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Selected reaction monitoring mass spectrometry for relative quantification of proteins involved in cellular life and death processes

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ABSTRACT

Monitoring of proteins involved in cellular life and death processes is of high scientific interest since it permits the elucidation of functional changes in a variety of diseases. In this study, we have developed a nanoLC–MS/MS assay for the simultaneous detection and quantification of 24 selected proteins that are known to be important for cellular homeostasis. The Selected Reaction Monitoring (SRM) assay applies heavy-labeled peptide analogues for the relative quantification of proteins with central functions in cellular stress and metabolism, including many mitochondrial proteins. The assay includes proteins involved in the quality control of mitochondrial proteins, oxidative stress, respiratory chain, and fatty acid degradation, as well as the cytosolic glyceraldehyde 3-phosphate dehydrogenase, lactate dehydrogenase and ribosomal proteins. The assay can thus quantitate the balance between mitochondrial and cytosolic pathways, which is relevant in many disease states, and can be studied by comparing patient and control samples. The measured validation parameters showed satisfactory results for the proteins included in the analysis. The linear range of the monitored proteins was 0.01–20 nM, with a median precision of less than 10%. The assay performed well in monitoring proteins in both cultured human skin fibroblast cells as well as in isolated peripheral blood mononuclear cells. We therefore believe that this assay is applicable for the study of cellular stress response in various types of cell defects and disease states.

1. Introduction

In this study we developed a quantitative nano-liquid chromatography mass spectrometry (nanoLC–MS) assay to investigate proteins taking part in cellular survival and death mechanisms. The Selected Reaction Monitoring (SRM) assay performed on triple quadrupole MS was used to monitor 24 proteins related to mitochondrial dysfunction and cell stress, as well as two reference proteins (Table 1).

Owing to the vital role of mitochondria, it is not surprising that mitochondrial dysfunction is involved in various pathologies, such as cancer, obesity, cardiomyopathies, neurodegenerative disorders, and metabolic syndrome, apart from the inherited disorders of mitochondrial functions, such as inherited defects in FAO, degradation of branched chain amino acids and respiratory chain defects [1–8]. The proteins included in the assay in this study cover several central functionalities of metabolism and cellular stress, and thus can serve to profile cellular defects, e.g. in relation to the above-mentioned disorders. The proteins were carefully selected based on literature and on previously performed untargeted proteomic studies describing the effects of deficiencies of mitochondrial enzymes, such as short-chain acyl-CoA dehydrogenase (ACADS) and ETHE1 [9–11], and from other studies of functional networks that control cellular survival and death mechanisms [12–15]. Overall, we aimed to cover proteins involved in mitochondrial protein quality control systems, antioxidant enzymes, and proteins involved in autophagy/mitophagy, mitochondrial dynamics, metabolic fuel regulation (Warburg effect), the respiratory chain, and apoptosis. Below, we outline the biological context and relevance of the proteins included in the developed assay. As shown in Table 1, the protein quality control system comprises three chaperones and one protease, which are important in assisting protein folding, intracellular protein trafficking, and in cellular protein quality control [16,17]. When cells are exposed to various stresses such as excess reactive oxygen species (ROS), the protein quality control system and the antioxidant enzymes and regulators such as mitochondrial superoxide dismutase (SOD2) and protein DJ-1 (PARK7) [18], can be considered as the first line of defense. The second line of defense is often referred to as the dynamic nature of the mitochondria which is controlled by the fusion of fission events [19].

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Table 1
– Proteins included in the presented SRM assay.

Protein	Accession	Description	UniProtKB	Gene
Hsp60	P10809	60 kDa heat shock protein, mitochondrial	CH60_HUMAN	*HSPD1*
ClpP	Q16740	ATP-dependent Clp protease proteolytic subunit, mitochondrial	CLPP_HUMAN	*CLPP*
TRAP1	Q12931	Heat shock protein 75 kDa, mitochondrial	TRAP1_HUMAN	*TRAP1*
mtHsp70	P38646	Stress-70 protein, mitochondrial	GRP75_HUMAN	*HSPA9*
SOD2	P04179	Superoxide dismutase [Mn], mitochondrial	SODM_HUMAN	*SOD2*
DJ1	Q99497	Protein DJ-1	PARK7_HUMAN	*PARK7*
LC3	Q9GZQ8	Microtubule-associated proteins 1A/1 B light chain 3B	MLP3B_HUMAN	*MAP1LC3B*
P62	P37198	Nuclear pore glycoprotein p62	NUP62_HUMAN	*NUP62*
Opa1	O60313	Dynamin-like 120 kDa protein, mitochondrial	OPA1_HUMAN	*OPA1*
Drp1	O00429	Dynamin-1-like protein	DNM1L_HUMAN	*DNM1L*
Mfn1	Q8IWA4	Mitofusin-1	MFN1_HUMAN	*MFN1*
Bax	Q07812	Apoptosis regulator BAX	BAX_HUMAN	*BAX*
SURF1	Q15526	Surfeit locus protein 1	SURF1_HUMAN	*SURF1*
BCS1L	Q9Y276	Mitochondrial chaperone BCS1	BCS1_HUMAN	*BCS1L*
MCAD	P11310	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	ACADM_HUMAN	*ACADM*
CPT1	P50416	Carnitine O-palmitoyltransferase 1, liver isoform	CPT1A_HUMAN	*CPT1A*
α-ETF	P13804	Electron transfer flavoprotein subunit alpha, mitochondrial	ETFA_HUMAN	*ETFA*
β-ETF	P38117	Electron transfer flavoprotein subunit beta, mitochondrial	ETFB_HUMAN	*ETFB*
IDH2	P48735	Isocitrate dehydrogenase [NADP], mitochondrial	IDHP_HUMAN	*IDH2*
LDH	P00338	L-lactate dehydrogenase A chain	LDHA_HUMAN	*LDHA*
GAPDH	P04406	Glyceraldehyde-3-phosphate dehydrogenase	G3P_HUMAN	*GAPDH*
SIRT3	Q9NTG7	NAD-dependent protein deacetylase sirtuin-3, mitochondrial	SIR3_HUMAN	*SIRT3*
VDAC	P21796	Voltage-dependent anion-selective channel protein 1	VDAC1_HUMAN	*VDAC1*
Tubb	P07437	Tubulin beta chain	TBB5_HUMAN	*TUBB*
RPS21	P63220	40S ribosomal protein S21	RS21_HUMAN	*RPS21*
RPS8	P62241	40S ribosomal protein S8	RS8_HUMAN	*RPS8*

Mitochondrial damage can be compensated by fusion of damaged mitochondria with healthy mitochondria. The proteins MFN1 in the inner mitochondrial membrane and OPA1 in the outer mitochondrial membrane control fusion events, whereas the protein Drp1 (DNM1L) mediates fission events [20,21]. Alternatively any damaged mitochondrial sections are isolated via fission and removed by an autophagy variant, which is also referred to as mitophagy [22]. The proteins p62 (NUP62) and LC3 (MAP1LC3B) can function as markers for mitophagy and autophagy and can give an estimate of the quality of mitochondria [23]. In cases where mitochondrial function can no longer be maintained, programmed cell death, apoptosis, is initiated. Apoptosis is tightly regulated by proteins such as the pro-apoptotic factor BAX [24].

Metabolic fuel regulation, also known as the metabolic shift or the Warburg effect [25], is here represented by several proteins. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and lactate dehydrogenase (LDH) are central enzymes in cytosolic carbon metabolism, and isocitrate dehydrogenase 2 [NADP] (IDH2) represents the citric acid cycle. GAPDH, the sixth enzyme in glycolysis, is tightly regulated under normal conditions, but is deregulated in events of metabolic and oxidative stress [26]. IDH2 is a vital part of the mitochondrial antioxidant system due to its ability to generate NADPH [27].

The respiratory chain located in the mitochondria provides the cell with energy in the form of ATP through OXPHOS [28], and its activity is partly dependent on the amount of proteins composing the respiratory complexes and their assembling factors [29], two of which are SURF1 of complex IV and BCS1L of complex III [30,31]. Fatty acid oxidation plays a pivotal role in energy homoeostasis and is an essential complement to glucose oxidation [32]. The fatty acid β-oxidation pathway is a cyclic process and medium-chain acyl-CoA dehydrogenase (MCAD) is positioned centrally in this degradation cycle; further, MCAD has been described to be regulated by feeding state [33]. β-Oxidation of long-chain fatty acids is dependent on the synthesis of carnitine esters [32], and carnitine palmitoyltransferase (CPT) 1 catalyzes a rate-controlling step [34]. However, the electron transfer proteins (ETF) (α- and β) can also contribute to the control of fatty acid β-oxidation flux [35] and are also involved in the mitochondrial metabolism of branched chain amino acids; therefore, these were also included in the protein assay.

The method of choice used to monitor the above-mentioned proteins was SRM on a triple quadrupole MS, which has gained increasing interest in recent years [36]. Hitherto, a limitation in the implementation of the triple quadrupole mass spectrometer in quantitative proteomics has been the development of robust SRM assays. The SRM methodology can provide protein quantitation, and measure a given set of proteins with high sequence specificity, reproducibility, and precision [37]. In the present study, we developed an SRM method for detecting 26 selected proteins. The method was supported by the use of heavy labeled peptide standards and was technically optimized and validated. We successfully applied this method on two different kinds of biological samples, namely cultivated skin fibroblasts and peripheral blood mononuclear cells. Our study shows that this method holds promise to be a strong tool in studies of mitochondrial and cellular stress in the future.

2. Materials and methods

2.1. Reagents and materials

Acetonitrile (AcN), methanol, formic acid and trifluoroacetic acid (TFA) were LC grade. Ultra LC/MS water (Baker analyzed) was used; heavy labeled peptide analogues were cysteine carbamidomethylated with heavy labeled arginine and lysine (JPT Technologies, Germany). Any-KD Tris-HCl Criterion TGX pre-cast polyacrylamide gel (Bio-Rad) were electrophoresed in Tris-Glycine 0.1% SDS (sodium dodecyl sulfate) buffer, and TCEP (tris(2-carboxyethyl)phosphine), ammonium bicarbonate, iodoacetamide, trypsin gold (Promega), and PepClean C_{18} spin columns (Pierce, Thermo Scientific) were applied for sample treatment.

2.2. Method development – peptide selection

The SRM method was developed using the open-source software Skyline [38]. The selected proteins for this study were imported into Skyline for tryptic in silico digestion, where the tryptic cleav-