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Resveratrol induces dynamic changes to the microglia transcriptome, inhibiting inflammatory pathways and protecting against microglia-mediated photoreceptor apoptosis

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ABSTRACT

Microglia activation is central to the pathophysiology of retinal degenerative disorders. Resveratrol, a naturally occurring non-flavonoid phenolic compound present in red wine has potent anti-inflammatory and immunomodulatory properties. However, molecular mechanisms by which resveratrol influences microglial inflammatory pathways and housekeeping functions remain unclear. Here, we first studied the immuno-modulatory effects of resveratrol on BV-2 microglial cells at the transcriptome level using DNA-microarrays and selected qRT-PCR analyses. We then analyzed resveratrol effects on microglia morphology, phagocytosis and migration and estimated their neurotoxicity on 661 W photoreceptors by quantification of caspase 3/7 levels. We found that resveratrol effectively blocked gene expression of a broad spectrum of lipopolysaccharide (LPS)-induced pro-inflammatory molecules, including cytokines and complement proteins. These transcriptomic changes were accompanied by potent inhibition of LPS-induced nitric oxide secretion and reduced microglia-mediated apoptosis of 661 W photoreceptor cultures. Our findings highlight novel targets involved in the anti-inflammatory and neuroprotective action of resveratrol against neuroinflammatory responses.

1. Introduction

Neurodegenerative diseases are a major challenge for medicine and public health [1]. Microglial cells, central nervous system (CNS) resident macrophages, play a major role in neurodegenerative disorders as they are the primary effectors of the neuronal innate immune system [2]. In response to inflammatory stimuli such as intracellular factors released by dying neurons such as photoreceptors or during an infection, microglial cells adopt an amoeboid morphology, proliferate rapidly and migrate to the affected site [3]. Here, they secrete a multitude of pro-and anti-inflammatory cytokines and chemokines, reactive oxygen (ROS) and trophic factors which leads to the re-establishment of tissue homeostasis [2,3]. However, microglia also contribute to the progression and severity of neurodegeneration [4]. Therefore, efforts to modulate microglia activation represent an attractive therapy approach for neurodegenerative diseases.

Resveratrol, also known as 3, 4′, 5-trihydroxystilbene, is a naturally occurring non-flavonoid phenolic compound present in red wine, grapes, berries, peanuts, soy beans, pomegranates and many other fruits and plants [5]. Resveratrol initially attracted interest as a potential explanation for the “French paradox” concept [6]. It was reported that the much lower incidence of cardiovascular diseases in French people, despite their high-fat diet, was most likely attributed to resveratrol consumption through red wine [6]. Resveratrol also possesses a wide range of other biological properties including potent anti-tumor, anti-inflammatory and anti-oxidative effects [7,8].

Although its immunomodulatory actions have been shown to be potentiated via NFkB [9], NLRP3 inflammasome [10], SIRT1-SOCS1 and SIRT1-PGC-1α signalling pathways [11], a genome-wide search for further molecular targets in microglia remains to be fully elucidated. The aim of this study was therefore to further define the transcriptomic effects of resveratrol in quiescent and LPS-activated BV-2 microglia cultures using DNA microarrays and qRT-PCR and then to define the outcome on microglia function.

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2. Methods

2.1. Reagents

Resveratrol was purchased from Carl Roth GmbH + Co KG (Karlsruhe, Germany). *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). CellTiter 96® Non-Radioactive Cell Proliferation (MTT) assay kit was purchased from Promega (Mannheim, Germany).

2.2. Cell culture

BV-2 microglia-like cells were cultured as described previously [12]. 661 W, a murine photoreceptor-like cell line, was a gift from Prof. Muayyad Al-Ubaidi (Department of Cell Biology, University of Oklahoma Health Sciences Centre) and the culture conditions have been described elsewhere [12].

2.3. MTT cytotoxicity assay

Cytotoxicity was analyzed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay to determine the optimal concentration of resveratrol that does not induce apoptosis on our target cells. Experiments were performed in triplicate and the data presented as percentage of cell viability when compared to the untreated cells whose percent viability was considered 100%. After 6 h of treatment, cells in a 96-well plate were treated with 15 μl of MTT and incubated for 2 h at 37 °C. The cells were then treated with 100 μl solubilization-solution and left overnight at room temperature in a humid environment. Absorbance measurements were made with a plate reader (Infinite F200 Pro, Tecan) at a wavelength of 570 nm.

2.4. RNA isolation, reverse transcription and quantitative real-time RT-PCR

RNA was extracted using the NucleoSpin® RNA Mini Kit (Macherey & Nagel, Dueren, Germany) instructions. RNA concentrations were determined spectrophotometrically using a NanoDrop 2000 (Thermo Scientific) and 1 μg was reverse transcribed into cDNA in a 20-μl final volume using RevertAid™ H Minus First strand cDNA Synthesis Kit (Thermo Scientific). The resulting cDNA was diluted to 50 μl final volume (final conc. 20 ng/μl) with RNase free water and used as a template for real-time PCR using 200 nM gene specific primers, 1× TaqMan Universal PCR Master Mix (Applied Biosystems) and 0.125 μl of dual-labelled UPL probe (Roche Applied Science, Basel, Switzerland). Primer sequences and UPL probe numbers are listed in Supplementary Table 1. Amplification of Atp5b served as a reference control gene. qRT-PCR reactions were performed as described recently [13].

2.5. DNA microarrays and bioinformatic data analysis

Duplicate microarrays were carried out with two independent RNAs from non-stimulated BV-2 microglia or cultures treated for 6 h with 25 and 50 μM resveratrol, 50 ng/ml LPS + vehicle (DMSO), or 50 ng/ml LPS + 25 or 50 μM resveratrol. DNA micro-array analysis was performed according to the Affymetrix standard protocol. Procedures involving preparation and labelling of cRNA, hybridization to Affymetrix 430 2.0 mouse genome arrays, washing, and scanning were performed as previously described [14]. The cut-off applied for genes considered to be differentially expressed was a log2 fold change of ≥ 2 or ≤ -2 and a $p < 0.05$. Integrative analysis of genome-wide expression activities was performed with the Gene Expression Dynamics Inspector (GEDI), a Matlab (Mathworks, Natick, MA) freeware program which uses self-organizing maps (SOMs) to translate high-dimensional data into a 2D mosaic [15]. Each tile of the mosaic represents an individual SOM cluster and is color-coded to represent high or low expression of the cluster's genes, thus identifying the underlying pattern.

2.6. Nitrite measurement, phalloidin staining, scratch wound-healing assay, phagocytosis, and caspase 3/7assays

Nitric oxide (NO) production, F-actin distribution, migration, phagocytosis of BV-2 cells and caspase activity of 661 W cells were determined as detailed before [12, 16].

2.7. Statistics

All data were analyzed using Prism Graph pad version 6.0. Real-time RT-PCR data were analyzed with the ΔΔCt method. Significance of difference between means was determined by one-way ANOVA and Tukey post hoc test. The data are expressed as mean ± standard deviation (SD).

3. Results

3.1. Cytotoxicity assessment of resveratrol by MTT

We first evaluated the potential toxicity of resveratrol by exposing BV-2 microglia for 24 h and then measuring cell viability by MTT assay. We observed that at 50 μM, resveratrol was well tolerated by the BV-2 cells with no adverse effects detected (Supplementary Fig. 1).

3.2. Resveratrol attenuates pro-inflammatory gene expression in microglia

We then investigated whether treatment of murine BV-2 microglial cells with resveratrol impacts microglial reactivity. We tested the mRNA expression of the pro-inflammatory cytokines IL-1β and IL-6 as well as the inducible form of nitric oxide synthase (iNOS) in LPS-activated microglia. Resveratrol significantly and dose-dependently suppressed mRNA levels of IL-1β (Fig. 1A), IL-6 (Fig. 1B), and iNOS (Fig. 1C). Resveratrol also effectively diminished the strong LPS-induced NO secretion into the culture medium (Fig. 1D).

3.3. Resveratrol triggers global changes on the microglial transcriptome

Our next objective was to evaluate the transcriptional profile of control and LPS-activated BV-2 cells treated with resveratrol for 6 h using Affymetrix Mouse Genome 430 2.0 GeneChips. For the stimulation groups, genes had to be significantly up or down-regulated with LPS (fold change > 2, p-value < 0.05) and followed by a significant (p-value < 0.05) down or upregulation by resveratrol treatment. We thereby identified 184 differentially regulated genes for the LPS + resveratrol group and 134 genes for the resveratrol only groups (Supplementary Table 2). We used the Gene Expression Dynamics Inspector (GEDI) to analyze and navigate through our global gene expression data in the different treatment groups. The GEDI maps showed a strong regulation of gene expression in stimulated versus non-stimulated microglia (Fig. 2A, rectangles). These dynamic changes in gene expression by resveratrol were more pronounced in the LPS-stimulated dataset as compared to the unstimulated groups (Fig. 2A). These findings demonstrate that resveratrol has an enormous impact on the global pattern of gene expression in microglia.

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