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Starvation of Jurkat T cells causes metabolic switch from glycolysis to lipolysis as revealed by comprehensive GC-qMS

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Abstract

T cells play a central role in the cellular part of the immune system and their metabolomic activity is strictly bound to the status of activation. Little is known about the metabolomic resilience of T helper cells in the resting status. Therefore we analyzed the metabolomic profile of non-activated Jurkat T cells under normal and starvation conditions by a two-dimensional GC-MS approach. We detected 52 organic and amino acids in the biological replicates covering the majority of central metabolic pathways. Under starvation conditions 21 analytes representing major metabolic pathways showed a significant down-regulation. For palmitoleic acid a significant up-regulation was detected. The annotation of differentially abundant metabolites to the pathways of TCA-cycle, amino acid metabolism and fatty acid biosynthesis indicating a metabolic switch from glycolytic to lipolytic energy generation upon starvation.

Keywords: Comprehensive GC-qMS; Metabolite profiling; Starvation; Jurkat T cells.

1. Introduction

In the postgenome era the focus shifts from the static blueprint to the factors that actually dominate the phenotype of a cell, proteins and metabolites. Both are linked to the genotype but are also regulated independently and therefore provide targets for the analysis of factors that can be both indicative and causative to a great variety of cellular processes [1]. Due to their central role for the cellular life most processes eventually influence the metabolome. In addition, the perspective on the metabolome as a mere consequence of catabolic and anabolic reactions is changing with the discovery of the different levels of crosstalk between metabolites and signalling pathways.

Furthermore, functions exerted by metabolites can be highly tissue specific. For example, glutamine is the most abundant amino acid in plasma and in skeletal muscle [2]. In contrast, glutamine serves in liver as a precursor for glucose and urea synthesis, whereas it is used in the brain in synthesis of neurotransmitters. Hence glutamine is reported to play a role in maintenance of skeletal muscle, immune systems function and glucose and glycogen metabolism [3]. As a consequence, the metabolome has to be analyzed in a cell-type specific manner. This is particularly true for the immune system in which the differentiation of cells leads to a great variety of cellular subtypes. In the present study Jurkat cells are used as a widely accepted model for the study of activation of T cells and the resulting effect on the metabolome [4].

T cells control the adaptive immune response to pathogens either by destruction of the antigen presenting cell directly (cytotoxic T cells) or by secretion of cytokines (T helper cells) which activate effector cells. T cells are activated by interaction of their T cell receptor (TCR) with an antigen-

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bound major histocompatibility complex containing an antigen-presenting cell [5, 6]. In addition to the TCR induction, T cells require additional activation signals from one or more costimulatory receptors, such as CD3 and CD28, to become fully active [7, 8].

Peripheral T cells which expand upon signal responses to foreign antigens spend the majority of their life span in a resting state. Therefore their metabolic resilience to suboptimal conditions is crucial for their survival. Besides changes in protein expression, changes in concentration of central metabolites during activation and differentiation of T cells are of particular interest. Since metabolites are the end products of cellular regulatory processes, their levels can be regarded as the ultimate response of biological systems to genetic or environmental changes. In order to tackle the question of metabolomic resilience of resting T cells we established a metabolomic analysis of Jurkat T cell grown under normal and starvation conditions as a model system to elucidate the cellular response towards suboptimal conditions.

For the analysis of the central carbon and nitrogen metabolites that are part of the glycolysis, pyruvate metabolism as well as the tricarboxylic acid cycle (TCA-cycle) and the amino acid synthesis methods are required that cope with the high polarity of the metabolites. GC-MS is a suitable technique for this purpose. Several GC-MS based metabolomics methods have been reported [9-13]. Most of these methods are based on one or more derivatization steps by oximation and/or silylation analysis prior to conversion of the polar functional groups. These methods are suitable for the analysis of cell extracts, body fluids and tissues and allow the measurement of a broad range of small, medium-polar to polar metabolites.

However, profiling analyses and quantification of target substances from complex biological samples is often hampered by insufficient chromatographic separation and/or matrix effects resulting from difficult sample matrices. Comprehensive two-dimensional gas chromatography (GCxGC) offers several advantages over one-dimensional gas chromatography, i.e. higher peak capacity, a broader dynamic range and lower detection limits. Metabolite profiling of differentially grown Jurkat T cells was therefore carried out on a comprehensive GCxGC quadrupole MS to characterize molecular changes in cellular systems by quantitative signatures of primary metabolites.

2. Material and Methods

2.1 *Materials and reagents*

Methanol and Chloroform were purchased from Merck (Darmstadt, Germany). Water was deionized and filtered through a 0.22 µm filter using a Millipore water generation system (Millipore, Schwalbach, Germany). Methoxyamine hydrochloride (MOX) and *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) were purchased from Sigma-Aldrich (Seelze, Germany).

2.2 *Cell culture*

Jurkat T cells clone E6-1 (TIB-152, ATCC, Manassas, VA, USA) were routinely cultured in RPMI-1640 medium (Biochrom AG., Berlin, Germany) containing 10% fetal bovine serum (Biochrom AG, Berlin, Germany), 1% L- Glutamine (Biochrom AG, Berlin, Germany) and 1% penicillin (100 U/mL)/ streptomycin (100 mg/mL) (PAA, Pasching, Austria) at an atmosphere of 5% CO_2, 95% humidity at 37°C in a CO_2 incubator (MCO-18AIC, Sanyo Electric Co Ltd, Gunma-ken, Japan). Jurkat cells were maintained between 1 x 10^5 and 1 x 10^6 cells/mL of medium in flasks with a surface area of 25 cm^2 (Greiner Bio-One, Solingen, Germany). For the experiment control cells were cultured in 75 cm^2 flasks as described earlier [14]. Starving cells were incubated in RPMI-1640 medium containing 1% L-Glutamine, 1% penicillin/streptomycin for three to four days in 75 cm^2 flasks under the conditions stated above.

Before and after starvation, cell numbers were recorded by cell counting after Trypan blue staining in a Neugebauer-chamber. After cultivation cells were spun down at 300*g* for 5 min, the medium was removed and the cell pellets were immediately shock-frozen in liquid nitrogen and stored at –80°C until further processing.

2.3 *Metabolite extraction*

In order to separate hydrophilic target analytes from lipophilic metabolites, phases of different polarity were obtained by performing a liquid-liquid extraction with ice cold methanol, chloroform and water. For comprehensive GCxGCqMS analysis frozen cell pellets were extracted using 0.5 mL methanol, 1 mL chloroform and 0.375 mL water. Cell disruption was performed for 5 min on ice using an ultrasonic homogenization device. The mixture was then centrifuged at 4000 *g* for 5 min at 4°C. 500 µL of the upper hydrophilic methanol/water phase was transferred into 2 mL reaction glass vials. The remaining lipophilic phase as well as protein precipitate was discarded. All samples were dried under a stream of nitrogen at 40 °C for a maximum of 2 h. First derivatization step of hydrophilic compounds was a methoximation using 200 µL MOX for 2 h at 80°C. After first derivatization step the fractions were dried under a stream of nitrogen at 40 °C and silylated using 100 µL MSTFA for 20 min at 90°C.

2.4 *GCxGC-qMS analysis and data processing*

Analyses were done in biological and technical duplicates by comprehensive gas chromatography in combination with a fast quadrupole mass spectrometer, GCxGCqMS. The dimension of the first column (Rtx-1; Restek) were, 30 m length, 0.25 mm i.D. with 0.25 µm film, of the second column (BPX50 (SGE)) 1 m length, 0.15 mm i.D. and 0.15 µm film. The oven was heated from 60°C to 290°C with a ramp of 5°C/mi. The modulation time was 6 sec and the hot pulse time 0.375 sec, respectively. The detection was performed in

Analyte	Fold change Starvation vs Normal	p-Value
4-Hydroxyproline	0.234	0.004
Glutamic acid	0.267	0.006
Fumaric acid	0.169	0.008
Glycolic acid	0.594	0.008
Methylsuccinic acid	0.380	0.009
Lactic acid	0.247	0.010
Succinic acid	0.235	0.010
Malic acid	0.267	0.011
Palmitoleic acid	2.738	0.012
Phenylalanine	0.391	0.012
Serine	0.518	0.013
Proline	0.188	0.014
Tyrosine	0.500	0.016
3-Hydroxybutyric acid	0.118	0.019
Urea	0.416	0.020
L-Glycine	0.238	0.026
a-Aminobutyric acid	0.343	0.031
2-Hydroxyglutaric acid	0.498	0.034
Isoluecine	0.440	0.036
5-Oxoproline	0.382	0.039
Aspartic acid	0.548	0.040
L-Alanine	0.242	0.048
Threonin	0.372	0.057
Asaparagin	0.064	0.067
Tryptophan	0.060	0.069
Glycerol	0.710	0.071
Methionine	0.314	0.073
Benzoic acid	0.612	0.113
Valine	0.446	0.121
Citric acid	0.728	0.133
Phenol	0.871	0.163
Leucine	0.486	0.168
Glutaric acid	0.330	0.199
3-Hydroxy-3-methylglutaric acid	0.249	0.204
Thymine	0.071	0.269
Oxalic acid	0.450	0.333
Palmitic acid	1.115	0.336
Caproic acid	0.733	0.366
Stearic acid	1.056	0.392
Myristic acid	0.690	0.397
Azelaic acid	0.871	0.427
Sebacic acid	0.879	0.458

Table 1. Summary of detected organic acids in Jurkat T cells after GCxGC-qMS analyses sorted according to ascending p-values. Italicized analyte names represent significant regaulated organic and amino acids.

Not detected in starvation cells
2-Hydroxybutyric acid
Sarcosine
2-Hydroxyisovaleric acid
3-Hydroxyisovaleric acid
Ethylhydracrylic acid
Phenylacetic acid
Maleic acid
3-Methylglutaconic acid
L-Glutamine
Hippuric acid
Indolelactic acid

full scan mode from 35 to 280 amu with 20.000 amu/sec. The chromatograms were recorded and processed by GCMSsolution software (Shimadzu). The identification of metabolites was performed by Excel based macro, taking spectra similarity, linear retention index and the relation between modulated peaks into account. Fold changes were calculated between the accumulated peak areas of modulated signals of starvation cultivation versus the normal cultivated Jurakt T cells. An unpaired, heteroscedastic, two-tailed Student's t-test was conducted on the peak areas for the comparison of starved and normal cultered cells. Metabolites with a p-value less than 0.05 and an average linear fold change higher than 1.5 were considered as significantly regulated.

3. Results and Discussion

Hydrophilic extracts gave reproducible characteristic chromatograms. In total, 42 metabolites from the classes of organic and amino acids could be detected and verified in the biological duplicates in normal and starvation cultivated cells. Identification of metabolites with a substance specific database was supported by use of linear retention time indices and the time-dependent relation between modulated GC -peaks. As a result of high retention time stability of the GCxGCqMS system, precise determination of linear retention time indices was possible. A typical chromatogram of an extract from Jurkat T cells grown under normal conditions is shown in Figure 1. In addition, eleven metabolites comprising among others sarcosine, phenylacetic and hippuric acid could be observed only in T cells grown under normal conditions. Obviously due to decelerated metabolism of T cells under starving conditions and generally lower signal intensities in the total ion chromatogram, these analytes were not detectable in samples derived from starving T cells (Table 1). Above all, also glutamine could only be observed in normal cultivated cells, even it was also supplied during cultivation,

which suggests an almost complete consumption cells under starvation.

Due to sample complexity several compounds were coeluting within the first separation dimension. Therefore, comprehensive GC-qMS was applied to increase the separation power of coeluting substances in the second dimension (Figure 2). Besides this, GCxGC separation leads to a better matrix separation and provides a basis for better spectra quality and library based identification of target analytes.

In metabolite extracts of control and starving Jurkat T cells clear differences in analyte intensities became evident. Due to decelerated metabolism of T cells under starving conditions, reduced intensities for several metabolites could be observed. Wilcoxon signed-rank test was employed to detect metabolites that are significantly different between control and starving Jurkat T cells. Using a threshold of $p < 0.05$ and a fold change >1.5, in total 11 metabolites were detected that showed statistical relevant differences (Figure 3, Table 1).

The most affected amino and organic acids were assigned showing varying dynamics during different growth conditions. Cellular concentrations of all identified amino acids decreased during growth under starving conditions.

For serine, phenylalanine, alanine, glycine, proline, isoleucine and tyrosine a significant decrease of cellular concentrations (fold changes 0.2 - 0.5) from normal to starvation conditions could be observed (Table 1). The effect of increased amino acid consumption during starvation could also be shown in mouse macrophages -like RAW cells [15]. Certain amino acids are now known to play important nutrient-sensing roles involving the (mTOR)-mediated signaling pathway [16]. mTOR is a component of a signaling pathway that couples insulin receptor stimulation and nutrient availability with protein synthesis via activation and phosphorylation of the ribosomal protein S6. Energy levels and nutrients regulate mTOR effectors such as S6K1 (S6 kinase 1) through the interaction with the mTOR complex. In cells growing in nutrient-rich conditions, the mTOR kinase activity is high, whereas under nutrient-poor conditions the mTOR kinase activity is decreased. It is not known how amino acids activate the mTOR complex, but it is probable that stimulation of a kinase or inhibition of a phosphatase that act upon mTOR as a substrate is involved [17-19].

In addition, various organic acids revealed the same effect of decreased signal intensities compared to Jurkat T cells grown under normal conditions (Table 1). A 0.2-fold change of signal intensities could be observed for fumaric and succinic acid after starvation cultivation ($p<0.05$). The intracellular decrease of TCA-cycle metabolites could be explained with consumption of these metabolites for energy generation of the cells during starvation period.

Significant differences could also be detected in pyruvate metabolism, resulting in lowered amounts of lactate as well as malate (fold changes 0.2 and 0.3, respectively).

For stearic acid, palmitic acid and especially its oxidation product palmitoleic acid higher signal intensities in the starvation culture could be observed (Fold changes 1.1, 1.1 and

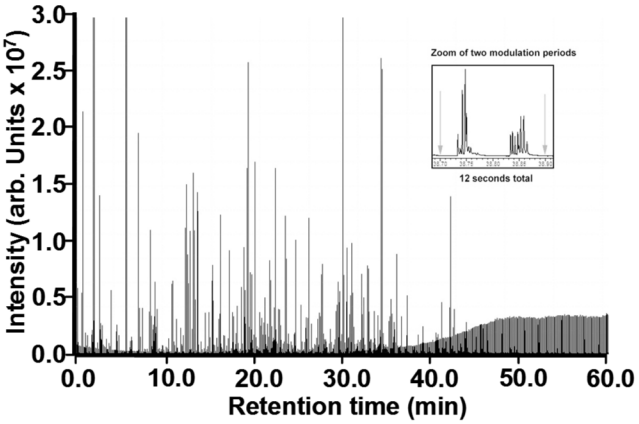

Figure 1. Comprehensive GC-qMS chromatogram of a hydrophilic extract from Jurkat T cells grown under normal conditions. Due to modulation by continuous freezing and hot pulse evaporation of analytes eluting from 1st dimension column peaks were cut into multiple fractions. Thermally desorbed analytes will be further separated on 2nd dimension column and reveal chromatographic peaks with a temporal distance according to the chosen modulation time (see insert; modulation time of 6s).

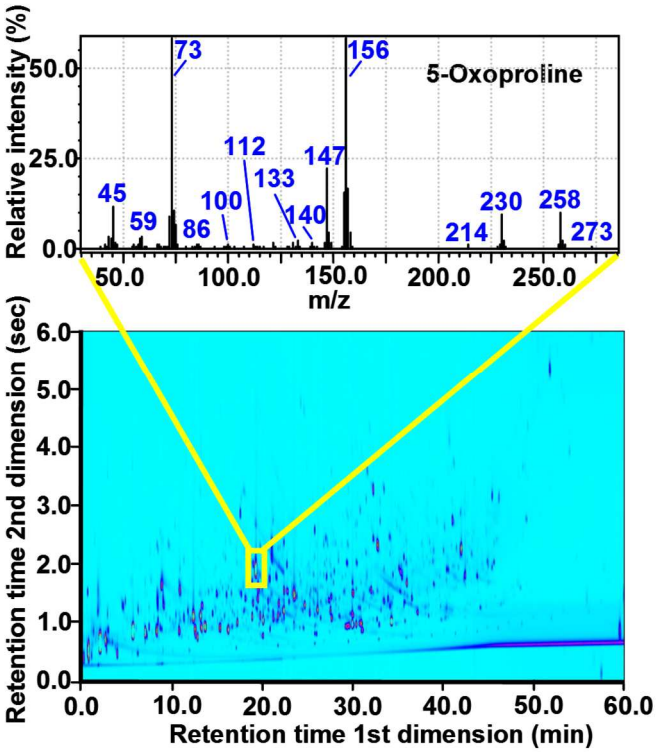

Figure 2. Comprehensive two-dimensional GC-qMS plot of a hydrophilic extract from Jurkat T cells grown under normal conditions. Additionally, the corresponding mass spectrum for 5-Oxoproline is shown as an example. Separation of analytes into two chromatographic dimensions by preferably orthogonal separation characteristics. Resulting 'blobs' in horizontal direction illustrating non-separated substances coeluting under "classical" chromatographic conditions. Referring mass spectra can be obtained from the modulated single peaks and used for compound identification.

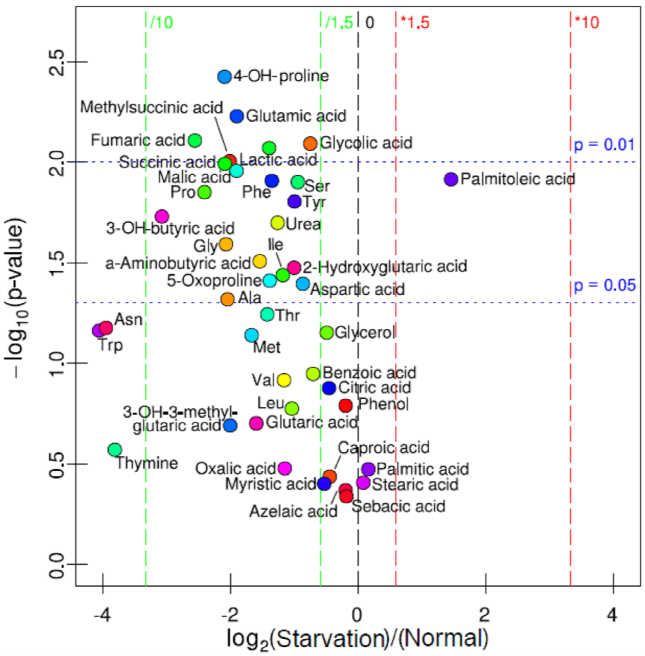

Figure 3. Volcano plot of for comparison of Jurkat T cell extracts after normal and starvation cultivation. As a reaction to cultivation of cells under starvation conditions and a resulting decelerated metabolism reduced signal intensities for several amino and organic acids could be detected. For three fatty acids an increase of signal intensities due to lipolytic shift could be observed.

2.7, respectively). This could be explained by a shift to lipolysis as one possible alternative for energy generation as a result of the energy deficit of starving Jurkat T cells. Thereby, triglycerides were hydrolyzed to provide free fatty acids as energy source [20, 21].

4. Concluding Remarks

The application of GCxGC-qMS allowed the reproducible detection of metabolites from the central carbon and amino acid metabolism of T cells. Starvation conditions induced significant changes in resting T cells metabolism, predominantly in the metabolic pathways of amino acid synthesis, the TCA cycle and fatty acid biosynthesis. These results will help to complement the puzzle of the metabotype of the variant status of immune cells. Since the involved pathways are swiftly affected the next step will be to elucidate if consumption of amino and organic acids as well as the lipolytic switch during starvation is a reversible process or not and indicating for the final fate of these cells.

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