases suggests that proper proteostasis maintenance is an important aspect of cardiac aging.

Proteostasis declines during aging

Experimental findings reveal that during aging, the gradual failure of stress-responsive and degradation pathways impair the proteostasis network resulting in the accumulation of cellular damage over time (Figure 2A). The importance of the factor time in bringing about the failure of proteostasis explains why the incidence of diseases, such as neurodegenerative and cardiovascular diseases, increases with age.
Figure 2 Aging reduces proteostatic function and increases risk for cardiac diseases. A) During aging, the function of the pathways of the proteostasis network declines. Consequently, protein aggregation and risk for cardiac disease development increases. B) Cardiac aging is associated with electrical and structural changes in the heart and a decline in the function of proteostatic pathways. This decline results in increased cellular stress. Due to a diminished stress response of the proteostatic pathway, cellular stress is detrimental for aged hearts and leads to development of cardiac diseases.
Increased proteotoxicity during aging

The relation between aging and proteostasis has been extensively studied in several model systems, particularly in Caenorhabditis elegans and Drosophila melanogaster. During aging in C. elegans, various proteins tend to aggregate and become proteotoxic, including proteins engaged in protein homeostasis and quality control, such as the proteasome, ribosomes and molecular chaperones, including HSP70 and HSP90. Moreover, many of these aging-related aggregate-prone proteins are included in amyloid plaques, neurofibrillary tangles and Lewy bodies in Alzheimer’s disease and aggregates in amyotrophic lateral sclerosis. Furthermore, aging affects the proteome of C. elegans by an upregulation of proteins involved in the UPS, oxidative stress defense and DNA replication and repair and downregulation of ER, mitochondrial and ribosomal proteins. The decline in ribosomal proteins corresponds with age-related diminishing of protein synthesis rate. Aged D. melanogaster show increased carbonylated and ubiquitinated proteins and a decline in proteasome function. Similar changes have been documented in mammals, as aged mice show differential expression of mitochondrial proteins, proteins involved in defense against oxidative stress and chaperones or proteins involved in the stress-response. In addition, aging of ex vivo human dermal fibroblasts also displays reduced expression of chaperone, proteosomal, ribosomal and mitochondrial proteins. Together, the findings from experimental studies indicate aging to prompt proteotoxicity, likely because of a hampered functioning of the proteostasis network.

Derailment of proteostasis network during aging

During aging, the function of the stress-responsive and protein degradation pathways of the proteostasis network declines. Aging thus attenuates the HSR, UPR ER and UPR mito upon cellular stress. The expression of multiple mitochondrial proteins, including chaperones and components of the electron transport chain, is reduced during aging and mitochondrial function and morphology are changed. In case of the UPR ER, aging attenuates HSPA5 induction and eIF2α phosphorylation, while GADD34 and CHOP expression are enhanced. Similar to the stress-responsive pathways, aging impairs both protein degradation pathways, i.e. the UPS and autophagy, by affecting expression and/or activation. Experimental findings reveal aging to be accompanied by expression of the low-activity 20S proteasomes, in contrast to the high-activity 26S proteasomes, which are mostly found in young animals. This divergence could possibly be explained by the dependency of the 26S proteasome assembly on
ATP, the latter being decreased during aging.\textsuperscript{69} Furthermore, aging attenuates the proper mounting of an autophagy response, which could have rescued, or delayed the effects of the age-related dysfunctionality of the UPS.\textsuperscript{73,74} Loss of autophagy also generally leads to increased ROS levels as the degradation of damaged, old or otherwise dysfunctional mitochondria is impaired. These dysfunctional mitochondria generate high levels of ROS, in turn promoting proteotoxic stress and accelerating detrimental effects on the cell.\textsuperscript{68} In addition, aging modulates mTOR activity, and depending on the species, gender and organ studied, mTOR activity is either up- or downregulated.\textsuperscript{75-77} As mTOR is an important negative regulator of autophagy, aberrant activity may attenuate autophagy during aging. Thus, aging affects the proper function of all stress-responsive and protein degradation pathways at various levels, thereby progressively disturbing the proteostasis network.

**Proteostasis and longevity**

While aging induces a gradual dysfunction of stress-response and protein degradation pathways, these pathways seem fortified in long-lived species.\textsuperscript{78,79} Thus, a higher expression of several chaperones, including HSP60 in mitochondria, HSPA5 and GRP94 in ER and HSP70 in cytosol, correlates with enhanced lifespan.\textsuperscript{78,79} Furthermore, long-lived species show enhanced autophagic and proteosomal function compared to short-lived species. Moreover, long-lived species appear to excessively enhance their proteotoxic defense in comparison to short-lived species, by inducing high levels of HSP and substantial activation of autophagic and proteosomal clearance.\textsuperscript{79} These observations suggest that long-lived species deploy their intrinsic defense mechanisms to the maximum to protect from age-related derailment of proteostasis.

Similar to long-lived species, boosting components of the proteostatic network prolong life of short-lived species. This is exemplified by the notion that both \textit{C. elegans}\textsuperscript{80,81} and \textit{D. melanogaster}\textsuperscript{82,83} have a longer lifespan when HSPs are overexpressed. Conversely, HSF1 knockdown reduces life span and features proteins that more promptly aggregate.\textsuperscript{55,84} Moreover, overexpression of mitochondrial chaperones HSPA9\textsuperscript{81} and HSP22\textsuperscript{82} or PGC1α increases lifespan and mitochondrial function or mitochondrial biogenesis in aged \textit{C. elegans} or \textit{D. melanogaster},\textsuperscript{85} respectively. Furthermore, enhancing UPR\textsubscript{ER} function to counteract the age-related decline in UPR\textsubscript{ER} activity by overexpression of XBP1\textsubscript{s} also resulted in an increased life span in \textit{C. elegans}.\textsuperscript{86} Similar to the stress-responsive pathways, enhancement of protein degradation pathways also promotes longevity, as shown by overexpression of proteasome subunits\textsuperscript{71,87} and induction of
autophagy.\textsuperscript{68,88,89} Moreover, inhibition of mTOR increases lifespan in mice and \textit{C. elegans}, possibly conveyed via induction of autophagy.\textsuperscript{90,91} Thus, experimental evidence reveals that aging diminishes the function of both the stress-responsive and the degradation pathways of the proteostasis network. This may result in progressive derailment of proteostasis, accumulation of cellular damage and, ultimately, age-related diseases, such as CVD. Importantly, experimental evidence suggests that boosting components of these pathways rescues the proteostasis network from aging effects and promotes longevity.

**Cardiac proteostasis in the elderly**

Age-related changes in the proteostasis network are also present in cardiomyocytes. The expression of heat shock proteins is reduced in aged cardiomyocytes,\textsuperscript{92} which may be due to an upregulation of Rho expression\textsuperscript{92} as activation of Rho prevents the mounting of HSR by attenuation of HSF1 binding to the promotor region of \textit{hsp} genes.\textsuperscript{93} Cardiac mitochondrial function also decreases during aging, due to a variety of mitochondrial changes, including loss of cristae, destruction of inner membranes, swelling, decreased expression of mitochondrial proteins and loss of ATP production.\textsuperscript{92,94} In turn, mitochondrial changes impair maintenance of a proper proton gradient and functional respiratory chain, which contributes to increased ROS production.\textsuperscript{94,95} Subsequently, increased amount of ROS in aged cardiomyocytes leads to excess protein damage\textsuperscript{94} and a less effective stress response. Importantly, ROS stimulates the lysosomal formation of lipofuscin aggregates,\textsuperscript{95,96} consisting of aggregated liposomal proteins and lipids, which effectively impair autophagy by preventing fusion of lysosomes to autophagosomes. Consequently, aging-related impairment of autophagy promotes accumulation of dysfunctional mitochondria, representing the major part of the extralysosomal waste in aged cardiomyocytes.\textsuperscript{95,97} Dysfunctional mitochondria are known to produce increased amount of ROS, thereby triggering a vicious circle of high ROS production and the subsequent low stress response. In addition, accumulation of dysfunctional mitochondria and aberrant proteins results in functional loss in cardiomyocytes.\textsuperscript{96} Moreover, impaired autophagy affects the UPS, as damaged or aged proteasomes are degraded by autophagy.\textsuperscript{96} In accord with the prominent role of autophagy, ATG5 knockout mice display both increased polyubiquitination and attenuated mitochondrial function.\textsuperscript{97} The impaired stress-responsive and protein degradation pathways make aged cardiomyocytes susceptible to cellular damage. As cardiomyocytes are post-mitotic
cells, and are therefore not constantly replaced by proliferation, the whole heart is highly susceptible to protein damage. During normal aging, the structure and function of the heart changes (Figure 2B), resulting in a decline of diastolic function in rest and systolic function during exercise, impairment of Ca\textsuperscript{2+} homeostasis, cardiac hypertrophy and fibrosis.\textsuperscript{94} Cardiac hypertrophy originates from changes in ion channels and Ca\textsuperscript{2+} homeostasis, and fibrosis during aging, which puts additional strain on the remaining cardiomyocytes to match cardiac demands.\textsuperscript{98,99} Interestingly, stimulation of autophagy in the aged heart decreases hypertrophy, improves contractile function, reduces protein damage and restores Ca\textsuperscript{2+} homeostasis.\textsuperscript{100} Conversely, impairment of autophagy predisposes the heart to early dilated cardiomyopathy development.\textsuperscript{97} Although cardiac aging is related to reduced activation of autophagy, most CVDs, including heart failure, myocardial infarction and atrial fibrillation, are related to excessive activation of autophagy (Table 1). Interestingly, reduced activity of autophagy is observed in CVDs caused by overexpression of (mutant) proteins, including dilated and desmin-related cardiomyopathy. These studies showed that (mutant) protein expression results in protein aggregates which are toxic for the cardiomyocytes. Protein aggregates are cleared by autophagy but chronic overexpression results in exhaustion of the autophagy machinery, ultimately resulting in accumulation of protein aggregates and loss of cardiomyocyte function. These observations indicate that in case of proteotoxicity, activators of autophagy may be beneficial, whereas other CVDs likely benefit from inhibitors of autophagy.

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Accumulating evidence reveals cardiac diseases to accelerate aging-induced derailment of proteostasis and atrial fibrillation (AF) is an excellent example of the adverse effects of cardiac aging on disease development. AF is the most common age-related clinical tachyarrhythmia, characterized by electrical and structural remodeling and atrial contractile dysfunction. Derailment of proteostasis is associated with the AF-related structural remodeling and, thereby, may create a substrate for both initiation and progression of AF. The HSR gets exhausted in patients with (longstanding) persistent AF, and induction of HSP expression was found to protect against AF progression. Furthermore, AF is associated with aberrant phosphatase and kinase activities. As several components of the proteostasis network, including components of the UPR (IRE1 and PERK), are regulated by these posttranslational modifications, the aberrant function may affect them. In addition, changes in phosphorylation-increased deacetylation of structural proteins by histone deacetylase 6 (HDAC6) contributes to reduced contractile function, structural remodeling and AF progression. Together, these findings suggest that proteostasis derailment is an important feature in the initiation and progression of AF, which may explain its prevalence increasing with age.

**Therapeutic targets to attenuate cardiac aging and disease**

Fortifying proteostasis may prevent the detrimental changes associated with aging and cardiac diseases, in line with observations of extended lifespan in model systems that overexress components of the proteostasis network. Thus, (pharmacological) boosting of the proteostasis network may represent an appealing strategy to prevent and/or treat cardiac diseases, including AF, cardiomyopathies and heart failure.

**Lifestyle interventions**

Sustained caloric restriction (without restriction of vitamins and micronutrients) is found to increase lifespan in various experimental model systems from yeast to mice and decreases the risk of cardiac diseases, by improving contractile function and declining cardiac hypertrophy and cardiomyopathy in rodents and monkeys. Autophagy induction is commonly believed to constitute an important effector of caloric restriction. However, sustained caloric restriction is not feasible for most humans. Except caloric restriction, exercise also reduces the risk of CVDs and has been proposed to slow cardiac aging. Exercise reduces cardiac fibrosis and hypertrophy and improves diastolic function, contractile function and Ca²⁺ homeostasis. Moreover, exercise has a positive effect on HSP expression, mitochondrial function and
indirectly, proteosome function.\textsuperscript{125} Interestingly, exercise induces autophagy in mice cardiac muscle.\textsuperscript{126} In addition, voluntary exercise in a desmin-related cardiomyopathy mouse model showed reduced protein aggregation and reduced disease progression.\textsuperscript{127} This indicates that regular exercise may be able to improve cardiac outcome in aging and cardiac disease progression.

\textbf{Pharmacological interventions}

\textit{Boosting the HSR}

Pharmacological induction of HSP expression has beneficial effects in several cardiac diseases.\textsuperscript{20,21,128-130} Geranylgeranylatedone (GGA) is an HSP inducer, used in Japan as anti-ulcer drug since 1984 without reported serious side effects,\textsuperscript{131} and is therefore currently the most promising compound to boost the HSR. GGA treatment in experimental models of cardiac disease attenuated structural damage and improved function of the heart.\textsuperscript{20,21,128,129} \textit{In vitro}, GGA protected against contractile dysfunction and cardiomyocyte damage by attenuation of sarcomere damage.\textsuperscript{20,21} \textit{In vivo}, GGA protected against desmin-related cardiomyopathy by attenuation of fibrosis, which may be due to increased aggregate clearance by GGA-induced augmented HSPB1 expression.\textsuperscript{128} In addition, \textit{in vivo} GGA treatment reduced inducibility of AF in heart failure\textsuperscript{132} and atrial ischemia.\textsuperscript{133} Another HSP inducer, BGP-15, was found to protect against contractile dysfunction in a \textit{D. melanogaster} model for AF and no serious side-effects were reported in a phase II clinical study for insulin resistance.\textsuperscript{134,135} Importantly, recent research found BGP-15 to protect from heart failure and AF in \textit{in vivo} mice models.\textsuperscript{136} Thus, both GGA and BGP-15 show promising cardioprotective effects probably by boosting the HSR (Table 2). As GGA is already on the market for decades, it features a favorable safety profile, while this is currently still unknown for BGP15.

\textit{Autophagy induction to counteract aging and proteotoxicity}

As autophagy shows reduced activation during aging and overexpression of (mutant) proteins, induction of autophagy may represent an interesting strategy to attenuate CVD onset and progression. Interestingly, rapamycin treatment induces similar effects as caloric restriction in mice.\textsuperscript{94} Rapamycin is an inhibitor of the nutrient-sensing mTOR, and its administration thus resulting in the activation of autophagy.\textsuperscript{94,100} The inhibition of mTOR activity increases lifespan \textit{in vivo},\textsuperscript{137} even in old mice,\textsuperscript{138} as rapamycin treatment resulted in induced voluntary exercise, reduced cardiac inflammation and hypertrophy, enhanced ejection fraction, and also in increased mitochondrial function.\textsuperscript{139} Moreover,
rapamycin treatment protected in vitro against Hutchinson-Gilford and Werner progeria syndromes, both accelerated aging disorders.\(^{140,141}\) In addition to clinical studies in which rapamycin is used to treat cancer\(^{142}\) and auto-immune disease\(^{143}\) (Table 2), experimental evidence suggests rapamycin mitigates progression of heart failure.\(^{144}\) Moreover, rapamycin-treatment attenuated contractile dysfunction and cardiac hypertrophy in aged mice with reversal of diastolic dysfunction and age-related cardiac proteomic changes.\(^{100}\) In addition, rapamycin treatment prevented diabetic cardiomyopathy in mice.\(^{145}\) Although rapamycin is an FDA approved drug, caution is needed as rapamycin may exert serious side effects, including suppression of the immune system,\(^{146}\) dermatological changes,\(^{146}\) lung toxicity\(^{147}\) and increased risk of diabetes development.\(^{148}\) Due to these adverse effects, multiple derivatives of rapamycin have been developed. Unfortunately, these derivatives do not only inhibit mTOR activity, but also increase Akt activity, which can drive cancer development.\(^{146}\)

**Inhibition of autophagy to counteract CVDs**

Various CVDs show excessive activation of autophagy (Table 1). Therefore, inhibition of autophagy may represent an interesting target for treatment of CVDs, which are not caused by the overexpression of a (mutant) protein. However, inhibitors of autophagy, such as bafilomycin, cause cellular toxicity.\(^{149}\) Thus, direct inhibition of autophagy may currently not be feasible. However, indirect inhibition of autophagy, by targeting upstream activation pathways may be applicable. One of the pathways implicated in cardiac disease and autophagy activation is the UPR\(_{ER}\).

**Inhibition of UPR\(_{ER}\)**

As the ER is the cellular compartment where most proteins (at least one-third) are synthesized and folded,\(^{33,150}\) this compartment is highly susceptible for proteotoxic stress. Reinforcing the UPR\(_{ER}\) with 4-phenyl butyrate (4PBA) in experimental models protects from progression of multiple diseases, including obesity, diabetes, cancer, cystic fibrosis and neurodegenerative disorders.\(^{151}\) Importantly, 4PBA is already an FDA approved drug to treat urea cycle disorders,\(^{152,153}\) with no reported serious side effects.\(^{154}\) Interestingly, 4PBA has several modes of action; it is an ammonia scavenger, a weak histone deacetylase inhibitor and a chemical chaperone.\(^{151}\) At the moment, 4PBA is in clinical trials for several disorders related to proteotoxicity, including amyotrophic lateral sclerosis (NCT00107770), Huntington's disease (NCT00212316), spinal muscular atrophy (NCT00528268), proteinuric nephropathies (NCT02343094)
and cystic fibrosis (NCT00590538) (Table 2). Results of these studies may inform us about possible effectiveness of 4PBA in respect to cardiac diseases.

**Enhancement of mitochondrial function**

Pharmacological enhancement of mitochondrial function does also have beneficial effects on proteostasis both in aging and cardiac diseases. Several compounds have been tested in mice models, such as addition of endogenous antioxidants (SOD, catalase and GPx) and synthetic antioxidants (Mn (III) porphyrin, Mn (III) salen, EUK-8

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and EUK:34). Although genetic overexpression of catalase in old mice mitochondria resulted in longer life span and a decreased risk for cardiac disease development, the endogenous antioxidants are not practical therapeutics due to their fast degradation and their potential to provoke inflammatory responses. The synthetic antioxidants show potential in protection against cardiac disease, however, the notion that they are non-targeted synthetic oxidants makes them less applicable. Targeted mitochondrial antioxidants, such as coenzyme Q (mitoQ) and plastoquinone (SkQ1) also increase mitochondrial function in in vivo rodent heart failure models. Nevertheless, these antioxidants also have a downside, as the window between antioxidant and pro-oxidant is quite narrow and their uptake is dependent on the mitochondrial membrane potential, which is often decreased in cardiac disease. Promising compounds for increasing mitochondrial function are the Szeto-Schiller peptides (Table 2). The uptake of these peptides in the mitochondria is not dependent on the mitochondrial membrane potential. Furthermore, Szeto-Schiller peptides have antioxidant properties, promote ATP production through increasing mitochondrial respiration, reduce mitochondrial swelling and inhibit the loss of cristae. In case of the heart, these peptides are able to increase diastolic function, prevent cardiac hypertrophy and could protect against ischemia reperfusion injury without pretreatment. Importantly, the Szeto-Schiller peptides were shown to have protective effects in heart failure and arrhythmias.

### Interconnections between the derailed stress-responsive and protein degradation pathways

As cellular stress can activate multiple stress-responsive pathways in parallel, due to cross-talk of these pathways, it is plausible that derailment of one stress-responsive pathway leads to derailment of the other pathways. In this thesis, we show AF to be associated with induction of the UPR\textsubscript{ER} and autophagy, due to ER stress, and induction of the UPR\textsubscript{mito} and mitochondrial dysfunction. In addition, we found that AF-induced activation of RhoA inhibits the induction of the HSR. This increased activity of RhoA in AF, by induction of F-actin stress fibers, contributes to contractile dysfunction. The autophagic pathway can degrade RhoA, thereby reducing the expression of F-actin stress fibers and the RhoA-mediated inhibition of the HSR. Induction of the HSR is important during cellular stress, as HSF1 induces transcription of the autophagy-related gene 7 (ATG7), ER stress-related chaperones, mitochondrial respiration proteins and mitochondrial chaperones, including HSP60 and HSP10, thereby promoting cell survival (Figure 3).
Previous research identified calpain to degrade structural proteins in AF, leading to myolysis. Increased activation of calpain, as in AF, stimulates autophagy, induces the UPR$_{ER}$ and activates Akt (Figure 3). This thesis shows enhanced activation of Akt during ER stress and in the kinome array in tachypaced dog atrial tissue, both showing detrimental effects. Akt is a pro-survival protein involved in a variety of cell signaling pathways. Phosphorylation and, thus, activation of Akt protects the heart against ischemia-reperfusion injury, due to protection of the mitochondrial network. However, chronic activation of Akt causes cardiac hypertrophy, which may (partly) be due to mitochondrial dysfunction and inhibition of respiration. In turn, decreased mitochondrial respiration activates Akt, initiating a vicious circle. Furthermore, enhanced Akt activity reduces Ca$^{2+}$ flux from the ER, thus decreasing the Ca$^{2+}$ concentration in mitochondria, which attenuates mitochondrial dysfunction and programmed cell death (Figure 3). However, it also produces cytosolic Ca$^{2+}$ overload, which is a trigger for AF. In addition to decreased ER flux, tachypacing-induced cytosolic Ca$^{2+}$ overload may also result from increased mitochondrial fission, i.e. fragmentation of the mitochondrial network. The increased mitochondrial fission we observed during tachypacing may therefore be an additional trigger for AF.

**Figure 3** Interconnection between stress-responsive and protein degradation pathways, which are derailed in AF. RhoA inhibits activation of the HSR, but can be degraded by autophagy, thereby reducing the inhibition of the HSR. HSF1 induces transcription of autophagy-related genes (ATG7), ER chaperones and mitochondrial chaperones and respiration proteins. Calpain activation induces autophagy, the UPR$_{ER}$ and activates Akt. Akt attenuates programmed cell death by reducing Ca$^{2+}$ from the ER and protects mitochondrial network formation and is activated by decreased mitochondrial respiration.
**The mitochondria-associated membranes**

In view of the results described in this thesis, the connection between the ER and the mitochondria, accomplished by the mitochondria-associated membranes (MAMs) is an emerging and highly interesting study subject in AF. The functions of the MAMs are versatile and include Ca\(^{2+}\) transport between the ER and mitochondria, stimulation of mitochondrial ATP synthesis, exchange of stress signals between the ER and mitochondria and the initiation and progression of autophagy.

The MAMs are formed by the connection of specific proteins on the membranes of the ER and mitochondria. Voltage-dependent anion channels (VDACs) on the mitochondrial membrane connect with inositol 1,4,5-triphosphate receptors (IP\(_3\)Rs) on the ER membrane and the chaperone GRP75 strengthens this connection. Another connection is between mitofusin 2 (Mfn2) on the ER, which makes a homotypic or heterotypic connection with Mfn2 or Mfn1, respectively, on the mitochondrial membrane. Moreover, B-cell receptor-associated protein 31 (Bap31) on the ER connects with Fission-1 homolog (Fis1) on the mitochondria (Figure 4). The MAM regulates several important functions, such as lipid and Ca\(^{2+}\) exchange between the two organelles. Especially the Ca\(^{2+}\) exchange is important, as this regulates mitochondrial respiration, buffers excessive cytoplasmic and ER Ca\(^{2+}\) and dysfunction can lead to impaired cell function and finally programmed cell death. The importance of the above mentioned proteins in MAM formation is exemplified by the notion that knockdown of Mfn2 not only reduces the amount of protein in the MAMs and Ca\(^{2+}\) exchange, but also affects mitochondrial fusion, respiration and membrane potential.\(^{172-176}\) On the other hand, overexpression of Fis1, increasing MAM formation, increases Ca\(^{2+}\) exchange to the mitochondria.\(^{174,175}\)

In addition to MAM formation, Mfn2 also contributes to mitochondrial respiration and fusion events, ER stress (increased levels of Mfn2 upon ER stress and Mfn2 deficiency causing ER stress) and induction of ER stress-related autophagy (Figure 4).\(^{172,176,176}\) Moreover, Mfn2 interacts with PERK at the MAMs,\(^{175}\) the latter inducing CHOP expression during ER stress,\(^{11,33,117,150}\) which, in turn, promotes mitochondrial Ca\(^{2+}\) overload.\(^{175}\) Due to the versatile role of Mfn2, it would be interesting to determine the role of this protein in AF pathogenesis. Studies on other cardiac disorders, such as heart failure and cardiac ischemia-reperfusion injury, show that aberrant expression of Mfn2 influences disease pathogenesis by altering the activation of the programmed cell death and autophagic pathways, leading to cardiac hypertrophy.\(^{177-180}\)

The ER is able to store a large amount of Ca\(^{2+}\) and the mitochondria are Ca\(^{2+}\) buffering organelles and both contribute significantly to cellular Ca\(^{2+}\) homeostasis, mainly
through the presence of Ca\textsuperscript{2+} binding proteins. One of these Ca\textsuperscript{2+} binding proteins, and abundantly present in the ER, is HSPA5. The binding of Ca\textsuperscript{2+} to HSPA5 regulates its chaperone activity. In addition to its chaperone properties, HSPA5 has been implicated in the regulation of the Ca\textsuperscript{2+} flux from the ER to the mitochondria.\textsuperscript{181} Moreover, HSPA5 may translocate form the ER to the mitochondria during ER stress,\textsuperscript{182} thereby preserving mitochondrial function.\textsuperscript{183} However, HSPA5 function is dependent on mitochondrial respiration, as it is an ATPase.\textsuperscript{184} Therefore, severe ER stress, which depletes the ATP in the ER lumen, signals to the mitochondria to stimulate mitochondrial respiration.\textsuperscript{184} In addition, ER stress also activates a mitochondrial-protection mechanism. The transcription factor ATF4, induced during ER stress, activates transcription of Parkin, which preserves mitochondrial network morphology and respiration.\textsuperscript{185-187} In this thesis, we showed that overexpression of HSPA5 or treatment with 4PBA protected against contractile dysfunction in experimental models of AF. This protective effect may be caused by the preservation of mitochondrial function and respiration, thereby protecting against ATP depletion. Consequently, normal ATP production by mitochondria ensures functioning of ATP-dependent chaperones, such as HSPA5, which may relieve ER stress.

The sarcoplasmic/endoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA) mediates Ca\textsuperscript{2+} influx into the ER lumen. An increase in cytosolic Ca\textsuperscript{2+} stimulates SERCA activity, thereby increasing Ca\textsuperscript{2+} uptake in the ER.\textsuperscript{184} A cytosolic Ca\textsuperscript{2+} overload, as in AF, can therefore lead to an ER Ca\textsuperscript{2+} overload, and impaired protein folding, thus stimulating the mitochondria.

**Figure 4 The mitochondria-associated membranes (MAMs).** The MAMs are formed by proteins in the membranes of the ER and the mitochondria. VDAC connects with IP\textsubscript{R} and GRP75 strengthens this connection. The VDAC-IP\textsubscript{R} connection is necessary for the Ca\textsuperscript{2+} flux to the mitochondria. The MCU on the IMM regulates the Ca\textsuperscript{2+} flux from VDAC to the mitochondrial matrix. The connection of Bap31 and Fis1 regulates the activation of the mitochondrial programmed cell death pathway. Mfn2 forms homo- or heterotypic connections with Mfn2 or Mfn1, respectively and Mfn2 is important for mitochondrial fusion and respiration, ER stress and autophagy induction. Furthermore, the ER and the mitochondria are sensitive to each other’s stress through the MAMs. Moreover, the MAMs are implicated to be the site where the formation of the autophagosomes occurs, as ATG14 and ATG5 are present at the MAMs at autophagy initiation.
to buffer Ca^{2+}. The Ca^{2+} flux from the ER to the mitochondria is mediated by the IP_{3}Rs,\textsuperscript{184} which are important channels for the Ca^{2+} flux as reducing IP_{3}R activity attenuated mitochondrial respiration.\textsuperscript{188} Mitochondrial Ca^{2+} must cross two membranes, the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM), to end up in the mitochondrial matrix, where it stimulates mitochondrial respiration.\textsuperscript{184} Ca^{2+} transport over the OMM is facilitated by VDACs, which is permeable to ions and small molecules and connects with IP_{3}Rs.\textsuperscript{175,184} Transport across the IMM is facilitated by the MCU, which is impermeable and transfers Ca^{2+} into the mitochondrial matrix against a steep electrochemical gradient (Figure 4).\textsuperscript{184} In AF, SERCA expression is decreased and IP_{3}R expression is increased,\textsuperscript{189-194} which may lead to depletion of ER Ca^{2+} into the mitochondria, resulting in mitochondrial Ca^{2+} overload. We showed in this thesis that a modest inhibition of the MCU protects against tachypacing-induced mitochondrial dysfunction. However, the involvement of VDAC in AF-induced mitochondrial dysfunction is not yet known, but aberrant functioning of VDAC plays a role in cardiac ischemia-reperfusion\textsuperscript{195,196} and desmin-related cardiomyopathy.\textsuperscript{197} Next to Ca^{2+} transfer, the MAMs are also implicated to play a role in autophagy. It has been suggested that the formation of autophagosomes occurs at the MAMs, evidenced by the presence of ATG14 and ATG5 at these sites during autophagy activation (Figure 4).\textsuperscript{172,198,199} The induction of autophagy is Ca^{2+} dependent, as reduced Ca^{2+} signaling by the IP_{3}R and reduction of cytosolic Ca^{2+} activated\textsuperscript{188} or inhibited\textsuperscript{200} autophagy, respectively. As we found increased activation of autophagy in experimental models of AF and AF patients, this might suggest that the amount of MAMs is increased in AF, resulting not only in increased autophagy, but also in increased mitochondrial Ca^{2+} uptake.

The close contact between the mitochondria and the ER makes these two organelles responsive to each other’s stress (Figure 4).\textsuperscript{30,185} Dysfunctional mitochondrial respiration causes protein damage,\textsuperscript{201} which, in turn, causes ER stress and activation of the UPR\textsubscript{ER}. Early during ER stress, the amount of MAMs increases, stimulating Ca^{2+} transfer to the mitochondria and, subsequently, ATP production needed for HSPA5 chaperone activity. However, chronic ER stress results in depletion of ER Ca^{2+} and in mitochondrial Ca^{2+} overload, the latter resulting in reduced mitochondrial respiration and, consequently, ATP production.\textsuperscript{30} In addition, ER stress increases autophagosome formation and impairment of autophagy increases the likelihood to develop ER stress.\textsuperscript{202} Moreover, also HSPA5 has been implicated in autophagy, as loss of HSPA5 suppresses autophagosome formation.\textsuperscript{203}
Thus, although the MAMs have versatile functions that regulate normal physiological function of the ER and the mitochondria, because of this the MAMs can also be involved in the pathophysiology of diseases. In the case of AF, the related ER stress may increase the amount of MAMs, leading to autophagy activation and mitochondrial Ca$^{2+}$ overload, resulting in myolysis and reduced mitochondrial respiration.

**Mitochondrial DNA as biomarker**

In this thesis, we show that AF induces ER stress and autophagy and reduces p62 expression. Induction of ER stress leads to transcription of mitochondrial DNA (mtDNA)\(^{204}\) and mtDNA itself is also able to regulate ER stress and autophagy.\(^{205}\)

In addition, p62, which is able to translocate to mitochondria during cellular stress, stabilizes mtDNA.\(^{206}\) As mitochondria are descendants of prokaryotes, mtDNA shares similarities to bacterial DNA,\(^{207}\) and can therefore act as damage-associated pattern, thus promoting inflammation. mtDNA that has escaped the autolysosomes causes an inflammatory reaction,\(^{207}\) while the autophagic pathway also provokes inflammation, as it regulates the activation of the inflammasome.\(^{208}\)

Accordingly, the activation of ER stress and autophagy and the reduction of p62 expression in AF, described in this thesis, may lead to destabilization of mtDNA and mtDNA escape from the autophagic pathway. This may result in an inflammatory reaction, and inflammation has been associated with AF pathogenesis and anti-inflammatory drugs show, to some extent, protective effects.\(^{209-211}\) Moreover, it may result in the release of mtDNA in the circulation, where it can be considered as a potential biomarker. Importantly, circulating mtDNA is already in use as biomarker in several cancers, including lung and breast cancer, and associates with cancer stage and progression.\(^{212-214}\)

**Cardiac hibernation**

Cardiac diseases, including AF, are often characterized by so-called ‘hibernation’ of cardiomyocytes. Features of hearts in cardiac hibernation resemble the cardiac features of natural hibernators, including hypocontractility and morphological changes.\(^{23-25}\) Interestingly, natural hibernators do not show structural damage and do not develop arrhythmias, although they encounter severe cellular stress.\(^{23,26-29}\) A main mechanism preventing abnormal heart function in hibernators is maintenance of normal Ca$^{2+}$ handling and increased uptake of Ca$^{2+}$ in the sarcoplasmic reticulum to prevent cytosolic Ca$^{2+}$ overload, even at very low temperatures.\(^{28,29,215}\) Moreover, SERCA
expression increases during hibernation, thereby preserving contractile function. However, in AF, which is characterized by cytosolic Ca\(^{2+}\) overload and contractile dysfunction, SERCA expression is decreased. Interestingly, a proteome assay showed that pathways related to cardiomyopathy pathogenesis in non-hibernators were downregulated during hibernation. Importantly, a mammalian hibernator showed markedly protective effects against induced myocardial ischemia during hibernation. This suggests that hibernators have intrinsic mechanisms which protect against development of cardiac diseases, including AF, which may be elucidated by examining the effects of tachypacing on natural hibernators. We have demonstrated that induction of the UPR\(_{ER}\) and especially autophagy might be part of these intrinsic mechanisms. Elucidation of the regulation of these protective mechanisms may help to understand how natural hibernators are protected against arrhythmias, such as AF, and identify mechanism-related new AF therapies.

**FUTURE PERSPECTIVES**

In summary, two important elements are involved in AF pathogenesis, namely the influence of aging and the connection of ER and mitochondria through the MAMs. Loss of proteostasis during aging compromises the proper functioning of cardiomyocytes, promoting development of cardiac diseases, including AF. In addition, existing cardiac disease further compromises proteostasis, thereby accelerating the progression of contractile dysfunction and disease. Targeting of key components of proteostasis may thus represent an interesting therapeutic approach to attenuate aging and cardiac disease onset in the elderly. Several compounds fortifying proteostasis are already marketed, such as GGA and 4PBA, and their clinical potential may be readily explored. Compounds targeting autophagy may be of high potential depending on outcomes of current research. However, regular exercise and caloric restriction throughout life may represent the easiest and cheapest intervention to boost cardiac proteostasis and thus slow cardiac aging, reduce the incidence of cardiac diseases and improve clinical outcome.

We discovered several pathways of the proteostasis network, including the HSR, UPR\(_{ER}\), UPR\(_{mito}\) and autophagy, to be involved in AF pathogenesis. Targeting these pathways with compounds proved to be protective in experimental models of AF. As these stress-responsive and protein degradation pathways are interconnected and often function in parallel, the effectiveness of a compound targeting one specific pathway may also target the other pathways. In addition, the versatile functions of the MAMs and the
implication of some of these function in AF, such as ER stress, autophagy, Ca\textsuperscript{2+} overload and mitochondrial dysfunction, makes the MAMs and their specific involvement in AF an additional highly interesting target. Furthermore, although the compounds proved to protect against the induction of AF in experimental models, their effectiveness on electrical and structural remodeling in AF patients is unknown. As structural remodeling is already present in the majority of the AF patients before the start of treatment, elucidating whether these compounds are able to reverse AF structural remodeling is a necessity.
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