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Linking energy metabolism to dysfunctions in mitochondrial respiration – A metabolomics *in vitro* approach

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ABSTRACT

The study presented here describes the application of metabolite profiling of highly polar, intracellular metabolites after incubation of a mammalian fibroblast cell line with inhibitors of mitochondrial function. A metabolomics approach was used to assess the complex response of the cellular energy metabolism. Metabolic profiles of phosphorylated and carboxylated intracellular metabolites were assessed by UPLC-MS/MS and used to predict the mode of mitochondrial toxicity. Based on distinct metabolic patterns, multivariate data analysis allowed for the discrimination of two groups of toxins: inhibitors of the electron transport in mitochondrial membranes (complex IV inhibitors) and uncouplers of oxidative phosphorylation. Beyond these known interferences, metabolic profiling was able to reveal additional inhibitory effects on the cellular energy metabolism. Most prominently, for three of the toxins, metabolic patterns also disclosed an enhanced activity of the glycerol phosphate shuttle inferring the inhibition of NADH dehydrogenase at complex I. Secondly, inhibition of the electron transport was accompanied by a limiting availability of citric acid cycle intermediates and aspartate. Concomitantly, specific perturbations of the purine nucleotide cycle were observed. We have shown here that metabolomic approaches may assist to predict complex modes of action of toxic compounds on cellular level as well as to unravel specific dysfunctions in the energy metabolism.

1. Introduction

Newly emerging technologies such as the comprehensive profiling of metabolites by means of nuclear magnetic resonance or mass spectrometry techniques (metabolomics) are now available and may assist in elucidation of modes of action of toxicity, early diagnose of diseases, as well as in the development of therapeutic strategies from simple cell culture studies (Kilk et al., 2009; Marrero et al., 2010; Munger et al., 2008; Uehara et al., 2009). Likewise, the profiling of metabolites involved in the central carbon and energy metabolism will significantly advance predictive toxicology. In this context, screening cell cultures under well-controlled testing conditions may improve our understanding about complex regulation in cellular metabolism on a systems level and may therefore provide a valuable alternative method for mechanistic investigations.

For animal welfare reasons, animal experiments have to be refined and reduced to a scientifically sound essential minimum. Every measure that can be undertaken in order to gain additional information from animal studies as compared to current study designs will refine animal experimentation and finally possibly reduce the number of test animals. Metabolomics is an emerging technique, which gains more and more importance in achieving these goals (Beger et al., 2010; Soga et al., 2006; van Ravenzwaay et al., 2010; van Vliet et al., 2008).

Accordingly, we have established a new method that involves optimized cell cultivation on membranes, quenching, and extraction of intracellular metabolites from adherent cell cultures. For metabolite analysis we used UPLC-MS/MS with modifications as published recently (Buescher et al., 2010). The method is applied to assess the cellular energy metabolism in mammalian cell cultures to study complex mitochondrial toxicity on a metabolomic scale.

Stable isotope labeling studies have shown that microorganisms respond to alterations of their environment on the metabolic level within a few seconds (Mashego et al., 2007; Noh et al., 2007; Yuan et al., 2006). Although similar studies have not yet been reported with mammalian cells, rapid turnover of metabolites involved in catabolic pathways, e.g. the glycolysis, can be anticipated by comparing enzyme characteristics of pro- and eukaryotic species (cf. turnover numbers given in the BRENDA enzyme database (Chang et al., 2009)). Since ATP turnover as well as NAD metabolism is inextricably coupled to those initial pathways any bias due to inadequate quenching will also affect the energy metabolism (Meyer

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et al., 2010). Thus, rapid quenching of enzymatic activity during sampling is a prerequisite for the correct assessment of metabolic profiles from metabolically active matter (Canelas et al., 2008; Lee do and Fiehn, 2008; Ritter et al., 2008; Villas-Boas et al., 2005; Winder et al., 2008). However, applying the most widely used method, i.e. quenching with cold solvents (e.g. methanol <−40 °C), leakage-free separation of intra- and extracellular metabolites with efficient quenching of cell metabolism is difficult to achieve (Villas-Boas et al., 2005; Wittmann et al., 2004). In addition, the conventional protocol for the detachment of adherent mammalian cells from their growth surface requires e.g., trypsinizing which inevitably changes the profile of cellular metabolites (Teng et al., 2009). For this reason Bennett and coworkers omitted any cell wash or trypsinizing and used filter-grown bacterial cultures instead (Bennett et al., 2008). Thereby intra- and extracellular metabolites could be quickly separated (∼1 s) by transferring the filter with adhering bacteria to cold methanol (−80 °C). The remarkable difference compared to the conventional quenching approach is that the quenching solvent was no longer separated from the cells during further processing by centrifugation but was used for extraction, thus circumventing the problematic issue of lost analytes due to cell leakage.

We used of membrane-grown cultures eliminating laborious cell scraping (Dietmair et al., 2010; Ritter et al., 2008; Teng et al., 2009; Yuan et al., 2009), and optimized it in order to enable metabolite screening of adherent mammalian cells. The method was applied to compare profiles of 129 polar metabolites from V79 fibroblasts which were exposed to potassium cyanide, sodium azide, 2,4-dinitrophenol, or carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP). All four substances are well known to inhibit the generation of ATP via mitochondrial electron transport phosphorylation (ETP), with the former two compounds impairing cytochrome oxidase (type IV inhibitor) and the latter two being strong uncouplers of oxidative phosphorylation (Chang et al., 2009 and citations therein). We hypothesized that intracellular levels of intermediates of the central carbon and energy metabolism would correspond to the type of inhibition by inhibition-specific metabolic patterns.

2. Materials and methods

2.1. Cell cultures

V79 A4 chinese hamster lung fibroblasts were maintained in modified Eagle medium (MEM) containing 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin (all purchased from Biochrom AG, Berlin, Germany). In the main experiments, adherent cultures were grown on membrane strainers (Nunc GmbH, Langenselbold, Germany; track-edge polycarbonate, 2.5 cm diameter) inserted in 6-well plates containing 1.5 mL MEM and 0.5% DMSO (with/without an inhibitor).

In the experimental setup using 6-well plates per treatment group strainers of five wells were used for replicate metabolome analysis and one for protein analysis (Fig. 1). Finally, another six strainers without cells were processed to assess medium-derived contribution to analytical signals. The media volume adhering to the membrane was determined to be about 50 μL (data not shown). Taking into account a theoretical volume of the cells of not more than 1 μL, the volume adhering to the strainer by far exceeds the volume of medium that my additionally adhere to the cells. Therefore, the extra portion of metabolites potentially sorbed by the cells is considered as being negligible.

Two sampling time points were chosen: 1 h ("immediate response") and 24 h ("chronic response"). To ensure sufficient cellular matter for an extraction but avoiding confluence, different cell densities of the exponentially growing culture were plated for the different sampling time points: 5.0×10^5 cells for 1 h exposure and 1.5×10^5 cells for the 24 h exposure were seeded into each strainer in a culture volume of 1 mL. Correspondingly, the respective cell numbers at the sampling time reached approximately 1.6×10^6 cells. 1 h prior to the 24 h sampling the medium (e.g., containing, DMSO, test substance, and D-glucose) was refreshed.

2.2. Test chemicals

Potassium cyanide (KCN), sodium azide (NaN_3), 2,4-dinitrophenol (DNP), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), and DMSO were purchased from Sigma–Aldrich (all p.a. grade, Taufkirchen, Germany).

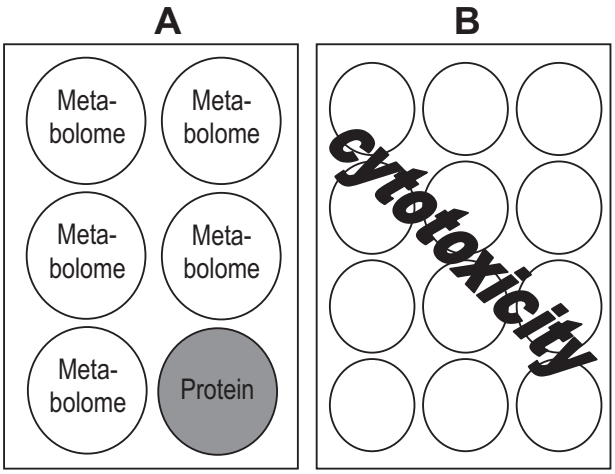

Fig. 1. Study design of *in vitro* toxicity tests using V79 cell cultures. (A) 6-well multi titer plate (MTP) using membrane strainers for metabolome and protein analysis, for time point 1 (1 h) 5.0 × 105 cells and for time point 2 (24 h) 1.5 × 105 cells were plated. (B) Cytotoxicity assessment and determination of total protein content (MTT and BCA test) in 12-well MTP format.

2.3. Cytotoxicity assay

The MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma–Aldrich, Taufkirchen, Germany, Product Number M5655; Mosmann, 1983) was chosen to determine a test substance concentration for the main experiment and was considered appropriate for compounds with unknown modes of toxicity. For comparison of metabolic profiles in response to the individual inhibitor test substance concentrations affording 50–80% cell viability relative to control samples after 24 h compound exposure using the were chosen (data of preliminary cytotoxicity experiment not shown). Based on these pretests, test substance concentrations used in the main experiments were 1 mM (CN^-), 1 mM (N_3^-), 500 μM (DNP), and 10 μM (FCCP), respectively. Further, in adjunct to the metabolomics experiments, cytotoxicity after 24 h exposure was determined in duplicates using the MTT assay and the total protein content was determined using the BCA assay (Fig. 1).

2.4. Protein determination

Paralleling the sampling time points for the metabolome analysis, total protein was determined according to the manufacturer's protocol (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Bonn Germany, Product Number 23225). Prior to the protein analysis the adherent cultures were washed with 2 mL PBS. The membrane was excised and transferred into 1.5 mL Eppendorf tubes with 1 mL PBS containing 1% Triton X-100 and 0.1% protease inhibitor (Sigma–Aldrich) and incubated for 1 h at 37 °C.

2.5. Preparation of an $U^{13}C$-yeast extract

Candida utilis DSMZ sp. 2361 (DSMZ, Braunschweig, Germany) was cultured on synthetic minimal medium (yeast nitrogen base without amino acids, Sigma–Aldrich, Taufkirchen, Germany, Product Number Y1250) and 10 g L^{-1} $U^{13}C$-D-glucose (uniformly ^{13}C-labeled with >99% ^{13}C-enrichment, Euroriso-Top GmbH, Saarbrücken, Germany). Cells were harvested during exponential growth at an optical density of 4.0 (600 nm, 1 cm cuvette). The cells were pelleted by centrifugation (0 °C, 1200 × *g*, 2 min). Prior to the extraction, the cell pellets were washed twice in cold (0 °C) 0.15 M ammonium acetate solution (Sigma–Aldrich, p.a. grade) containing 10 g L^{-1} $U^{13}C$-D-glucose. The extraction was carried out by a bead milling procedure (FastPrep24, MP Biomedicals Europe, 20 s milling, speed 6.5 m/s, using an adapter for 15 mL Falcon-Tubes) using 7.5 mL of cold dichloromethane/ethanol (−80 °C, 2:1 (v/v)) and 2.5 mL of 1.5 M aqueous ammonium acetate solution (0 °C). After phase separation, the upper aqueous phase was collected. Upon addition of another 2.5 mL 1.5 M ammonium acetate solution, the extraction step was repeated and the upper phase was combined with the first extract. The final cell extract was stored at −80 °C until further use (Fig. 1 supplementary information).

2.6. Sampling and extraction

For sampling, the membranes with adhering V79 cells were excised in the well, then transferred quickly into a pre-chilled extraction tube (Precellys Steel Kit 2.8 mm, Peqlab Biotechnologie GmbH, Erlangen, Germany) and quenched immediately in a −80 °C cold organic solvent (900 μL of a 2:1 (v/v) mixture of dichloromethane and ethanol (both LC grade)). The extraction tubes were stored up to one week at −80 °C until further processing. Prior to the extraction, 150 μL of the