European Journal of Pharmaceutical Sciences 123 (2018) 135–142

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

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

The role of autophagy induction in the mechanism of cytoprotective effect of resveratrol

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ARTICLE INFO

Keywords:
Autophagy
Caspase 3 activation
Cytoprotection
Non-transformed cells
Serum deprivation
Resveratrol
Mitochondrial membrane potential

ABSTRACT

We aimed at studying the potential mechanisms in the preventive effect of resveratrol on serum deprivation induced caspase 3 activation on non-transformed cells.

Methods: Apoptosis was induced by serum deprivation in primary mouse embryonic fibroblasts. Caspase 3 activation, reactive oxygen species production and depolarization of the mitochondrial membrane were measured by fluorescence methods. The involvement of intracellular receptors and autophagy in the effect of resveratrol were analyzed by using specific agonists and antagonists. The role of autophagy was further examined by Western Blot analysis of its protein markers, LC3-II and p62 as well as by acridine orange staining of acidic vacuoles.

Results: We found that neither aromatic hydrocarbon receptors nor estrogen receptors play an important role in the cytoprotective effect of resveratrol. Reactive oxygen species production was not significantly altered by either serum deprivation or resveratrol treatment. In the presence of serum deprivation resveratrol however, induced a significant depolarization in mitochondrial membrane potential. The autophagy inhibitor, chloroquine not only eliminated the preventive effect of resveratrol, but also turned it to deleterious suggesting the prominent role of autophagy induction in the cytoprotective effect. Resveratrol did not alter LC3-II expression, but facilitated p62 degradation in serum deprived cells, suggesting its ability to augment the late phase of autophagy and thus promote the autophagic flux.

Conclusion: We have demonstrated that resveratrol can protect primary fibroblasts against serum deprivation induced apoptosis by provoking mild mitochondrial stress and consequent up-regulation of autophagic flux.

1. Introduction

Trans-resveratrol (3,4′,5-trihydroxystilbene) is commonly used as a dietary supplement for prevention or treatment of various health problems, *e.g.* cardiovascular, neurological diseases and cancer. It was reported to possess multiple pharmacological properties such as cardioprotective, antioxidant (for review see Kovacic and Somanathan, 2010), anti-inflammatory, chemopreventive (for review see Hsieh and Wu, 2010) and anticancer activities (for review see Kalantari and Das, 2010). However, in the literature many of its effects are rather contradictory, *i.e.* both its cytoprotective and proapoptotic (Baarine et al., 2011; Li et al., 2014) as well as pro- or antioxidant activities were shown (for review see de la Lastra and Villegas, 2007). These conflicting effects can derive from the variety of models using different cell types, treatment duration and dosage (Barger et al., 2008).

We aimed at studying its effects on non-transformed cells since due to the significantly different survival pathways in normal and cancer cells may be one of the reasons of the contradictory findings in the literature. In our previous studies we showed resveratrol dose-dependently prevented serum deprivation induced caspase activation in primary fibroblasts confirming its cytoprotective properties on non-transformed cells. The mechanism of its effect is only partially understood, crucial role of p38 kinase pathway activation was demonstrated since its inhibition entirely abolished the anti-apoptotic effect of resveratrol (Ulakcsai et al., 2015).

In the literature a plethora of molecular targets of resveratrol among them estrogen receptor, aromatic hydrocarbon (Ah) receptor, mitochondrial respiratory chain and modulation of reactive oxygen species (ROS) generation were suggested, though their contribution to its cytoprotective effect is not fully understood.

Several previous studies showed resveratrol binding to estrogen receptors resulting in either agonist (Gehm et al., 1999) or antagonist

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https://doi.org/10.1016/j.ejps.2018.07.039
Received 20 March 2018; Received in revised form 13 July 2018; Accepted 18 July 2018
Available online 20 July 2018

effects (Lu and Serrero, 1999). It was also reported to be a competitive AhR antagonist that prevents the transactivation property of the receptor and inhibits the agonist induced gene expression (Casper et al., 1999). Resveratrol, in high concentration, was evidenced to inhibit the mitochondrial function, decrease cellular ATP levels, and activate AMP-activated protein kinase (AMPK) (Lionaki et al., 2015, Lovelace and Polyak, 2015). Its influence on mitochondrial respiratory chain function and ROS production were proposed to be responsible for some of its pharmacological effects (Q. Zhang et al., 2016). However, its anti- or prooxidant activity was suggested to be dependent on its concentration, the treatment duration and cellular redox status (Gueguen et al., 2015). Recent evidence also indicated that resveratrol can provoke autophagy in many kinds of cells causing either cytoprotective or proapoptotic outcome depending on the cell type and treatment protocol (Gu et al., 2016; Puissant et al., 2010). Mammalian target of rapamycin (mTOR) signaling pathway plays a central role in the regulation of autophagy (for review Kim and Guan, 2015; Paquette et al., 2018) and cardioprotective effect of resveratrol was reported to be partly mediated by mTOR dependent activation of autophagy (Gurusamy et al., 2010). Resveratrol has also been suggested to inhibit the mTOR pathway potentially serving as a neuroprotective mechanism following injuries (Zhou et al., 2018).

In the present study we aimed at elucidating whether estrogen or Ah receptors, mitochondrial respiratory chain and ROS modulation are involved in the previously reported caspase activation preventing effect of resveratrol. Furthermore, the involvement of autophagy and its regulator mTOR pathway were also investigated. Autophagy inhibitor chloroquine, mTOR inhibitor rapamycin, levels of LC3 and p62, marker proteins of early and late autophagy, respectively and staining for acidic vacuoles were used to characterize the process.

Mouse embryonic fibroblast (MEF) culture is widely used for translational studies because it is a simple model for testing basic pharmacological mechanisms due to its easy accessibility, rapid growth rates, and the lack of transformation. Moreover, its use raises less ethical concerns compared to primary cultures of human origin (Qiu et al., 2016). Primary mouse fibroblasts have previously been determined to express estrogen and Ah receptors and were used as model cultures to investigate various roles of these receptors (Alexander et al., 1997; Harper et al., 1991; Marsh et al., 2017). Based on these attributes MEF cells were used as non-transformed cell model in our experiments.

2. Materials and Methods

2.1. Reagents and Animals

Resveratrol, caspase 3 activity assay kit using fluorogenic caspase 3 substrate (Ac-DEVD-AMC), buffer components, benzo(a)pyrene, trimethoxyflavone, fulvestrant, chloroquine, tamoxifen, rapamycin, dichlorofluorescin diacetate (DCFDA) and hydroethidine (HE) and Western Blot reagents, such as acrylamide/bis-acrylamide 30% solution, ammonium persulfate, tetramethylethylenediamine, Tris, glycine and tricine were purchased from Sigma (St. Louis, MO, USA). JC-1 mitochondrial membrane potential detection dye was supplied by ThermoFisher Scientific (Waltham, MA, USA), β-estradiol hemihydrate was a kind gift of Gedeon Richter Pharmaceutical Inc. (Budapest, Hungary). Cell culture mediums and fetal bovine serum were supplied by Corning (Tewksbury, MA, USA) and ThermoFisher Scientific, respectively. Polyvinylidene fluoride (PVDF) transfer membrane and autoradiography film were obtained from Santa Cruz (Dallas, TX, USA) and Pierce ECL Western Blotting substrate was purchased from ThermoFisher Scientific.

Primary antibodies against LC3A/B and SQSTM1/p62 were supplied by Cell Signal Technology (Danvers, MA, USA) and that against GAPDH was purchased from R&D Systems (Minneapolis, MN, USA). Secondary anti-rabbit and anti-mouse antibodies were obtained from ThermoFisher Scientific.

Test compounds were dissolved in dimethyl sulfoxide (DMSO) and used in cell culture medium to provide 0.5% final DMSO concentration. Control cells were treated with the same concentration of DMSO. Resveratrol was used in 200 μM concentration that could completely prevent caspase activation according to our previous studies (Ulakcsai et al., 2015). Concentration of other test compounds was chosen according to literature data.

Pregnant NMRI mice for cell culture establishment were supplied by Toxicoop, Gödöllő, Hungary. All animal procedures were approved by the Ethical Committee of the Semmelweis University (22.1/1375/7/2010) and were in accordance with the EU Council Directives on laboratory animals (86/609/EEC).

2.2. Cell Culture Conditions

Mouse embryonic fibroblast culture was established according to CSH protocol (Nagy et al., 2006). Cells were maintained in DMEM supplemented with 10% fetal bovine serum and used between passage 3 and 7.

One day before the experiment cells were seeded to 6 cm Petri dishes (3×10^5 cells/dish). Twenty-four hours later fetal bovine serum was withdrawn from the cell culture medium to induce cell death. Resveratrol treatment was initiated simultaneously with serum deprivation. Benzo(a)pyrene, trimethoxyflavone, tamoxifen, fulvestrant, estradiol, chloroquine and rapamycin were applied simultaneously with serum deprivation and/or resveratrol treatment.

2.3. Caspase 3 Activity

For caspase activity assay after 3-hour treatment period cells were rinsed with PBS and harvested by trypsin-EDTA, and cytosol extract was prepared by hypotonic lysis with 0.6% Nonidet P40 according to Andrews and Faller (Andrews and Faller, 1991). Caspase 3 activity was measured by commercially available kit according to the manufacturer instructions using a Fluoroskan Ascent FL Microplate spectrofluorometer (ThermoFisher Scientific). Caspase 3 activity is normalized to the protein content of the sample measured by Lowry's method (Lowry et al., 1951).

2.4. Analysis of ROS Production and JC-1 Assay for Mitochondrial Membrane Potential

After 3-hour treatment period cells were rinsed with PBS and harvested by trypsin-EDTA. Cells were resuspended in PBS containing 1 μM HE, 2 μM DCFDA or 5 μM JC-1, respectively. After 30 min incubation at 37 °C cells were collected by centrifugation (450*g*, 5 min, room temperature), washed by PBS and fluorescence was recorded at 485 nm (excitation)/538 nm (emission) for DCFDA, 530 nm (excitation)/590 nm (emission) for HE and both 485 nm (excitation)/538 nm (emission) and 485 nm (excitation)/590 nm (emission) for JC-1 using a Fluoroskan Ascent FL Microplate spectrofluorometer. Depolarization of mitochondrial membrane potential is presented as increase in the green/red fluorescence ratio.

2.5. Staining of Acidic Vacuoles

Cells were cultured on glass coverslips in 24-well tissue culture plates and subjected to 3-hour serum deprivation in the absence or presence of 200 μM resveratrol. After completion of the treatment period the cells were washed by PBS and stained by acridine orange (1 μg/mL in PBS) for 15 min at 37 °C. After incubation the coverslips were washed in PBS and the cells were visualized by epifluorescent microscope (Olympus Corporation, Tokyo, Japan) using fluorescein filter set.