Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt

Lehrstuhl für Experimentelle Genetik

Development and biochemical verification of a new method to isolate and quantify mitochondria from cell culture and liver tissue biopsies

Sabine Schmitt

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender:	Univ.-Prof. Dr. J. J. Hauner
Prüfer der Dissertation:	1. apl.-Prof. Dr. J. Adamski 2. Univ.-Prof. Dr. M. Klingenspor

Die Dissertation wurde am 21.05.2015 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 01.10.2015 angenommen.

Table of contents

1. Summary 1

1.1. Summary 1

1.2. Zusammenfassung 4

2. Aim of this thesis 8

3. Introduction 9

3.1 The structure and functions of mitochondria: a complex and dynamic system 9

3.2. The role of mitochondria in disease, pharmacology and toxicology 13

3.2.1. Mitochondrial dysfunction: Causes and consequences 13

3.2.2. Principles in mitochondrial pharmacology 15

3.3. Analyses of isolated mitochondria: Potential and challenges 16

3.3.1 The isolation of mitochondria 17

3.3.2 The normalization of comparative mitochondrial analyses 19

4. Design of the study 21

4.1. The isolation of mitochondria from cell culture and small amounts of tissue 21

4.1.1. The homogenization by the pump controlled cell rupture system 21

4.1.2. The purification of the PCC isolated mitochondria 23

4.2. Quantification of the isolated mitochondria by flow cytometry 23

4.3. Biochemical verification of the integrity and purity of the isolated mitochondria 25

4.3.1. The mitochondrial membrane potential 25

4.3.2. Calcium induced mitochondrial swelling 26

4.3.3. The respiratory control ratio 26

4.3.4. Electron microscopy 27

4.3.5. Miscellaneous 27

5. Results 28

5.1. Summary of Publication 1 28

5.2. Summary of Publication 2 31

5.3. Summary of Publication 3 33

6. Discussion 35

6.1. Analyses of isolated mitochondria: a piece in the puzzle to understand and combat mitochondrial disorders 35

6.2. Strength and applicability of the new isolation method 37

6.2.1. The "pump controlled cell rupture system" (PCC) enables the isolation of functionally and structurally intact mitochondria from cell suspensions 37

6.2.2. PCC enables the isolation of mitochondria from tissue biopsies 38
6.2.3. Purification of the PCC isolated mitochondria for molecular analyses 39
6.3. Impact of the quantification of mitochondria 40
7. Conclusion 44
8. List of publications 45
9. Contributions 46
10. Abbreviations 49
11. References 50
12. Acknowledgement 59

1. Summary

1.1. Summary

Mitochondria are essential organelles for cellular homeostasis and mitochondrial dysfunction can impair cellular function causing severe diseases, such as myopathy, neurodegenerative diseases, diabetes and cancer. Similarily, targeting of specific mitochondrial structures by pharmacological agents can manipulate mitochondrial functions to either stabilize or disrupt cellular homeostasis. The comparative functional and molecular analysis of isolated mitochondria from healthy and pathologic situations is an important step towards the identification of such mitochondrial targets. An essential prerequisite for such analyses are comparable and reproducible isolations of functionally and structurally intact mitochondria.

Several protocols for the isolation of mitochondria have been established in the past 70 years. Despite all adaptations, they basically consist of two main steps: (I) the homogenization, *i.e.* the destruction of the tissue and the plasma membrane, to liberate the mitochondria and (II) the separation of the liberated mitochondria from other cellular compartments. The separation and further purification of the released mitochondria is mostly accomplished by differential and density gradient centrifugation. It is readily controllable and only slight modifications are necessary for mitochondria of different origin. The homogenization is also without difficulty for soft and homogenous tissue, such as rat liver, which additionally provides a high amount of starting material. Using a Teflon/glass potter, only five to seven strokes are sufficient to yield a suspension of largely broken cells. This mild homogenization preserves the integrity of the mitochondrial membranes, but yields sufficient mitochondria for further purification steps. Therefore, “standard” isolated rat liver mitochondria are regarded as the „gold standard“, concerning their functionality and purity. However, if the starting material is limited, as it is the case for cell suspensions or tissue biopsies, the homogenization is much more critical. In this case, the homogenization needs to be tightly controlled to be rough enough for an efficient rupture of the plasma membrane but mild enough to preserve mitochondrial integrity. The prerequisite for such a high controllability is the ability to finely adjust a consistent destructive force. However, using the existing homogenization methods, such as a potter, nitrogen cavitation, detergents

or enzymatic digestion, the destructive force cannot be precisely adjusted or is not affecting all cells evenly. Furthermore, these methods are operator dependent thereby increasing the inter-experimental variability.

In this study, a new homogenization method, the “pump controlled cell rupture system” (PCC) has been developed. Using a high precision pump, cells or small pieces of tissue are pumped with a consistent velocity through a clearance that can be accurately adjusted in steps of 2 µm. Therefore, the intensity of the shear forces which affect the cells or the tissue during the homogenization can be tightly controlled, leading to a highly reproducible homogenization. Furthermore, due to the employment of the high precision pump this method is operator independent.

The applicability of the new homogenization method was tested on 13 different cell lines and five different types of tissue. Similar to the “standard” isolation, the homogenized samples were separated by differential centrifugation into a nuclear, crude mitochondrial and cytosolic fraction and the crude mitochondria were further purified by density gradient centrifugation. The hereby isolated mitochondria proved to be functionally and structurally intact. Subjected to a discontinuous Nycodenz® density gradient, the obtained mitochondria met the purity, required for molecular analyses, such as proteomics or lipidomics. In comparison to the “gold standard”, *i.e.* “standard” isolated rat liver mitochondria, the PCC isolated mitochondria revealed a similar quality. Furthermore, the homogenization was very efficient, as only five million cells or 20-50 mg of tissue were sufficient to yield enough mitochondria for further analyses. PCC also enabled the isolation of mitochondria from small amounts of tissue stored frozen. Although several mitochondrial functions are impaired due to the freezing-thawing process, the isolated mitochondria revealed a fairly high structural integrity and purity, which may allow for specific molecular analyses.

Comparative analyses of isolated mitochondria rely on normalizations. Typically this is based on equal amounts of mitochondrial protein, as it can be fast and readily determined. However, as mitochondria of different origin or metabolic situations vary significantly in their morphology and molecular composition, they might also differ in their total protein content. This is supported by the differential behavior of mitochondria upon density gradient centrifugation, depending on their origin.

In order to investigate whether mitochondria can differ in their protein content, a new method for the quantification of isolated mitochondria was developed. To this end,

suspensions of fluorescently labelled mitochondria with known protein concentration were subjected to a flow cytometer. Based on the measured volume, determined by an internal standard, the number of fluorescent particles and the known protein concentration, the number of mitochondria per mg protein and therefrom the mitochondrial protein content was calculated.

For rat liver mitochondria, this approach resulted in mitochondrial protein contents comparable to earlier reports using alternative methods. Similar protein contents were determined for rat heart and kidney mitochondria, whereas lower protein contents were determined for rat brain mitochondria and for mitochondria from different rat hepatocellular carcinoma (HCC) cell lines. This result challenges mitochondrial comparisons that rely on equal protein amounts as a typical normalization method. Therefore, rat liver mitochondria and mitochondria from the rat hepatoma cell line McA 7777 were compared regarding their complex II activity and vulnerability. Significant discrepancies were obtained by either normalizing to protein amount or to absolute mitochondrial number. Importantly, only normalization on an equal number of mitochondria unveiled the lower complex II activity and higher susceptibility of McA 7777 mitochondria compared to liver mitochondria. These findings demonstrate that solely normalizing to protein amount may obscure essential molecular differences between mitochondrial populations.

Taken together, the methods developed in the course of this study enable the isolation of functionally and structurally intact mitochondria from various cell types and tissue biopsies in a highly reproducible manner as well as the quantification of the isolated mitochondria, thereby allowing for comparative analyses of mitochondria from different pathologic and metabolic situations.

1.2. Zusammenfassung

Mitochondrien spielen eine zentrale Rolle in der zellulären Homöostase. Daher können mitochondriale Dysfunktionen schwerwiegende Konsequenzen für die Zelle haben und zu Erkrankungen wie Myopathie, Neurodegeneration, Diabetes und Krebs führen. Auf Grund ihrer essentiellen Funktionen sind Mitochondrien aber auch eine interessante Zielstruktur für neue Therapieansätze. So kann durch die gezielte Manipulation von mitochondrialen Strukturen und Funktionen Einfluss auf das Überleben der Zelle genommen werden. Vergleichende Analysen von isolierten Mitochondrien aus gesunden und pathologisch veränderten Zellen, hinsichtlich ihrer Funktion und molekularen Zusammensetzung, können wichtige Hinweise auf entsprechende Zielstrukturen liefern. Eine essentielle Voraussetzung für solche Analysen ist die reproduzierbare Isolierung von funktional und strukturell intakten Mitochondrien.

In den letzten 70 Jahren wurden zahlreiche Protokolle zur Isolierung von Mitochondrien aus unterschiedlichen Zell- und Gewebetypen etabliert und in Abhängigkeit des Ausgangsmaterials modifiziert. Unabhängig von diesen Modifikationen ist die Isolierung von Mitochondrien jedoch immer aus zwei wesentlichen Schritten aufgebaut: (I) der Homogenisierung der Probe, bei der die Zellmembran zerstört wird um die Mitochondrien aus der Zelle freizusetzen und (II) der Abtrennung der Mitochondrien von anderen Zellbestandteilen. Die Abtrennung und weitere Aufreinigung der freigesetzten Mitochondrien erfolgt typischerweise mittels differentieller Zentrifugation bzw. mittels eines Dichtegradienten. Hierbei sind nur geringfügige Änderungen notwendig um eine optimale Aufreinigung von Mitochondrien unterschiedlicher Herkunft zu erreichen. Handelt es sich bei dem Ausgangsmaterial um weiches und homogenes Gewebe, wie z.B. Lebergewebe, ist auch die Homogenisierung unproblematisch. In diesem Fall sind fünf bis sieben Hübe mit einem Teflon/Glas Potter ausreichend um einen Großteil der Zellmembranen zu zerstören. Da die hierbei auftretenden Scherkräfte relativ gering sind und zudem nur kurz wirken, wird die Integrität der mitochondrialen Membranen jedoch erhalten. Zudem ist das Ausgangsgewicht bei der Rattenleber so hoch, dass auch bei nicht vollständigem Zellaufschluss ausreichend Mitochondrien gewonnen werden können um eine weitere Aufreinigung zu ermöglichen. Isolierte Rattenlebermitochondrien gelten daher als Goldstandard hinsichtlich ihrer Funktionalität und Reinheit. Steht weniger Ausgangsmaterial zur Verfügung, wie z.B. bei kultivierten Zellen oder Biopsien, ist die Homogenisierung deutlich komplizierter, da der

Zellaufschluss sehr effizient sein muss. Die damit verbundenen höheren Scherkräfte oder die erhöhte Dauer können jedoch zu einer Schädigung der Mitochondrien führen. Die Herausforderung bei Proben mit einer geringen Ausgangsmenge ist daher, die perfekte Balance zwischen einer effizienten Ruptur der Plasmamembran und einer minimalen Beeinträchtigung der mitochondrialen Struktur und somit Funktion zu finden. Dies setzt voraus, dass die zur Homogenisierung eingesetzte Methode präzise einstellbar und kontrollierbar ist. Die Intensität der Membran zerstörenden Kraft lässt sich bei den bisher verwendeten Methoden wie Teflon/Glas- oder Glas/Glas Potter, Stickstoffbombe, Detergenzien oder enzymatischem Verdau jedoch entweder nicht genau einstellen und/oder nur schwer kontrollieren.

In dieser Arbeit wurde eine neue Homogenisierungsmethode, das “pump controlled cell rupture system” (PCC) entwickelt. Hierbei werden mit Hilfe einer Hochpräzisionspumpe Zellen oder kleine Gewebestückchen durch einen Spalt gepresst, welcher in 2 µm Schritten exakt eingestellt werden kann. Da die Flussgeschwindigkeit und die Spaltgröße sehr fein justierbar und zudem stabil sind, lassen sich die Scherkräfte, welche während der Homogenisierung auf die Zellen oder das Gewebe wirken somit genau einstellen und kontrollieren. Damit kann ein effizienter Zellaufschluss erreicht werden ohne die mitochondrialen Membranen zu zerstören. Die Methode ist zudem operatorunabhängig, wodurch eine reproduzierbar hohe Qualität erreicht werden kann.

Die neue Homogenisierungsmethode wurde an 13 verschiedenen Zelllinien und fünf verschiedenen Gewebetypen getestet. Die homogenisierten Proben wurden dann, wie auch bei klassischen Methoden, mittels differentieller Zentrifugation in eine nukleäre, eine grobe mitochondriale und eine zytosolische Fraktion unterteilt. Die hierbei isolierten Mitochondrien waren funktionell und strukturell intakt. Nach einer weiteren Aufreinigung mittels eines 2-stufigen Nycodenz® Dichtegradienten wiesen die Mitochondrien eine für molekulare Analysen, wie z.B. Proteom- oder Lipidomanalysen, benötigte Reinheit auf. Insgesamt war die Qualität der PCC isolierten Mitochondrien mit der von klassisch isolierten Rattenlebermitochondrien, d.h. dem „Goldstandard“, vergleichbar. Weiterhin konnte die Effizienz des Zellaufschlusses so erhöht werden, dass bereits fünf Millionen Zellen bzw. 20-50 mg Gewebe ausreichend waren um ausreichend Mitochondrien für weitere Analysen zu erhalten. Mittels PCC konnten auch Mitochondrien aus Gewebe isoliert werden, welches nach der Entnahme bei -80°C gelagert war. Auf Grund des Einfrier- und Auftauprozesses

werden zwar einige mitochondriale Funktionen zerstört, jedoch wiesen die isolierten Mitochondrien noch eine hohe strukturelle Integrität und eine relativ hohe Reinheit auf, wodurch spezifische molekulare Analysen möglich sind.

Um isolierte Mitochondrien hinsichtlich ihrer Funktionen oder molekularen Zusammensetzung vergleichen zu können wird ein Normalisierungsfaktor benötigt. Dies erfolgt meist anhand gleicher Proteinmenge, da diese schnell und leicht bestimmt werden kann. Da Mitochondrien, in Abhängigkeit ihrer Herkunft oder metabolischen Situation deutliche Unterschiede in ihrer Morphologie und molekularen Zusammensetzung aufweisen, könnte es jedoch auch Unterschiede im mitochondrialen Proteingehalt geben. Ein Hinweis dafür ist, dass Mitochondrien unterschiedlicher Herkunft sich in ihrer Dichte unterscheiden.

Um zu untersuchen, ob sich Mitochondrien in ihrem Proteingehalt unterscheiden, wurde in dieser Arbeit eine Methode entwickelt, die es ermöglicht Mitochondrien zu quantifizieren. Hierfür wurden isolierte Mitochondrien mit einem Fluoreszenzfarbstoff gefärbt, die Proteinkonzentration gemessen und die Anzahl mittels Durchflusszytometrie bestimmt. Das gemessene Volumen wurde mit Hilfe eines internen Standards ermittelt und damit die Anzahl an Mitochondrien pro mg Protein bzw. der Proteingehalt pro Mitochondrium berechnet.

Mit Hilfe dieser neuen Methode wurde für Lebermitochondrien ein Proteingehalt ermittelt, der bereits mit anderen Methoden bestimmt wurde. Für Rattenherz- und Rattennierenmitochondrien wurde ein Proteingehalt festgestellt der mit dem von Rattenlebermitochondrien vergleichbar ist. Dahingegen war der Proteingehalt von Rattengehirnmitochondrien und Mitochondrien aus verschiedenen Rattenhepatom Zelllinien deutlich geringer. Diese Ergebnisse deuten darauf hin, dass mitochondriale Analysen nicht immer anhand gleicher Proteinmenge normalisiert werden können. Um dies zu überprüfen wurden exemplarisch Rattenlebermitochondrien und Mitochondrien aus der Rattenhepatomzelllinie McA 7777 hinsichtlich ihrer Komplex II Aktivität und Empfindlichkeit miteinander verglichen. In Abhängigkeit der Normalisierung, Proteinmenge bzw. Anzahl an Mitochondrien, konnten deutliche Unterschiede festgestellt werden. Interessanterweise konnte nur bei einer gleichen Anzahl an Mitochondrien beobachtet werden, dass die McA 7777 Mitochondrien eine geringere Komplex II Aktvität besitzen und dieser zudem bei einer geringeren Dosis inhibiert werden kann. Damit konnte gezeigt werden, dass bei einer

Normalisierung anhand gleicher Proteinmenge wichtige Unterschiede zwischen Mitochondrien unterschiedlicher Herkunft unentdeckt bleiben können.

Insgesamt konnte gezeigt werden, dass die in dieser Arbeit entwickelten Methoden eine reproduzierbare Isolierung von funktionell und strukturell intakten Mitochondrien aus verschiedenen Zelltypen und aus Biopsien sowie die Bestimmung des mitochondrialen Proteingehalts ermöglichen. Damit sind die wichtigsten Voraussetzungen für eine vergleichende Analyse von Mitochondrien aus unterschiedlichen metabolischen und pathologischen Situationen erfüllt.

2. Aim of this thesis

The aim of this thesis was to establish an isolation method for mitochondria from cell suspensions and small amounts of tissue samples that allows for comparative analyses of mitochondria of different origin and from different metabolic or pathologic situations. Prerequisites for such analyses are isolated mitochondria with a reproducible and comparable high quality, *i.e.* biochemical functionality, structural integrity, and purity.

The initial step in mitochondrial isolation is the homogenization of the cells or the tissue, *i.e.* the rupture of the plasma membrane. This step is very critical, as the used mechanical or chemical means can also damage the mitochondrial membranes. Especially if the starting material is limited, e.g. cultured cells and tissue biopsies, the homogenization has to be tightly controlled, thus enabling an efficient cell rupture without impairing mitochondrial structure and function. However, the existing methods lack this controllability. Therefore, this study focuses on the homogenization, to make it more controllable, reproducible, readily adjustable on a variety of samples, and operator independent.

Another critical issue for comparative analyses of mitochondrial suspensions is the normalization. Mitochondrial function, morphology and molecular composition vary substantially depending on the cell type or the metabolic situation. It is conceivable that these variations are accompanied by differences in the overall mitochondrial protein content. This would challenge mitochondrial comparisons based on same amounts of protein as typical normalization method. Therefore, the second aim was the development of a fast method to quantify isolated mitochondria, allowing for normalization based on an equal number of mitochondria.

3. Introduction

3.1 The structure and functions of mitochondria: a complex and dynamic system

Mitochondria play a key role in cellular homeostasis (Alirol and Martinou 2006; Zorov, Isaev et al. 2007) (Figure 1). Most importantly, they generate up to 90% of the cellular ATP (Mootha, Bunkenborg et al. 2003), are essential for several anabolic and catabolic processes, serve as a calcium reservoir (Vasington 1963) and are involved in the regulation of apoptosis (Newmeyer, Farschon et al. 1994; Marzo, Brenner et al. 1998). The multitude of mitochondrial functions is implemented by the organization of the organelle into the four compartments matrix, inner membrane, intermembrane space, and outer membrane (Figure 1). Each of the compartments has unique tasks, mirrored by a specific molecular composition. In addition, there are several mitochondrial functions that rely on the cooperation of two or more compartments.

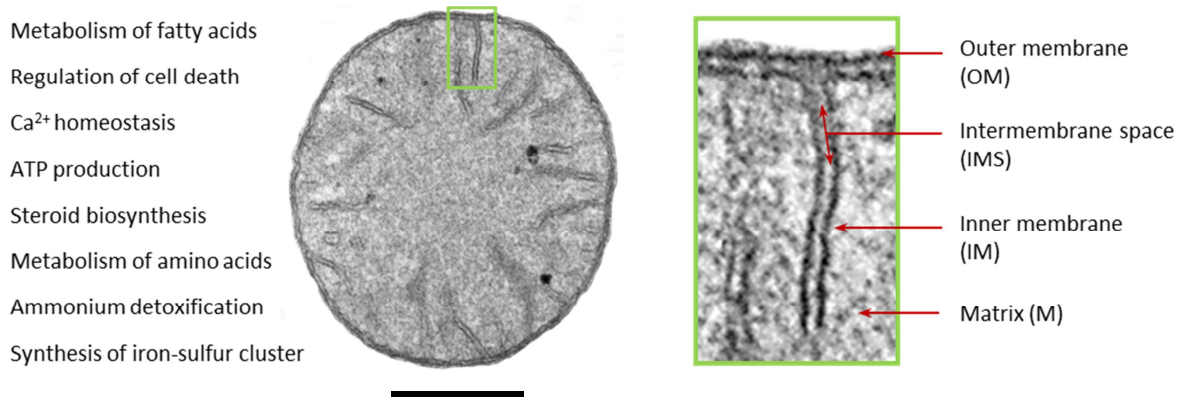

Figure 1 **Overview of the main mitochondrial functions and structures.** Mitochondria are essential for cellular homeostasis as they generate the majority of cellular ATP, are involved in several anabolic and catabolic processes and play a crucial role in the regulation of cell death. This multitude of mitochondrial functions is facilitated by the organization of the organelle into four compartments: outer membrane, intermembrane space, inner membrane and matrix. Electron micrograph of a rat liver mitochondrion. Bar equals 500 nm.

The mitochondrial outer membrane (OM) delineates the organelle from the cytosol, but also forms the connection to the rest of the cell. It consists of 52% proteins and 48% of lipids (Guidotti 1972), being structurally similar to other cell membranes. Among its proteins are receptors, channels and transporters which are required for the exchange of metabolites, for mitochondrial fission and fusion and the communication with other cell compartments (Becker, Gebert et al. 2009), thereby regulating mitochondrial function in coordination with

the cellular demands. The most abundant protein of the outer membrane is the voltage dependent anion channel (VDAC) accounting for ~ 50% of the total protein content (Mannella 1998; Goncalves, Buzhynskyy et al. 2007). VDAC makes significant contributions to the permeability of the outer membrane for small molecules with a size up to 5 kD (Colombini 1979; Colombini 1980; Zalman, Nikaido et al. 1980). However it does not allow for the diffusion of proteins (Wojtczak and Zaluska 1969). Therefore, the intermembrane space (IMS) which is located between the inner and the outer membrane reveals similar concentrations of small molecules, e.g. ions and sugars, as the cytosol (Herrmann and Riemer 2010), but a specific protein composition. Most importantly, it contains the pro-apoptotic proteins cytochrome c (Cyt C), apoptosis inducing factor (AIF), Diablo and Endonuclease G (Munoz-Pinedo, Guio-Carrion et al. 2006). As the release of these proteins into the cytosol triggers the induction of apoptosis (Susin, Lorenzo et al. 1999; Du, Fang et al. 2000; Verhagen, Ekert et al. 2000; Li, Luo et al. 2001; Suzuki, Imai et al. 2001), the permeabilization of the mitochondrial outer membrane (MOMP) plays a major role in the regulation of cell death (Chipuk, Bouchier-Hayes et al. 2006). The most important function of the mitochondrial inner membrane is the generation of ATP via oxidative phosphorylation (Kennedy and Lehninger 1949; Schneider and Potter 1949). The oxidative phosphorylation consists of two separate, but coupled, processes. In a first step, the four complexes (CI, CII, CIII and CIV) of the respiratory chain transfer electrons from $NADH/H^+$ or $FADH_2$ to oxygen. Simultaneously, three of the four complexes (CI, CIII and CIV) shuttle protons from the mitochondrial matrix to the intermembrane space, creating a proton gradient which causes a membrane potential of about 180 mV, being negative on the inside. In a second step, this proton gradient is used by complex V to generate ATP (Mitchell 1961). As a prerequisite for this coupling, the inner membrane has to be impermeable for ions. The impermeability of the mitochondrial inner membrane for solutes and ions is predominantly achieved by cardiolipin (Stoffel and Schiefer 1968; Colbeau, Nachbaur et al. 1971), a phospholipid, which is primarily located in the mitochondrial inner membrane. Further structural and molecular characteristics of this membrane are an increased surface, due to several invaginations, termed cristae (Palade 1952), and the high protein to lipid ratio of about 75% to 25% (Guidotti 1972). The most abundant proteins are members of the complexes of the respiratory chain, of the ATP synthase and the adenine nucleotide transporter (ANT). Furthermore, it contains specific transporters to shuttle nuclear encoded mitochondrial

proteins and metabolites, such as pyruvate, calcium and phosphate into the mitochondrial matrix. The mitochondrial matrix accomplishes a variety of functions. Among them are anabolic and catabolic processes, such as the tricarboxylic acid cycle (Kennedy and Lehninger 1949) and the β-oxidation of fatty acids, the synthesis of iron-sulfur cluster and the detoxification of urea. It furthermore serves as a calcium reservoir (Thayer and Miller 1990; Friel and Tsien 1994; Werth and Thayer 1994; Robb-Gaspers, Rutter et al. 1998; David and Barrett 2000; Kaftan, Xu et al. 2000; David and Barrett 2003; Isaeva and Shirokova 2003; Maack, Cortassa et al. 2006; Veitinger, Veitinger et al. 2011; Drago, De Stefani et al. 2012; Fluegge, Moeller et al. 2012) and contains molecules for the reduction of reactive oxygen species (ROS). As a relict of their bacterial origin, mitochondria possess remnants of their own DNA (mtDNA) which is also located in the matrix, encoding for 13 proteins of the respiratory chain, for 2 rRNAs and for 22 tRNAs (Orth and Schapira 2001). The multitude of functions of the mitochondrial matrix requires quantities of proteins. Therefore, it is not surprising that this compartment has a very high protein concentration of about 560 mg per ml (Srere 1980) and contains up to 67% of the total mitochondrial protein content (Schnaitman and Greenawalt 1968).

Depending on the cell type and the metabolic situation, certain mitochondrial functions are required to variable extents, mirrored by pronounced differences in molecular composition and morphology (Voet Daniel 1992). In cardiomyocytes for example, mitochondria are particularly important for the generation of ATP via oxidative phosphorylation. Therefore, they exhibit a large number of densely packed cristae (Figure 2A), increasing the surface of the inner membrane to a maximum. In hepatocytes, mitochondria play an important role in the metabolism of fatty acids, in gluconeogenesis, in heme biosynthesis and in the detoxification of urea. All of these processes occur in the mitochondrial matrix, wherefore this compartment is much more prominent in liver than in heart mitochondria (Figure 2B).

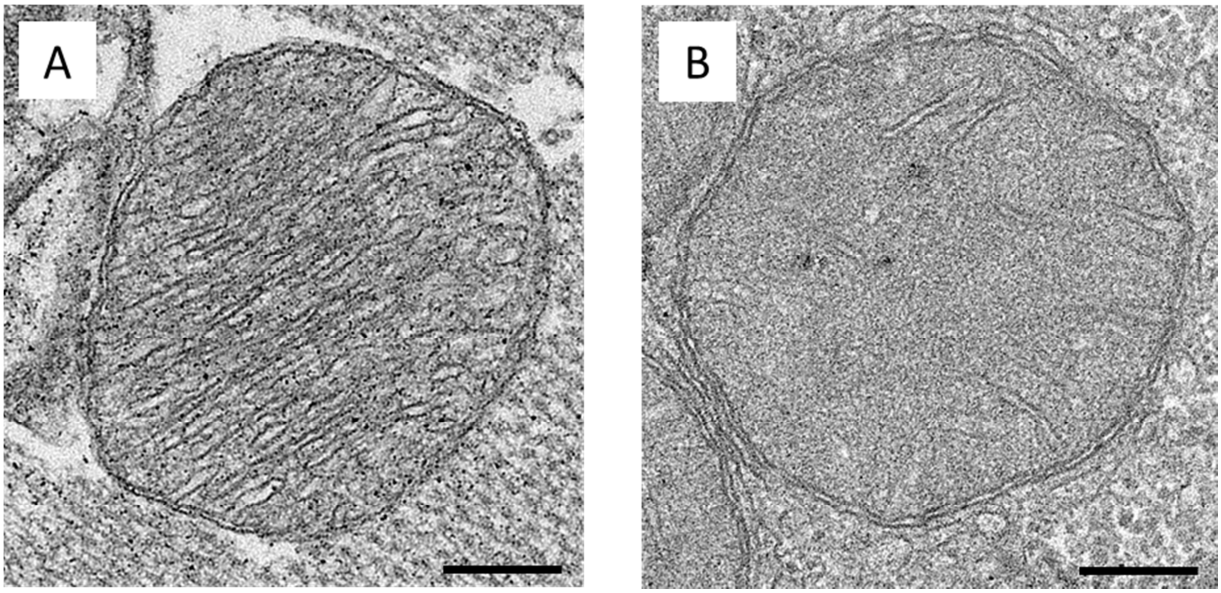

Figure 2 **Mitochondrial functions are dependent on the cell type, causing distinct mitochondrial morphologies.** Electron micrographs of (A) rat heart and (B) rat liver mitochondria. Mitochondria in cardiomyocytes reveal a high number of densely packed cristae, reflecting the importance of these organelles for ATP production. In contrast, liver mitochondria are more important for anabolic and catabolic processes and the detoxification of urea, thus possessing a prominent matrix and a reduced number of cristae compared to the mitochondria from cardiomyocytes. Bars equal 200 nm.

One of the most impressive examples for the mitochondrial adaptability upon different metabolic conditions has been described by the group of Gottfried Schatz. If baker's yeast is grown under anaerobic conditions they form poorly differentiated thread-like 'promitochondria' (Criddle, Paltauf et al. 1969; Plattner and Schatz 1969). Promitochondria have a dramatically changed enzymatic composition compared to "normal" mitochondria (Criddle and Schatz 1969) and transform to mitochondria when the cells are back-shifted to aerobic conditions (Plattner, Salpeter et al. 1970).

The natural adaptability of the mitochondrial structure, molecular composition and function facilitates the various cell types to fulfill their specific functions and to act on changing metabolic situations. However, uncontrolled alterations within these organelles can also lead to mitochondrial dysfunction which contributes to several disparate pathologies (Smith, Hartley et al. 2012).

3.2. The role of mitochondria in disease, pharmacology and toxicology

3.2.1. Mitochondrial dysfunction: Causes and consequences

Mitochondrial dysfunctions can be divided into two subclasses, depending on whether they are attributed to a direct or indirect impairment of the organelle (Smith, Hartley et al. 2012). Primary mitochondrial disorders are usually caused by mutations in the mitochondrial DNA or in nuclear genes that encode for mitochondrial proteins. Recent epidemiological data suggest that the prevalence of such genetically induced mitochondrial disorders is at least 1 in 5000, and could be much higher (Schaefer, Taylor et al. 2004). In several cases, the affected proteins are involved in ATP synthesis, causing an energetic crises. Due to their high demand in energy, typically muscle cells and neurons are affected, leading to mitochondrial myopathies or encephalomyopathies. MELAS (mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes) (Pavlakis, Phillips et al. 1984) for example, is induced by mutations in mitochondrial genes either encoding for a protein of complex I or for a tRNA which leads to a defective assembly of oxidative phosphorylation complexes. Another example for a genetically induced primary mitochondrial disorder is Friedreichs Ataxia (Rotig, de Lonlay et al. 1997; Munnich and Rustin 2001), which accounts for ~50% of all inherited forms of ataxia. It is caused by a mutation in the nuclear encoded mitochondrial protein Frataxin, which plays an important role in the synthesis of iron-sulfur clusters. The deficiency in iron sulfur cluster synthesis leads to an enhanced transport of iron into the mitochondrial matrix, causing oxidative stress and cell death. Beside the genetic mutations, xenobiotics are also able to directly impair mitochondrial function. There are several agents that disrupt ATP synthesis by either inhibiting one of the OxPhos complexes, *e.g.* cyanide, which acts on complex IV, or by increasing the permeability of the inner membrane, *e.g.* nonsteroidal anti-inflammatory drugs (NSAID) (Masubuchi, Nakayama et al. 2002; Li, Qi et al. 2009). The dissipation of the proton gradient without ATP generation can result in the generation of heat, and, in extreme conditions, a malignant hyperthermia syndrome can occur (Duchen 2004). Mitochondria are also very susceptible to genotoxic agents as the mtDNA, in contrast to nuclear DNA, contains a lower number of introns, is devoid of protecting histones and has a less efficient DNA repair system (Yakes and Van Houten 1997).

Secondary mitochondrial dysfunction is caused by pathological events that originate outside mitochondria (Smith, Hartley et al. 2012), and can occur at the very beginning or at an

advanced stage of the disease. They have been associated with frequent diseases, such as heart failure in consequence of ischemia/reperfusion, neurodegenerative disorders or diabetes. As with primary mitochondrial disorders, the causes for mitochondrial dysfunction are diverse, yet, the consequences are very similar: disruption of ATP synthesis, an imbalance in calcium homeostasis and/or oxidative stress. In many cases these processes occur in parallel or are induced by each other resulting in the mitochondrial permeability transition (MPT) and/or the mitochondrial outer membrane permeabilization (MOMP) (Figure 3).

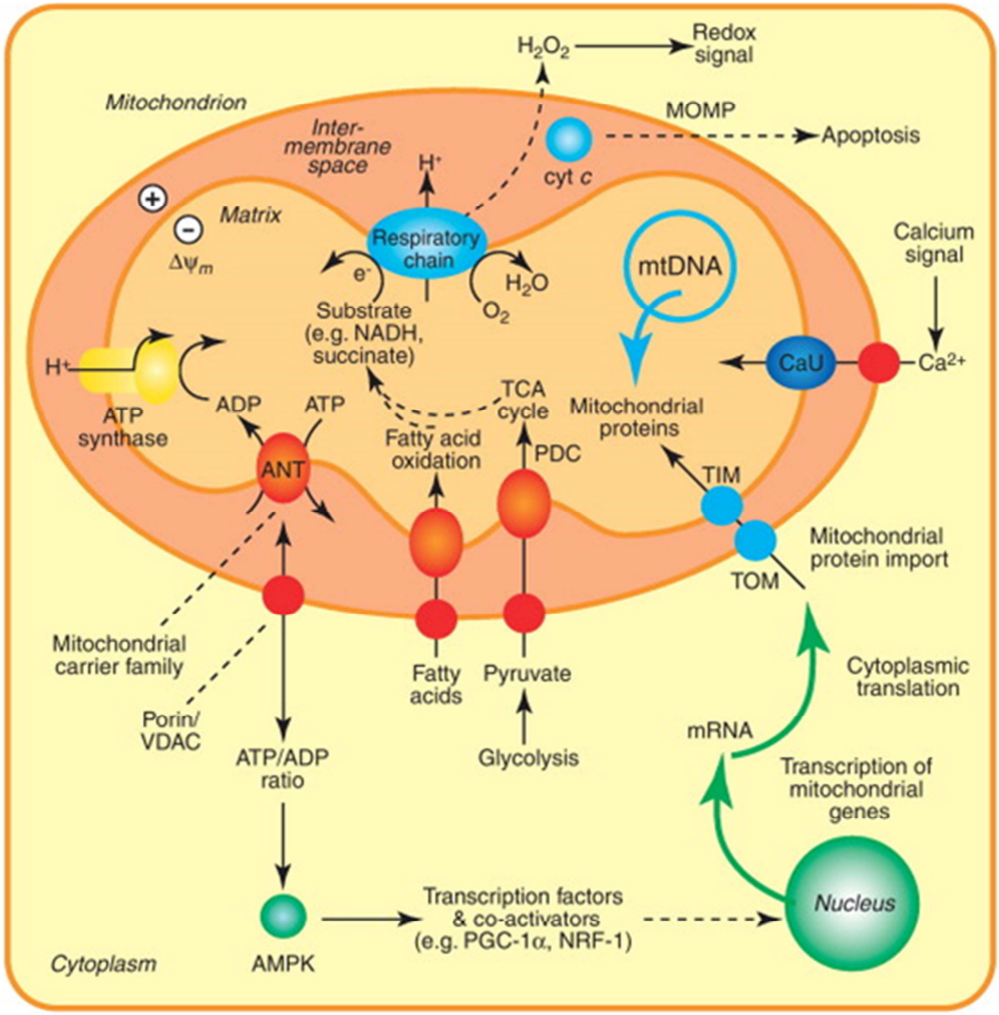

Figure 3 **Mitochondrial dysfunction.** Disruption of mitochondrial function can be caused by primary events, such as mutation to mitochondrial or nuclear genes. Secondary mitochondrial dysfunction arises due to causes outside the mitochondrion. There are often common factors to mitochondrial dysfunction such as increased oxidative stress, disruption to calcium homeostasis and defective mitochondrial ATP synthesis. Frequently, these occur together or lead into each other. The combination of elevated mitochondrial matrix calcium and oxidative stress leads to induction of the mitochondrial permeability transition pore (mPT), which further disrupts mitochondrial function. Reused with kind permission from: Robin A.J. Smith, Richard C. Hartley, Helena M. Cocheme and Michael P. Murphy. (2012) Mitochondrial Pharmacology. Trends in Pharmacological Sciences Vol. 33, No. 6:341-352.

The MPT is defined as the sudden increase in the permeability of the mitochondrial inner membrane (Hunter, Haworth et al. 1976) to ions and solutes of up to 1.5 kD (Haworth and Hunter 1979). This causes a breakdown of the mitochondrial membrane potential (MMP) and thus a disruption of ATP synthesis. As a second consequence, the influx of solutes into the mitochondrial matrix is followed by H_2O, the volume of the mitochondrial matrix increases and the inner membrane unfolds. This process, termed mitochondrial swelling leads to a rupture of the mitochondrial outer membrane (MOMP), as the surface of the mitochondrial inner membrane exceeds that of the outer membrane. MOMP than leads to the release of proapoptotic proteins, such as cytochrome c (Liu, Kim et al. 1996) or apoptosis inducing factor (AIF) (Susin, Lorenzo et al. 1999; Daugas, Susin et al. 2000), from the intermembrane space into the cytosol, thereby inducing cell death. A further elicitor of MOMP is the oligomerization of the outer membrane proteins Bax or Bak (Jurgensmeier, Xie et al. 1998; Eskes, Desagher et al. 2000; Gogvadze 2011).

The similarity of the cell death inducing mitochondrial events offers the possibility to treat patients with a wide range of primary and secondary mitochondrial disorders by stabilizing mitochondrial structures and functions, involved in these events.

Cancer has also been associated with mitochondrial dysfunction. However, in contrast to other mitochondrial disorders it is not an increase but a decrease in cell death that leads to the progression of the disease. Thus, molecular and functional alterations that are causative for primary or secondary mitochondrial disorders are a potential target for cancer chemotherapy (Galluzzi, Larochette et al. 2006; Pilkington, Parker et al. 2008; Berridge, Herst et al. 2009; Ralph and Neuzil 2009; Fulda, Galluzzi et al. 2010; Fulda and Kroemer 2011; Wenner 2011).

3.2.2. Principles in mitochondrial pharmacology

Mitochondria can be targeted in a direct or an indirect manner. An indirect way is the modulation of proteins outside mitochondria, controlling the number and activity of the organelle (Smith, Hartley et al. 2012). Among these regulating proteins are the NAD+-dependent deacetylases belonging to the sirtuin family (Fernandez-Marcos and Auwerx 2011). For example, the nuclear pool of Sirtuin3 can deacetylate and thereby activate the forkhead transcription factor, FOXO3a, upregulating the expression of mitochondrial

antioxidant enzymes such as MnSOD (Sundaresan, Gupta et al. 2009). However, changes in the acetylation status of histones and of other transcription factors and coactivators, such as PGC-1a, also affect the transcription of mitochondrial genes, and this complicates the interpretation of sirtuin activity on mitochondria (Smith, Hartley et al. 2012). Thus, the manipulation of signaling pathways outside of mitochondria is very complex and can cause a variety of side effects. Targeting mitochondria directly might be more specific thereby reducing the number of adverse reactions. It can be accomplished in two ways, either by targeting specific mitochondrial proteins or by targeting small molecules specifically to the mitochondria using their high membrane potential (Smith, Hartley et al. 2012). The major therapeutic application of mitochondria-targeted therapies so far has been the use of antioxidants to block mitochondrial damage in cardiomyocytes, *e.g.* to prevent cardiac injury after ischemia/reperfusion (Smith and Murphy 2010; Smith, Hartley et al. 2011). Targeting of specific mitochondrial structures, e.g. the components of the mitochondrial permeability transition pore or the Oxphos-complexes, is an important strategy in combating cancer (Rohlena, Dong et al. 2011). Analyses of isolated mitochondria are required to define such mitochondrial target structures and to verify the direct impact of potential mitochondria-targeting drugs.

3.3. Analyses of isolated mitochondria: Potential and challenges

Many aspects depict mitochondria as major research focus. First, it was important to elucidate the fundamental biochemical functions of mitochondria and the involved molecules and structures. Indeed, former studies on isolated mitochondria led to seminal discoveries about mitochondrial biology, including the chemiosmotic theory of oxidative phosphorylation (Mitchell 1961) and elucidation of the Krebs cycle (Williams 1965). Although the majority of mitochondrial structures and functions are known, the exact mechanisms of some processes, *e.g*. the induction of the mitochondrial permeability transition (MPT), are still elusive. Secondly, analyses of isolated mitochondria from different metabolic and pathologic situations are necessary to investigate their molecular and functional adaptations, which in turn are the basis for the development of specific and effective therapies. Finally, sensitivity profiles of isolated mitochondria can be useful to estimate the efficiency but also the potential toxicity of newly developed therapies.

Depending on the type of analyses, the structural and functional integrity and the purity of the isolated mitochondria are of variant importance. However, in any case, the mitochondrial quality needs to be reproducible in order to draw firm conclusions on the determined mitochondrial peculiarities and enzymatic activities. If isolated mitochondria of different origin are compared, the comparability of their quality and the normalization are further critical aspects.

3.3.1 The isolation of mitochondria

The general approach for the isolation of mitochondria has been established in the late 1940s (Hogeboom, Claude et al. 1946; Hogeboom, Schneider et al. 1948; Schneider, Claude et al. 1948) and requires two steps: (I) an initial homogenization of the sample, *i.e.* the rupture of the plasma membrane, to liberate the mitochondria from the cell and (II) a subsequent purification of the mitochondria from other cell components. Differential centrifugation and density gradient centrifugation are mostly used for the purification and only slight modifications are necessary to optimize it for different types of mitochondria. In contrast, the initial homogenization needs to be adapted on the respective mitochondrial source of origin (Pallotti and Lenaz 2007; Schulz, Lichtmannegger et al. 2015). Isolation of mitochondria from yeast for example, requires an additional step to remove the cell wall (Daum, Bohni et al. 1982; Boldogh and Pon 2007) and very tough tissue, such as the heart, has to be minced into small pieces (Scholte, Weijers et al. 1973; Saks, Chernousova et al. 1975; Schulz, Lichtmannegger et al. 2015). This preprocessing is required to allow for a reduction of the forces during the homogenization thereby preventing mitochondrial damage, but still causing an efficient destruction of the cell membrane. For rat liver, the homogenization is readily feasible, as the tissue is very soft and homogenous and provides several grams of starting material. Therefore, it can be very mild, using only five to seven strokes with a Teflon/glass potter, still yielding enough mitochondria for further purification and ensuring mitochondrial integrity. These “standard” isolated rat liver mitochondria, *i.e.* homogenized with a Teflon/glass potter, separated by differential centrifugation and purified by Percoll^{TM} density gradient purification, are therefore regarded as the „gold standard“, concerning their functionality and purity (Fuller and Arriaga 2004). However, if the starting material is limited, *e.g.* cultured cells or tissue biopsies, the homogenization

needs to be tightly controlled to achieve a perfect balance between an efficient rupture of the plasma membrane and a minimal impairment of the mitochondrial membranes, thereby preserving mitochondrial function.

Using a potter, the shear forces are determined by: (I) the clearance between the glass tube and the pestle, (II) the surface character of the glass tube and the pestle, (III) the type of movement, *e.g.* up and down and/or rotation and (IV) the velocity of the movement. In order to adjust these parameters for different types of samples, a vast assortment of homogenizers of different sizes, forms and with different surface character has been manufactured. However, the clearance between the tube and the pestle is not precisely determined and varies during the homogenization and the velocity of the movement is, at least partially, dependent on the operator. Therefore, the reported number of strokes needed to homogenize cell suspensions varies dramatically, ranging from 20 to 40, and is often not precisely specified (Almeida and Medina 1998; Frezza, Cipolat et al. 2007; Pallotti and Lenaz 2007; Wieckowski, Giorgi et al. 2009). Nitrogen cavitation has the advantage to be operator independent and the pressure that causes the disruption of the cell membrane is stable. However, in contrast to a potter, a long exposition to the mechanical disruption is necessary, impairing the integrity of the mitochondrial membranes. Furthermore, the intensity of the pressure cannot be finely adjusted. Detergents and enzymatic digestion are very useful as a preprocessing for fibrous tissue, as skeletal muscle, to break the cellular connections. Yet, they lack the needed controllability for the subsequent mitochondrial isolation. As with these classical methods the homogenization cannot be precisely controlled or adjusted, mitochondria isolated from cultured cells frequently reveal a relatively low quality, at least if compared to the “gold standard” (Figure 4 B, C, D, compared to A).

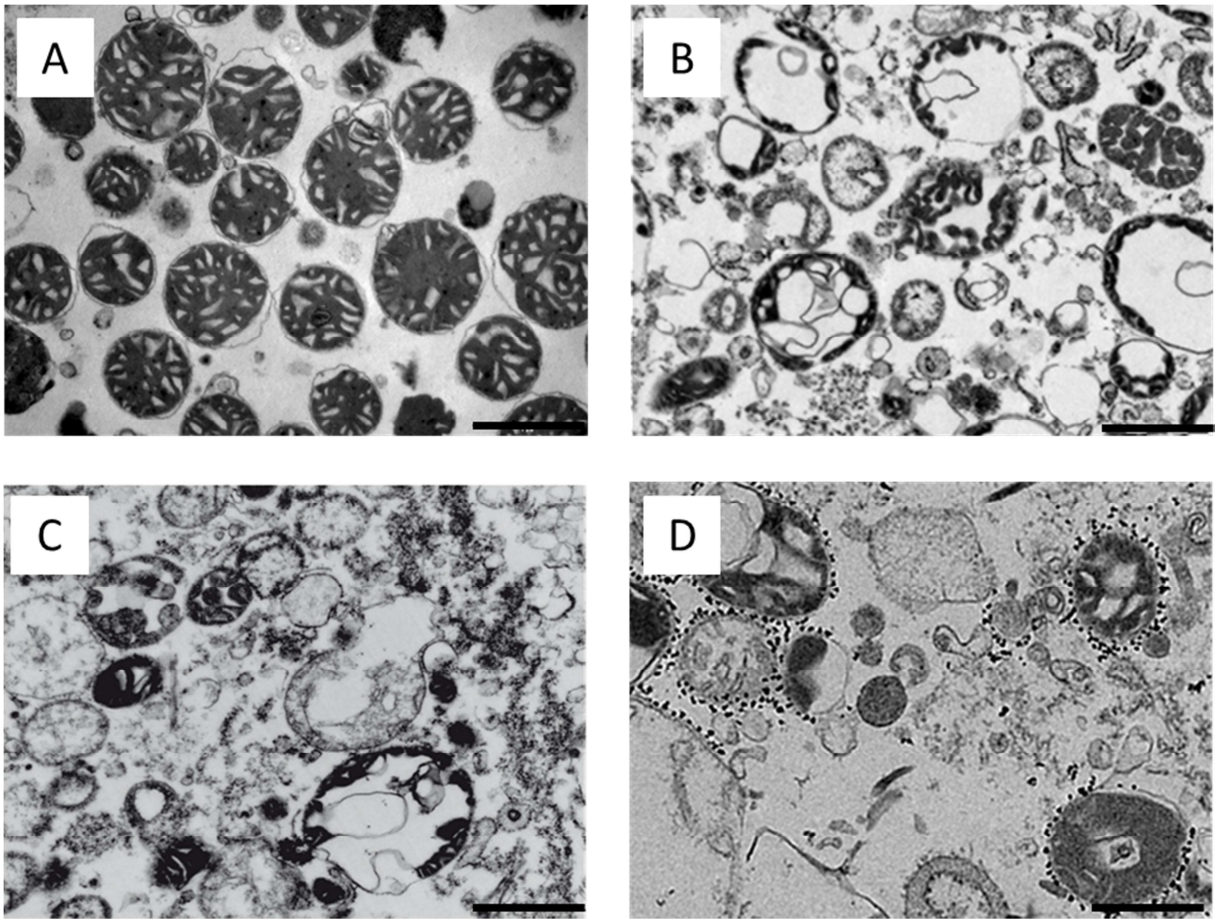

Figure 4 **„Standard“ isolation methods are applicable to liver and other soft tissues but frequently result in mitochondrial suspensions of poor quality if applied to cell suspensions.** Electron micrographs of mitochondria from (A) rat liver, isolated by six strokes with a Teflon/glass potter and purified by Percoll^{TM} density gradient centrifugation, (B) U251 human glioma cells, isolated with 40-50 strokes with a Teflon/glass potter, (C) U251 human glioma cells, isolated by nitrogen cavitation (20 min, 10 bar) and 20 strokes with a Teflon/glass potter, (D) 1205LU cells, isolated with a syringe and purified by magnetic beads. Bars equal 1 µm.

3.3.2 The normalization of comparative mitochondrial analyses

Comparative biochemical analyses of isolated mitochondria, *e.g.* proteomics, immuno-blotting or enzymatic measurements are usually normalized to equal amounts of mitochondrial protein, which can be determined in a fast, inexpensive and readily feasible fashion. This normalization is based on the assumption that the mitochondrial protein content is invariable, independent of the organelles origin and the metabolic situation. However, as mitochondria are very dynamic organelles, revealing massive adaptations in their total number, their morphology and protein composition (Hackenbrock 1966; Hackenbrock 1968; Hackenbrock 1968; Hostetler, Zenner et al. 1976; Morton, Cunningham et al. 1976; Ernster and Schatz 1981; Cuezva, Krajewska et al. 2002; Rossignol, Gilkerson et

al. 2004), it is conceivable that they also differ in their overall protein content. Alterations in molecular composition and structure have especially been described in terms of different metabolic situations. Yeast for example, switches from respiro-fermentative to respiratory metabolism simply upon change of the nutritive carbon source (Dejean, Beauvoit et al. 2002). This simulated "diauxic shift" is associated with tremendous mitochondrial adaptations regarding their protein composition and structure structure (DeRisi, Iyer et al. 1997; Zischka, Braun et al. 2006). Similar to these findings, marked differences have been described for the molecular composition of brain mitochondria compared to rat liver mitochondria (Veltri, Espiritu et al. 1990; Vijayasarathy, Biunno et al. 1998; Mootha, Bunkenborg et al. 2003). Brain tissue relies on glucose as the major metabolite (Löffler 1990), whereas liver, especially in the postresorption phase, relies on fatty acids. As cultured cells are typically grown in media that favour glycolysis, their mitochondria might be fundamentally different from mitochondria of the respective tissue. Similar differences might occur between cancer mitochondria and the mitochondria from the respective healthy tissue. Evidently, a pronounced difference in the overall protein content of mitochondria from different sources would challenge the validity of potential proteinaceous targets/differences identified by comparisons solely based on equal mitochondrial protein amount. Over- and underestimations of the true amount of such proteins per mitochondrion would result. Moreover, discrepancies in the effectiveness of mitochondrially targeted drugs in cultured cells and *in vivo* testing may arise simply from the fact that the metabolic situation, and consequently the net amount of the proteins to be targeted, differs between cells in culture media and solid tissues.

4. Design of the study

4.1. The isolation of mitochondria from cell culture and small amounts of tissue

Mitochondrial isolation was carried out in two steps: (I) the homogenization of the sample with the new developed “pump controlled cell rupture system” (PCC) and (II) the separation of the released mitochondria by differential centrifugation. The further purification was achieved by density gradient centrifugation or zone electrophoresis in a free flow electrophoresis device (ZE-FFE).

4.1.1. The homogenization by the pump controlled cell rupture system

For the homogenization, we employed the “Balch homogenizer”, an accurately carved metal apparatus that was designed for high precision cell breakage (Balch and Rothman 1985) more than 30 years ago. It has been successfully used for the homogenization of different cell types (Balch and Rothman 1985; German and Howe 2009; Barysch, Jahn et al. 2010), but scarcely in the context of mitochondrial isolations (Bhaskaran, Butler et al. 2011). In principle, the homogenization occurs by pressing the sample through a clearance within the Balch homogenizer (Figure 5). The clearance can be adjusted in steps of 2 µm by spherical tungsten carbide balls of precisely defined diameters. In order to further increase the stability of the shear forces, we coupled this Balch homogenizer to a high precision pump. This enables a constant and operator independent flow rate, thus increasing the reproducibility of the homogenization. The homogenization parameters used for the tested cell lines and tissues are shown in table 1 and 2.

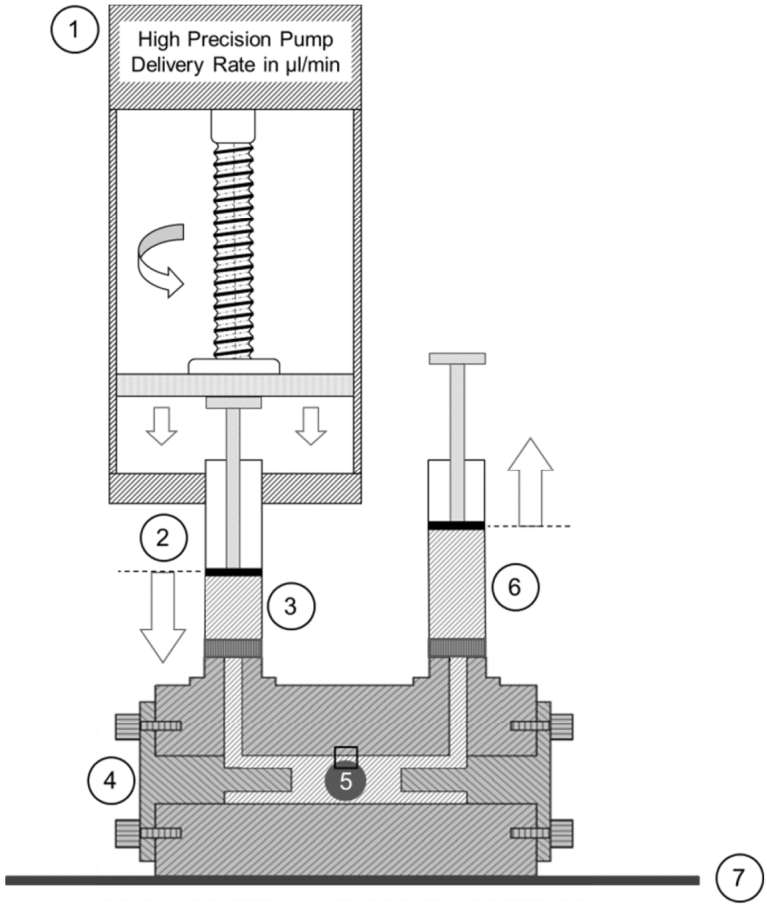

Figure 5 **Scheme of the pump controlled cell rupture system (PCC).** A high precision pump (1) ensures, via gastight syringes (2), the continuous sample delivery (3) in a constant rate to the "Balch-homogenizer" (4). Cell breakage occurs upon passage through a defined clearance (square), which is adjusted by selecting tungsten carbide balls of different diameters (5). The cell homogenate is collected in a second syringe (6) and can be re-subjected to the homogenizer, which is thermally equilibrated by a cooling plate (7). Reproduced with kind permission from: Schmitt S, Saathoff F, Meissner L, Schropp EM, Lichtmannegger J, Schulz S, Eberhagen C, Borchard S, Aichler M, Adamski J, Plesnila N, Rothenfusser S, Kroemer G, Zischka H. (2013) A semi-automated method for the isolating functionally active mitochondria from cultured cells and tissue biopsies. Anal Biochem 443:66-74

Table 1 **Preprocessing and optimized parameters for the homogenization of tissues tested in the course of this thesis.**

	Preprocessing	Input	Flow rate (µl/min)	Number of strokes	Clearance (µm)
Rat liver	Minced	30-40 mg	700	1	18
Rat kidney					
Mouse cortex	Strained through a 100 µm mesh size sieve	20-30 mg		3	10
Mouse striatum					
Mouse hippocampus					

Table 2 **Optimized parameters for the homogenization of the different cell types tested in the course of this thesis.**

	Input (cells/ml)	Flow rate (µl/min)	Number of strokes	Clearance (µm)
Primary hepatocytes	$3x10^6$	700	3	10
McA 7777	$5x10^6$		3	10
H4IIE	$7x10^6$		3	6
Fao	$5x10^6$		3	6
HepG2	$5x10^6$		6	6
Huh6	$5x10^6$		3	10
HepT1	$5x10^6$		3	6
HEK 293	$5x10^6$		6	6
HeLa	$5x10^6$		6	6
BeWo	$5x10^6$		3	10
1205Lu	$5x10^6$		3	10
Panc02	$5x10^6$		6	6
MEF	$5x10^6$		6	6

4.1.2. The purification of the PCC isolated mitochondria

Applying differential centrifugation, the homogenate was separated into a nuclear, crude mitochondrial and cytosolic fraction. For further purification, the crude mitochondrial fraction was either subjected to a discontinuous Nycodenz® density gradient or to zone electrophoresis in a free flow electrophoresis device (ZE-FFE). In ZE-FFE, particles are not separated upon differences in density but upon differences in their surface charge to size ratio (Hannig 1978; Krivánková 1998).

4.2. Quantification of the isolated mitochondria by flow cytometry

For the normalization of mitochondrial comparisons on an equal number, mitochondria were quantified by flow cytometry (Figure 6). Mitochondria are similar in their light scattering properties to the most common contaminations, *i.e.* lysosomes and peroxisomes and even to the background signal. Therefore they were specifically stained with 10N-nonyl acridine orange (NAO), a fluorescent dye that binds with high affinity to cardiolipin (Petit, Maftah et al. 1992), a phospholipid that is predominantly located in the mitochondrial inner membrane. Suspensions of isolated and stained mitochondria with known protein

concentration were mixed with two internal standards and subjected to a flow cytometer. Two internal standards, *i.e.* TruCOUNT™ beads (BD Biosciences, USA) and Fluoresbrite® microspheres (diameter 0.94 µm, Polysciences Europe GmbH, Germany) were necessary to determine the measured volume. For the TruCOUNT™ beads the number is precisely pre-determined by the manufacturer, and thus, solutions with known TruCOUNT™bead concentrations can be generated. The large difference in size as well as in optical density of TruCOUNT™ beads and mitochondria prevented to record both with the same gain settings in sideward scatter (SSC-A) in the LSRII flow cytometer (BD Biosciences, USA). Sideward scatter as a trigger signal is necessary to clearly separate mitochondria from other particles and intrinsic instrumental noise signals. Therefore a second internal standard for volume determination had to be introduced, the smaller Fluoresbrite® beads. While a higher sensitivity setting in SSC-A quantitatively detected the NAO-stained mitochondria and the Fluoresbrite® beads, a lower sensitivity setting did so for the TruCOUNT™ and the Fluoresbrite® beads. Thus, two consecutive measurements from the same sample were required. In the first measurement, the concentration of Fluoresbrite® microspheres was calculated from the analyzed volume determined by the number of determined TruCOUNT™beads. In the second measurement, the concentration of NAO-stained mitochondria was calculated from the analyzed volume determined by the number of determined Fluoresbrite® microspheres. The absolute number of mitochondria per mg protein was subsequently calculated from the concentration of NAO-stained mitochondria and the determined protein concentration of this mitochondrial suspension. In control experiments the NAO staining efficiency and specificity for intact mitochondria was verified by measuring either unstained mitochondria or stained mitochondria before and after destruction by sonication. As a further control NAO stained lysosomes were analyzed.

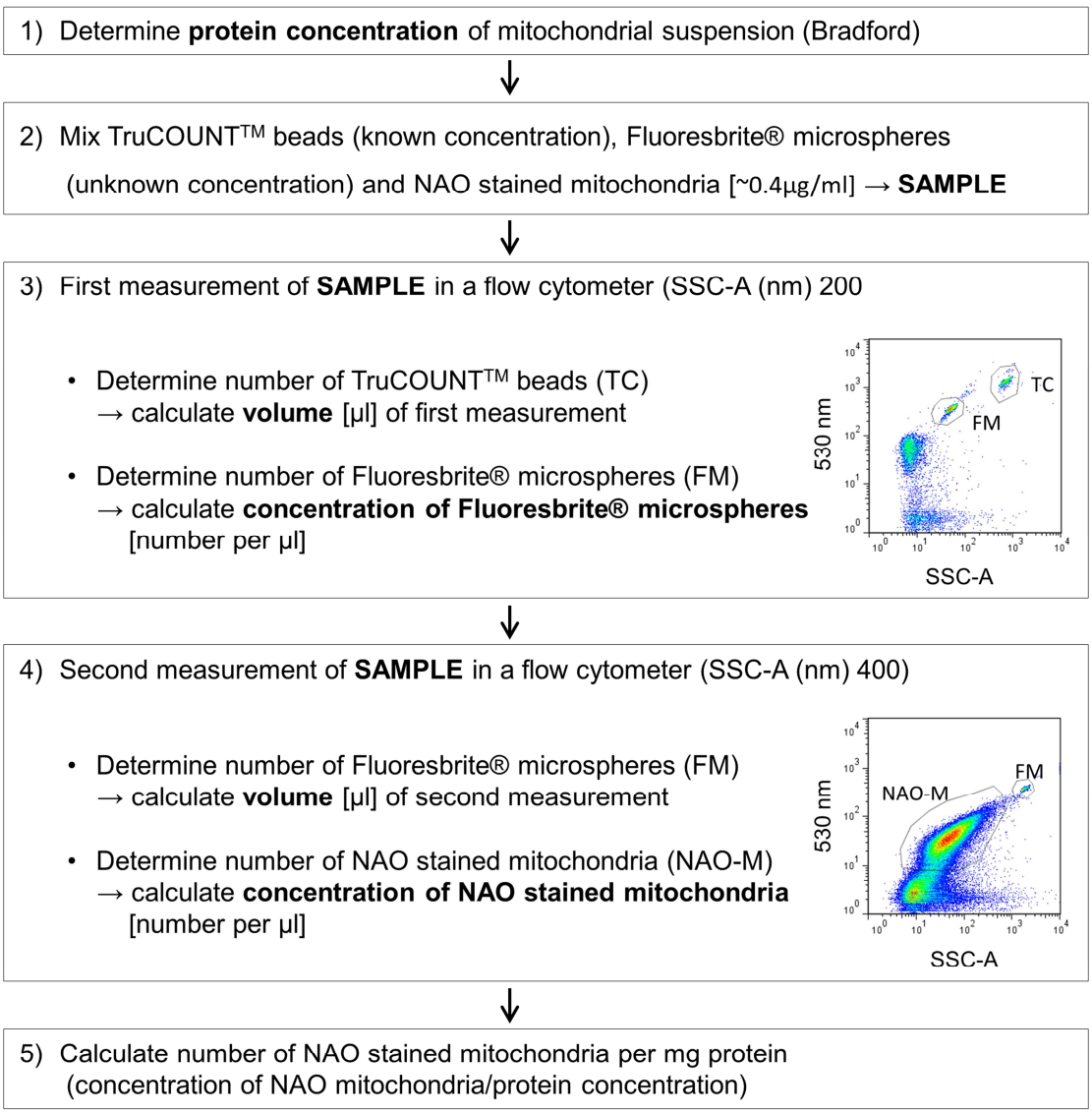

Figure 6 **Schematic flowchart for the quantification of mitochondria by flow cytometry.** Reproduced with kind permission from: Schmitt S, Schulz S, Schropp EM, Eberhagen C, Smmons A, Beisker W, Aichler M and Zischka H. (2014) Why to compare absolute numbers of mitochondria. Mitochondrion 19 Pt A:113-23

4.3. Biochemical verification of the integrity and purity of the isolated mitochondria

4.3.1. The mitochondrial membrane potential

The integrity of the mitochondrial inner membrane and the functionality of the respiratory chain were investigated by the determination of the mitochondrial membrane potential

using the fluorescent dye Rhodamine 123 (Rh123) (Emaus, Grunwald et al. 1986; Zamzami, Metivier et al. 2000; Baracca, Sgarbi et al. 2003). Isolated mitochondria were energized by succinate, the substrate for complex II of the respiratory chain. Rotenone, an inhibitor of complex I was added to prevent the reflux of electrons from CII to CI. Depending on the integrity of the inner membrane and the functionality of the electron transport chain, this leads to an electrochemical potential across the mitochondrial inner membrane. Therefore, the lipophilic, positively charged dye Rh123 accumulates in the matrix, leading to a self-quenching and thus a low fluorescence. Upon a dissipation of the membrane potential, *e.g.* by adding the protonophore FCCP, Rh123 is equally distributed leading to an increase in fluorescence.

4.3.2. Calcium induced mitochondrial swelling

Mitochondrial swelling was monitored by the optical density of the isolated mitochondria (Zischka, Larochette et al. 2008; Zischka, Lichtmannegger et al. 2011). The optical density of a mitochondrial suspension is dependent on the light scattering properties of the organelles. Prompted by the MPT, the volume of the matrix increases and the mitochondrial inner membrane unfolds, leading to a rupture of the outer membrane. Due to this "mitochondrial swelling", the light scattering properties of the mitochondria change and a lower optical density is observed (Nicholls 1992). Calcium, a classical inducer of the MPT (Hunter, Haworth et al. 1976), was used as a control.

4.3.3. The respiratory control ratio

The oxygen consumption of the isolated mitochondria was determined employing a Clark-type oxygen electrode (Oxygraph, Hansatech Instruments UK) (Fleischer 1979). Succinate was used as a substrate and the respiratory control ratio was calculated by the oxygen consumption of mitochondria in the presence of rotenone, succinate and ADP to the oxygen consumption after addition of oligomycin. Oligomycin is an inhibitor of the ATP synthase (Penefsky 1985). Thus, the proton gradient is not used and the respiratory chain slows down, leading to a decrease in oxygen consumption. Any damage on the inner membrane that allows for the flux of protons causes a dissipation of the membrane potential, an increased

activity of the respiratory chain, followed by an increase in oxygen consumption. If this occurs, no difference in oxygen consumption between state III (rotenone, succinate and ADP) and state IV (rotenone, succinate, ADP and oligomycin) can be detected.

4.3.4. Electron microscopy

Electron microscopy of the isolated mitochondria was done as previously described (Zischka, Larochette et al. 2008). Briefly, samples were fixed in 2.5 % glutaraldehyde, postfixation and prestaining was done with osmium tetroxide. After dehydration with ethanol and propylene oxide, samples were embedded in Epon. Ultrathin sections were stained with uranylacetate and lead citrate and examined with an EM 10 CR transmission electron microscope (Zeiss, Germany).

4.3.5. Miscellaneous

Protein concentrations were determined by the Bradford assay (Bradford 1976). Immunoblotting analysis was performed according to Laemmli (Laemmli 1970). 10 µg protein were subjected to SDS-PAGE and separated proteins were transferred onto a PVDF membrane (Towbin, Staehelin et al. 1979). Mitochondrial citrate synthase (Saggerson and Carpenter 1986; Williams, Coakley et al. 1998) and complex II activity (Kiebish, Han et al. 2008) were measured as previously described, respectively.

5. Results

5.1. Summary of Publication 1

A semi-automated method for isolating functionally intact mitochondria from cultured cells and tissue biopsies

Sabine Schmitt a,1, Friederike Saathoff b,1, Lilja Meissner c, Eva-Maria Schropp a, Josef Lichtmannegger a, Sabine Schulz a, Carola Eberhagen a, Sabine Borchard a, Michaela Aichler d, Jerzy Adamski e, Nikolaus Plesnila c, Simon Rothenfusser b, Guido Kroemer f,g,h,i,j, Hans Zischka a

a Institute of Molecular Toxicology and Pharmacology, Helmholtz Center Munich, German Research Center for Environmental Health, D-85764 Neuherberg, Germany
b Division of Clinical Pharmacology, Department of Medicine IV, Ludwig Maximilian University Munich, D-80336 Munich, Germany
c Institute for Stroke and Dementia Research, University of Munich, Medical Center–Campus Grosshadern, D-81377 Munich, Germany
d Research Unit Analytical Pathology–Institute of Pathology, Helmholtz Center Munich, German Research Center for Environmental Health, D-85764 Neuherberg, Germany
e Genome Analysis Center–Institute of Experimental Genetics, Helmholtz Center Munich, German Research Center for Environmental Health, D-85764 Neuherberg, Germany
f INSERM, U848, 94805 Villejuif, France
g Université Paris Descartes/Paris V, Sorbonne Paris Cité, 75270 Paris, France
h Metabolomics Platform, Institut Gustave Roussy, 94805 Villejuif, France
i Equipe 11 labellisée Ligue contre le Cancer, Centre de Recherche des Cordeliers, 75006 Paris, France
j Pôle de Biologie, Hôpital Européen Georges Pompidou, AP–HP, 75908 Paris, France

1 both authors share first authorship

Published in

Analytical Biochemistry (2013) 443:66-74

Analyses of isolated mitochondria are widely used to investigate their role in disease progression, to develop new therapies and to test the potential toxicity of drugs. A prerequisite for such analyses is a high and comparable quality of the isolated mitochondria. Of the two main steps required for isolating mitochondria, *i.e.* the homogenization of the sample and the purification of the thereby released mitochondria, it is the initial homogenization that makes or breaks the isolation of functionally active and pure mitochondria. The homogenization is supposed to efficiently break the plasma membrane without damaging the mitochondrial membranes. For rat liver, a very soft and homogenous tissue, this can be accomplished by very few strokes with a Teflon/glass potter. Furthermore, the input amounts to several grams of wet weight, wherefore an optimization of the efficiency of the homogenization is not required. Together this allows for a mild homogenization and subsequent purification steps yielding in functionally and structurally intact mitochondria with a high purity. Therefore, “standard” isolated rat liver mitochondria, *i.e.* homogenization with a Teflon/glass potter and purification by differential and density gradient centrifugation, are regarded as the “gold standard” in mitochondrial research. However, especially if the starting material is limited it is important to precisely adjust and control this step in order to increase the yield without impairing mitochondrial function.

Classical homogenization tools such as Teflon/glass or Glass/glass potter, nitrogen cavitation, detergents or enzymatic digestion do not allow for such a precise adjustment and/or controllability. Furthermore, these methods are operator dependent, thereby increasing inter-experimental variability.

This article describes a new method to homogenize cell suspensions or small amounts of tissue in a highly controllable and reproducible manner. The homogenization is performed with a unit called “pump controlled cell ruptures system” (PCC). PCC consists of the “Balch homogenizer”, an accurately carved metal apparatus designed for high precision cell breakage and a high precision pump. The sample is pumped through a clearance within the “Balch homogenizer”. As the size of the clearance and the flow rate can be accurately adjusted, the shear forces which occur during the homogenization can be tightly controlled. In addition, this method is operator independent, thereby decreasing inter-experimental variability.

The applicability of this new method was tested with different types of cultured cells, primary hepatocytes as well as different types of fresh and frozen stored tissues. Subsequent to the homogenization by PCC, the respective homogenate was separated by differential centrifugation into a nuclear, crude mitochondrial, and cytosolic fraction. The quality of the obtained mitochondria was verified and compared to the “gold standard”, *i.e.* “standard” isolated rat liver mitochondria.

Only five million cells or 20-40 mg of tissue were sufficient to yield mitochondria for further analyses, indicating a very efficient cell breakage. This was supported by immuno-blot analysis, as the signals for mitochondrial proteins were most prominent in the crude mitochondrial fraction, partially visible in the nuclear fraction and completely absent in the cytosolic fraction. The lack of mitochondrial proteins, especially proteins of the intermembrane space, in the cytosolic fraction indicates a structural integrity of the mitochondrial outer membrane, which was substantiated by electron microscopy. The isolated mitochondria revealed a stable optical density. This points to an intact inner membrane, as a permeabilization of this membrane would lead to mitochondrial swelling and therefore a decrease in optical density. A further evidence for the structural and functional integrity of the PCC isolated mitochondria was their ability to build up a time stable, (*i.e.* > 30 min) membrane potential and the coupling of the respiration to the production of ATP, as indicated by the respiratory control ratio.

Immuno-blot analysis and electron micrographs of the isolated mitochondria indicated a fairly high purity, with only slight contaminations of lysosomes, peroxisomes and endoplasmic reticulum. For further purification, the crude mitochondrial fraction was subjected to a Nycodenz® density gradient or zone-electrophoresis in a free flow electrophoresis device (ZE-FFE). This led to an enrichment of mitochondrial proteins and a reduction of peroxisomal, ER, and especially lysosomal proteins. However, both purification methods caused a marked overall loss of mitochondria, thus requiring further optimization.

PCC was also applicable to tissues stored frozen. The isolated mitochondria from rat liver or kidney tissue were fairly pure and displayed an apparently intact outer membrane, as demonstrated by electron microscopy and immunblot analysis.

Interestingly, when PCC isolated mitochondria from primary rat hepatocytes and rat hepatoma cells were compared massive structural alterations between the different types of mitochondria were observed. Mitochondria from rat liver or rat primary hepatocytes displayed a condensed matrix that occupies nearly the whole mitochondrial volume and triangle shaped defined cristae. In contrast, the cristae of the mitochondria from rat hepatoma cells were widened and unstructured and the matrix seemed to be reduced in quantity. The mitochondria from rat primary hepatocytes and rat hepatoma cells were isolated with the same clearance, number of strokes and flow rate. This implicates that the morphological differences are not due to the isolation but might refer to differences in the molecular composition of the respective mitochondria.

In summary, the controllability, adjustability and reproducibility are key advantages of PCC over the existing homogenization methods. Thus, PCC enables the isolation of functionally and structurally intact mitochondria from cell suspensions and small amounts of tissue, comparable in quality to the “goldstandard”, *i.e.* standard isolated rat liver mitochondria.

5.2. Summary of Publication 2

Isolation of mitochondria from cultured cells and liver tissue biopsies for molecular and biochemical analyses

Sabine Schmitt a, Carola Eberhagen a, Susanne Weber b
Michaela Aichler c, Hans Zischka a

a Institute of Molecular Toxicology and Pharmacology, Helmholtz Center Munich, German Research Center for Environmental Health, D-85764 Neuherberg, Germany

b Institute of Experimental Genetics, Helmholtz Center Munich, German Research Center for Environmental Health, D-85764 Neuherberg, Germany

c Research Unit Analytical Pathology–Institute of Pathology, Helmholtz Center Munich, German Research Center for Environmental Health, D-85764 Neuherberg, Germany

Published in

Methods in Molecular Biology (2015) 1295:87-97

This article provides a detailed hands-on protocol of the recently reported semi-automated method for the isolation of mitochondria from cell culture and liver tissue biopsies. In addition it includes several notes on the critical steps in the isolation process and a refined protocol for the purification of the isolated mitochondria. The homogenization of the respective samples was implemented by the “pump controlled cell rupture system” (PCC). Two steps of differential centrifugation were applied to receive a nuclear, a cytosolic and a crude mitochondrial fraction. This mitochondrial fraction already revealed a fairly high purity, sufficient for functional analyses. However, molecular analyses, such as proteomics or lipidomics require higher mitochondrial purities. Therefore, we refined the purification of the isolated mitochondria by subjecting them to a discontinuous Nycodenz® density gradient. Typical contaminations of isolated mitochondrial fractions are lysosomes, endoplasmic reticulum (ER) and peroxisomes, which have a lower or slightly higher density. Therefore we applied a two-step Nycodenz® density gradient to enrich the mitochondria at the phase boundary whereas cytosolic proteins, lysosomes and ER are expected to stay on, or in the upper phase and nuclei and peroxisomes to stay in, or pass the lower phase. As already indicated by electron micrographs, mitochondria of different origin seem to differ in their molecular composition. Indeed, we found the mitochondria from the rat hepatoma cell line McA 7777 to behave differently from rat liver mitochondria. Subjected to a density gradient with a 24% and an 18% Nycodenz layer, the hepatoma mitochondria gathered at the phase boundary whereas the liver mitochondria passed the lower layer and pelleted at

the bottom of the tube. We therefore used a 33%/18% gradient for the liver mitochondria to enrich them at the phase boundary. Applying this purification step we found an increase in mitochondrial proteins, a marked decrease in the lysosomal protein Lamp2 and the nuclear protein Histone 3 and a clearly visible reduction of the peroxisomal protein PMP70 and the ER protein Bip as demonstrated by immunblot analysis. The very high proportion of mitochondria was also demonstrated by electron microscopy.

5.3. Summary of Publication 3

Why to compare absolute numbers of mitochondria

Sabine Schmitt a, Sabine Schulz a, Eva-Maria Schropp a, Carola Eberhagen a,
Alisha Simmons a, Wolfgang Beisker a, Michaela Aichler b, Hans Zischka a

a Institute of Molecular Toxicology and Pharmacology, Helmholtz Center Munich, German Research Center for Environmental Health, D-85764 Neuherberg, Germany
b Research Unit Analytical Pathology–Institute of Pathology, Helmholtz Center Munich, German Research Center for Environmental Health, D-85764 Neuherberg, Germany

Published in

Mitochondrion (2014) 19 Pt A:113-123

Electron micrographs reveal massive differences in mitochondrial morphology, depending on their origin and metabolic situation. This is especially apparent when isolated mitochondria are compared. Isolated rat liver mitochondria, for example, have an intensely stained matrix that fills most of the organelle and their inner membrane forms defined and triangle shaped cristae. In contrast, mitochondria from the rat hepatoma cell line McA 7777, grown in a medium that promotes glycolysis, reveal widened and undefined cristae. Furthermore, the matrix of these mitochondria, even though intensly stained, seems to be reduced in quantity. If the same cell line is grown in a medium that forces the cell towards oxidative phosphorylation, the mitochondria reveal a more liver mitochondria like structure, but with a relatively pale matrix. Proteins are typically stained by the contrast agents used for the electron micrographs. The lower appearance or intensity of stained mitochondrial substructures in the McA 7777 mitochondria compared to the rat liver mitochondria indicated a substantial depletion of their protein content. This was supported by their different behaviour upon density gradient centrifugation. If subjected to a two-step Nycodenz® density gradient of 24% and 18%, the McA 7777 mitochondria gathered at the phase boundary, whereas the rat liver mitochondria passed the 24% phase. This indicates that the McA 7777 mitochondria have a lower density which could be explained by a lower proportion of proteins. Similar differences are apparent between rat liver and rat brain mitochondria, with brain mitochondria revealing a decreased quantity of matrix and a lower density.

In order to verify the differences in protein content we established a new method to determine the number and thereafter the protein content of isolated mitochondria by flow

cytometry. As mitochondria cannot be distinguished from background signals by their light scattering properties they were stained with 10N-nonyl acridine orange (NAO). NAO is a fluorescent dye that binds with high affinity to cardiolipin, a phospholipid predominantly located in the mitochondrial inner membrane. The fluorescently labelled mitochondria were counted by flow cytometry and the measured volume was determined by an internal standard. Subsequently, the number of mitochondria per ml and the determined protein concentration of the mitochondrial suspension were used to calculate the number of mitochondria per mg protein and therefrom the mitochondrial protein content.

The new quantification method was applied to rat liver, kidney, heart and brain mitochondria and to mitochondria from the two rat hepatocellular carcinoma cell lines McA 7777 and H4IIE. For rat liver mitochondria the determined protein content was in accordance to earlier reports, using alternative methods. Similar protein contents were determined for rat heart and kidney mitochondria. In contrast, however, lower protein contents were determined for rat brain mitochondria and for mitochondria from the rat hepatocellular carcinoma cell lines. In order to verify the differences between rat liver and the McA 7777 hepatoma mitochondria, the abundance and activity of the mitochondrial citrate synthase was determined. Both, the abundance and the activity of this house keeping enzyme was lower in the McA 7777 mitochondria compared to rat liver mitochondria if normalized to same amounts of protein but similar if normalized to an equal number of mitochondria. These results substantiate the differences in protein content and challenge a comparison of these mitochondria based on equal protein amounts, the typical normalization method. Moreover, the activity and susceptibility towards inhibition of complex II of rat liver and McA 7777 mitochondria was compared and significant discrepancies were obtained by either normalizing to an equal protein amount or to absolute mitochondrial number. Importantly, the latter normalization, in contrast to the former, demonstrated a lower complex II activity and higher susceptibility towards inhibition in McA 7777 mitochondria compared to liver mitochondria. These findings demonstrate that solely normalizing to protein amount may obscure essential molecular differences between mitochondrial populations.

6. Discussion

6.1. Analyses of isolated mitochondria: a piece in the puzzle to understand and combat mitochondrial disorders

Mitochondrial functions are essential for cellular homeostasis (Alirol and Martinou 2006; Zorov, Isaev et al. 2007), wherefore mitochondrial dysfunction can cause severe diseases. Interestingly, despite the variety of triggers, the consequences, which finally lead to the demise of the cell are very consistent (Smith, Hartley et al. 2012). These are the mitochondrial permeability transition (MPT) and/or the mitochondrial outer membrane permeabilization (MOMP) that in turn are mostly elicited by dysfunctions in ATP synthesis, oxidative stress and calcium dyshomeostasis. This might offer the possibility to combat a range of primary and secondary mitochondrial disorders with only few types of pharmaceuticals that interfere with one of these major mitochondrial dysfunctions. In order to find such pharmaceuticals, a better understanding of these processes is needed. Investigations of isolated mitochondria are indispensable as to understand the processes within the organelle. The MPT for example can be elicited by several different agents that share no common feature (Petronilli, Cola et al. 1993), questioning a single mechanism. Furthermore, it is unclear whether the induction of the MPT by different stimuli causes different stages of mitochondrial destruction, thereby leading to different cell death scenarios, like apoptosis or necrosis (Green and Kroemer 2004; Bernardi, Krauskopf et al. 2006; Grimm and Brdiczka 2007; Kroemer, Galluzzi et al. 2007; Tsujimoto and Shimizu 2007; Rust, Wild et al. 2009). Glycochenodeoxycholic acid (GCDCA) and taurochenodeoxycholic acid (TCDCA) are the predominant cell toxic bile salts that accumulate in cholestatic patients (Rust, Bauchmuller et al. 2005; Rust, Wild et al. 2009; Hohenester, Gates et al. 2010) and have been reported to impair the inner mitochondrial membrane potential (Rolo, Palmeira et al. 2004) and to induce the MPT (Botla, Spivey et al. 1995; Gores, Miyoshi et al. 1998; Rolo, Palmeira et al. 2004). Using isolated rat liver mitochondria, Schulz et al. demonstrated that this mitochondrio-toxicity is bile salt species-, dose-, and time-dependent. Low doses and short exposure caused structural changes with only partial loss of cytochrome C but no impairment of the mitochondrial membrane potential (MMP). In contrast, higher doses, longer exposure and co-treatment with low doses of calcium led to MMP-loss and elicited the MPT, causing massive mitochondrial damage. We therefore hypothesized that upon low

doses of bile salts hepatocytes undergo apoptosis due to limited mitochondrial impairment, whereas long-term or massive bile salt stress causes irreversible mitochondrial destruction, leading to necrosis (Schulz, Schmitt et al. 2013). This is in line with the clinically observed switch from apoptosis to necrosis in consequence to elevated doses of bile salts (Patel, Bronk et al. 1994; Palmeira and Rolo 2004; Rolo, Palmeira et al. 2004). Furthermore, unlike the calcium elicited MPT, the bile salt induced MPT happened to be rather independent of the mitochondrial membrane potential (MMP) and could not be prevented by Cyclosporin A (CsA), indicating a different mechanism (Schulz, Schmitt et al. 2013).

Beside the investigation of the final stages of mitochondrial dysfunctions, analyses of isolated mitochondria from normal and pathological situations are a valuable tool to investigate the changes in mitochondrial function and molecular composition that occur at disease onset as well as their role in disease progression (Zischka, Lichtmannegger et al. 2011).

With respect to cancer cells, analyses of isolated mitochondria are of special interest. Among the hallmarks of cancer cells are their ability to evade apoptosis as well as alterations in their bioenergetics (Warburg 1926) and biosynthesis (Hanahan and Weinberg 2000; Vander Heiden, Cantley et al. 2009; Hanahan and Weinberg 2011). As the mitochondrion is the major organelle implicated in these processes (Pathania, Millard et al. 2009), investigation of the molecular and functional differences between normal and cancer mitochondria may be useful to develop new mitochondria targeted anticancer strategies.

Certainly, solely investigations of isolated mitochondria are limited, as they obscure cellular differences in drug uptake and metabolism as well as in the overall mitochondrial content. However, analyses of isolated mitochondria are indispensable to gain insights into the molecular and functional mechanisms that are in involved in mitochondrial dysfunction as well as in mitochondrial sensitivity against toxic or pharmacological agents.

For such analyses, a highly reproducible and comparable quality of the isolated mitochondria in terms of functionality, structural integrity and purity is of major importance.

6.2. Strength and applicability of the new isolation method

6.2.1. The "pump controlled cell rupture system" (PCC) enables the isolation of functionally and structurally intact mitochondria from cell suspensions

The isolation of mitochondria is well established for several types of rat and mouse tissue, such as liver, kidney and heart. Although in vivo models resemble the pathologic situation best, in a first approach cell culture models have several advantages. First of all it is more feasible to investigate the impact of specific changes in substrate availability on mitochondrial function. It is also less complicate to genetically modify cells, *e.g.* specifically "knockdown", "knockout" or "upregulate" specific proteins in cultured cells compared to animal models. Furthermore, cell culture allows for the parallel treatment of different concentrations and combinations of various drugs. However, so far, there are no generally applicable protocols for the isolation of mitochondria from cell suspensions, as the existing protocols are largely based on try and error adaptations to the specific cell type of interest. In order to liberate the mitochondria from the cell, the plasma membrane needs to be ruptured. This step, referred to as the homogenization of the sample is rather critical as it carries the risk of damaging the mitochondrial membranes. As the starting amount is much lower using cultured cells instead of whole organs this step needs to be more precisely controlled and adjusted to allow for an efficient destruction of the cell membrane without impairing mitochondrial structure. Classical homogenization methods, however, either lack the controllability and/or adjustability.

Therefore, this study intended to develop a new method for the homogenization of cell suspensions. Using the "pump controlled cell rupture system" (PCC), cells are pumped with a precise flow rate through a defined clearance. In order to separate the thereby liberated mitochondria from other cell compartments, two steps of differential centrifugation were performed. In a first centrifugation (800xg, 5 min) mitochondria were separated from unbroken cells, cell debris and nuclei. In a second centrifugation (9000xg, 10 min), mitochondria were pelleted, thereby separating them from cytosolic proteins. Only five million cells were sufficient to yield mitochondria for further analyses, indicating a very efficient cell breakage. This was supported by immuno-blot analysis, as the signals for mitochondrial proteins were most prominent in the crude mitochondrial fraction, partially visible in the nuclear fraction and completely absent in the cytosolic fraction. The lack of mitochondrial proteins, especially proteins of the intermembrane space, in the cytosolic

fraction indicate a structural integrity of the mitochondrial outer membrane. This was substantiated by electron microscopy. Furthermore, the isolated mitochondria revealed a stable optical density for at least 60 min. This points to an intact inner membrane, as a permeabilization of this membrane would lead to mitochondrial swelling and therefore a decrease in optical density (Nicholls 1992), as observed by adding 100 µM calcium, a classical inducer of mitochondrial swelling (Yang and Cortopassi 1998). The structural integrity of the inner membrane as well as the functionality of the isolated mitochondria was verified by their ability to build up a membrane potential. Therefore, the isolated mitochondria were energized with succinate and the membrane potential was monitored using the positively charged fluorescent dye Rh123 (Zamzami, Metivier et al. 2000). Using FCCP as an internal control, we observed a mitochondrial membrane potential that remained stable for at least 60 min. The functionality of the isolated mitochondria was substantiated by the coupling of the activity of the respiratory chain to the activity of complex V. Taken together, PCC enables the isolation of structurally and functionally intact mitochondria from cell suspensions in a highly reproducible manner. Furthermore, as few as five million cells were sufficient to yield mitochondria for downstream analyses and the parameters of PCC (number of strokes and flow rate and clearance) could be readily adapted to a variety of different cell types.

6.2.2. PCC enables the isolation of mitochondria from tissue biopsies

PCC was also applicable to small amounts of rat liver and kidney tissues as well as from different mouse brain areas. Using a similar protocol as for cultured cells, only 20 – 40 mg of tissue was sufficient to yield mitochondria for further analyses. The structural integrity of the outer membrane of the hereby obtained mitochondria was verified by electron microscopy and immuno-blotting. Furthermore, they were functionally intact, indicated by a time-stable membrane potential. The efficient isolation of mitochondria from small amounts of tissue could be of high interest with respect to the investigation of mitochondria from tissue biopsies. Frequently, biopsies cannot be processed freshly but are stored frozen. Therefore, small amounts of rat liver and kidney were stored frozen, thawed and subjected to PCC. Using 100-150 mg of rat liver and kidney tissue, PCC enabled the isolation of fairly pure and intact mitochondria. Interestingly, in contrast to isolations from fresh tissues, the isolated mitochondria featured the ‘‘orthodox state’’ (Hackenbrock 1966) lacking the typical cristae

structure. This demonstrates the structure-altering effect on mitochondria due to storage. Still, the isolated mitochondria presented a fully surrounding, apparently uninterrupted outer membrane. This was confirmed by immuno-blot analysis, as mitochondrial proteins were enriched in the crude mitochondrial fraction, but nearly absent in the cytosolic fraction. Obviously, mitochondria isolated from tissues stored frozen may not be used for functional studies because storage/thawing will affect their functional integrity. However, such samples may still be useful, for example, to test for the specific presence of selected biomarkers.

6.2.3. Purification of the PCC isolated mitochondria for molecular analyses

Immuno-blot analyses and electron micrographs of the PCC isolated mitochondria from cultured cells as well as from fresh and frozen stored tissues indicated a fairly high purity, with some contaminations of lysosomes, peroxisomes and endoplasmic reticulum (ER) and only slight contaminations of cytosolic and nuclear proteins. In order to further increase the purity, the crude mitochondrial fraction was subjected to a two-step Nycodenz® density gradient or zone-electrophoresis in a free flow electrophoresis device (ZE-FFE). Application of the density gradient led to an enrichment of mitochondrial proteins and a reduction of nuclear, cytosolic, peroxisomal, ER, and lysosomal proteins. Especially peroxisomes and ER proteins are hard to eliminate from mitochondrial fractions. Peroxisomes as they have a very similar density as mitochondria and ER proteins as mitochondria and the endoplasmic reticulum are connected by so called mitochondria associated membranes (MAM) (Vance 1990). A further reduction of peroxisomal, lysosomal and nuclear contaminations might be possible, using a finely graduated density gradient. However, the two-step density gradient already caused a marked overall loss of mitochondria and more layers would decrease the mitochondrial yield even further. Additionally, a finely graduated density gradient carries the risk to select for a specific type of mitochondria. Nevertheless, subjecting mitochondria to density gradient centrifugation increases their purity substantially, still yielding sufficient mitochondria for molecular analyses and can be readily accomplished. In contrast, ZE-FFE is hardly available in most laboratories, more time-consuming, and higher amounts of sample are required. Similar to density gradient centrifugation, it led to an enrichment of mitochondria and a marked reduction of nuclear, cytosolic and especially lysosomal

contaminations. Purification by ZE-FFE is based on a separation of particles due to their surface charge to size ratio (Hannig 1978; Krivánková 1998). As peroxisomes are very similar to mitochondria in terms of their size and surface charge, they cannot be separated from mitochondria by ZE-FFE (Hannig and Heidrich 1974). However, ZE-FFE offers an unmatched advantage as it allows for the separation of intact mitochondria from mitochondria with a damaged outer membrane (Heidrich 1971; Zischka, Larochette et al. 2008). This is, as the surface charge of the mitochondrial inner membrane is more negative than that of the mitochondrial outer membrane (Heidrich, Stahn et al. 1970; Hackenbrock and Miller 1975). The separation of mitochondrial populations upon differences in the extent of outer membrane damage is a valuable tool to investigate the toxic effect of MPT inducing agents (Zischka, Larochette et al. 2008; Schulz, Schmitt et al. 2013).

6.3. Impact of the quantification of mitochondria

Comparative analyses of isolated mitochondria are typically normalized to equal amounts of protein. This is generally useful as the protein concentration can be readily determined. However, this normalization assumes that mitochondrial protein contents are equal, independent of the cell type or the metabolic or pathologic situation.

Using PCC we found marked differences in the morphology of rat liver (whole tissue and primary hepatocytes) mitochondria and mitochondria from the rat hepatoma cell line McA 7777 that challenge such invariabilities in mitochondrial protein content. Liver mitochondria revealed an intensly stained matrix, occupying most of the mitochondrial space and well defined triangle shaped cristae. In contrast, mitochondria from McA 7777 cells grown in high glucose medium displayed undefined cristae and an intensly stained matrix that seemed to be reduced in quantity, thereby increasing the proportion of the intermembrane space. Mitochondria from the same cell line, but cultivated in medium depleted of glucose and enriched in galactose and glutamine demonstrated a more liver mitochondria like structure with better defined cristae and a less condensed matrix. The latter condition forces the cells towards OxPhos (Marroquin, Hynes et al. 2007), resembling the metabolic situation of hepatocytes (Löffler 1990). It is known that the isolation procedure affects mitochondrial morphology, causing a switch from the orthodox to the condensed form (Hackenbrock 1966; Schnaitman, Erwin et al. 1967). However, mitochondria

from primary hepatocytes and the McA 7777 cells were isolated with exactly the same PCC parameters (3 strokes, 10 µm clearance, 700 µl/min). This indicates that the observed structural alterations are not solely due to the homogenization but can be attributed to differences in the mitochondrial molecular composition. In fact, the mitochondrial molecular composition has been described to vary depending on the cell type or metabolic situation (Criddle, Paltauf et al. 1969; Plattner and Schatz 1969; Plattner, Salpeter et al. 1970; Veltri, Espiritu et al. 1990; Voet Daniel 1992; Vijayasarathy, Biunno et al. 1998). Conceivably, such dramatic morphological changes could correlate with variations in the overall mitochondrial protein content. Indeed, the contrast agents uranyl acetate and lead citrate, used for electron microscopy both stain proteins (Mulisch and Welsch 2010), indicating that the decrease in intensly stained areas could be due to a decrease in protein content. Furthermore, McA 7777 mitochondria revealed a lower density than rat liver mitochondria as observed upon density gradient centrifugation.

In order to quantitatively determine the net protein content of mitochondrial populations, we developed a method to directly quantify the isolated mitochondria by flow cytometry in a fast and reproducible manner. To this end, isolated mitochondria were specifically stained with the fluorescent dye 10N-nonyl acridine orange (NAO) to distinguish them from other cell organelles, *e.g.* lysosomes. The mitochondrial protein content was then calculated from the measured volume and the protein concentration of the subjected mitochondrial suspension. Using this approach we found significantly lower protein contents in rat brain mitochondria and mitochondria from the hepatocellular carcinoma cell line McA 7777 compared to reference rat liver mitochondria. In order to confirm these results, the enzyme activity of the mitochondrial citrate synthase (CS) was determined in rat liver and McA 7777 mitochondria. This “housekeeping” enzyme activity is frequently considered not to be subjected to fluctuations in pathological situations (Pallotti and Lenaz 2007). Thus, CS activity is often used as a surrogate marker for mitochondrial content (Dalziel, Moore et al. 2005; Garrabou, Soriano et al. 2007). Applying the same amount of protein, we observed significantly higher activities for the McA 7777 compared to rat liver mitochondria, strengthening the above notion of a higher number of these hepatoma mitochondria per mg protein compared to reference rat liver mitochondria. Or expressed *vice versa*, McA 7777 mitochondria have a markedly lower protein content compared to rat liver mitochondria. Consequently, comparative analyses of these mitochondria solely based on equal amounts

of protein may arrive at misleading results. Indeed, comparing the activity and sensitivity of complex II from these mitochondria, conflicting results were observed. Whereas a comparable activity was determined upon normalization on equal amounts of mitochondrial protein, the McA 7777 mitochondria revealed a lower activity if an equal number of mitochondria was applied. Furthermore, the sensitivity of CII against an inhibitor of the UbQ binding site of CII, thenoyltrifluoroacetone (TTFA) (Rohlena, Dong et al. 2011), was significantly higher in McA 7777 than in rat liver mitochondria, as became apparent when the comparisons were normalized to mitochondrial number, but not, when normalized to equal protein amounts.

These results indicate that it is appropriate to consider other parameters than protein amount for normalization, if mitochondria from divergent origin or metabolic situations are compared. Citrate synthase activity has frequently been used in this respect. However, differing citrate synthase activities have been reported if isolated mitochondria from variant rat tissues (Saggerson and Carpenter 1986) were compared. A further potential option for mitochondrial quantifications may be based on their mtDNA content. Whereas the ratio of mtDNA to nDNA may be used to estimate the number of mt-genomes per cell (Phillips, Sprouse et al. 2014), it is questionable whether this approach is reasonable for the quantification of isolated mitochondria. In fact, the mtDNA content is highly dynamic, being regulated in a cell-specific manner by mechanisms that are not completely understood (Phillips, Sprouse et al. 2014). In this respect, Veltri et al. reported that the mtDNA content is organ-specific, with heart displaying the lowest mtDNA content expressed per gram mitochondria followed by kidney and liver and brain with the highest mtDNA content (Veltri, Espiritu et al. 1990). Differences in mtDNA content have also been observed for tumor mitochondria, *e.g.* from hepatocellular carcinomas, in comparison to the respective normal tissue (Hsu, Lee et al. 2013). Consequently, mitochondrial comparisons based on equal amounts of mtDNA may also arrive at misleading results. In contrast to enzymatic measurements or amount of mtDNA, determination of the absolute number of mitochondria offers the unmatched advantage to be independent of changes in the mitochondrial molecular composition. As this is especially the case when mitochondria from different origin or metabolic situations are compared, it is reasonable to normalize such comparisons on equal numbers of mitochondria, instead of enzyme activity or equal amounts of mtDNA or protein. The determined differences in mitochondrial protein content are not solely

important with respect to normalizing mitochondrial comparisons. It also raises the question about the causes of these changes. Is the mitochondrial protein content diminished if certain mitochondrial functions, *e.g.* the generation of ATP, are of minor importance for the cell? This could be an explanation for the low protein content of mitochondria from the hepatoma cell lines grown in high glucose medium. However, we could not observe an increase in mitochondrial protein content if the cells were grown in medium that forces them towards oxidative phosphorylation. Another reason for a proteinaceous reduction in mitochondria could be a high proliferation rate. In contrast to hepatocytes, the cultured McA 7777 cells display a high proliferation rate, irrespective of the growth medium. As mitochondria are apportioned to the daughter cells (Valero 2014), they undergo fission processes to increase their number. Cells with an increase in mitochondrial fission have been described to contain smaller mitochondria (Mishra and Chan 2014), possibly leading to decreases in mitochondrial protein content. Although we did not observe any differences in mitochondrial size, comparing rat liver and rat hepatoma mitochondria, it might be possible that the rate of fission and fusion influences the protein content of mitochondria as protein synthesis or degradation are associated with these processes. Fission and fusion occur also in resting cells and are closely related to mitochondrial turnover (Bernhardt, Muller et al. 2015), offering a possible explanation for the decreased protein content in brain mitochondria. Certainly, these hypotheses regarding the regulation of the mitochondrial protein content are highly speculative and demand for intensive studies. It also remains for future studies to investigate the consequences of such alterations. This might be of special interest regarding new mitochondria targeted anticancer therapies. If cancer cell mitochondria contain less protein, they may be more susceptible, *e.g.* towards inhibitors of oxidative phosphorylation, as implied by the comparison of complex II activity and vulnerability of rat liver and hepatoma mitochondria. However, normal cells which also contain mitochondria with reduced protein content, such as brain cells, might be very sensitive to such treatments, as well. Moreover, it is also possible that many mitochondrial proteins which are reduced in amount are less important for cancer cell survival and that especially proteins which are not reduced in amount, are the most promising targets. The methods established in this thesis are valuable tools to investigate such issues, as they enable the isolation, quantification and biochemical characterization of mitochondria from biopsies and cell lines grown under diverse conditions.

7. Conclusion

In the course of this thesis, a new method (PCC) for the isolation of mitochondria from cultured cells and small amounts of tissue has been developed and proven to be of general applicability. The major advantage of this new approach over existing techniques is the opportunity to precisely adjust and control the destructive forces during the initial cell disruption. It is therefore possible to acquire an efficient cell membrane breakage without impairing the mitochondrial membranes, yielding structurally and functionally intact mitochondria. PCC is also applicable for tissues stored frozen and only small amounts of sample are necessary to yield sufficient mitochondria for downstream analyses. Thus it might be useful not only for experimental but also for clinically relevant diagnostic applications. Due to the employment of a high precision pump this operator independent homogenization is highly reproducible and can be readily adapted to other cell lines, as corroborated by recent studies (Foerster, Braig et al. 2014; Tan, Baty et al. 2015; Weber, Salabei et al. 2015).

Comparative analyses of isolated mitochondria from normal and pathologic or different metabolic situations are valuable to gain insights into mitochondrial functions and dysfunctions and to develop new mitochondria targeted strategies. Such comparisons are typically based on equal amounts of mitochondrial protein, assuming an invariability of the mitochondrial protein content. However, as mitochondria can differ markedly in their morphology and molecular composition it is conceivable that they vary also in their overall protein content. In the course of this thesis, a new method was developed for the quantification of isolated mitochondria by flow cytometry. The mitochondrial protein content was then calculated from the measured volume and the protein concentration of the subjected mitochondrial suspension. Using this new approach we found significantly lower protein contents in rat brain mitochondria and mitochondria from the hepatoma cell line McA 7777 compared to reference rat liver mitochondria. Subsequently, rat liver and McA 7777 mitochondria were compared regarding their complex II activity and vulnerability. Only normalization on an equal number of mitochondria demonstrated that the hepatoma mitochondria reveal a lower complex II activity but higher vulnerability. Thus, if mitochondria of different origin or markedly different situations are compared, normalization solely to protein amount may obscure molecular and functional differences.

8. List of publications

First author:

1. **S. Schmitt**, F. Saathoff, L. Meissner, E.M. Schropp, J. Lichtmannegger, S. Schulz, C. Eberhagen, S. Borchard, M. Aichler, J. Adamski, N. Plesnila, S. Rothenfusser, G. Kroemer, and H. Zischka. A semi-automated method for isolating functionally intact mitochondria from cultured cells and tissue biopsies. Anal Biochem (2013) 443:66-74
2. **S. Schmitt**, C. Eberhagen, S. Weber, M. Aichler, and H. Zischka. Isolation of mitochondria from cultured cells and liver tissue biopsies for molecular and biochemical analyses. Methods Mol Biology (2015) 1295:87-97
3. **S. Schmitt**, S. Schulz, E.M. Schropp, C. Eberhagen, A. Simmons, W. Beisker, M. Aichler, and H. Zischka. Why to compare absolute numbers of mitochondria. Mitochondrion (2014) 19 Pt A:113-23.
4. Meeting abstract (poster) at EMBO Workshop MAC ´13, Stockholm Sweden
5. Meeting abstract (poster) at Conference AussieMit ´14, Perth Australia

Co-author:

1. H. Zischka, J. Lichtmannegger, **S. Schmitt**, N. Jägemann, S. Schulz, D. Wartini, L. Jennen, C. Rust, N. Larochette, L. Galluzzi, V. Chajes, N. Bandow, V.S. Gilles, A.A. DiSpirito, I. Esposito, M. Goettlicher, K.H. Summer, and G. Kroemer. Liver mitochondrial membrane crosslinking and destruction in a rat model of Wilson disease. J Clin Invest (2011) 121:1508-18.
2. J. Horstkotte, T. Perisic, M. Schneider, P. Lange, M. Schroeder, C. Kiermayer, R. Hinkel, T. Ziegler, P.K. Mandal, R. David, S. Schulz, **S. Schmitt**, J. Widder, F. Sinowatz, B.F. Becker, J. Bauersachs, M. Naebauer, W.M. Franz, I. Jeremias, M. Brielmeier, H. Zischka, M. Conrad, and C. Kupatt. Mitochondrial thioredoxin reductase is essential for early postischemic myocardial protection. Circulation (2011) 124:2892-902.
3. S. Schulz, **S. Schmitt**, R. Wimmer, M. Aichler, S. Eisenhofer, J. Lichtmannegger, C. Eberhagen, R. Artmann, F. Tookos, A. Walch, D. Krappmann, C. Brenner, C. Rust, and H. Zischka. Progressive stages of mitochondrial destruction caused by cell toxic bile salts. Biochim Biophys Acta (2013) 1828:2121-33.
4. F. Foerster, S. Braig, C. Moser, R. Kubisch, J. Busse, E. Wagner, E. Schmoeckel, D. Mayr, **S. Schmitt**, S. Huettel, H. Zischka, R. Mueller, and A.M. Vollmar. Targeting the actin cytoskeleton: selective antitumor action via trapping PKCε. Cell Death Dis (2014) 5:e1398.
5. S. Schulz, J. Lichtmannegger, **S. Schmitt**, C. Leitzinger, C. Eberhagen, C. Einer, J. Kerth, M.Aichler, and H.Zischka. A protocol fo the parallel isolation of intact mitochondria from rat liver, kidney, heart, and brain. Methods Mol Biology (2015) 1295:75-86

9. Contributions

The present work was directly supervised by PD Dr. Hans Zischka with assistance and guidance of:

Prof. Dr. Jerzy Adamski, Dr. Michaela Aichler, Dr. Wolfgang Beisker, Sabine Borchard, Carola Eberhagen, Prof. Dr. Martin Klingenspor, Prof. Dr. Guido Kroemer, Luise Jennen, Josef Lichtmannegger, Dr. Lilja Meissner, Gabriele Mettenleiter, Prof. Dr. Nikolaus Plesnila, Prof. Dr. Simon Rothenfusser, Dr. Friederike Saathoff, Eva-Maria Schropp, Dr. Sabine Schulz, Alisha Simmons and Susanne Weber.

In detail, their assistance relates to:

Adamski, Jerzy:	Advisory supervision and examiner of the Technical University Munich.
Aichler, Michaela:	Support on electron microscopy.
Beisker, Wolfgang:	Introduction to flow cytometry and support on the setup for quantification of mitochondria by flow cytometry.
Borchard Sabine:	Bachelor student in the AG Zischka. Repetition of mitochondrial isolations from cell culture as well as of the subsequent determination of the mitochondrial membrane potential.
Eberhagen, Carola:	Cell culture and repetition of mitochondrial isolations from cell culture.
Klingenspor, Martin:	Advisory supervision and co-examiner of the Technical University Munich.
Kroemer, Guido:	Advisory supervision in terms of isolating mitochondria from cultured cells and tissue biopsies.
Jennen, Luise:	Support in the processing of electron microscopy.
Lichtmannegger, Josef:	Animal handling and help with the isolation of mitochondria from rat liver. Support on the isolation of primary hepatocytes.

Meissner, Lilja:	Preparation of different mouse brain tissues.
Mettenleiter, Gabriele:	Support on the processing of electron microscopy.
Plesnila, Nikolaus:	Advisory supervision in terms of isolating mitochondria from cultured cells and tissue biopsies.
Rothenfusser, Simon:	Advisory supervision in terms of isolating mitochondria from cultured cells and tissue biopsies.
Saathoff, Friederike:	Experimental and scientific support in terms of isolating mitochondria from cultured cells and tissue biopsies. Preparation (40%) of the manuscript: A semi-automated method for isolating functionally intact mitochondria from cultured cells and tissue biopsies.
Schropp, Eva-Maria:	Bachelor student of the AG Zischka. Repetition of mitochondrial isolations from cell culture as well as of the subsequent determination of the mitochondrial membrane potential. Assistance in the setup of the quantification of mitochondria by flow cytometry.
Schulz, Sabine:	Support on the simultaneous determination of the mitochondrial membrane potential and mitochondrial swelling.
Simmons Alisha:	Bachelor student of the AG Zischka. Determination of the complex I and complex II activity of rat liver mitochondria and mitochondria from the rat hepatoma cell line McA 7777.
Weber Susanne:	Isolation of mitochondria from BeWo cells.
Zischka, Hans:	Advisory supervision in all aspects of the manuscripts as well as of this thesis.

Candidate´s contributions to the included publications

Publication 1: A semi-automated method for isolating functionally intact mitochondria from cultured cells and tissue biopsies.

- Idea of the manuscript together with supervisor and Friederike Saathoff.
- Performance of experiments (70%) together with co-authors.
- Experimental design (60%) together with Friederike Saathoff.
- Data analysis.
- Writing and finalizing of the manuscript (50%) together with co-authors.

Publication 2: Isolation of mitochondria from cultured cells and liver tissue biopsies for molecular and biochemical analyses.

- Idea of the manuscript together with supervisor.
- Performance of experiments (80%) together with co-authors.
- Writing and finalizing of the manuscript (80%) together with supervisor.

Publication 3: Why to compare absolute numbers of mitochondria.

- Performance of experiments (70%) together with co-authors.
- Experimental design.
- Data analysis.
- Writing and finalizing of the manuscript (70%) together with supervisor.

10. Abbreviations

AIF	Apoptosis inducing factor
ANT	Adenine nucleotide transporter
CS	Citrate Synthase
CsA	Cyclosporin A
Cyt C	Cytochrome C
FCCP	Carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone
GCDCA	Glycochenodeoxycholate
MMP	Mitochondrial membrane potential
MOMP	Mitochondrial outer membrane permeabilization
MPT	Mitochondrial permeability transition
NAO	10N-nonyl acridine orange
OxPhos	Oxidative phophorylation
PCC	Pump controlled cell rupture system
RCR	Respiratory control ratio
Rh123	Rhodamine 123
TCDCA	Taurochenodeoxycholate
TTFA	Thenoyltrifluoroacetone
VDAC	Voltage dependent anion channel
ZE-FFE	Zone electrophoresis in a free flow electrophoresis device

11. References

Alirol, E. and J. C. Martinou (2006). "Mitochondria and cancer: is there a morphological connection?" Oncogene **25**(34): 4706-4716.

Almeida, A. and J. M. Medina (1998). "A rapid method for the isolation of metabolically active mitochondria from rat neurons and astrocytes in primary culture." Brain Res Brain Res Protoc **2**(3): 209-214.

Balch, W. E. and J. E. Rothman (1985). "Characterization of protein transport between successive compartments of the Golgi apparatus: asymmetric properties of donor and acceptor activities in a cell-free system." Arch Biochem Biophys **240**(1): 413-425.

Baracca, A., G. Sgarbi, et al. (2003). "Rhodamine 123 as a probe of mitochondrial membrane potential: evaluation of proton flux through F(0) during ATP synthesis." Biochim Biophys Acta **1606**(1-3): 137-146.

Barysch, S. V., R. Jahn, et al. (2010). "A fluorescence-based in vitro assay for investigating early endosome dynamics." Nat Protoc **5**(6): 1127-1137.

Becker, T., M. Gebert, et al. (2009). "Biogenesis of mitochondrial membrane proteins." Curr Opin Cell Biol **21**(4): 484-493.

Bernardi, P., A. Krauskopf, et al. (2006). "The mitochondrial permeability transition from in vitro artifact to disease target." FEBS J **273**(10): 2077-2099.

Bernhardt, D., M. Muller, et al. (2015). "Simultaneous impairment of mitochondrial fission and fusion reduces mitophagy and shortens replicative lifespan." Sci Rep **5**: 7885.

Berridge, M. V., P. M. Herst, et al. (2009). "Targeting mitochondrial permeability in cancer drug development." Mol Nutr Food Res **53**(1): 76-86.

Bhaskaran, S., J. A. Butler, et al. (2011). "Breaking Caenorhabditis elegans the easy way using the Balch homogenizer: an old tool for a new application." Anal Biochem **413**(2): 123-132.

Boldogh, I. R. and L. A. Pon (2007). "Purification and subfractionation of mitochondria from the yeast Saccharomyces cerevisiae." Methods Cell Biol **80**: 45-64.

Botla, R., J. R. Spivey, et al. (1995). "Ursodeoxycholate (UDCA) inhibits the mitochondrial membrane permeability transition induced by glycochenodeoxycholate: a mechanism of UDCA cytoprotection." J Pharmacol Exp Ther **272**(2): 930-938.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal Biochem **72**: 248-254.

Chipuk, J. E., L. Bouchier-Hayes, et al. (2006). "Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario." Cell Death Differ **13**(8): 1396-1402.

Colbeau, A., J. Nachbaur, et al. (1971). "Enzymic characterization and lipid composition of rat liver subcellular membranes." Biochim Biophys Acta **249**(2): 462-492.

Colombini, M. (1979). "A candidate for the permeability pathway of the outer mitochondrial membrane." Nature **279**(5714): 643-645.

Colombini, M. (1980). "Structure and mode of action of a voltage dependent anion-selective channel (VDAC) located in the outer mitochondrial membrane." Ann N Y Acad Sci **341**: 552-563.

Criddle, R. S., F. Paltauf, et al. (1969). "Identification of Mitochondrial Inner Membranes in Anaerobically Grown Baker's Yeast." J Gen Physiol **54**(1): 57-65.

Criddle, R. S. and G. Schatz (1969). "Promitochondria of anaerobically grown yeast. I. Isolation and biochemical properties." Biochemistry **8**(1): 322-334.
Cuezva, J. M., M. Krajewska, et al. (2002). "The bioenergetic signature of cancer: a marker of tumor progression." Cancer Res **62**(22): 6674-6681.
Dalziel, A. C., S. E. Moore, et al. (2005). "Mitochondrial enzyme content in the muscles of high-performance fish: evolution and variation among fiber types." Am J Physiol Regul Integr Comp Physiol **288**(1): R163-172.
Daugas, E., S. A. Susin, et al. (2000). "Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis." FASEB J **14**(5): 729-739.
Daum, G., P. C. Bohni, et al. (1982). "Import of proteins into mitochondria. Cytochrome b2 and cytochrome c peroxidase are located in the intermembrane space of yeast mitochondria." J Biol Chem **257**(21): 13028-13033.
David, G. and E. F. Barrett (2000). "Stimulation-evoked increases in cytosolic [Ca(2+)] in mouse motor nerve terminals are limited by mitochondrial uptake and are temperature-dependent." J Neurosci **20**(19): 7290-7296.
David, G. and E. F. Barrett (2003). "Mitochondrial Ca2+ uptake prevents desynchronization of quantal release and minimizes depletion during repetitive stimulation of mouse motor nerve terminals." J Physiol **548**(Pt 2): 425-438.
Dejean, L., B. Beauvoit, et al. (2002). "cAMP-induced modulation of the growth yield of Saccharomyces cerevisiae during respiratory and respiro-fermentative metabolism." Biochim Biophys Acta **1554**(3): 159-169.
DeRisi, J. L., V. R. Iyer, et al. (1997). "Exploring the metabolic and genetic control of gene expression on a genomic scale." Science **278**(5338): 680-686.
Drago, I., D. De Stefani, et al. (2012). "Mitochondrial Ca2+ uptake contributes to buffering cytoplasmic Ca2+ peaks in cardiomyocytes." Proc Natl Acad Sci U S A **109**(32): 12986-12991.
Du, C., M. Fang, et al. (2000). "Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition." Cell **102**(1): 33-42.
Duchen, M. R. (2004). "Mitochondria in health and disease: perspectives on a new mitochondrial biology." Mol Aspects Med **25**(4): 365-451.
Emaus, R. K., R. Grunwald, et al. (1986). "Rhodamine 123 as a probe of transmembrane potential in isolated rat-liver mitochondria: spectral and metabolic properties." Biochim Biophys Acta **850**(3): 436-448.
Ernster, L. and G. Schatz (1981). "Mitochondria: a historical review." J Cell Biol **91**(3 Pt 2): 227s-255s.
Eskes, R., S. Desagher, et al. (2000). "Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane." Mol Cell Biol **20**(3): 929-935.
Fernandez-Marcos, P. J. and J. Auwerx (2011). "Regulation of PGC-1alpha, a nodal regulator of mitochondrial biogenesis." Am J Clin Nutr **93**(4): 884S-890.
Fleischer, S. (1979). "Long-term storage of mitochondria to preserve energy-linked functions." Methods Enzymol **55**: 28-32.
Fluegge, D., L. M. Moeller, et al. (2012). "Mitochondrial Ca(2+) mobilization is a key element in olfactory signaling." Nat Neurosci **15**(5): 754-762.
Foerster, F., S. Braig, et al. (2014). "Targeting the actin cytoskeleton: selective antitumor action via trapping PKCvarepsilon." Cell Death Dis **5**: e1398.
Frezza, C., S. Cipolat, et al. (2007). "Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts." Nat Protoc **2**(2): 287-295.

Friel, D. D. and R. W. Tsien (1994). "An FCCP-sensitive Ca2+ store in bullfrog sympathetic neurons and its participation in stimulus-evoked changes in [Ca2+]i." J Neurosci **14**(7): 4007-4024.
Fulda, S., L. Galluzzi, et al. (2010). "Targeting mitochondria for cancer therapy." Nat Rev Drug Discov **9**(6): 447-464.
Fulda, S. and G. Kroemer (2011). "Mitochondria as therapeutic targets for the treatment of malignant disease." Antioxid Redox Signal **15**(12): 2937-2949.
Fuller, K. M. and E. A. Arriaga (2004). "Capillary electrophoresis monitors changes in the electrophoretic behavior of mitochondrial preparations." J Chromatogr B Analyt Technol Biomed Life Sci **806**(2): 151-159.
Galluzzi, L., N. Larochette, et al. (2006). "Mitochondria as therapeutic targets for cancer chemotherapy." Oncogene **25**(34): 4812-4830.
Garrabou, G., A. Soriano, et al. (2007). "Reversible inhibition of mitochondrial protein synthesis during linezolid-related hyperlactatemia." Antimicrob Agents Chemother **51**(3): 962-967.
German, C. L. and C. L. Howe (2009). "Preparation of biologically active subcellular fractions using the Balch homogenizer." Anal Biochem **394**(1): 117-124.
Gogvadze, V. (2011). "Targeting mitochondria in fighting cancer." Curr Pharm Des **17**(36): 4034-4046.
Goncalves, R. P., N. Buzhynskyy, et al. (2007). "Supramolecular assembly of VDAC in native mitochondrial outer membranes." J Mol Biol **369**(2): 413-418.
Gores, G. J., H. Miyoshi, et al. (1998). "Induction of the mitochondrial permeability transition as a mechanism of liver injury during cholestasis: a potential role for mitochondrial proteases." Biochim Biophys Acta **1366**(1-2): 167-175.
Green, D. R. and G. Kroemer (2004). "The pathophysiology of mitochondrial cell death." Science **305**(5684): 626-629.
Grimm, S. and D. Brdiczka (2007). "The permeability transition pore in cell death." Apoptosis **12**(5): 841-855.
Guidotti, G. (1972). "Membrane proteins." Annu Rev Biochem **41**: 731-752.
Hackenbrock, C. R. (1966). "Ultrastructural bases for metabolically linked mechanical activity in mitochondria. I. Reversible ultrastructural changes with change in metabolic steady state in isolated liver mitochondria." J Cell Biol **30**(2): 269-297.
Hackenbrock, C. R. (1968). "Chemical and physical fixation of isolated mitochondria in low-energy and high-energy states." Proc Natl Acad Sci U S A **61**(2): 598-605.
Hackenbrock, C. R. (1968). "Ultrastructural bases for metabolically linked mechanical activity in mitochondria. II. Electron transport-linked ultrastructural transformations in mitochondria." J Cell Biol **37**(2): 345-369.
Hackenbrock, C. R. and K. J. Miller (1975). "The distribution of anionic sites on the surfaces of mitochondrial membranes. Visual probing with polycationic ferritin." J Cell Biol **65**(3): 615-630.
Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.
Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.
Hannig, K. (1978). "Continuous free-flow electrophoresis as an analytical and preparative method in biology." J Chromatogr **159**(1): 183-191.
Hannig, K. and H. G. Heidrich (1974). "The use of continuous preparative free-flow electrophoresis for dissociating cell fractions and isolation of membranous components." Methods Enzymol **31**: 746-761.

Haworth, R. A. and D. R. Hunter (1979). "The Ca2+-induced membrane transition in mitochondria. II. Nature of the Ca2+ trigger site." Arch Biochem Biophys **195**(2): 460-467.
Heidrich, H. (1971). "The sideness of mitochondrial membranes and their sub-particles as characterized by preparative free flow electrophoresis." FEBS Lett **17**(2): 253-256.
Heidrich, H. G., R. Stahn, et al. (1970). "The surface charge of rat liver mitochondria and their membranes. Clarification of some controversies concerning mitochondrial structure." J Cell Biol **46**(1): 137-150.
Herrmann, J. M. and J. Riemer (2010). "Oxidation and reduction of cysteines in the intermembrane space of mitochondria: multiple facets of redox control." Antioxid Redox Signal **13**(9): 1323-1326.
Hogeboom, G. H., A. Claude, et al. (1946). "The distribution of cytochrome oxidase and succinoxidase in the cytoplasm of the mammalian liver cell." J Biol Chem **165**(2): 615-629.
Hogeboom, G. H., W. C. Schneider, et al. (1948). "Cytochemical studies of mammalian tissues; isolation of intact mitochondria from rat liver; some biochemical properties of mitochondria and submicroscopic particulate material." J Biol Chem **172**(2): 619-635.
Hohenester, S., A. Gates, et al. (2010). "Phosphatidylinositol-3-kinase p110gamma contributes to bile salt-induced apoptosis in primary rat hepatocytes and human hepatoma cells." J Hepatol **53**(5): 918-926.
Hostetler, K. Y., B. D. Zenner, et al. (1976). "Abnormal membrane phospholipid content in subcellular fractions from the Morris 7777 hepatoma." Biochim Biophys Acta **441**(2): 231-238.
Hsu, C. C., H. C. Lee, et al. (2013). "Mitochondrial DNA alterations and mitochondrial dysfunction in the progression of hepatocellular carcinoma." World J Gastroenterol **19**(47): 8880-8886.
Hunter, D. R., R. A. Haworth, et al. (1976). "Relationship between configuration, function, and permeability in calcium-treated mitochondria." J Biol Chem **251**(16): 5069-5077.
Isaeva, E. V. and N. Shirokova (2003). "Metabolic regulation of Ca2+ release in permeabilized mammalian skeletal muscle fibres." J Physiol **547**(Pt 2): 453-462.
Jurgensmeier, J. M., Z. Xie, et al. (1998). "Bax directly induces release of cytochrome c from isolated mitochondria." Proc Natl Acad Sci U S A **95**(9): 4997-5002.
Kaftan, E. J., T. Xu, et al. (2000). "Mitochondria shape hormonally induced cytoplasmic calcium oscillations and modulate exocytosis." J Biol Chem **275**(33): 25465-25470.
Kennedy, E. P. and A. L. Lehninger (1949). "Oxidation of fatty acids and tricarboxylic acid cycle intermediates by isolated rat liver mitochondria." J Biol Chem **179**(2): 957-972.
Kiebish, M. A., X. Han, et al. (2008). "Lipidomic analysis and electron transport chain activities in C57BL/6J mouse brain mitochondria." J Neurochem **106**(1): 299-312.
Krivánková, L. (1998). "Continuous free-flow electrophoresis." Electrophoresis **19**(7): 1064-1074.
Kroemer, G., L. Galluzzi, et al. (2007). "Mitochondrial membrane permeabilization in cell death." Physiol Rev **87**(1): 99-163.
Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature **227**(5259): 680-685.
Li, L. Y., X. Luo, et al. (2001). "Endonuclease G is an apoptotic DNase when released from mitochondria." Nature **412**(6842): 95-99.

Li, Y., X. M. Qi, et al. (2009). "The relationship between diphenylamine structure and NSAIDs-induced hepatocytes injury." Toxicol Lett **186**(2): 111-114.
Liu, X., C. N. Kim, et al. (1996). "Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c." Cell **86**(1): 147-157.
Löffler, G. a. P., P.E. (1990). Biochemie und Pathobiochemie. Berlin Heidelberg New York, Springer-Verlag.
Maack, C., S. Cortassa, et al. (2006). "Elevated cytosolic Na+ decreases mitochondrial Ca2+ uptake during excitation-contraction coupling and impairs energetic adaptation in cardiac myocytes." Circ Res **99**(2): 172-182.
Mannella, C. A. (1998). "Conformational changes in the mitochondrial channel protein, VDAC, and their functional implications." J Struct Biol **121**(2): 207-218.
Marroquin, L. D., J. Hynes, et al. (2007). "Circumventing the Crabtree effect: replacing media glucose with galactose increases susceptibility of HepG2 cells to mitochondrial toxicants." Toxicol Sci **97**(2): 539-547.
Marzo, I., C. Brenner, et al. (1998). "The central role of the mitochondrial megachannel in apoptosis: evidence obtained with intact cells, isolated mitochondria, and purified protein complexes." Biomed Pharmacother **52**(6): 248-251.
Masubuchi, Y., S. Nakayama, et al. (2002). "Role of mitochondrial permeability transition in diclofenac-induced hepatocyte injury in rats." Hepatology **35**(3): 544-551.
Mishra, P. and D. C. Chan (2014). "Mitochondrial dynamics and inheritance during cell division, development and disease." Nat Rev Mol Cell Biol **15**(10): 634-646.
Mitchell, P. (1961). "Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism." Nature **191**: 144-148.
Mootha, V. K., J. Bunkenborg, et al. (2003). "Integrated analysis of protein composition, tissue diversity, and gene regulation in mouse mitochondria." Cell **115**(5): 629-640.
Morton, R., C. Cunningham, et al. (1976). "Alteration of mitochondrial function and lipid composition in Morris 7777 hepatoma." Cancer Res **36**(9 pt.1): 3246-3254.
Mulisch, M. and U. Welsch (2010). Romeis Mikroskopische Technik. Heidelberg, Spektrum Akademischer Verlag Heidelberg.
Munnich, A. and P. Rustin (2001). "Clinical spectrum and diagnosis of mitochondrial disorders." Am J Med Genet **106**(1): 4-17.
Munoz-Pinedo, C., A. Guio-Carrion, et al. (2006). "Different mitochondrial intermembrane space proteins are released during apoptosis in a manner that is coordinately initiated but can vary in duration." Proc Natl Acad Sci U S A **103**(31): 11573-11578.
Newmeyer, D. D., D. M. Farschon, et al. (1994). "Cell-free apoptosis in Xenopus egg extracts: inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria." Cell **79**(2): 353-364.
Nicholls, D., Ferguson SJ (1992). Bioenergetics. San Diego, Academic Press.
Orth, M. and A. H. Schapira (2001). "Mitochondria and degenerative disorders." Am J Med Genet **106**(1): 27-36.
Palade, G. E. (1952). "The fine structure of mitochondria." Anat Rec **114**(3): 427-451.
Pallotti, F. and G. Lenaz (2007). "Isolation and subfractionation of mitochondria from animal cells and tissue culture lines." Methods Cell Biol **80**: 3-44.
Palmeira, C. M. and A. P. Rolo (2004). "Mitochondrially-mediated toxicity of bile acids." Toxicology **203**(1-3): 1-15.
Patel, T., S. F. Bronk, et al. (1994). "Increases of intracellular magnesium promote glycodeoxycholate-induced apoptosis in rat hepatocytes." J Clin Invest **94**(6): 2183-2192.

Pathania, D., M. Millard, et al. (2009). "Opportunities in discovery and delivery of anticancer drugs targeting mitochondria and cancer cell metabolism." Adv Drug Deliv Rev **61**(14): 1250-1275.

Pavlakis, S. G., P. C. Phillips, et al. (1984). "Mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes: a distinctive clinical syndrome." Ann Neurol **16**(4): 481-488.

Penefsky, H. S. (1985). "Mechanism of inhibition of mitochondrial adenosine triphosphatase by dicyclohexylcarbodiimide and oligomycin: relationship to ATP synthesis." Proc Natl Acad Sci U S A **82**(6): 1589-1593.

Petit, J. M., A. Maftah, et al. (1992). "10N-nonyl acridine orange interacts with cardiolipin and allows the quantification of this phospholipid in isolated mitochondria." Eur J Biochem **209**(1): 267-273.

Petronilli, V., C. Cola, et al. (1993). "Physiological effectors modify voltage sensing by the cyclosporin A-sensitive permeability transition pore of mitochondria." J Biol Chem **268**(29): 21939-21945.

Phillips, N. R., M. L. Sprouse, et al. (2014). "Simultaneous quantification of mitochondrial DNA copy number and deletion ratio: a multiplex real-time PCR assay." Sci Rep **4**: 3887.

Pilkington, G. J., K. Parker, et al. (2008). "Approaches to mitochondrially mediated cancer therapy." Semin Cancer Biol **18**(3): 226-235.

Plattner, H., M. M. Salpeter, et al. (1970). "Promitochondria of anaerobically grown yeast. IV. Conversion into respiring mitochondria." Proc Natl Acad Sci U S A **66**(4): 1252-1259.

Plattner, H. and G. Schatz (1969). "Promitochondria of anaerobically grown yeast. 3. Morphology." Biochemistry **8**(1): 339-343.

Ralph, S. J. and J. Neuzil (2009). "Mitochondria as targets for cancer therapy." Mol Nutr Food Res **53**(1): 9-28.

Robb-Gaspers, L. D., G. A. Rutter, et al. (1998). "Coupling between cytosolic and mitochondrial calcium oscillations: role in the regulation of hepatic metabolism." Biochim Biophys Acta **1366**(1-2): 17-32.

Rohlena, J., L. F. Dong, et al. (2011). "Anticancer drugs targeting the mitochondrial electron transport chain." Antioxid Redox Signal **15**(12): 2951-2974.

Rolo, A. P., C. M. Palmeira, et al. (2004). "Role of mitochondrial dysfunction in combined bile acid-induced cytotoxicity: the switch between apoptosis and necrosis." Toxicol Sci **79**(1): 196-204.

Rossignol, R., R. Gilkerson, et al. (2004). "Energy substrate modulates mitochondrial structure and oxidative capacity in cancer cells." Cancer Res **64**(3): 985-993.

Rotig, A., P. de Lonlay, et al. (1997). "Aconitase and mitochondrial iron-sulphur protein deficiency in Friedreich ataxia." Nat Genet **17**(2): 215-217.

Rust, C., K. Bauchmuller, et al. (2005). "Phosphatidylinositol 3-kinase-dependent signaling modulates taurochenodeoxycholic acid-induced liver injury and cholestasis in perfused rat livers." Am J Physiol Gastrointest Liver Physiol **289**(1): G88-94.

Rust, C., N. Wild, et al. (2009). "Bile acid-induced apoptosis in hepatocytes is caspase-6-dependent." J Biol Chem **284**(5): 2908-2916.

Saggerson, E. D. and C. A. Carpenter (1986). "Carnitine palmitoyltransferase in liver and five extrahepatic tissues in the rat. Inhibition by DL-2-bromopalmitoyl-CoA and effect of hypothyroidism." Biochem J **236**(1): 137-141.

Saks, V. A., G. B. Chernousova, et al. (1975). "Studies of energy transport in heart cells. Mitochondrial isoenzyme of creatine phosphokinase: kinetic properties and regulatory action of Mg2+ ions." Eur J Biochem **57**(1): 273-290.
Schaefer, A. M., R. W. Taylor, et al. (2004). "The epidemiology of mitochondrial disorders--past, present and future." Biochim Biophys Acta **1659**(2-3): 115-120.
Schnaitman, C., V. G. Erwin, et al. (1967). "The submitochondrial localization of monoamine oxidase. An enzymatic marker for the outer membrane of rat liver mitochondria." J Cell Biol **32**(3): 719-735.
Schnaitman, C. and J. W. Greenawalt (1968). "Enzymatic properties of the inner and outer membranes of rat liver mitochondria." J Cell Biol **38**(1): 158-175.
Schneider, W. C., A. Claude, et al. (1948). "The distribution of cytochrome c and succinoxidase activity in rat liver fractions." J Biol Chem **172**(2): 451-458.
Schneider, W. C. and V. R. Potter (1949). "Intracellular distribution of enzymes; the distribution of oxalacetic oxidase activity in rat liver and rat kidney fractions." J Biol Chem **177**(2): 893-903.
Scholte, H. R., P. J. Weijers, et al. (1973). "The localization of mitochondrial creatine kinase, and its use for the determination of the sidedness of submitochondrial particles." Biochim Biophys Acta **291**(3): 764-773.
Schulz, S., J. Lichtmannegger, et al. (2015). "A protocol for the parallel isolation of intact mitochondria from rat liver, kidney, heart, and brain." Methods Mol Biol **1295**: 75-86.
Schulz, S., S. Schmitt, et al. (2013). "Progressive stages of mitochondrial destruction caused by cell toxic bile salts." Biochim Biophys Acta **1828**(9): 2121-2133.
Smith, R. A., R. C. Hartley, et al. (2012). "Mitochondrial pharmacology." Trends Pharmacol Sci **33**(6): 341-352.
Smith, R. A., R. C. Hartley, et al. (2011). "Mitochondria-targeted small molecule therapeutics and probes." Antioxid Redox Signal **15**(12): 3021-3038.
Smith, R. A. and M. P. Murphy (2010). "Animal and human studies with the mitochondria-targeted antioxidant MitoQ." Ann N Y Acad Sci **1201**: 96-103.
Srere, P. A. (1980). "The infrastructure of the mitochondrial matrix." Trends Biochem Sci **5**(5): 2.
Stoffel, W. and H. G. Schiefer (1968). "Biosynthesis and composition of phosphatides in outer and inner mitochondrial membranes." Hoppe Seylers Z Physiol Chem **349**(8): 1017-1026.
Sundaresan, N. R., M. Gupta, et al. (2009). "Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice." J Clin Invest **119**(9): 2758-2771.
Susin, S. A., H. K. Lorenzo, et al. (1999). "Molecular characterization of mitochondrial apoptosis-inducing factor." Nature **397**(6718): 441-446.
Suzuki, Y., Y. Imai, et al. (2001). "A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death." Mol Cell **8**(3): 613-621.
Tan, A. S., J. W. Baty, et al. (2015). "Mitochondrial genome acquisition restores respiratory function and tumorigenic potential of cancer cells without mitochondrial DNA." Cell Metab **21**(1): 81-94.
Thayer, S. A. and R. J. Miller (1990). "Regulation of the intracellular free calcium concentration in single rat dorsal root ganglion neurones in vitro." J Physiol **425**: 85-115.

Towbin, H., T. Staehelin, et al. (1979). "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications." Proc Natl Acad Sci U S A **76**(9): 4350-4354.
Tsujimoto, Y. and S. Shimizu (2007). "Role of the mitochondrial membrane permeability transition in cell death." Apoptosis **12**(5): 835-840.
Valero, T. (2014). "Mitochondrial biogenesis: pharmacological approaches." Curr Pharm Des **20**(35): 5507-5509.
Vance, J. E. (1990). "Phospholipid synthesis in a membrane fraction associated with mitochondria." J Biol Chem **265**(13): 7248-7256.
Vander Heiden, M. G., L. C. Cantley, et al. (2009). "Understanding the Warburg effect: the metabolic requirements of cell proliferation." Science **324**(5930): 1029-1033.
Vasington, F. D. (1963). "Calcium ion uptake by fragments of rat liver mitochondria and its dependence on electron transport." J Biol Chem **238**: 1841-1847.
Veitinger, S., T. Veitinger, et al. (2011). "Purinergic signalling mobilizes mitochondrial Ca(2)(+) in mouse Sertoli cells." J Physiol **589**(Pt 21): 5033-5055.
Veltri, K. L., M. Espiritu, et al. (1990). "Distinct genomic copy number in mitochondria of different mammalian organs." J Cell Physiol **143**(1): 160-164.
Verhagen, A. M., P. G. Ekert, et al. (2000). "Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins." Cell **102**(1): 43-53.
Vijayasarathy, C., I. Biunno, et al. (1998). "Variations in the subunit content and catalytic activity of the cytochrome c oxidase complex from different tissues and different cardiac compartments." Biochim Biophys Acta **1371**(1): 71-82.
Voet Daniel, V. J. G. (1992). Biochemie. Weinheim, Germany, VCH.
Warburg, O. (1926). Über den Stoffwechsel der Tumore. Berlin.
Weber, S., J. K. Salabei, et al. (2015). "Aldo-keto Reductase 1B15 (AKR1B15): a mitochondrial human aldo-keto reductase with activity toward steroids and 3-keto-acyl-CoA conjugates." J Biol Chem **290**(10): 6531-6545.
Wenner, C. E. (2011). "Targeting mitochondria as a therapeutic target in cancer." J Cell Physiol.
Werth, J. L. and S. A. Thayer (1994). "Mitochondria buffer physiological calcium loads in cultured rat dorsal root ganglion neurons." J Neurosci **14**(1): 348-356.
Wieckowski, M. R., C. Giorgi, et al. (2009). "Isolation of mitochondria-associated membranes and mitochondria from animal tissues and cells." Nat Protoc **4**(11): 1582-1590.
Williams, A. J., J. Coakley, et al. (1998). "Automated analysis of mitochondrial enzymes in cultured skin fibroblasts." Anal Biochem **259**(2): 176-180.
Williams, G. R. (1965). "Dynamic Aspects of the Tricarboxylic Acid Cycle in Isolated Mitochondria." Can J Biochem **43**: 603-615.
Wojtczak, L. and H. Zaluska (1969). "On the impermeability of the outer mitochondrial membrane to cytochrome c. I. Studies on whole mitochondria." Biochim Biophys Acta **193**(1): 64-72.
Yakes, F. M. and B. Van Houten (1997). "Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress." Proc Natl Acad Sci U S A **94**(2): 514-519.
Yang, J. C. and G. A. Cortopassi (1998). "Induction of the mitochondrial permeability transition causes release of the apoptogenic factor cytochrome c." Free Radic Biol Med **24**(4): 624-631.

Zalman, L. S., H. Nikaido, et al. (1980). "Mitochondrial outer membrane contains a protein producing nonspecific diffusion channels." J Biol Chem **255**(5): 1771-1774.
Zamzami, N., D. Metivier, et al. (2000). "Quantitation of mitochondrial transmembrane potential in cells and in isolated mitochondria." Methods Enzymol **322**: 208-213.
Zischka, H., R. J. Braun, et al. (2006). "Differential analysis of Saccharomyces cerevisiae mitochondria by free flow electrophoresis." Mol Cell Proteomics **5**(11): 2185-2200.
Zischka, H., N. Larochette, et al. (2008). "Electrophoretic analysis of the mitochondrial outer membrane rupture induced by permeability transition." Anal Chem **80**(13): 5051-5058.
Zischka, H., J. Lichtmannegger, et al. (2011). "Liver mitochondrial membrane crosslinking and destruction in a rat model of Wilson disease." J Clin Invest **121**(4): 1508-1518.
Zorov, D. B., N. K. Isaev, et al. (2007). "The mitochondrion as janus bifrons." Biochemistry (Mosc) **72**(10): 1115-1126.

12. Acknowledgement

Foremost, I am very grateful to my supervisor PD Dr. Hans Zischka for the continuous support on my PhD study and research, for his enthusiasm, and for sharing his expertise in intensive and fruitful discussions. Thanks for giving me this interesting topic and for giving me the opportunity to continue to work on it for the next year.

My sincere thanks is given to Prof. Dr. Jerzy Adamski for insightful comments in the course of the thesis committee meetings and for accepting to act as examiner for this work.

Prof. Dr. Martin Klingenspor is thanked for fruitful discussions and for accepting to act as co-examiner for this thesis.

I would like to thank Dr. Friederike Saathoff for her experimental and scientific support and her enthusiasm on isolating mitochondria from cultured cells. It was a pleasure to work with you on this project.

Special thanks to all members and students of the working group: Sabine for guiding my first steps in the lab of the AG Zischka as well as for fruitful discussions; Carola and Lichti for their support on all types of mitochondrial isolations and for sharing their experiences; Claudia, Christin, Eva, Sabine, Yashar, Salva, Lukas, Yuliya, Alisha and Andreas for experimental support. It was a pleasure to work with you.

Thanks to all cooperation partners: Prof. Dr. Axel Walch, Dr. Michaela Aichler, Luise Jennen and Gabriele Mettenleiter for the scientific and technical support on electron microscopy, Dr. Wolfgang Beisker for his guidance and support on flow cytometry, Prof. Dr. Roland Kappler for providing the human hepatoma cell lines Huh6 and HepT1, Prof. Dr. Nikolaus Plesnila, Dr. Lilja Meissner, Prof. Dr. Guido Kroemer, Prof. Dr. Simon Rothenfusser and Susanne Weber for experimental and scientific assistance and guidance on the publications this thesis is based on.

I would like to thank my family and friends who supported me in any respect during the completion of the project.

This work is dedicated to Andy.