Experimental Gerontology 102 (2018) 3–11

Contents lists available at ScienceDirect

Experimental Gerontology

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

Resveratrol modulates response against acute inflammatory stimuli in aged mouse brain

V. Palomera-Ávalos[a], C. Griñán-Ferré[a], V. Izquierdo[a], A. Camins[a], C. Sanfeliu[b], A.M. Canudas[a], M. Pallàs[a,*]

[a] Department of Pharmacology, Toxicology and Therapeutic Chemistry (Pharmacology Section), Institute of Neuroscience, CIBERNED, University of Barcelona, Avda. Joan XXIII s/n, 08028 Barcelona, Spain
[b] Institut d'Investigacions Biomèdiques de Barcelona (IIBB), CSIC, and IDIBAPS, 08036 Barcelona, Spain

ARTICLE INFO

Section Editor: Christian Humpel

Keywords:
Inflammation
Cytokines
eukaryotic Initiation Factor 2 (eIF2)
mTOR
iNOS
Aging

ABSTRACT

With upcoming age, the capability to fight against harmful stimuli decreases and the organism becomes more susceptible to infections and diseases. Here, the objective was to demonstrate the effect of dietary resveratrol in aged mice in potentiating brain defenses against LipoPolySaccharide (LPS). Acute LPS injection induced a strong proinflammatory effect in 24-months-old C57/BL6 mice hippocampi, increasing InterLeukin *(Il)-6*, Tumor Necrosis Factor-alpha (*Tnf-α*), *Il-1β*, and C-X-C motif chemokine (*Cxcl10*) gene expression levels. Resveratrol induced higher expression in those cytokines regarding to LPS. Oxidative Stress (OS) markers showed not significant changes after LPS or resveratrol, although for resveratrol treated groups a slight increment in most of the parameters studies was observed, reaching signification for NF-kB protein levels and i*NOS* expression. However, Endoplasmic Reticulum (ER) stress markers demonstrated significant changes in resveratrol-treated mice after LPS treatment, specifically in eIF2α, BIP, and ATF4. Moreover, as described, resveratrol is able to inhibit the mechanistic Target of Rapamycin (mTOR) pathway and this effect could be linked to (eIF2α) phosphorylation and the increase in the expression of the previously mentioned proinflammatory genes as a response to LPS treatment in aged animals. In conclusion, resveratrol treatment induced a different cellular response in aged animals when they encountered acute inflammatory stimuli.

1. Introduction

Aging is a progressive process that is strongly associated with activation of inflammatory mechanisms. With upcoming age, the organism's capability to fight against harmful stimuli decreases, becomes more susceptible to infections and diseases. Chronic low-grade inflammation is present in aged organisms and is related to the loss in efficacy of homeostatic mechanisms and with as decrease in resilience (Mattsson et al., 2016; Faye et al., 2017; Sampedro-Piquero et al., 2017). Age-related changes in immune homeostasis are characterized by the activation of several key inflammatory mediators, including Nuclear Factor-kappa Beta (NF-κB) and cytokines, among others. This inflammatory landscape, which characterizes advanced age, is termed inflammaging (Franceschi et al., 2000a,b; Cevenini et al., 2013). This phenomenon is caused by a continuous antigenic load and stress. Inflammaging has been also related to changes associated with age, such as genotoxic and oxidative events and gut microbiota composition (Kim et al., 2016). In addition to inflammation, aging is strongly associated with Oxidative Stress (OS) in general and with Endoplasmic Reticulum (ER) stress in particular.

It is accepted that the main traits of the harmful event gated to aging include the incapacity to confront this event with a variety of stressors and with the inflammaging characteristics defined previously (Franceschi et al., 2000a,b; Leon et al., 2017). The persistence of inflammatory stimuli over time, as occurs in old individuals, favors susceptibility to age-related diseases. This means that with upcoming age, there is a higher probability of developing an inflammatory pathogenesis, such as atherosclerosis, Alzheimer Disease (AD), and diabetes (Negash et al., 2013; Mattsson et al., 2016). In line with this perspective, several paradoxes of healthy centenarians, such as increased levels in plasma cytokines, are illustrative and explain longevity in the absence of disease (Franceschi et al., 2000a,b; Paolisso et al., 2000).

Resveratrol (3,5,4′-trihydroxystilbene) is a polyphenol contained in plant species and it is especially enriched in red fruits (Pallàs et al., 2013). Resveratrol possesses known antioxidant, anti-inflammatory, and other beneficial effects in several systems, including the brain. The

* Corresponding author.
E-mail address: pallas@ub.edu (M. Pallàs).

https://doi.org/10.1016/j.exger.2017.11.014
Received 17 June 2017; Received in revised form 16 November 2017; Accepted 21 November 2017
Available online 24 November 2017
0531-5565/

pleiotropic action is recognized of resveratrol in cardiovascular and neurodegenerative diseases and other inflammatory conditions (Abraham and Johnson, 2009). Calabrese et al. (2010) also classified resveratrol as a hormetic modulator in disease conditions, including inflammation, infection, or neurodegenerative processes.

Resveratrol is of particular interest in the modulation of diseases with an inflammatory component because several studies found it to inhibit the production of Reactive Oxygen Species (ROS) by neutrophils, monocytes, and macrophages (Rotondo et al., 1998; Abraham and Johnson, 2009), as well as the activation of several transcription factors including NF-κB and Activator Protein-1 (AP-1).

However, little is known about the preconditioning effects of resveratrol against acute insults (Calabrese et al., 2010). The endotoxin LipoPolySaccharide (LPS) is a major component of the outer membranes of Gram-negative bacteria and a chief member of pathogen-associated molecular patterns (Kallapura et al., 2014), which are largely responsible for the majority of the toxic inflammatory reactions described by means of ROS and Reactive Nitrogen Species (RNS); it is also widely used as an immunostimulatory tool to induce systemic inflammation and sepsis. LPS, through a cascade mechanism, stimulates Toll-Like Receptor 4 (TLR4) which acts on NF-κB, leading to cytokine and interferon activation pathways, which are the main inflammation mediators (Lu et al., 2008). Moreover, LPS through activation of TLR4 is reported to activate ER stress (Masson et al., 2015) that, at the same time, regulates the translation and transcription of proteins related with inflammation and OS, such as eukaryotic Initiation Factor 2α (eIF2α) (Toshchakov et al., 2016).

In this study, we investigated changes in cellular response related to dietary resveratrol with respect to acute LPS insult by measuring the protein expression levels of inflammaging markers such as cytokines, NF-κB, levels of OS, and ER stress markers in aged mice.

2. Animals and treatment

Male C57BL/6J mice 22 months of age were randomized in two experimental groups and, following 8 weeks of treatment, were euthanized under anesthesia. The normal diet (ND; $n = 9$) group had ad libitum access to a standard chow diet (2018 Teklad Global 18% Protein Rodent Diet; Harlan Teklad, Madison, WI, USA) and tap water; ResVeratrol group (RV; $n = 9$) had access to standard chow diet enriched with the polyphenol (1 g/kg, W/W). Resveratrol chow was formulated to provide daily doses of approximately 160 mg/kg to the mice.

The mice were treated according to European Community Council (ECC) Directive 86/609/EEC and the procedures established by the Department d'Agricultura, Ramaderia i Pesca of the Generalitat de Catalunya, Spain. Every effort was made to minimize animal suffering and to reduce the number of animals used in this study.

LPS extracted from *Escherichia coli* 0111:B4 (Sigma-Aldrich, USA) was dissolved in distilled water. The LPS solution was injected intraperitoneally (i.p.) at a dose of 100 μg/kg. After treatment with RV and ND, the mice were separated in four groups: ND-saline solution; RV-saline solution; ND-LPS; and RV-LPS. Immediately after the injections, the mice were placed in their home cages, where they remained during 3 h until euthanize.

3. Brain isolation and Western blot analysis

Mice were euthanized 3 h after the LPS or saline solution injection and the brain was quickly removed from the skull. Hippocampus was dissected and frozen in powdered dry ice and maintained at − 80 °C for further use. Tissue samples were homogenized in lysis buffer containing phosphatase and protease inhibitors (Cocktail II; Sigma), and cytosol and nuclear fractions were obtained as described elsewhere. Protein concentration was determined by the Bradford method. Twenty micrograms of protein were separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (8–15%) and transferred onto PolyVinylidene DiFlouride (PVDF) membranes (Millipore). The membranes were blocked in 5% non-fat milk in Tris-Buffered Saline solution containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature, followed by overnight incubation at 4 °C with primary antibodies diluted in TBS-T and 5% Bovine Serum Albumin (BSA) as follows: NF-κB (1:1000; Cell Signaling); NRF1 (1:500; Santa Cruz); NRF2 (1:1000; Cell Signaling), CATalase (1:1000; Cell Signaling); SOD1 (1:1000; Novus Biologicals); GPX1 (1:1000; Novus Biologicals); IRF3 (1:1000; Novus Biological); BIP (1:1000; Cell Signaling); ATF4 (1:1000; Cell Signaling); phospho-eIF2α (Ser51) (1:1000, Cell Signaling); total eIF2α (1:1000; Cell Signaling); mTOR (1:1000; Cell Signaling); total p70S6K1 (1:500; Santa Cruz); phospho-p70S6K1 (1:500; Santa Cruz), Tubulina (1:5000; Cell Signaling) and GAPDH (1:5000; Millipore). Membranes were then washed and incubated with secondary antibodies for 1 h at room temperature. Immunoreactive proteins were visualized utilizing an Enhanced ChemiLuminescence-based detection kit (ECL kit; Millipore) and digital images were acquired employing a ChemiDoc XRS + System (Bio-Rad). Band intensities were quantified by densitometric analysis using Image Lab software (Bio-Rad) and values were normalized to Tubulina and GAPDH.

4. RNA extraction and gene expression determination

Total RNA isolation was carried out by means of Trizol reagent following the manufacturer's instructions. RNA content in the samples was measured at 260 nm, and sample purity was determined by the A260/280 ratio in a NanoDrop™ ND-1000 (Thermo Scientific). Samples were also tested in an Agilent 2100B Bioanalyzer (Agilent Technologies) to determine the RNA integrity number. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed as follows: 2 μg of messenger RNA (mRNA) was reverse-transcribed using the High Capacity (complementary DNA) cDNA Reverse Transcription kit (Applied Biosystems). Real-time quantitative PCR (qPCR) was utilized to quantify the messenger RNA (mRNA) expression of inflammatory genes InterLeukin 6 and 1 (*Il-6* and *Il-1β*), Tumor Necrosis Factor alpha (*Tnf-α*), InterFeroN (INF) gamma (*Ifn-γ*), C-X-C motif chemokine (*Cxcl10*), Aldehyde dehydrogenase 2 (*Aldh2*), inducible Nitric Oxide Synthase (*iNOS*), Cyclooxygenase 2 (*Cox2*), and Hemooxygenase 1 (*Hmox1*), Toll-Like Receptor (*Tlr4*), MetalloThionein-1 (*Mt1*), Quinone oxidoreductase-1 (*Nqo1*), and Glutathione S-transferase-1 (*Gstp1*). Normalization of expression levels was performed with actin for SYBR Green and TATA-binding protein (*Tbp*) and *Gapdh* for TaqMan.

The primers used are presented in Table 1. Real-time PCR was performed on the Step One Plus Detection System (Applied Biosystems) employing the SYBR Green PCR Master Mix (Applied Biosystems). Each reaction mixture contained 7.5 μL of complementary DNA (cDNA), containing 2 μg, 0.75 μL of each primer (whose concentration was 100 nM), and 7.5 μL of SYBR Green PCR Master Mix (2 ×).

Data were analyzed utilizing the comparative Cycle threshold (Ct) method (ΔΔCt), where the actin transcript level was employed to normalize differences in sample loading and preparation. Each sample ($n = 4$–5) was analyzed in triplicate, and results represented the *n*-fold difference of transcript levels among different samples.

5. Statistical analysis

Data are expressed as means ± Standard error of the mean (SEM). Means were compared with two-way ANalysis Of VAriance (ANOVA) and post-hoc analysis. Statistical significance was considered when p values were < 0.05. Statistical outliers were carried out with the Grubbs' test and were removed from analysis.