Redox Biology 13 (2017) 244–254

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research Paper

# Pharmacologic concentrations of linezolid modify oxidative phosphorylation function and adipocyte secretome

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## ARTICLE INFO

*Keywords:*
Linezolid
Oxidative phosphorylation
Adipocyte
Secretome
Apolipoprotein E
Adipokine

## ABSTRACT

The oxidative phosphorylation system is important for adipocyte differentiation. Therefore, xenobiotics inhibitors of the oxidative phosphorylation system could affect adipocyte differentiation and adipokine secretion. As adipokines impact the overall health status, these xenobiotics may have wide effects on human health. Some of these xenobiotics are widely used therapeutic drugs, such as ribosomal antibiotics. Because of its similarity to the bacterial one, mitochondrial translation system is an off-target for these compounds. To study the influence of the ribosomal antibiotic linezolid on adipokine production, we analyzed its effects on adipocyte secretome. Linezolid, at therapeutic concentrations, modifies the levels of apolipoprotein E and several adipokines and proteins related with the extracellular matrix. This antibiotic also alters the global methylation status of human adipose tissue-derived stem cells and, therefore, its effects are not limited to the exposure period. Besides their consequences on other tissues, xenobiotics acting on the adipocyte oxidative phosphorylation system alter apolipoprotein E and adipokine production, secondarily contributing to their systemic effects.

## 1. Introduction

The oxidative phosphorylation system (OXPHOS) includes the electron transport chain (ETC) respiratory complexes I to IV (CI-CIV) and the ATP synthase (complex V, CV). Several OXPHOS subunits are encoded in the mitochondrial DNA (mtDNA). This molecule also codes for 2 ribosomal (rRNAs) and 22 transfer (tRNAs) RNAs needed for the expression of the 13 mtDNA-encoded polypeptides. Mature cells require OXPHOS function to perform many of their activities but it is also important for cell differentiation.

Genetic manipulation of the OXPHOS system affects adipocyte differentiation. This manipulation appears to be accompanied by a defect in adipokine secretion. Thus, it has been reported that a mutated thymidine kinase 2 (TK2) knock-in mouse showed mtDNA depletion in white adipose tissue and reduced fat accumulation. There was also a severe reduction in leptin mRNA and circulating protein levels [1]. Mouse 3T3-L1 cells knocked down for the mitochondrial transcription factor A (TFAM) showed a decrease in mtDNA copy number, levels of ETC subunits, CI and CIV activities, and oxygen consumption. These cells also showed a diminished adiponectin expression at both gene and secreted protein levels [2]. Adiponectin mRNA expression was also decreased in TFAM knocked down human mesenchymal stem cells (hMSCs) [3]. Mouse 3T3-L1 or adipose tissue derived stem (mASCs) cells that lacked the CR6/gadd45-interacting protein Gadd45gip1/

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http://dx.doi.org/10.1016/j.redox.2017.05.026
Received 19 May 2017; Received in revised form 29 May 2017; Accepted 30 May 2017
Available online 31 May 2017

Crif1, a translation/assembly factor for mtDNA-encoded polypeptides, expressed lower levels of mtDNA-encoded subunits and displayed disrupted adipocyte differentiation, accompanied by a reduced adiponectin expression [4].

Physiologic manipulation of OXPHOS can also affect adipokine secretion. Oxygen is the ultimate electron acceptor in the ETC and hypoxia restrained adipogenic differentiation in both mouse preadipocytes and hMSCs [3,5]. It has been found that adiponectin and leptin release was increased and decreased, respectively, in human adipocytes differentiated at 10% oxygen compared with 21% [6].

We have previously shown that some OXPHOS xenobiotics could alter the adipocyte differentiation of human adipose tissue-derived stem cells (hASCs) [7]. In addition to genetic and physiologic intervention, chemical manipulation of OXPHOS can also affect adipokine secretion. It was found that CI inhibitor rotenone decreased adiponectin secretion of rat adipocytes and adiponectin mRNA expression in hMSCs [3,8]. Capsaicin, another CI inhibitor, decreased leptin and increased adiponectin expression in 3T3-L1 adipocytes [9]. The CIII inhibitor antimycin A reduced adiponectin mRNA levels in mouse 3T3-L1 cells [10]. The CV inhibitor oligomycin diminished adiponectin mRNA levels and secreted adiponectin in mouse 3T3-L1 cells [2,10].

Some OXPHOS xenobiotics are widely used therapeutic drugs. For example, nucleoside reverse transcriptase inhibitors (NRTIs) are used against the human immunodeficiency virus (HIV), the agent causing the acquired immune deficiency syndrome (AIDS). However, these drugs can also inhibit the DNA polymerase gamma (POLG), the enzyme required for mtDNA replication. Thus, OXPHOS function was reported as a common target of NRTI toxicity [11]. NRTIs reduced adipocyte differentiation and leptin secretion by hASCs-derived adipocytes [7]. They also decreased adiponectin mRNA expression and protein secretion in mouse 3T3-L1 and 3T3-F442A cells and in primary human subcutaneous preadipocytes [12–14]. Systemic adiponectin levels were also reduced in patients under antiretroviral therapy [15]. The ribosomal antibiotic linezolid (LIN) is considered a promising option in the treatment of multidrug resistant tuberculosis [16]. Unfortunately, side effects, such as myelosuppression, lactic acidosis and optical and peripheral neuropathy have been associated to this antibiotic [16]. At concentrations below the steady-state peak serum concentration [17], LIN inhibited hASCs mitochondrial protein synthesis and decreased the triglycerides (TGs) amount and secreted leptin in hASCs-derived adipocytes [7].

Adipokines modulate many metabolic pathways and have a broad range of systemic actions [18]. The previous observations indicated that an OXPHOS blockage modifies the secretion of some adipokines. Then, an adipocyte OXPHOS disruption caused by off-target effects of therapeutic drugs might affect people health status. Therefore, we analyzed the effect of LIN on adipocyte secretome to uncover factors whose secretion was altered.

## 2. Materials and methods

### 2.1. Chemicals

All reagents used were of research or cell culture quality. Chloramphenicol (CAM) and LIN were purchased from Sigma-Aldrich (St Louis, MO, USA).

### 2.2. Cells, growth and differentiation conditions

StemPro® Human Adipose-Derived Stem Cells (#R7788-115, Gibco™, Thermo Fischer Scientific) derive from human adipose tissue collected during liposuction procedures and were cryopreserved at passage 1 from primary cultures. Each lot of hASCs originates from a single donor of human lipoaspirate tissue, and the two previously characterized lots used here [7], are named hASCs-1 and hASCs-2. In some experiments, we used hASCs derived from 13 different donors, after a signed informed consent was obtained [19].

These cells were generally grown in MesenPRO RS™ medium (Gibco™, Thermo Fischer Scientific), containing 5 mM glucose and 2% fetal bovine serum (FBS) complemented with MesenPRO RS™ growth supplement and 2 mM L-glutamine prior to use. To avoid undesired phenotypic effects, cells were grown in the absence of antibiotics [20].

To induce adipogenic differentiation, confluent hASCs were incubated for 21 days with StemPro® Adipogenesis Differentiation Kit (Gibco™), as previously described [7].

### 2.3. Analysis of adipocyte differentiation

Intracellular lipids were stained with the hydrophilic stain Nile Red that, when partitioned in a hydrophobic environment, becomes fluorescent. For quantitative determination of Nile Red fluorescence, the NovoStar MBG Labtech microplate instrument was used (Ex: 485 nm / Em: 572 nm).

Adipogenesis Detection Kit (Abcam) was used to quantify TGs accumulation in cells according to the manufacturer's instructions. In this assay, TGs are solubilized and hydrolyzed to glycerol, which is subsequently oxidized to convert the probe to generate color ($\lambda_{max}$ = 570 nm). A NovoStar MBG Labtech microplate instrument was used for measurements.

For quantitative determination of adiponectin and leptin in cell culture supernatants, Human Adiponectin ELISA kit (Millipore) and Leptin Human ELISA Kit (Abcam) were used [7], according to the manufacturer's instructions.

For immunocytochemistry, the cultured cells were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 for 10 min. After blocking with 0.1% bovine serum albumin (BSA), the washed cells were incubated for 1 h at room temperature with a primary antibody against APOE (Abcam). Subsequently, the cells were incubated with fluorescence-labeled secondary Alexa Fluor® 594 (Molecular Probes) at room temperature for 30 min, protected from light. The cells were further incubated with 1 μM 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining. Between incubations, samples were washed with phosphate buffered saline (PBS) containing 0.05% Tween.

### 2.4. Analysis of mitochondrial function

The enzymatic activity of citrate synthase (CS) was assayed following previously described protocols [21]. Protein concentration was determined by the Bradford protocol (#500-0006; Bio-Rad) [22]. CIV activity and levels were determined using the Complex IV Human Specific Activity Microplate Assay Kit (Mitosciences, Abcam®) according to the manufacturer's instructions.

### 2.5. Genetics characterization and gene expression analysis

*APOE* genotyping was performed by polymerase chain reaction (PCR) amplification and sequencing using primers and conditions described elsewhere [23,24]. These sequences were obtained using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applera Rockville, MD, USA) and an ABI Prism 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA).

For quantitative determination of the percentage of 5-methylcytosine (5-mC) in hASCs genome, the MethylFlash™ Methylated DNA Quantification Kit (Epigentek) was used, following the manufacturer's instructions.

To assess the methylation levels of the *APOE* gene, bisulfite conversion of genomic DNA (500 ng each) was carried out using the EZ DNA Methylation™ Kit (Zymo Research) according to the manufacturer's protocol. PCR was carried out with 100 ng of bisulfite-converted DNA, using the Pyromark PCR Kit (Qiagen) and the primers described elsewhere [25]. PCR products were purified using streptavidin-coated