Biochimica et Biophysica Acta 1820 (2012) 1–8

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Effects of resveratrol on HepG2 cells as revealed by ^{1}H-NMR based metabolic profiling

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ARTICLE INFO

Article history:
Received 18 July 2011
Received in revised form 4 October 2011
Accepted 11 October 2011
Available online 18 October 2011

Keywords:
Resveratrol
HepG2
Sirt1
Metabolic profiling
NMR spectroscopy
Metabolomics

ABSTRACT

Background: Resveratrol, a polyphenol found in plant products, has been shown to regulate many cellular processes and to display multiple protective and therapeutic effects. Several *in vitro* and *in vivo* studies have demonstrated the influence of resveratrol on multiple intracellular targets that may regulate metabolic homeostasis.
Methods: We analysed the metabolic modifications induced by resveratrol treatment in a human hepatoblastoma line, HepG2 cells, using a ^{1}H-NMR spectroscopy-based metabolomics approach that allows the simultaneous screening of multiple metabolic pathways.
Results: Results demonstrated that cells cultured in the presence or absence of resveratrol displayed different metabolic profiles: the treatment induced a decreased utilisation of glucose and amino acids for purposes of energy production and synthesis associated to a decreased release of lactate in the culture medium and an increase in succinate utilisation. At the same time, resveratrol treatment slowed the cell cycle in the S phase without inducing apoptosis, and increased Sirt1 expression, also affecting its intracellular localisation.
Conclusions: Our results show that the metabolomic analysis of the exometabolome of resveratrol-treated HepG2 cells indicates a metabolic switch from glucose and amino acid utilisation to fat utilisation for the production of energy, and seem in agreement with an effect mediated *via* AMPK- and Sirt1-activation.
General significance: NMR-based metabolomics has been applied in a hepatocyte cell culture model in relation to resveratrol treatment; such an approach could be transferred to evaluate the effects of nutritional compounds with health impact.

1. Introduction

Resveratrol (3,5,4′-trihydroxystilbene) is a polyphenol found in grapes, grape products and other plant products that has been the subject of intense scientific interest in recent years on account of its multiple protective and therapeutic effects [1–5]. Reported health benefits include cardioprotection, antiaging effects, defence against metabolic and neurodegenerative diseases, cancer prevention and therapy [6,7]. Some important cellular processes, such as cell cycle regulation and apoptosis are deeply influenced by this compound and its preventive and/or therapeutic properties against cancer have often been ascribed to antiproliferative and pro-apoptotic effects [8].

More recent research on the molecular mechanisms by which resveratrol might exert many of its biological effects has emphasised the importance of its interaction with sirtuins. In particular, resveratrol has been shown to increase Sirt1 activity and to enhance Sirt1-dependent metabolic processes both *in vivo* and *in vitro* [9–11].

Resveratrol can affect different metabolic pathways depending on the organ or cell type, on the cell state, and on the duration and dosage of treatment. At the same time, it has been shown that resveratrol has numerous intracellular targets and affects the expression and activity of different transcription factors/cofactors, thus regulating metabolic homeostasis and resulting in multiple pleiotropic effects. Among the numerous metabolic effects exhibited by resveratrol, the topic of "calorie restriction mimicry" has aroused considerable interest and several studies on cell cultures have been performed forcing the system toward either a fed or a fasted condition by increasing or decreasing nutritional supplies in the culture medium [10,12–14].

These considerations prompted us to study the metabolic modifications induced by resveratrol treatment in an *in vitro* system, using a metabolomic approach that allows the simultaneous screening of multiple metabolic pathways. Considering that the liver is one of

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doi:10.1016/j.bbagen.2011.10.005

the main target organs of resveratrol action *in vivo*, we analysed the effects of resveratrol on HepG2 cells, a human hepatoblastoma line that, although actively proliferating, exhibits many features specific to human differentiated hepatocytes, and is also widely used in resveratrol metabolic studies [15–17].

We first verified in our experimental conditions whether cellular responses to the treatment were similar to those reported in the literature, particularly in regard to antiproliferative and apoptotic effects [17,18]. Next, we studied the metabolic profile in relation to resveratrol treatment. Changes in the exo-metabolome were evaluated using ^{1}H-NMR spectroscopy on medium and Multivariate Data Analysis (MVDA). The utilisation of substrates and the concurrent release of metabolites into the extracellular space reflect the metabolic pathways that operate during a given set of physiological activities and in response to metabolic perturbations, either chemical or physical, leading to the characterisation of a specific metabolic profile (or fingerprint). Previously, this approach allowed us to discriminate two different metabolic profiles in HepG2 cells in relation to different phases of cell growth [19].

2. Materials and methods

2.1. Cell culture and viability

Human HepG2 cells were purchased from the American Type Culture Collection (ATCC) and were used within twenty passages. Cells, suspended in RPMI 1640 medium (Sigma) supplemented with 10% foetal calf serum, 2 mM L-glutamine, 1% sodium pyruvate, 100 μg/mL streptomycin and 100 U/mL penicillin, were seeded in culture plates at 8×10^{4} cells/cm^{2} at 37 °C in a humidified incubator in the presence of 5% CO_2. Resveratrol (Sigma), 20–200 μM in DMSO, was added to the cells and cultures were incubated for 24 or 48 h. Cells cultured in medium containing only DMSO were used as controls. At each time point, viable cells were estimated by a Trypan blue exclusion test. To evaluate the percentage of damaged cells, LDH leakage was monitored using an LDH Cytotoxicity Assay Kit (BioVision Inc.) on cells cultured in multiwells in the presence or absence of different amounts of resveratrol. DO was measured at 450 nm with a microplate reader (Bio-RAD).

2.2. Cytofluorimetric assay and DAPI staining

HepG2 cells after 24 or 48 h of treatment and control cells were extensively washed with PBS and 1×10^{6} aliquots were permeabilised with cold 70% ethanol for 30 min. The cells were then treated in the dark at room temperature with a DNA-staining solution (50 μg/ml propidium iodide in 0.1% sodium citrate containing 0.1% Triton X100). Cell cycle phase distribution was analysed by flow cytometry (FACScan flow cytometry, Becton Dickinson Immunocytometry System). Data from 10,000 events per sample were collected and analysed using Cell Quest software.

In parallel experiments, cells were cultured on coverslips and stained with DAPI (Sigma), 1:10,000 in PBS and observed under an epifluorescence microscope (Axioplan 2, Zeiss).

2.3. Sirt1 detection

2.3.1. Western blot analysis

To prepare the whole-cell lysates, cells were washed with ice-cold PBS, harvested and sonicated in lysis buffer (PBS containing 100 μg/mL PMSF and 5 μg/mL leupeptin). Proteins (80 μg) were separated on 10% SDS-PAGE and then transferred to a nitrocellulose membrane (Bio-Rad). The membrane was first blocked with 5% non-fat milk and then blotted with a rabbit polyclonal antibody to Sirt1 (Sigma) diluted 1:500 in Tris-bufferd saline (10 mM Tris–HCl, 150 mM NaCl, pH 7.4) for 2 h. After washing, the membranes were incubated with an anti-rabbit secondary antibody (1:10,000) for 1 h. The immunocomplexes were visualised by NBT-BCIP detection of alkaline phosphatase-conjugated secondary antibodies (Sigma). Quantitative analysis of the bands was performed on digitised images using the ImageJ 3.0 software. β-actin protein levels were determined to normalise the data.

2.3.2. Immunofluorescence

Cells grown on coverslips were fixed in methanol for 10 min at −20 °C, and non-specific staining was blocked with 10% normal goat serum in PBS. Cells were then incubated for 2 h at room temperature with a polyclonal antibody to Sirt1 (Sigma), 1:80 in PBS with 1% BSA. They were then stained with Alexa Fluor 555-conjugated goat anti-rabbit IgG for 1 h at room temperature (Molecular Probes, Invitrogen). After rinsing, the coverslips were mounted on slides in aqueous medium and examined under an epifluorescence microscope (Axioplan 2, Zeiss). Negative controls were performed by exposing slides under similar conditions while omitting the primary antibody.

2.4. Oil Red O staining

Cells were fixed in 10% formaldehyde in PBS for 1 h (or overnight) and stained with a working solution (5 mM) of Oil Red O (Sigma) for 10 min at room temperature. For quantitative analysis of cellular triglycerides, after exhaustive washings with water samples were dried and 60% isopropyl alcohol was added. The extracted dye was immediately removed by gentle pipetting and its absorbance was monitored by a spectrophotometer (Genesys) at 500 nm.

2.5. ^{1}H NMR spectroscopy and multivariate analysis

2.5.1. ^{1}H NMR spectroscopy

^{1}H NMR Spectra were acquired at a temperature of 298 K using a Bruker Avance 400 spectrometer (Bruker BioSpin GmbH, Germany) equipped with a magnet operating at 9.4 T, where the ^{1}H nucleus resonates at 400.13 MHz. The probehead was a 5 mm diameter multinuclear PABBO BB-1H/D (Z108618/0044) equipped with z-gradient. The pulse sequence adopted for spectra acquisition was a presaturation–single 90° detection pulse–acquire–delay sequence that is a modification of the presaturation ZGPR sequence available as a standard sequence in the spectrometer library. The modification makes it possible to set the presaturation delay independently of the D1 relaxation delay, while in the standard sequence the solvent signal is saturated for the entire duration of the relaxation delay. Since our spectra were acquired under full relaxation conditions, with a very long relaxation interval (9.5 s), our sequence allowed us to irradiate the solvent nuclei for a given time (3 s) leaving a true relaxation delay of 6.5 s. The acquisition time needed to collect the 32 K points was about 5.5 s, allowing proton nuclei other than solvent nuclei to relax for 15 s, complying with the full relaxation condition after a 90° pulse. The duration of the detection pulse was calibrated prior to the acquisition of each spectrum, the spectral width was set to 5995.02 Hz (12 ppm), 64 scans were collected for each spectrum. 1H NMR spectra were processed using the 1H-Manager ver. 12.0 software (Advanced Chemistry Development, Inc., Toronto, Ontario, Canada). The assignment of peaks to specific metabolites was achieved using an internal library of compounds and the literature [20,21, www.hmdb.ca] and confirmed by standard two-dimensional (2D) ^{1}H–^{1}H correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), and ^{1}H–^{13}C heteronuclear single quantum correlation (HSQC).

2.5.2. Multivariate analysis

Multivariate data analysis was carried out using Unscrambler 9.8 Software (CAMO, Oslo, Norway). Spectral data were mean centred and scaled before analysis [22] Principal components analysis (PCA) was used to examine inherent clustering and to identify outliers.