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Thermogenic flux induced by lignoceric acid in peroxisomes isolated from HepG2 cells and from X-adrenoleukodystrophy and control fibroblasts

Anna Petroni1,2 | Rita Paroni3 | Anna Maria Aloisi4 | Milena Blasevich1,2 | Nabil Haman5 | Dimitrios Fessas5

Abstract
This work analyzes the thermogenic flux induced by the very long-chain fatty acid (VLCFA) lignoceric acid (C24:0) in isolated peroxisomes. Specific metabolic alterations of peroxisomes are related to a variety of disorders, the most frequent one being the neurodegenerative inherited disease X-linked adrenoleukodystrophy (X-ALD). A peroxisomal transport protein is mutated in this disorder. Due to reduced catabolism and enhanced fatty acid (FA) elongation, VLCFA accumulates in plasma and in all tissues, contributing to the clinical manifestations of this disorder. During peroxisomal metabolism, heat is produced but it is considered lost. Instead, it is a form of energy that could play a role in molecular mechanisms of this pathology and other neurodegenerative disorders. The thermogenic flux induced by lignoceric acid (C24:0) was estimated by isothermal titration calorimetry in peroxisomes isolated from HepG2 cells and from fibroblasts obtained from patients with X-ALD and healthy subjects. Heat flux induced by lignoceric acid in HepG2 peroxisomes was exothermic, indicating normal peroxisomal metabolism. In X-ALD peroxisomes the heat flux was endothermic, indicating the requirement of heat/energy, possibly for cellular metabolism. In fibroblasts from healthy subjects, the effect was less pronounced than in HepG2, a kind of cell known to have greater FA metabolism than fibroblasts. Our hypothesis is that heat is not lost but it could act as an activator, for example on the heat-sensitive pathway related to TRVP2 receptors. To investigate this hypothesis we focused on peroxisomal metabolism, considering that impaired heat generation could contribute to the development of peroxisomal neurodegenerative disorders.

Keywords
beta-oxidation, heat, isothermal calorimetry, peroxisomal disorders, very long-chain fatty acid (VLCFA), X-linked adrenoleukodystrophy

Abbreviations: ALDP, ABC half-transporter; VLCFA, very long-chain fatty acid; X-ALD, X-linked adrenoleukodystrophy.
1 | INTRODUCTION

Peroxisomes are organelles with complex and differentiated enzymatic activities. The importance of peroxisomes emerged due to the growing understanding that the alterations of specific metabolic pathways, particularly those concerning lipid metabolism, were associated with various peroxisomal disorders, characterized by a variety of genotypes and clinical manifestations, most of which lead to progressive neurodegeneration (De Duve & Bauduin, 1966; Igarashi et al., 1976; H. W. Moser, 1997).

X-linked adrenoleukodystrophy (X‐ALD) is the most common peroxisomal disorder (Bezman et al., 2001; H. W. Moser, 1997). The mutated gene ABCD1 (Xq28) encodes for the peroxisomal ABC transporter (ALDP; J. Mosser et al., 1993).

Because of ALDP dysfunction, the degradation of very-long-chain fatty acids (VLCFA) to shorter fatty acid (FA), in a process called peroxisomal beta‐oxidation, is impaired, with consequent abnormal accumulation of the VLCFA lignoceric acid (C24:0) and hexacosanoic acid (C26:0) in plasma and all tissues (Singh, Moser, Goldfischer, & Moser, 1984; Tsuji, Ohno, Miyatake, Suzuki, & Yamakawa, 1984). VLCFA accumulation is also partly due to the enhancement of their elongation from shorter activated FA (Kemp, Berger, & Aubourg, 2012; Van den Bosch, Schutgens, Wanders, & J. M. Tager, 1992).

VLCFA are considered the biochemical markers of the pathology. Their abnormal accumulation is ubiquitous, although it mainly affects the nervous system, adrenal cortex, and testis, indicating a secondary alteration in steroid hormone homeostasis (Assies, van Geel, & Barth, 1998; Petroni, Cappa, Blasevich, Solinas, & Uziel, 2004). Other metabolic processes, including oxidative processes, the development of secondary inflammatory pathways, typical of neurodegenerative, and in general long‐lasting disorders, contribute to the clinical manifestation of X‐ALD (Uttara, Singh, Zamboni, & Mahajan, 2009).

Nevertheless, the complete molecular mechanisms leading to the progressive neurodegenerative clinical manifestations of X‐ALD are not clearly understood.

During metabolic processes, peroxisomes do not produce ATP but develop heat, which is considered to be dispersed in the cell (Schulz, 1996). Heat is a form of energy that could be useful for cellular maintenance and it might have a role in the pathogenesis of peroxisomal disorders (Petroni, 2013).

To investigate heat/energy expenditure of peroxisomes, we used isothermal microcalorimetry, a sensitive tool that can provide a variety of kinetic and thermodynamic information on complex biological systems and thus can help in the description of complex metabolic steps.

For this purpose, we first tested the procedures in peroxisomes isolated from HepG2 cells, as described (Santos, Ojeda, Garrido, & Leighton, 1985; Watkins, Ferrel, Pedersen, & Hoeffer, 1991), and then applied them to fibroblasts obtained from patients with X‐ALD and healthy subjects (controls).

Since VLCFA peroxisomal beta‐oxidation is impaired in X‐ALD fibroblasts, the aim of our research was to analyze the thermogenic flux induced by the VLCFA lignoceric acid (24:0) in peroxisomes isolated from cultured X‐ALD and control fibroblasts to determine if impaired heat generation plays a role in X‐ALD lipid metabolism.

2 | MATERIALS AND METHODS

2.1 | Patients and cell culture

Human fibroblasts (control and X‐ALD) were used as in a previous study and according to established institutional guidelines (Petroni et al, 2004). In brief, skin biopsies from three males affected by X‐ALD were obtained thanks to the kind assistance of Dr. Graziella Uziel (Istituto Neurologico Carlo Besta, Milan) and Dr. Marco Cappa (Ospedale Bambino Gesù, Rome, Italy). The age of the patients was 20 to 45 years; controls were healthy males without metabolic disease and of matching age. The diagnosis of X‐ALD was based on molecular genetic testing, clinical examination, brain MRI, and plasma VLCFA.

The standards and the materials for tissue culture and analyses were from Sigma‐Aldrich (St. Louis, MO). Fibroblasts were subcultivated and grown in minimum essential medium containing 10% heat‐inactivated fetal bovine serum (100 U/ml), 100 µg/ml streptomycin, and l‐glutamine, and they were maintained at 37°C, in 95% air and 5% CO2. Cells were frozen according to standard procedures and kept in liquid nitrogen.

Fibroblasts, used at passages below seven, were thawed and expanded. Cells were used at confluency, which was reached within 1 week. Cells were fed every 2 to 3 days with fresh growth medium. The normal growth was constantly checked under an optical microscope. Protein/lipid measurements and cell counting were performed at confluency.

2.2 | Homogenization of HepG2 cells and fibroblasts from X‐ALD patients and controls

HepG2 cells and fibroblasts from confluent 10 cm diameter dishes were harvested by trypsinization, washed with culture medium and then with phosphate buffered saline (PBS). The cell suspensions were centrifuged at 1,000 g for 8 min and the pellet was resuspended in PBS and centrifuged at 1,000 g for 8 min. The resulting pellet was homogenized in 250 mM sucrose, 1 mM Tris (Cl), pH 7.5, and 1 mM ethylenediaminetetraacetic acid with a Dounce or Potter‐Elvejhem tissue grinder, followed by centrifugation at 500 g for 5 min. The subsequent steps for peroxisome isolation and evaluation of the peroxisomal marker enzyme catalase were according to Santos et al. (1985) and Watkins et al. (1991). The experiments for calorimetry and VLCFA measurement were performed with protein concentrations ranging from 35 to 400 µg/ml according to Bradford (1976).

2.3 | Lignoceric acid incubation and isothermal titration calorimetry

Lignoceric acid was dissolved in ethanol, dried under N2 stream, dissolved in alpha‐cyclodextrin in 10 mM Tris‐HCl and sonicated
according to standard procedures (Watkins et al., 1991). In all the reported experiments, the final concentration of lignoceric acid was 10 µM.

The preparations, diluted to the chosen protein concentration, were placed in the calorimetry instrument for 2 hr, after which the aliquot of lignoceric acid was added.

Details of the calorimetric method and instrument are reported elsewhere (Fessas & Schiraldi, 2017; Gardikis et al., 2017). Measurements were performed at 37°C. A 5 ml suspension containing a peroxisome preparation with protein concentration ranging from 35 to 40 µg/ml was loaded in the measurement vessel under bland steering. After a 2-hour instrumental equilibration, lignoceric acid was added by injection to achieve the final concentration of 10 microM within the calorimetric vessel. The error on the overall thermal effect was below 6% based on three replications for each experiment.

2.4 | VLCFA measurement

At the end of the incubations, the preparations were washed with PBS, lipids were extracted according to Folch, Lees, and Sloane (1957). FA methyl esters were prepared according to Petroni, Blasevich, and Uziel (2003) and injected into a gas chromatograph (DANI GC 1000) with a Supelco Omegawax column (30 m). Further identification was carried out by liquid chromatography–mass spectrometry according to Banni et al. (2004).

2.5 | Statistical analysis

Statistical evaluation was performed by two-way analysis of variance followed by Student’s t test.

### 3 | RESULT

3.1 | Isothermal titration calorimetry

We used HepG2 peroxisomes for comparison because they have very active VLCFA beta-oxidation. Figure 1 shows the isothermal titration calorimetry thermograms (heat flow vs. time) of peroxisomes isolated from HepG2 cells and from X-ALD and healthy fibroblasts. Isolated peroxisomes were incubated in independent experiments with lignoceric acid (10 µM) at the peroxisomal protein concentration of 40 µg/ml. When peroxisomes were incubated with lignoceric acid vehicle or only buffer without the organelles, no signal was detected by microcalorimetry.

The thermogenic curve from HepG2 peroxisomes showed that heat flux was exothermic, with a consistent amplitude, indicating normal heat production.

In contrast, the curves of peroxisomes from X-ALD fibroblasts, incubated in the same conditions, showed an opposite effect with heat adsorption (endothermic) and a delay in the onset time of heat flux.

The observed endothermic flux reveals the requirement of this form of energy by the organelles and its detection was related to the reduction of heat production in this system. Indeed, the heat flux of peroxisomes isolated from controls showed a possible balance between exothermic and endothermic activity.

The metabolic thermogenic curves of control and X-ALD peroxisomes showed less amplitude than those of HepG2 peroxisomes. This is in agreement with HepG2 cellular characteristics, in that they present elevated lipid metabolic activity, in particular concerning VLCFA beta-oxidation, in comparison with fibroblasts (Watkins et al., 1991).

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**FIGURE 1** Representative ITC thermograms (heat flow vs. time) of peroxisomes isolated from HepG2 cells and from X-ALD and control fibroblasts, at a protein concentration of 40 µg/ml. X-ALD1 and X-ALD2 represent two independent experiments. The experiments were performed more than three times for each kind of cell. ITC: isothermal titration calorimetry; X-ALD: X-linked adrenoleukodystrophy.
3.2 VLCFA measurement

VLCFA beta-oxidation, expressed as 22:0/24:0, was evaluated with the same concentration of agonist, at 200 µg/ml proteins (Figure 2). The ratio was significantly reduced in X-ALD preparations with respect to control and HepG2 organelles. This is in agreement with the extensive literature, since VLCFA beta-oxidation is impaired but not absent in the X-ALD phenotype.

4 DISCUSSION

The main result of our study is the endothermic effect induced by lignoceric acid in X-ALD peroxisomes in comparison to controls and HepG2 peroxisomes. This indicates a reduction of heat/energy production and the requirement of energy by X-ALD organelles. The reduction was related to VLCFA beta-oxidation impairment, as shown here and reported in the literature (Igarashi et al., 1976).

In stimulated HepG2 the effect was exothermic, indicating normal heat/energy production, and was more enhanced than in control fibroblasts. This was related to normal VLCFA beta-oxidation, which is more efficient in the HepG2 cell line than in fibroblasts (Watkins et al., 1991).

VLCFA accumulation in all tissues, in particular in the brain, has been considered a major determinant in X-ALD pathogenesis. Their accumulation is due to reduced peroxisomal beta-oxidation and the enhancement of FA elongation. Peroxisomal beta-oxidation could be a heat-dependent system. Heat could sustain this pathway or cellular metabolism in general, influencing for example the velocity or the rate of specific metabolic pathways.

Specific elongases are required for FA elongation. These pathways, enhanced in X-ALD, could require a low level of heat/energy, thus being more facilitated with respect to beta-oxidation metabolism.

We can also speculate that the observed thermodynamic modifications could play a role in the activity of the ABC peroxisomal transporters, affecting dimerization processes of the mutated ALDP and related proteins and mechanisms underlying X-ALD pathogenesis.

Heat is generated in a variety of cellular metabolic pathways, although the energy reserve is rarely considered in biological studies, even though it could modulate different parameters. The heat produced by peroxisomes is considered lost (Petroni, 2013; Schulz, 1996), whereas it could have a role in X-ALD pathogenesis.

5 CONCLUSION

This is the first publication reporting data on a possible role of heat flux in X-ALD cell metabolism. The molecular mechanisms underlying this disorder and related neurodegenerative disorders are not clearly understood. The extensive literature has demonstrated that the serious dysfunctions characterizing this pathology are partly caused by VLCFA accumulation. However, the modification of heat generation during peroxisomal metabolism should be considered for an understanding of X-ALD and neurodegenerative disorders.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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