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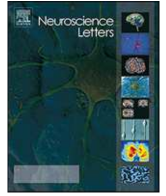

Resveratrol reduces oxidative stress and cell death and increases mitochondrial antioxidants and XIAP in PC6.3-cells

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ABSTRACT

Resveratrol, a polyphenol derived e.g. from red grapes, has been shown to mediate several positive biological actions such as protection of cells against oxidative stress. It can also influence cell signaling, but the mechanisms behind its antioxidant properties are largely unknown. Here we show that RSV reduces oxidative stress and enhances cell survival in PC6.3 cells depending on the concentration. In these cells, RSV increased the levels of antioxidants, SOD2 and TRX2, and of X chromosome-linked inhibitor of apoptosis protein. RSV also activated NFκB signaling as shown using luciferase reporter constructs. These findings show that RSV regulates oxidative stress and mitochondrial antioxidants in neuronal cells. This may contribute to cell protection in various brain disorders.

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Resveratrol (3,4′,5-trihydroxystilbene; RSV) is a polyphenol compound primarily found in grapes and red wine. RSV has a large number of biological actions including protection of cells against oxidative stress [6]. The capability of RSV to protect against ischemic brain damage has been reported, and RSV could be a useful compound in the treatment of neurodegenerative diseases like Huntington's, Parkinson's and Alzheimer's disease as well [2,7–9,19,20,25].

Mechanisms behind antioxidant properties of RSV are not fully understood but it has been reported that it affects to activation of certain genes and proteins: it increases the activity of AMP-activated kinase (AMPK), Silent Information Regulator 1 (SIRT-1), and peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α) [5,17]. PGC-1α in turn has shown to be co-induced with several intracellular antioxidant enzymes [24]. RSV is also able to affect survival pathways like NFκB and mitogen activated protein kinases (MAPKs) [1,11,16]. However as shown previously the biological outcome of RSV treatments may depend on the cell type and the concentration of the compound used. Thus higher concentrations of RSV have been shown to trigger apoptosis instead of survival in tumor cell and in cultured endothelial cells [12,13]. In this work we have studied this in more detail and investigated the connection between RSV and oxidative stress in PC6.3 cells that is a neuron-like cell line from the peripheral nervous system. Data showed that RSV at lower concentrations had a beneficial effect in PC6.3 cells, counteracting oxidative stress and increasing cell viability. Treatment with RSV increased levels of mitochondrial antioxidants and activated NFκB signaling that may contribute to cell protection.

Neuron-like PC6.3 cells were cultured in RPMI 1640 medium, containing 2 mM Glutamax with 10% Horse Serum (HS) and 5% Fetal Calf Serum (FCS). Approximately 30×10^3 cells per well in collagen-coated 96-well dish (Costar) plates were stimulated with 50 μM xanthine (X; Sigma) and 50 mU/ml xanthine oxidase (XO; Sigma) for 24 h in 1% FCS to induce oxidative stress in the presence and absence of 30 min pretreatment with 50 and 75 μM RSV. Cell viability was determined by MTT assay. Briefly, 0.5 mg/ml MTT-solution (Thiazolyl Blue Tetrazolium Bromide, Sigma) was added to the cells for the last 2 h (+37 °C). Then medium was removed and isopropanol-/HCl-solution was added. The dye formed was measured at 560 nm, and the absorbance was linear to the number of viable cells.

PC6.3-cells in 6 cm plates (Nunc) were stimulated with 100 μM X and 200 mU/ml XO for 3 h in the presence and absence of 30 min pretreatment with 50 and 75 μM RSV. 10 μM dihydroethid-

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ium (DHE; Molecular Probes) was added for the last 15 min, and then cells were suspended into PBS and immediately examined by fluorescent activated cell sorter (FACS) Aria (BD Biosciences). DHE-positive cells were calculated with excitation at 488 nm and emission at 595 nm.

Total RNA was extracted from control and treated PC6.3-cells. PCR was carried out as described previously [15]. To quantify SOD2, TRX2 and XIAP levels, we analyzed the cDNA samples by using the following primers: XIAP: FW 5′-TGC TGG ACT CTA CTA CA-3′ and RV 5′-GAC TTG ACT CAT CCT GCG A-3′; SOD2: FW 5′-GCC TGC ACT GAA GTT CAA TG-3′ and RV 5′-ATC TGT AAG CGA CCT TGC TC-3′; TRX2: FW 5′-GGA CTT TCA TGC ACA GTG-3′ and RV 5′-CGT CCC CGT TCT TGA T-3′. β-Actin from same cDNA samples was used to control the total cDNA levels with the following primers: FW 5′-CAC ACT GTG CCC ATC TAT GA-3′ and RV 5′-CCA TCT CTT GCT CGA AGT CT-3′.

Cells were lysed in a buffer containing 50 mM Tris–HCl (pH 7.4), 1% NP-40, 0.25% natriumdeoxycholate, 150 mM NaCl, 1 mM EDTA and protease inhibitors (Roche). Protein concentration was determined by BC Assay: protein quantitation kit (Uptima, Interchim). 20–40 μg proteins were separated using SDS-PAGE gels by electrophoresis and transferred to a nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences UK Limited), blocked for 1 h in room temperature in Tris-buffered saline (TBS) and 5% skim milk, and incubated overnight at +4 °C with primary antibodies diluted blocking buffer. Used antibodies were against SOD2 (AbFrontier) 1:15,000, TRX2 (AbFrontier) 1:1000 or XIAP (BD Transduction Laboratories) 1:5000. Anti-actin (Sigma) 1:1000 was used as a loading control. Next day membrane was incubated with horseradish peroxidase conjugated secondary antibodies (1:2500, Pierce) for 1 h and detection was performed using SuperSignal®West Pico Chemiluminescent Substrate (Pierce). Quantifications were performed using ImageJ.

PC6.3-cells in 6-well plates (3.5 cm) were transfected with 0.5 μg NFκB Luciferase Reporter and 0.01 μg *Renilla* luciferase pRL-TK control plasmid. Cells were stimulated with 50 μM and 75 μM RSV for 24 and 48 h. Then cells were lysed in Passive lysis buffer (Promega). For assay the expression of both *Renilla* and firefly luciferase, we used the dual luciferase substrate (Promega), and the activities were measured by luminometer (TD-20/20, Luminometer Turner Designs). The values for firefly luciferase were normalized to the *Renilla* luciferase activity.

One-way ANOVA with pots hoc-tests was used for statistical comparisons of our experiments.

Based on the perceived antioxidant properties of RSV, we first investigated the capability of RSV pretreatment to reduce oxidative stress in our system. Neuron-like PC6.3-cells were pretreated with 50 or 75 μM RSV, and after 30 min were stimulated with 100 μM xanthine and 200 mU/ml xanthine oxidase for 3 h. 10 μM DHE was added for the last 15 min, and the DHE-positive cell number was calculated by FACSAria and expressed as relative number of control. The data showed that both 50 μM and 75 μM RSV reduce the generation of intracellular ROS both in control cells and after exposure to oxidative stress by X + XO (Fig. 1a). The effect was about the same with both RSV concentrations.

We also examined cell viability with MTT assay following 24 h X + XO (50 μM + 50 mU/ml) treatment, and found out that both RSV concentrations (50 and 75 μM) reduced cell death caused by X + XO for about 25% (Fig. 1b). At dose of 100 μM this protective effect of RSV disappeared. Longer (6 h) pretreatment with RSV (50 μM) gave the same protection as was observed using a shorter (30 min) pretreatment.

The ability of RSV to decrease ROS levels in neuron-like PC6.3 cells, suggested us that RSV may have an influence on intracellular antioxidants, such as SOD2 and TRX2. Therefore, we evaluated the levels of these antioxidants and results showed that RSV (50 μM; 24 h) increases both mRNA and protein levels of mitochondrial antioxidants SOD2, and TRX2 (Fig. 2a–d). 75 μM RSV gave essentially the same results as 50 μM. RSV also enhanced mRNA and protein levels of anti-apoptotic protein XIAP (Fig. 2a–d). After a longer treatment (72 h), the effect of RSV on SOD2 and TRX2 was even stronger, and the increase was detectable already with smaller concentrations (10 and 25 μM) (Fig. 3).

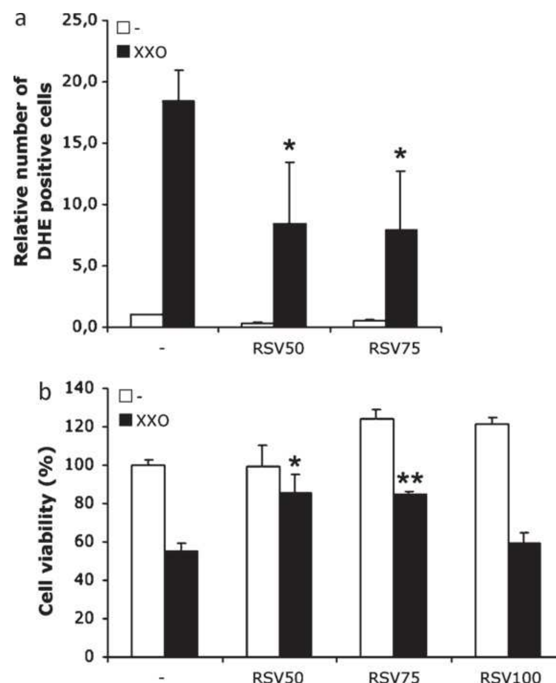

Fig. 1. 50–75 μM RSV pretreatment decreases cell death and production of ROS after oxidative stress. (a) Influence of RSV on ROS production. PC6.3 cells were pretreated with 50 μM and 75 μM RSV for 30 min, and then stimulated with 100 μM xanthine (X) and 200 mU/ml xanthine oxidase (XO) for 3 h. ROS levels were determined using the dye DHE and FACSAria, and presented here as a quantification of the data. X + XO treated cells are shown as black columns. 50 μM and 75 μM RSV treatment decreased the production of ROS both alone and after exposure to X + XO. Values are ±SEM, $n = 4$. $p < 0.05$ for XXO vs. XXO + RSV 50 μM and for XXO vs. XXO + RSV 75 μM. (b) Effect of RSV on cell viability. PC6.3 cells were pretreated with 50, 75 and 100 μM RSV for 30 min, and then stimulated with 50 μM xanthine (X) and 50 mU/ml xanthine oxidase (XO) for 24 h. X + XO treated cells are shown as black columns. Cell viability was determined using the MTT assay. 50 μM and 75 μM RSV pretreatment protected cells against death caused by X + XO, but the 100 μM RSV concentration did not show protective effect. Values are means ± SEM, $n = 4–6$. $p < 0.05$ for XXO vs. XXO + RSV 50 μM, and $p < 0.01$ for XXO vs. XXO + RSV 75 μM.

Next we investigated the NFκB levels since many of the intracellular antioxidants are known to be regulated by the NFκB system. The NFκB Luciferase Reporter construct was used to study the activation level of NFκB in PC6.3 cells by stimulation with 50 μM and 75 μM RSV. After 24 h, 50 μM RSV had a small positive effect on the activity of NFκB, but 75 μM RSV did not (Fig. 4). After 48 h, both 50 μM and 75 μM RSV increased the activity of NFκB about 2.5 fold. The activation of NFκB may contribute to the increase in antioxidants SOD2 and TRX2 mediated by RSV.

ROS levels are normally in balance with cellular antioxidants, but increased ROS levels are found in neurodegenerative diseases such as Huntington's, Parkinson's, and Alzheimer's diseases [4,10,21]. We observed that RSV reduces the basal levels of ROS and significantly attenuates the increase in ROS caused by xanthine and xanthine oxidase, and enhances the viability of the cells. Previously it has been reported that RSV can induce SOD2 in human fibroblasts [22]. We show here that RSV elevated the mitochondrial antioxidants, SOD2 and TRX2, in PC6.3 cells both alone, and during oxidative stress, when given as a pretreatment. The less prominent effect of RSV in the oxidative stress condition compared to RSV alone may be a consequence of the compensation mechanisms of

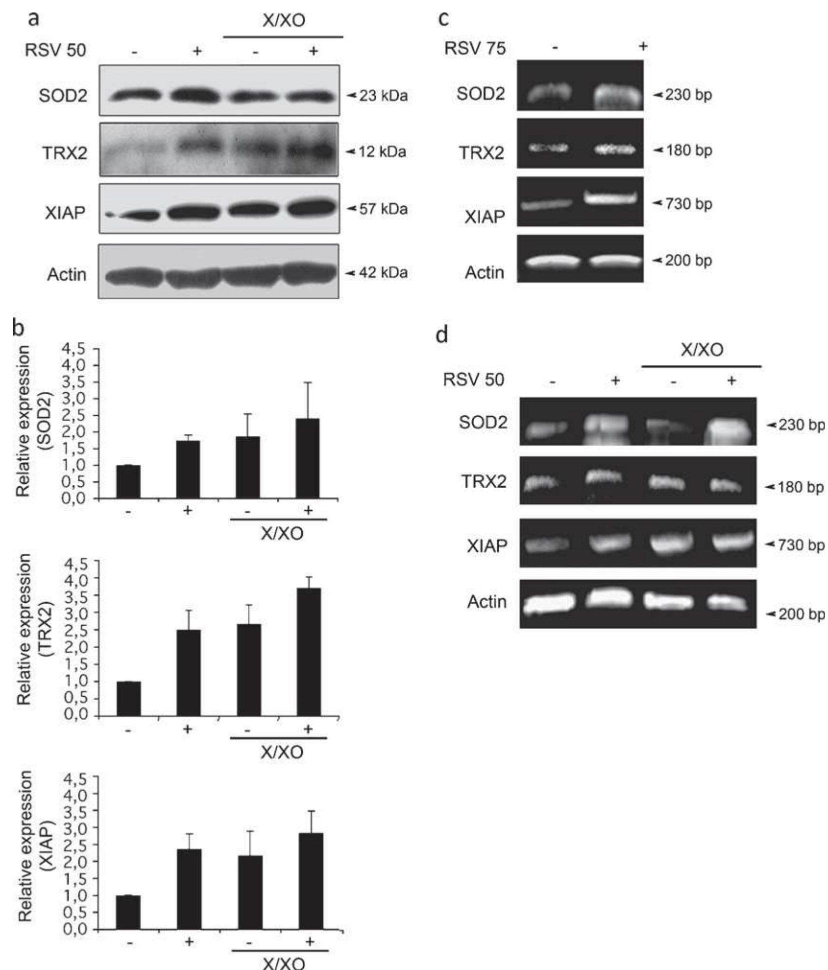

Fig. 2. (a) Effect of RSV on protein levels of SOD2, TRX2 and XIAP. PC6.3 cells were pretreated with 50 μM RSV for 30 min, and then stimulated with X + XO for 24 h. Protein levels were determined using Western Blotting analysis. RSV treatment increased the protein levels of SOD2, TRX2 and XIAP both in control cells and in cells exposed to X + XO. (b) Quantification of SOD2, TRX2 and XIAP proteins using ImageJ. Values are means ± SEM, $n = 3$. The influence of RSV on the protein levels of SOD2, TRX2 and XIAP proteins is rising. (c) Influence of 75 μM RSV treatment (24 h) on mRNA levels of SOD2, XIAP and TRX2. mRNA levels were measured using PCR. RSV treatment increased the mRNA amounts of SOD2, TRX2 and XIAP. (d) Effect of RSV together with 50 μM xanthine (X) and 50 mU/ml xanthine oxidase (XO). Cells were pretreated with RSV and 30 min after that, stimulated with X + XO for 24 h. RSV (50 μM; 24 h) elevated the mRNA levels of SOD2, TRX2 and XIAP both alone and during exposure to oxidative stress by X + XO.

the cells. Maybe these mechanisms attempt to increase the levels of antioxidants to reach the balance between oxidants and antioxidants within the cells, and that's why the relative change caused by RSV cannot be seen as clearly while oxidative stress prevails. Anyhow, the beneficial effects of RSV may be related to increases in antioxidants, SOD2 and TRX2. In addition to these, RSV also elevated both the mRNA and protein levels of the anti-apoptotic protein XIAP in our study. At least regarding SOD2 and TRX2, the effect of RSV seems to be time dependent, i.e. it will increase with the length of treatment. It remains to be studied which time and concentration of RSV would be optimal for the impact as an antioxidant agent in these cells.

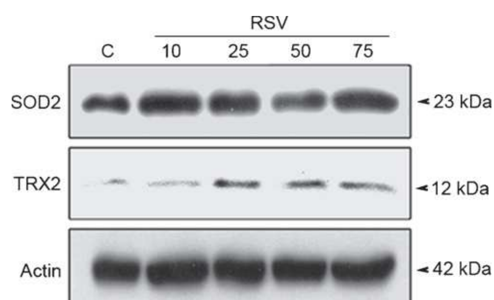

Fig. 3. Longer RSV treatment gives further increase in the proteins levels of SOD2 and TRX2. PC6.3 cells were treated with 10, 25, 50 and 75 μM RSV for 72 h.

Based on previous observations that XIAP increases SOD2 and TRX2 in neuronal cells via NFκB activation [15], in present work we evidenced that RSV enhances NFκB suggesting therefore that increased mitochondrial antioxidants may be mediated by NFκB. It is known that RSV suppresses NFκB i.e. in carcinoma cells [3,18]. In neuronal cells there is not much evidence concerning RSV and NFκB regulation, but it is known that RSV can have diverse effects depending on cell types [23]. The same RSV concentration can be protective in certain cell types and pernicious to others, and high concentrations trigger apoptosis whereas low concentrations promote cell survival [12,13]. In this study, we observed only a small increase in NFκB signaling after exposing cells to 50 μM RSV for 24 h. The 75 μM concentration RSV did not enhance the activation of NFκB after 24 h treatment at all. However, after 48 h of RSV treatment NFκB activity increased about 2.5 fold with both concentrations. This would speak for the hypothesis that the levels of antioxidants are more efficiently elevated after a longer RVS treatment. Apart from NFκB, other signaling pathways are probably involved in counteracting oxidative stress. Earlier it has been shown that RSV is able to activate AMP-activated kinase (AMPK), Silent Information Regulator 1 (SIRT-1), and PGC-1α [5,17].

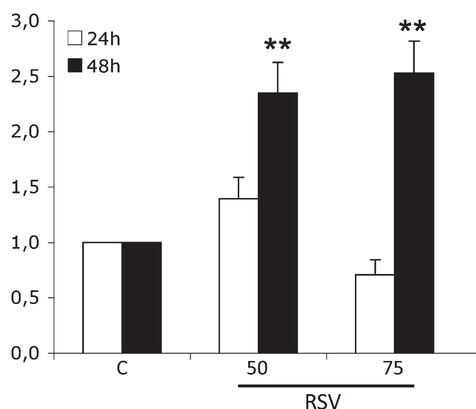

Fig. 4. After 24 h, 50 μM RSV had a small positive effect on the activity of NFκB, but 75 μM RSV did not. After 48 h, both 50 μM and 75 μM RSV increased the activity of NFκB. Cells were transfected with NFκB-Luciferase reporter plasmid and stimulated with 50 and 75 μM RSV for 24 h and 48 h. Co-transfection with *Renilla* expression vector was used as control to assay transfection efficiency. Results are presented as fold induction of Luciferase activity after of transfection. Values are means ± SEM, $n = 3$. $p < 0.01$ for RSV 50 μM vs. control (C), and for RSV 75 μM vs. control (C) after 48 h of stimulation.

It remains to be studied whether some of these pathways are influenced by RSV in PC6.3 cells. PGC-1α has previously been linked to intracellular antioxidant proteins and the action of RSV [17,24].

In this work, we demonstrate that RSV protects neuron-like PC6.3-cells from cell death caused by oxidative stress, and RSV reduced the generation of intracellular ROS. The precise timing and optimal dose of RSV may vary between cell types. Treatment of PC6.3 cells with 50–75 μM RSV increased viability if cells, but 100 μM concentration showed no significant effect. RSV is known to cross the blood–brain barrier (BBB) [6], and to have beneficial effects in acute brain insults and in some neurodegenerative disorders. In contrast to neurons, others have reported death-inducing effects of RSV i.e. in endothelial cells and in tumor cells including glioma cells [14,23]. As shown here, RSV at low concentrations promotes cell viability while at high concentrations it may exert some adverse effects that need to be taken into account. RSV is considered as a drug candidate for brain disorders, but the use of it will need careful analysis of its kinetics, actions and pharmacological and toxicological profiles.

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