Cardiolipin metabolism in Barth syndrome
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Chapter 8

Conclusions and future perspectives
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The research that is described in this thesis was primarily focussed on Barth syndrome (BTHS). BTHS is a severe X-linked disorder, clinically characterized by (cardio)myopathy, neutropenia and abnormal growth. It is caused by mutations in the tafazzin gene, which encodes several splice variants by means of alternative splicing. At the start of this project it was known that biochemically, BTHS cells display decreased levels of the mitochondrial phospholipid cardiolipin (CL) and have a reduced incorporation of linoleic acid in CL [1]. Based on sequence homology, it was hypothesized that tafazzin is involved in the remodeling of CL acyl chains [2]. The remodeling process to form mature CL follows the primary synthesis of (immature) CL, which is comprised of the condensation of one molecule phosphatidylglycerol (PG) and one molecule CDP-diacylglycerol (CDP-DAG), a reaction catalyzed by the enzyme CL synthase [3].

The work presented here was aimed to clarify the metabolism of CL and relate this to the pathophysiology of BTHS. The identification of the human gene coding for CL synthase is reported, followed by the characterization of its enzymatic properties. The results obtained suggested that acyl chain remodeling, e.g. by tafazzin, is required and likely occurs at the level of CL and not at the level of its precursors PG and CDP-DAG (chapter 3).

Using a newly developed validated HPLC-mass spectrometry method for the analysis of CL and monolyso-CL (MLCL; i.e. CL lacking one acyl chain) cells and tissues from BTHS patients were found to have decreased levels of CL and a lower degree of unsaturation when compared to controls (chapter 4). Furthermore, the levels of MLCL appeared to be markedly increased in BTHS cells, confirming earlier studies in tafazzin-deficient yeast cells (see chapter 5). Furthermore, the MLCL/CL ratio, which we recently introduced as a diagnostic parameter for BTHS in bloodspots [4], is indeed also a powerful diagnostic determinant in cells and tissues (chapter 4).

To determine the functionality of the different tafazzin splice variants with respect to CL metabolism the different human tafazzin splice variants were expressed in a Saccharomyces cerevisiae mutant that lacks the orthologue of tafazzin. This work led to the conclusion that only two tafazzin splice variants, i.e. full length tafazzin and tafazzin lacking exon5, are functional in CL metabolism (chapter 5). In order to further establish the functionality of the tafazzin variants, their mRNA expression was determined in various human tissues as well as in control and BTHS fibroblasts (chapter 6). All tafazzin variants, except the variant lacking exon6,7, turned out to be equally expressed. The expression of the tafazzin variants did not correlate to the CL profile, indicating that other factors, for example the microenvironment in the membrane, influence the CL profile. The tafazzin mRNA levels were not higher in tissues that are affected in BTHS patients. The increased mRNA expression of the presumed active tafazzin variants in fibroblasts of BTHS patients supported the notion that these represent the relevant variants with respect to CL metabolism (chapter 6).

In chapter 7, we compared the proteome of tafazzin-deficient Saccharomyces cerevisiae to that of wild type cells and identified several proteins involved in mitochondrial biogenesis and (oxidative) stress defense that were differentially expressed. By means of the increased mitochondrial biogenesis (e.g mitochondrial
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protein import and mitochondrial DNA stability) and stress defense, the tafazzin-deficient mitochondria likely compensate for the disturbed mitochondrial function, which is caused by the abnormal CL composition.

Although new insights are described in this thesis with respect to the function of the human tafazzin variants in CL metabolism and the pathophysiology underlying BTHS, many questions remain unanswered. Most important will be to establish whether the enzymatic function of the human tafazzins, especially full-length tafazzin and the variant lacking exon5, is identical to that of the tafazzin from *Drosophila melanogaster*, which is reported to transfer linoleic acid from phosphatidylcholine (or phosphatidylethanolamine) to MLCL [5]. Since the development of an enzymatic assay has proven to be difficult, the first goal should be to achieve stable expression of the human tafazzin variants in BTHS cells by means of viral transduction, followed by detailed characterization of the (ML)CL profile to functionally characterize the different variants. In addition, it would also be valuable to characterize the (ML)CL profile of the BTHS patients with mutations in exon5 of the tafazzin gene (recently reported in the “Human Tafazzin (TAZ) Gene Mutation & Variation Database”; www.barthsyndrome.org). These patients have the clinical phenotype that is classically associated with BTHS, although they are expected to have normal expression of the tafazzin lacking exon5, which is presumed to be active. The CL analysis in material from these patients should give an indication to what extent tafazzin lacking exon5 is sufficient for CL remodeling and may direct research towards alternative functions of other splice variants.

Given the high variability of the BTHS phenotype, even within families with the same mutation, a second line of research that should be explored further is the pathophysiology of BTHS and the potential role of modifier genes/proteins. Recently, several groups have focused on cellular pathways that are disturbed as a consequence of the deficiency of tafazzin (i.e. BTHS) [6-9]. Although it seems likely that these pathways (oxidative phosphorylation, mitochondrial apoptosis, oxidative stress response) are involved in the pathology of BTHS, it remains elusive to what extent they contribute to the pathology of BTHS and further research in this direction is warranted. The proteins that were identified in the yeast proteome screen described in chapter 7 represent candidates that could influence the BTHS phenotype. Single nucleotide polymorphisms (SNP) analysis as well as expression or functional studies of the human orthologues of these proteins in control versus BTHS cells could clarify this issue.

Indispensable for the future of BTHS research is the generation of a relevant mammalian (e.g. mouse) model. Until now, however, none of the attempts to create a BTHS mouse was successful and researchers are using other organisms as a model for BTHS such as yeast, fly and zebrafish. Although these models are valuable for many specific research purposes, they also have important limitations, especially with respect to the elucidation of the mammalian (patho)physiology. Based on experiments in zebrafish [10] and preliminary data in collaboration with the group of Dr. Michael Schlame on mouse embryonic stem cells in which tafazzin was deleted, it is clear that tafazzin plays a crucial role during development but that it might be restricted to certain cell types and/or tissues. Using a mouse model one could investigate the specific spatial and temporal function of tafazzin in more detail.
Additionally, the mouse could be used to clarify whether tafazzin deficiency results in hormonal disturbances, which could explain the abnormal growth of most of the BTHS patients [11] as well as the remarkable fat and salt craving which is reported by numerous BTHS patients and/or their parents.

A research area that has received little attention until now is the regulation of CL metabolism. As shown in chapter 6, tafazzin mRNA expression seemed to be upregulated and specific tafazzin variants appeared to be preferentially spliced in BTHS cells. The mechanisms behind these regulatory steps, however, are unknown. Although it was shown that CL biosynthesis is regulated at the transcriptional level, for instance by peroxisome proliferator-activated receptor-alpha activation [12], it remains to be established whether tafazzin is also regulated at this level and whether this regulatory mechanism could be employed as a target for the treatment of BTHS by increasing CL metabolism.

To summarize, the identification of CL abnormalities in cells of BTHS patients by Peter Vreken and colleagues in 2000 [1] has significantly advanced the field of CL research. Although further characterization of CL metabolism, notably the tafazzin-mediated acyl chain remodeling, by our group and others, has increased the state of knowledge on CL, the pathophysiology of BTHS still remains elusive. Future research in this direction will hopefully result in more detailed understanding of BTHS and the development of treatment strategies for this severe disorder.

References