Evaluation of the cytoprotective effect of resveratrol and its mechanism *in vitro* on primary fibroblasts

Doctoral short thesis

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1. Introduction

Resveratrol (3,5,4'-trihydroxy-stilbene) is a phytoalexin compound, which can be found in numerous plants, mainly in the skin and seeds of red grapes. It was reported to possess multiple pharmacological properties including anti-aging, anti-oxidative, anti-inflammatory, anti-carcinogenic, neuro- and cardioprotective effects. It is known to have an effect on apoptosis as well, however, in the literature its rather contradictory properties, i.e. cytoprotective and pro-apoptotic activities, were reported. The cause of opposite effects may lie in different experimental conditions, such as the duration or dosage of treatment and cell types used in the various models. Characteristically, resveratrol has an opposite impact on apoptosis in non-transformed and transformed cells.

The targets of resveratrol and the mechanisms governing its effects are currently unclear. It was reported to affect different metabolic and signaling pathways, exhibit pro- or anti-oxidative activities, and modify the functions of several transcription factors and cofactors. In the literature a plethora of molecular targets of resveratrol among them aromatic hydrocarbon (Ah) receptor and estrogen receptor were suggested. A crucial role of SIRT1 was suggested in the beneficial effects of resveratrol, but association between its effects and other signaling pathways, such as PI3-kinase/Akt, p38 MAPK/JNK/ERK and mTOR kinase pathways was also reported in several studies. Mitochondrial respiratory chain and modulation of reactive oxygen species (ROS) generation have been also proposed to be responsible for its pharmacological effects. Influence of resveratrol on autophagy is widely investigated as well.

2. Aims

My aims were:

1. To study the protective effect of resveratrol against cell damage in primary non-transformed cell culture
    - Investigate the preventive effect of resveratrol on serum deprivation induced caspase 3 activation in primary mouse embryonic fibroblasts.

- Study the rescue effect of resveratrol on the already up-regulated caspase 3 activity, adding the compound to the cell culture medium after 3-hour serum deprivation.

2. To evaluate the specific mechanisms involved in the effect of resveratrol on serum deprivation induced caspase 3 activation
   - Determine the involvement of p38 MAPK, JNK, ERK, PI3, mTOR-kinases and SIRT1 signaling pathways.
   - Study the role of aromatic hydrocarbon and estrogen receptors in the effect of resveratrol.
   - Investigate the association between the antioxidant properties of resveratrol and its effect on serum deprivation induced caspase 3 activation.
   - Examine the role of mitochondrial depolarization and ROS generation.
   - Study the role of autophagy in the effect of resveratrol on serum deprivation induced caspase activation.

3. Methods

3.1 Concentration of test compounds was chosen according to literature data. All animal procedures were approved by the ethics committee of the Semmelweis University (22.1/606/001/2010, February 5, 2010) and were in accordance with the EU Council directives on laboratory animals (86/609/EEC).

3.2 Primary mouse embryonic fibroblast culture was established according to CSH protocol. 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 ($3x10^5$cells/dish). Twenty-four hours later FBS was withdrawn from the cell culture medium to induce cell death. Resveratrol treatment was initiated simultaneously with serum deprivation. Inhibitors of various signaling pathways, N-acetylcysteine, benzo(a)pyrene, trimethoxyflavone, tamoxifen, fulvestrant, estradiol, chloroquine were applied simultaneously with serum deprivation and/or resveratrol treatment. When the rescue effect of resveratrol was investigated, resveratrol was added to the cell culture medium after 3-hour serum deprivation.

3.3 For caspase activity assay after specified treatment periods (3, 4.5 and 6 hour) cells were rinsed with PBS and harvested by trypsin-EDTA, and cytosol extract was prepared by hypotonic lysis with 0.6% Nonidet P40. In order to evaluate direct caspase inhibitory effect of resveratrol, resveratrol was added directly to cytosol extract of serum-deprived fibroblasts immediately before measuring caspase 3 activity. Ac-VAD-CMK, a nonselective direct caspase inhibitor, was used in 20 μM concentration as positive control. Caspase 3 activity was measured by commercially available kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer instructions using a Fluoroskan Ascent FL Microplate spectrofluorometer (ThermoFisher Scientific, Waltham, MA, USA). Caspase 3 activity is normalized to the protein content of the sample measured by Lowry's method.

3.4 Analysis of cell viability by the CytoxOne® lactate dehydrogenase release kit (Promega, Fitchburg, WI, USA) was performed according to the manufacturer instructions using a Fluoroskan Ascent FL Microplate spectrofluorometer with an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Calculation of results were made as per the manufacturer's instructions.

3.5 For analysis of ROS production and mitochondrial membrane potential changes 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 (450g, 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.

3.6 For 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.

3.7 For Western Blot analysis total cell lysates were prepared using RIPA buffer. Total protein concentration was determined by Bradford's method. After denaturation by heating at 95 °C for 5 min in Laemmli buffer 30 μg protein samples were separated in 15% SDS-polyacrylamide gels and then transferred onto PVDF membranes. Membranes were then blocked with 2% non-fat dry milk or 5% BSA dissolved by Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h and then probed with primary LC3A/B, p62 and GAPDH antibodies overnight at 4 °C. Then the membranes were washed with TBST three times for 10 min and then incubated with 1:2000-diluted HRP conjugated secondary antibodies (rabbit and mouse IgG) for 1 h at room temperature. The specific proteins were detected on autoradiography films using ECL reagent. The densitometric analysis was performed using Image J software (National Institute of Health, Bethesda, MD, USA). Density ratios of LC3-II to LC3-I and p62 to GAPDH were used for calculations.

3.8 Data are expressed as proportional changes compared to control and are given as mean ± standard deviation of at least 3 parallel measurements. Data are represented as fold increase relative to control value. Paired *t* test and one-way ANOVA was used for data analysis followed by Sidak *post-hoc* test for multiple comparisons. Corrected $p < 0.05$ was considered statistically significant. Individual experiments were repeated at least three times. Figures represent the mean of multiple tests.

4. Results

4.1 Primary mouse fibroblasts were exposed to serum deprivation, which after 3-6 hours induced significant caspase 3 activation ($P < 0.001$). In order to evaluate the protective effect of resveratrol, the cells were treated with several concentrations (10, 25, 50, 75, 100, 200 μM) of resveratrol simultaneously with serum deprivation. Resveratrol prevented caspase 3 activation in a dose-dependent manner, with 50% inhibitory concentration (IC50) = 66.3 ± 13.81 μM. Caspase 3 activation following 3 hour serum deprivation was completely inhibited at 200 μM

resveratrol concentration (Figure 1), and thus this level was used in the further experiments. This protective effect was also obtained after up to 6 hours of serum deprivation. Serum deprivation induced caspase activation and protective effect of resveratrol were evident after 3 hour serum deprivation, and thus 3 hour serum deprivation was used in the further experiments.

To verify whether resveratrol regulates the cellular response or directly interacts with caspase 3, resveratrol was added directly to the cytosol extract rather than to cell culture medium. Resveratrol showed no direct caspase inhibitory effect.

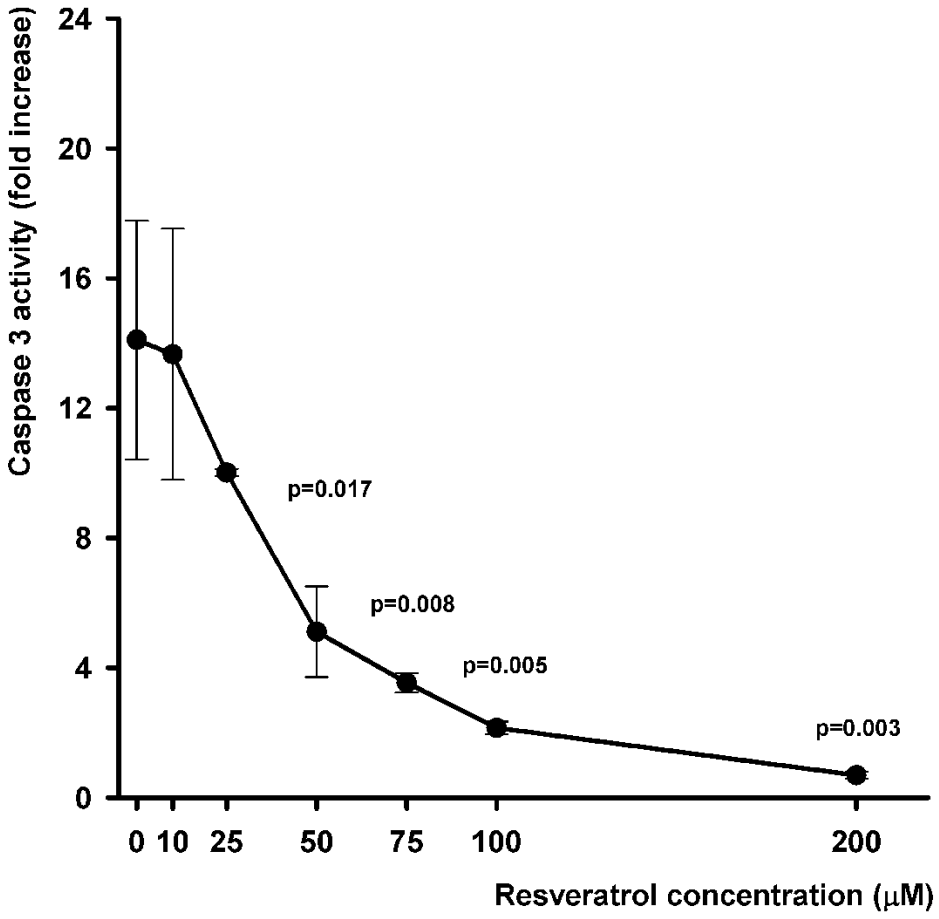

***Figure 1*: Resveratrol dose-dependently prevented caspase 3 activation after 3 h serum deprivation. Control value of caspase 3 activity in serum supported cells: 1.76 ± 0.097 nmol/mg/3 h. P values refer to the comparison with caspase 3 activity of serum deprived, resveratrol untreated cell group.**

4.2 We further investigated whether resveratrol reduced the already up-regulated caspase 3 activity. Primary fibroblasts were exposed to serum deprivation for 3 hours, after which the culture medium was treated with 200 μM resveratrol for an additional 2 hours. Resveratrol significantly reduced the already activated caspase 3. It prevented not only its further increase but also reduced it to a level below that observed after 3-hour serum deprivation. These experiments indicate that in addition to cytoprotection resveratrol may also have rescue effect on cells.

4.3 Lactate dehydrogenase release was measured to evaluate whether the inhibition of caspase 3 activation by resveratrol was accompanied by increased cell viability. Cell viability decreased by 24 hour serum deprivation was significantly improved by 200 μM resveratrol treatment.

4.4 In order to investigate the signaling cascades involved in the protective effects of resveratrol, we carried out experiments in the presence of specific inhibitors of p38, JNK, ERK, PI3K, mTOR kinase pathways, and SIRT1. Among them, only the p38 MAPK inhibitor SB202190 decreased the protective effect of resveratrol on caspase 3 activation (Figure 2).

4.5 In order to investigate the target receptors involved in the protective effects of resveratrol, we carried out experiments in the presence of specific Ah receptor agonist benzo(a)pyren or antagonist trimethoxyflavone. Their influence on the preventive effect of resveratrol on caspase activation was negligible.

4.6 To study the role of estrogen receptors in the effect of resveratrol the most commonly used antiestrogen, tamoxifen was first applied. It did not influence the cytoprotective effect of resveratrol, furthermore in the absence of resveratrol, tamoxifen by itself powerfully prevented serum deprivation induced caspase activation. The more selective and pure estrogen receptor antagonist fulvestrant and agonist estradiol interestingly acted alike, namely they significantly potentiated the caspase activation after serum deprivation, but neither treatment affected significantly the protective property of resveratrol.

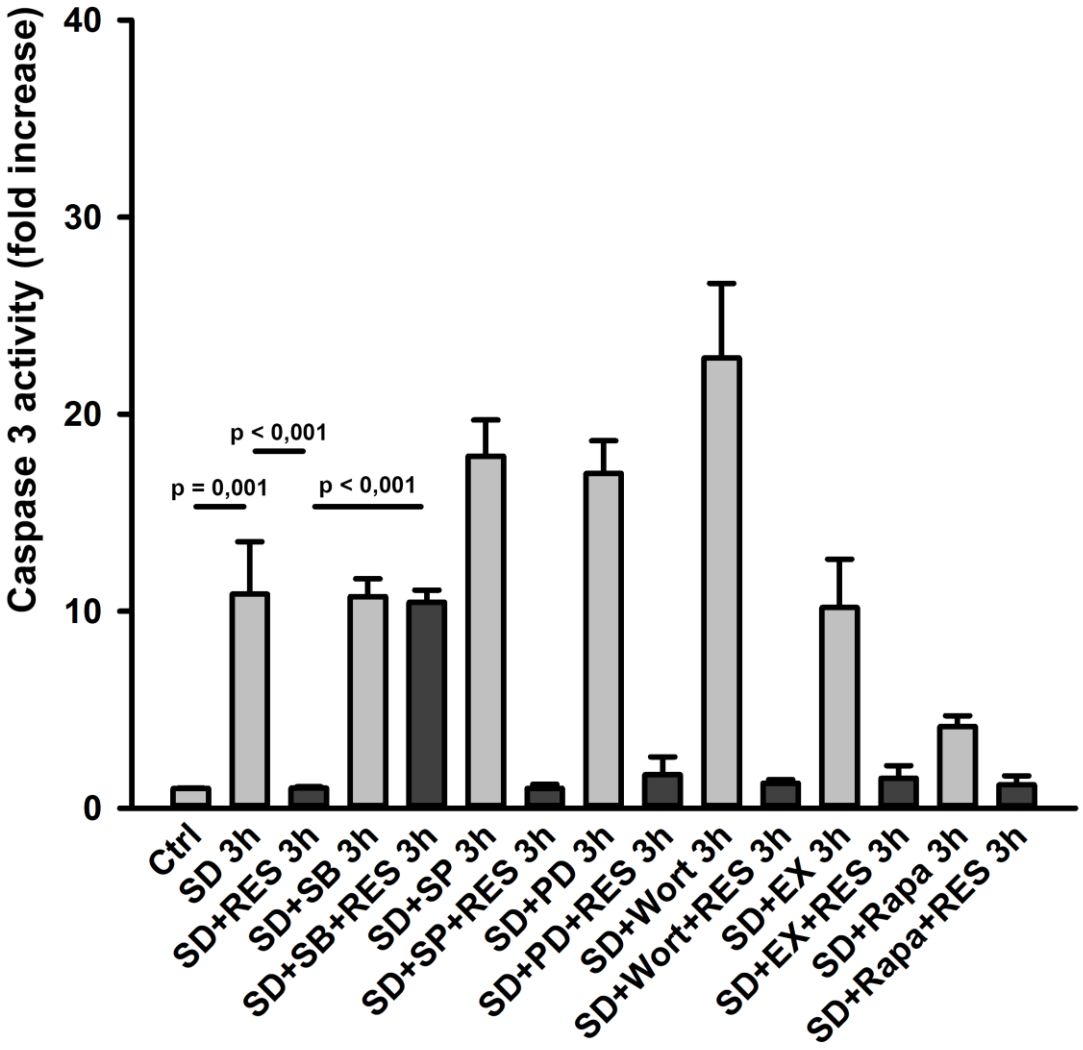

Figure 2: The effect of p38, JNK, ERK, PI3k, SIRT-1 and mTOR inhibitors on 3-h serum deprivation-induced (SD) caspase 3 activation and the protective action of 200 μM resveratrol (RES).

4.7 Considering that p38 kinase pathway is activated by mild intracellular stress and pro- and antioxidant properties of resveratrol had been previously described, we hypothesized that oxidative stress could be involved in caspase 3 activation induced by serum deprivation and/or the protective effect of resveratrol. To clarify if the antioxidant property of resveratrol may play a key role in its cytoprotective effect, we investigated the effect of N-acetylcysteine, a well-known antioxidant agent, on caspase 3 activation. Contrary to our expectations, it did not prevent caspase 3 activation but exacerbated it. However, resveratrol abolished the combined effect of serum deprivation and N-acetylcysteine on caspase 3 activation suggesting that its antioxidant property alone could not be responsible for its cytoprotective effect.

4.8 We have also studied the mitochondrial membrane potential and the generation of ROS as markers of intracellular stress. The mitochondrial membrane potential was not influenced by serum deprivation and resveratrol showed only a tendency for depolarizing mitochondrial membrane in serum supplemented cells. In the presence of serum deprivation however it induced a significant deterioration in mitochondrial membrane potential. Serum deprivation, resveratrol or their combination did not significantly alter the ROS production.

4.9 As resveratrol was previously reported to induce autophagy the role of the process was also studied in the protective behavior of the compound. Staining the cells by acridine orange showed that both serum deprivation and resveratrol, as well as their combination increased the number of acidic vacuoles consistent with enhanced formation of autophagosomes (Figure 3).

The well-known autophagy inhibitor, chloroquine completely eliminated the protective effect of resveratrol. Moreover, caspase activation was exaggerated when both resveratrol and chloroquine were present in the culture medium. Chloroquine alone did not change the serum deprivation induced caspase activation.

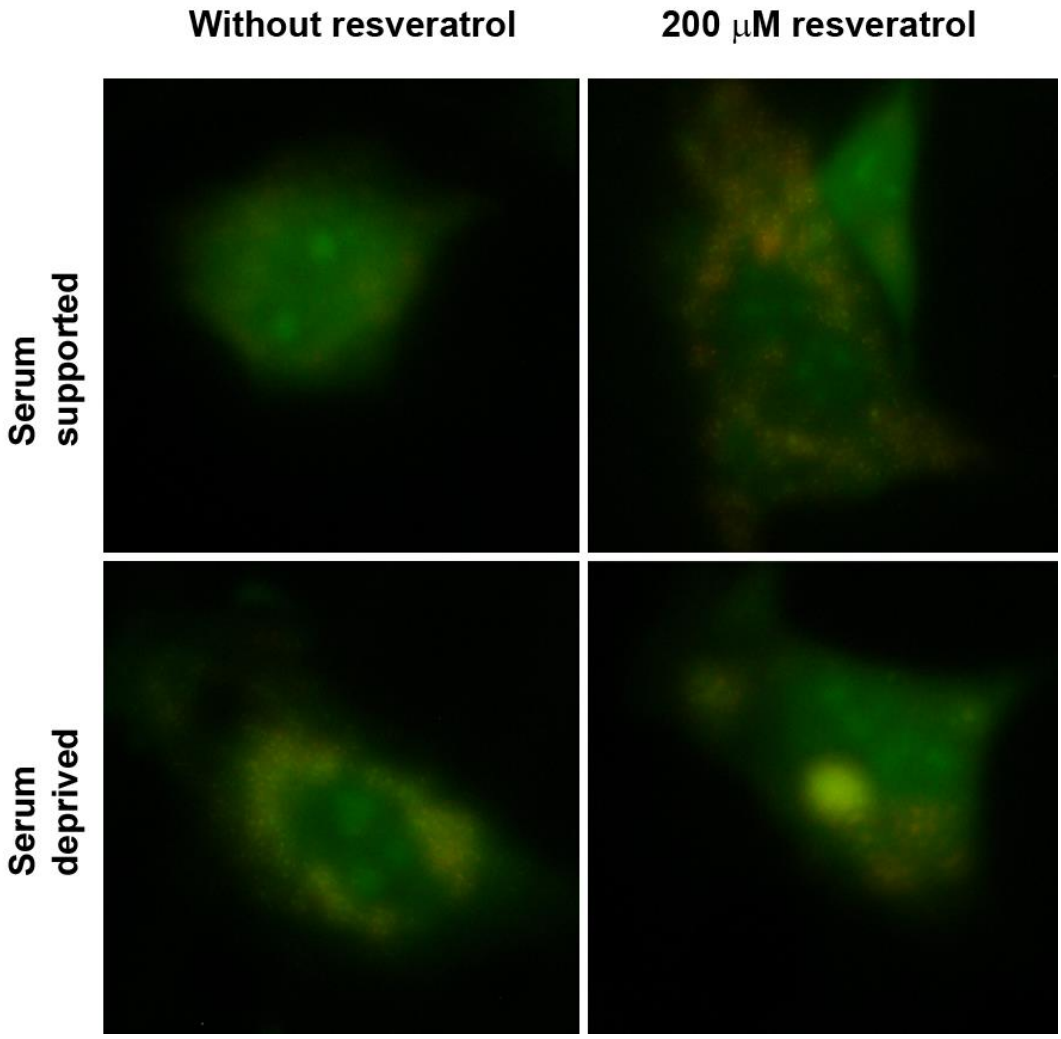

Figure 3: The effect of resveratrol on autophagy process. A: The effect of serum deprivation, 200 μM resveratrol (RES) and their combination on the formation of acidic vacuoles. Yellow to orange dots in the cytoplasm represent acidic vacuoles.

Expression of proteins LC3 and p62, markers of early and late autophagy, respectively, was detected by Western Blotting to further characterize the effect of serum deprivation and resveratrol on autophagy. Serum deprivation alone significantly increased the ratio of LC3-II/LC3-I which was not further altered by concomitant resveratrol treatment. Serum deprivation alone did not influence the level of p62, while resveratrol treatment of serum deprived cells significantly enhanced p62 degradation suggesting the improvement of autophagic flux by the compound (Figure 4).

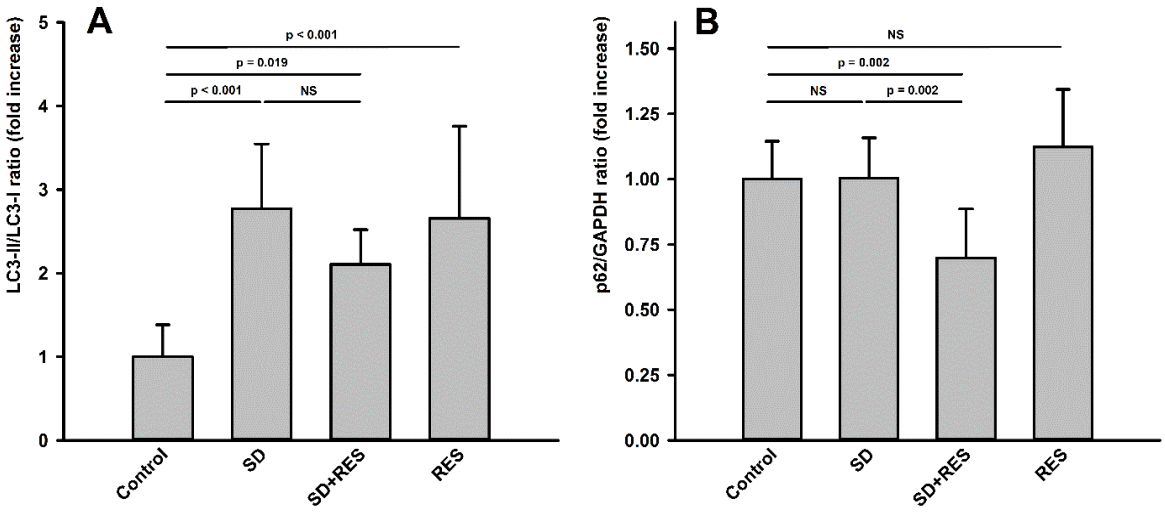

Figure 4: Expression of protein markers of autophagy. The effect of serum deprivation (SD), 200 μM resveratrol (RES) and their combination on the ratio of LC3-II/LC3-I (A) and degradation of p62 protein (B).

5. Conclusions

5.1 Resveratrol prevented serum deprivation-induced caspase 3 activation in primary mouse embryonic fibroblasts and increased their viability. In this study, cytoprotective effect of resveratrol was considerable in 100-200 μM concentration range. In these experiments resveratrol treatment was initiated simultaneously with serum deprivation and its cytoprotective effect was demonstrated. However, resveratrol was able to significantly reduce the already activated caspase 3 added to culture medium after 3-hour serum deprivation. To the best of our knowledge this is the first report demonstrating that resveratrol abolishes the already elevated caspase 3 activity induced by serum deprivation, suggesting its rescue effect as well.

5.2 It has already been suggested that several kinase pathways have a role in the cytoprotective effects of resveratrol. Activation of PI3-kinase/Akt, p38 MAPK/JNK/ERK, mTOR signaling, and molecular pathways involving SIRT1 were previously suggested as downstream routs of resveratrol. Our present findings indicate that the most critical signaling pathway in the protective effect of resveratrol against serum deprivation-induced caspase 3 activation is the activation of

p38. The reports about the effects of resveratrol on p38 kinase pathway are rather contradictory. P38 kinase seems to have a dual role as a regulator of cell fate, mediating either survival or death depending on the cell type, stimuli, and/or p38 isoform. In the present experiments, we showed that p38 MAPK had a cytoprotective rather than proapoptotic role.

5.3 Association between the effects of resveratrol and Ah receptors was reported in several studies. In our present experiments compounds known to have direct effect on Ah receptor – its agonist benzo(a)pyrene and antagonist trimethoxyflavone – did not alter the protective effect of resveratrol against serum deprivation induced caspase activation, indicating that the protective effect of resveratrol is independent of inhibition or activation of Ah receptors.

5.4 Many reports identified resveratrol as an estrogen receptor modulator and many of its effects are suggested to be based on this activity, however these data are often contradictory. Molecular dynamic simulations revealed that resveratrol acts rather as a selective estrogen receptor modulator and its actual effect is highly dependent on the cellular environment and presence of co-regulator proteins. In our present experiments, tamoxifen did not affect the protective influence of resveratrol on caspase activation, moreover similarly to resveratrol it was able to prevent serum deprivation induced caspase activation. To clearly understand the role of estrogen receptors in the caspase activation preventing effect of resveratrol the pure estrogen receptor antagonist fulvestrant and agonist estradiol were also tested. The results of these experiments suggest that the present effect of resveratrol is not associated with the activation of estrogen receptors.

5.5 Both anti- and prooxidant properties of resveratrol have already been reported. Whether it has anti- or prooxidant effects was suggested depending on its dose, the treatment duration and cellular redox status. Importantly, the effect of resveratrol can differ in the presence or absence of cellular stress. Several articles discuss antioxidant properties of resveratrol as the cause of its cytoprotective effect. Considering that another antioxidant, namely N-acetylcysteine exacerbated rather than prevented serum deprivation-induced caspase activation and resveratrol abolished their combined effect, antioxidant properties of resveratrol alone cannot explain its protective action.

5.6 Based on our previous findings, that p38 stress kinase pathway is critical for the cytoprotective effect of resveratrol, we investigated the role of mitochondrial dysfunction and ROS generation as possible sources of cellular stress in its protective effect. We found that neither serum deprivation

nor resveratrol alone influence mitochondrial membrane potential, but resveratrol caused significant mitochondrial depolarization in serum deprived cells. In line with our results resveratrol was previously reported to regulate mitochondrial respiratory chain function. Although mitochondrial dysfunction is regarded as the major source of ROS, in the present study we found that neither serum deprivation nor resveratrol treatment caused significant alteration in the ROS production. These results might be explained by the direct antioxidant effect of the compound that may compensate the ROS generation induced by the mitochondrial damage. By this way the induced intracellular stress may be kept in a low range that activates the cytoprotective machineries, such as autophagy, instead of direct cellular damage. This process can mimic the effect of ischemic preconditioning by increasing the stress resistance induced by a mild damage. Activation of various signal transduction pathways including p38 kinase signaling was reported as molecular mechanisms involved in preconditioning that is in line with our previous findings.

5.7 Because mitochondrial membrane depolarization and activation of p38 kinase pathway are commonly associated to autophagy and both tamoxifen and resveratrol were reported to modulate autophagy our interest turned to this latter. Autophagy can be a protective mechanism and can enhance the stress-resistance by eliminating damaged cell organelles. In the present study chloroquine an established late phase autophagy inhibitor entirely eliminated the inhibitory effect of resveratrol on serum deprivation induced caspase activation, which suggests the importance of autophagy in its cytoprotective function. Moreover, chloroquine not only abolished but inverted the effect of resveratrol on caspase activity, which reached even a higher level compared to that in case of serum deprivation alone. As in the absence of resveratrol chloroquine with or without serum deprivation did not affect caspase activation, its aggravation is likely the consequence of turning the effect of resveratrol from the protective to a harmful direction. The involvement of autophagy in the protective effect of resveratrol was further confirmed by examining the levels of LC3 and p62, marker proteins of early and late autophagy, respectively. Our data suggest that serum deprivation itself upregulated the autophagosome formation, but it was not accompanied by an increase in the autophagic flux. Resveratrol affected this latter process, indicated by increased degradation of p62, thus improving the late phase of autophagy may contribute to its cytoprotective activity. We can thus speculate that up-regulation of autophagy secondary to serum deprivation is a counterregulatory process aiming at elimination of damages, but it may not be enough to overcome the apoptotic process. Facilitation of autophagy thus can be supposed to be

cytoprotective by counteracting the apoptosis activation. Resveratrol may enhance this cytoprotective mechanism via facilitation of autophagic flux.

5.8 Based on our present data we can hypothesize that resveratrol causes a mild mitochondrial damage and intracellular stress in serum deprived primary fibroblasts, which can induce autophagy *via* p38 dependent pathway and protect cells from apoptosis by removing dysfunctional cell constituents. Our present hypothesis might explain some of the contradictory data regarding the pro- and anti-apoptotic effect of resveratrol. It induces a mild damage *per se* that can result in either potentiation of stress resistance pathways or direct cytotoxicity depending on the actual circumstances e.g. its concentration, the cell type studied, the intensity of damaging insult or mitochondrial redox status (Figure 5).

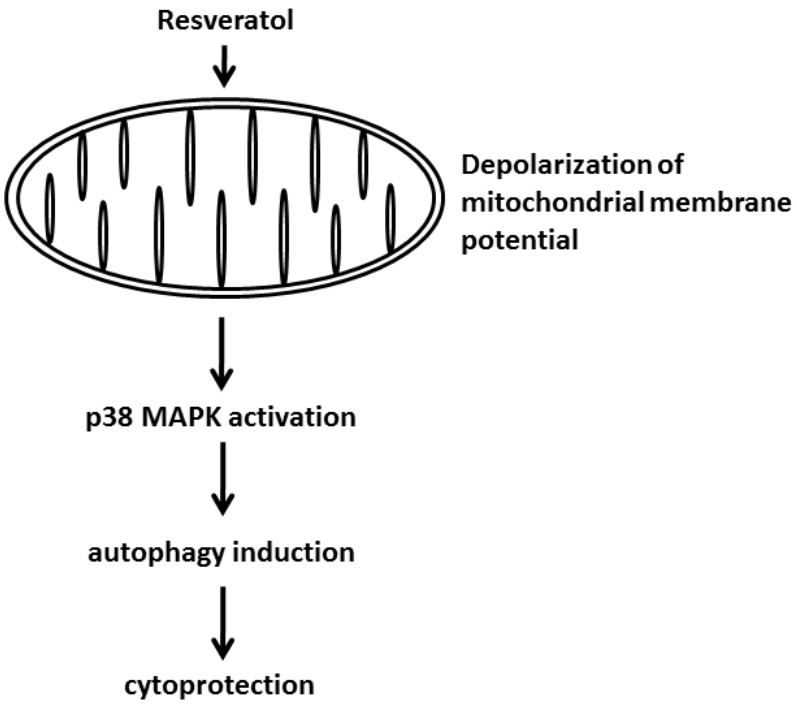

***Figure 5*: Proposed mechanisms of protective effect of resveratrol on serum deprivation induced caspase 3 activation. Based on our hypothesis resveratrol causes a mild mitochondrial damage and intracellular stress in serum deprived primary fibroblasts. The ROS generation induced by the mitochondrial damage might be compensated by the direct antioxidant effect of the compound and intracellular stress may be kept in a low range that activates the cytoprotective machineries, such as autophagy, instead of direct cellular damage. We can hypothesize that mild intracellular stress caused by resveratrol which can induce autophagy *via* p38 dependent pathway and protect cells from apoptosis.**

6. Publications

Publications in the topic of dissertation:

Ulakcsai Z, Bagaméry F, Vincze I, Szökő É, Tábi T.

Protective effect of resveratrol against caspase 3 activation in primary mouse fibroblasts.

CROATIAN MEDICAL JOURNAL. 2015 Apr;56(2):78-84.

IF: 1,483

Ulakcsai Z, Bagaméry F, Szökő É, Tábi T.

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

EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES. 2018 Oct 15;123:135-142.

IF: 3,466 (2017)

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