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Research paper

Differential proteomic and oxidative profiles unveil dysfunctional protein import to adipocyte mitochondria in obesity-associated aging and diabetes

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ABSTRACT

Human age-related diseases, including obesity and type 2 diabetes (T2DM), have long been associated to mitochondrial dysfunction; however, the role for adipose tissue mitochondria in these conditions remains unknown. We have tackled the impact of aging and T2DM on adipocyte mitochondria from obese patients by quantitating not only the corresponding abundance changes of proteins, but also the redox alterations undergone by Cys residues thereof. For that, we have resorted to a high-throughput proteomic approach based on isobaric labeling, liquid chromatography and mass spectrometry. The alterations undergone by the mitochondrial proteome revealed aging- and T2DM-specific hallmarks. Thus, while a global decrease of oxidative phosphorylation (OXPHOS) subunits was found in aging, the diabetic patients exhibited a reduction of specific OXPHOS complexes as well as an up-regulation of the anti-oxidant response. Under both conditions, evidence is shown for the first time of a link between increased thiol protein oxidation and decreased protein abundance in adipose tissue mitochondria. This association was stronger in T2DM, where OXPHOS mitochondrial- *vs*. nuclear-encoded protein modules were found altered, suggesting impaired mitochondrial protein translocation and complex assembly. The marked down-regulation of OXPHOS oxidized proteins and the alteration of oxidized Cys residues related to protein import through the redox-active MIA (Mitochondrial Intermembrane space Assembly) pathway support that defects in protein translocation to the mitochondria may be an important underlying mechanism for mitochondrial dysfunction in T2DM and physiological aging. The present draft of redox targets together with the quantification of protein and oxidative changes may help to better understand the role of oxidative stress in both a physiological process like aging and a pathological condition like T2DM.

Abbreviations: 2D, Second-Dimension; ACN, acetonitrile; BMI, Body Mass Index; BN, Blue-Native; DAPs, Differentially Abundant Proteins; ETC, Electron Transport Chain; FDR, False Discovery Rate; GO, Gene Ontology; IAA, Iodoacetamide; IR, Insulin Resistance; iTRAQ, isobaric Tags for Relative and Absolute Quantification; LC-MS, Liquid-Chromatography coupled to Mass Spectrometry; MCX, Mixed-mode Cationic eXchange; MIA, Mitochondrial Intermembrane space Assembly; mtDNA, mitochondrial DNA; oxCys, oxidized Cys; OXPHOS, Oxidative Phosphorylation; RIPA, RadioImmunoPrecipitation Assay buffer; ROS, Reactive Oxygen Species; SD, Standard Deviation; SVF, Stromal-Vascular Fraction; T2DM, Type 2 Diabetes Mellitus; TCA, TriCarboxilic Acid cycle; TEAB, TriEthylAmmoniumBicarbonate; TFA, TriFluoroacetic Acid; VAT, Visceral Adipose Tissue; WB, Western-Blot; Zc, standardized log2 ratio at the Category level; Zp, standardized log2 ratio at the Peptide level; Zq, standardized log2 ratio at the Protein level

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1. Introduction

The mitochondrion harbors crucial cellular processes including ATP supply, metabolites generation for cytosolic processes, amino acid catabolism, ketogenesis and urea cycle, ion homeostasis, calcium cycling, oxygen sensing and other cell signaling pathways such as autophagy/mitophagy and apoptosis [1], and therefore it is considered a central integrator of the homeostatic signals in the organism [2]. Additionally, mitochondria enclose main cellular generators of reactive oxygen species (ROS), such as the components of the respiratory chain, a number of redox enzymes and potent anti-oxidative defense systems [1,3] which make this organelle a key player in cellular redox homeostasis.

Obesity and obesity-associated pathologies have long been related to mitochondrial dysfunction [4]. Obesity is the most prevalent metabolic disease worldwide and is caused by an augmentation of the body fat due to a disproportion between energy uptake and expenditure [5]. White adipose tissue, the main adiposity regulator, is not merely a fat reservoir, but also a complex, essential endocrine organ [6]. Detrimental accumulation of the major intra-abdominal fat depot, the visceral adipose tissue (VAT), significantly increases the risk for metabolic comorbidities, like insulin resistance (IR) and type 2 diabetes mellitus (T2DM) [7].

Obesity-driven IR clinical manifestations initiate when insulin target cells, such as adipocytes, inadequately respond to insulin, and over time T2DM pathology emerges [8]. Some of the key factors contributing to IR are: i) excessive nutrient supply to adipocytes, which leads to ROS production; ii) pro-inflammatory processes; iii) endoplasmic reticulum stress; iv) cell aging; and v) altered mitochondrial dynamics (such as fusion and fission events) [9–11]. Thus, mitochondrion is both the origin and target of multiple metabolic signals whose integration maintains insulin sensitivity. Importantly, it has been suggested that mitochondrial redox signaling has a key role in white adipose tissue, regulating different processes such as adipocyte differentiation or adiponectin secretion through the modulation of redox-sensitive transcriptional factors [12,13]. Not surprisingly, given that white adipose tissue is the greatest endocrine organ in the human body any alteration in adipocyte mitochondria could result in significant homeostatic disturbances.

Although most mitochondrial functions are ubiquitous to all tissues, tissue-specific functions and tissue-specific control of mitochondrial capacity have been extensively reported [14]. Despite that adipose tissue in involved in regulating energy expenditure and insulin signaling [15], there is limited understanding of the relevance of mitochondria in this organ, mainly due to the scarcity of this organelle in white adipocytes [16].

We have linked obesity to the down-regulation of mitochondrial functions [17]. In this work we have extended our previous results, exploring the adipocyte mitochondrial proteome to further investigate, in obesity, the influence of a physiological process, aging, and a pathological process, T2DM. For that, we have relied on a high-throughput multiplexed proteomic approach with isobaric labeling followed by liquid chromatography coupled to mass spectrometry (LC-MS) analysis, which allows the simultaneous identification and quantification of proteins. It is noteworthy that very few proteomic studies based on human mitochondria have been reported [18–21]. Using visceral adipocytes, Lindinger et al. [20] identified 62 mitochondrial proteins associated with obesity. Similarly to our study, the authors resorted to MS for protein identification, although the quantification relied on immunoblot analysis.

Since oxidation and reduction of thiol proteins are one of the major mechanisms by which reactive oxidants integrate into cellular signal pathways [22], a number of proteomic technologies have been recently developed for the enrichment, identification, and characterization of thiol-based redox modifications, what is known as redox proteomics [23,24]. Cys is considered the most important redox-responsive amino acid due to its chemical properties and the range of its different oxidation states, together with its involvement in highly conserved functional positions in proteins. Additionally, due to their relevance and specificity, reversible Cys modifications have been suggested to play a main role in the regulation of protein activity and signal transduction [25,26]. In spite of their enormous potential, the function of oxidative modifications under physiological and pathophysiological conditions remains largely unknown. For these reasons, in this work we have further coupled our proteomic analysis with the study of dynamic alterations of oxidized Cys residues (oxCys) using a GELSILOX-based method [27].

Here, the application of state-of-the-art redox proteomics approaches to obese adipose tissue has uncovered a significant increase of Cys oxidation levels in oxidative phosphorylation (OXPHOS) subunits and, remarkably, an inverse correlation between Cys oxidation levels and protein abundance in both aging and T2DM conditions. Our results suggest impaired assembly of mitochondrial complexes together with defective import of nuclear-encoded proteins to the mitochondrion in diabetic patients. As far as we know, this work constitutes the most comprehensive depiction of the human mitochondrial proteome to date, as well as the first assessment of redox changes in adipocyte mitochondria.

2. Material and methods

2.1. Ethic statement

The study was conducted according to the recommendations of the Declaration of Helsinki and was approved by the Ethics Committees of Hospital Clínico San Carlos and Hospital Gregorio Marañón (Madrid, Spain). Signed informed consent was obtained from all subjects.

2.2. Biological samples

VAT samples were collected from 32 obese women (Body Mass Index, BMI≥40 kg/m^2) who underwent bariatric surgery. All the patients were of Caucasian origin. The surgeon aimed to obtain the samples at the beginning of the surgery and from the same anatomical location (*omentum*) in all patients. None of the non-diabetic patients suffered T2DM or other obesity-associated comorbidity (hypertension, dyslipidemia, obstructive sleep apnea syndrome or cardiovascular disease). Inclusion criterion for the diabetic group was suffering T2DM for at least two years. T2DM was defined by fasting plasma glucose $\geq$ 7 mmol/L and HbA1c $\geq$ 6.5%. All T2DM subjects were being treated with oral anti-diabetic drugs and in one case with insulin in order to control the comorbidities. Exclusion criteria encompassed: i) clinically significant hepatic, neurological, or other major systemic disease, including malignancy; ii) history of drug or alcohol abuse, defined as > 80 g/day, or serum transaminase activity more than twice the upper normal range limit; iii) elevated serum creatinine concentrations; iv) acute illnesses and current evidence of chronic inflammatory or infectious diseases; and v) mental illness rendering the subjects unable to understand the scope of the analysis.

2.3. Separation protocol

After sample collection, fresh VAT was suspended in Hank's Balanced Medium 199 (Cat. 22350, GIBCO®-Life Technologies) and immediately processed for adipocytes and stromal-vascular fraction (SVF) separation according to standard procedures. Briefly, 6g of adipose tissue were washed three times with PBS and minced until 1–2 mm^3 pieces were obtained. Digestion was carried out in 0.2% Collagenase type I (M0A3689, Worthington Biochemical Corp.) solution in PBS for 60 min in water bath at 37 °C. The enzyme was inactivated with fetal bovine serum (FBS) and the suspension was centrifuged at 1,000 rpm for 10 min. Three different phases could be