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The SIRT1 activator resveratrol protects SK-N-BE cells from oxidative stress and against toxicity caused by α-synuclein or amyloid-β (1-42) peptide

Diego Albani,*[,1] Letizia Polito,*[,§,1] Sara Batelli,* Stefania De Mauro,* Claudia Fracasso,* Giuliana Martelli,* Laura Colombo,† Claudia Manzoni,† Mario Salmona,† Silvio Caccia,* Alessandro Negro‡ and Gianluigi Forloni*

*Department of Neuroscience, Mario Negri Institute for Pharmacological Research, Milan, Italy

†Department of Biochemistry and Molecular Pharmacology, Mario Negri Institute for Pharmacological Research, Milan, Italy

‡Department of Biochemistry, University of Padua, Padua, Italy

§Golgi Cenci Research Center, Abbiategrasso, Milan, Italy

Abstract

Human sirtuins are a family of seven conserved proteins (SIRT1-7). The most investigated is the silent mating type information regulation-2 homolog (SIRT1, **NM_012238**), which was associated with neuroprotection in models of polyglutamine toxicity or Alzheimer's disease (AD) and whose activation by the phytocompound resveratrol (RES) has been described. We have examined the neuroprotective role of RES in a cellular model of oxidative stress, a common feature of neurodegeneration. RES prevented toxicity triggered by hydrogen peroxide or 6-hydroxydopamine (6-OHDA). This action was likely mediated by SIRT1 activation, as the protection was lost in the presence of the SIRT1 inhibitor sirtinol and when SIRT1 expression was down-regulated by siRNA approach. RES was also able to protect SK-N-BE from the toxicity arising from two aggregation-prone proteins, the AD-involved amyloid-β (1-42) peptide (Aβ42) and the familiar Parkinson's disease linked α-synuclein(A30P) [α-syn(A30P)]. Alpha-syn(A30P) toxicity was restored by sirtinol addition, while a partial RES protective effect against Aβ42 was found even in presence of sirtinol, thus suggesting a direct RES effect on Aβ42 fibrils. We conclude that SIRT1 activation by RES can prevent in our neuroblastoma model the deleterious effects triggered by oxidative stress or α-syn(A30P) aggregation, while RES displayed a SIRT1-independent protective action against Aβ42.

Keywords: alpha-synuclein, amyloid-β-protein, autophagy, oxidative stress, resveratrol, SIRT1.

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Human sirtuins are coded by seven different genes (SIRT1 to SIRT7) whose individual functions are still under investigation (Haigis and Guarente 2006; Michan and Sinclair 2007). The best known member of the family is silent mating type information regulation 2 homolog (SIRT1, NM_012238, NP_036370) a NAD^+- dependent class III histone deacetylase that is the human homolog of the yeast Sir2 protein, which was linked to lifespan elongation of *Saccharomyces cerevisiae* and *Caenorhabditis elegans* (Tissenbaum and Guarente 2001; Belenky *et al.* 2007). Several investigations have shed light on the SIRT1 protein structure, enzymatic activity, physiological functions and cellular localization (Finnin *et al.* 2001; Saunders and Verdin 2007), but so far no straightforward data link SIRT1 and human longevity (Flachsbart *et al.* 2006). Nevertheless, SIRT1 appears to have a role in basic processes related to mammal senescence such as food and glucose metabolism, adipose tissue homeostasis or DNA repair (Cohen *et al.* 2004; Wang *et al.* 2006a; Qiang *et al.* 2007; Rodgers and Puigserver 2007). Sirtuins have been involved in neurodegeneration too. It is well known that a reduction of calorie intake is beneficial in preventing neurodegenerative processes, and SIRT1

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Address correspondence and reprint requests to Dr Diego Albani, Department of Neuroscience, Mario Negri Institute for Pharmacological Research, Via La Masa 19, 20156 Milan, Italy.
E-mail: albani@marionegri.it

[1]These authors contributed equally to the work.

Abbreviations used: 6-OHDA, 6-hydroxydopamine; AD, Alzheimer's disease; Aβ42, amyloid-β (1-42) peptide; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DS, Down syndrome; H_2O_2, hydrogen peroxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetylcysteine; PD, Parkinson's disease; RES, resveratrol; α-syn, alpha-synuclein.

up-regulation is one of the key features of calorie restriction (Bordone *et al.* 2007). A protective role of SIRT1 was suggested in models of Huntington's disease (Parker *et al.* 2005) and of Alzheimer's disease (AD) (Chen *et al.* 2005; Qin *et al.* 2006b; Kim *et al.* 2007), while to date no SIRT1 involvement has been reported in counteracting alpha-synuclein (α-syn) toxicity, one of the proteins linked to Parkinson's disease (PD) genetics. However, SIRT2 down-regulation correlated with a reduction of α-syn toxicity (Outeiro *et al.* 2007).

The SIRT1 activator resveratrol (RES), a phytocompound found in red wine, has proved beneficial against cardiovascular diseases and cancer (Bradamante *et al.* 2004; Aggarwal and Shisodia 2006). RES was reported to have also neuroprotective effects both *in vitro* and *in vivo*, mainly against oxidative stress and Aβ protein toxicity (Gélinas and Martinoli 2002; Marambaud *et al.* 2005; Wang *et al.* 2006b; Jang *et al.* 2007; Kim *et al.* 2007; Kumar *et al.* 2007).

To characterize the neuroprotective role of RES against oxidative stress and protein aggregation and to find out whether the protection was dependent on SIRT1 we developed an *in vitro* model using the neuroblastoma cell line SK-N-BE, positively modulating SIRT1 enzymatic activity by RES and negatively with the inhibitor sirtinol and by siRNA methodology.

Materials and methods

SK-N-BE cells

Cells were cultured at 37°C, 5% CO_2 in D-MEM supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). Cells differentiation was induced by retinoic acid 10 μM for 5–7 days.

TAT-α-syn(A30P) and Aβ42 preparation

Human α-syn cDNA was cloned starting from a cDNA library (Clontech, Palo Alto, CA, USA) by RACE-PCR, inserted into pRSET expression vectors (Invitrogen) and sequenced. TAT-α-syn(A30P) was generated by fusing the sequence of the minimal translocation domain of the HIV-1 virus Tat protein (YGRKKRR) in frame before α-syn N-terminal and the point mutation A30P was produced by PCR. The fusion protein, structurally identical to the native protein, was then purified by standard techniques (Dietz and Bahr 2004).

Aβ42 (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLM VGGVVIA) was synthesized by solid-phase peptide synthesis (SPPS) and purified by reverse phase HPLC. Peptide identity was confirmed by MALDI-TOF analysis (Salmona *et al.* 2003).

Atomic force microscopy

Aβ42 was incubated in presence or absence of RES (ratio 1 : 1.5) at 37 °C for increasing time intervals (0.5, 6 and 24 h). Each sample was diluted to 1 μM with phosphate-buffered saline (PBS) 10 mM, pH 7.4 and incubated for 0.5 min on a freshly cleaved Muscovite mica disk. After the incubation time, the disk was washed with H_2O and dried under gentle nitrogen stream. The sample was mounted onto a Multimode AFM with a NanoScope V system operating in Tapping Mode using standard phosphorus-doped silicon probes (T: 3.5–4.5 μm, L: 115–135 μm, W: 30–40 μm, K: 20–80 N/m) (Veeco Instruments, Plainview, NY, USA).

Oxidative stress challenge, TAT-α-syn(A30P) and Aβ42 treatment

30×10^3 cells were seeded in a 96-well plate. Next day, the medium was changed, a freshly prepared H_2O_2 dilution (or 6-hydroxydopamine [6-OHDA]) was added for 24 h (final concentration H_2O_2 or 6-OHDA: 75 μM). The toxic action of α-syn was reproduced by addition of TAT-α-syn(A30P) 3.0 μM for 24 h, while cell were incubated with Aβ42 peptide 10 μM for 24 h. Viable cells were then estimated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based viability assay according to the manufacturer's instructions (Promega Corp., Madison, WI, USA).

Resveratrol, sirtinol and *N*-acetylcysteine treatment

Cells were seeded in a 96-well plate at 30×10^3/well and after 6 h a freshly prepared dilution of RES (final concentration 7.5 μM) was added to the medium. The next day, (15 h from the first RES addition) a second RES dose was added and after further 4 h (19 h from the first RES addition) a toxic challenge was started for another 24 h interval (43 h from the first RES addition). Cell viability was then measured by MTT assay run in quadruplicate.

When required, sirtinol was added 2 h before RES. To test another antioxidant agent, 0.6 mM *N*-acetylcysteine (NAC) was added, following RES experimental scheme.

RES and sirtinol were dissolved in ethanol (vehicle, < 0.1% vol/vol of final volume). No viability difference was measured between vehicle-treated and untreated cells.

Resveratrol uptake

To assess RES uptake, 3.5×10^6 SK-N-BE cells were seeded and 6 h after RES 7.5 μM was added. After 15 h from RES addition, a second RES 7.5 μM dose was added. After 15, 19 and 44 h from the first RES treatment, RES intracellular content was measured. Cells were harvested, counted by erythrosine-dye exclusion assay and centrifuged. The washed pellet was frozen and then resuspended in a solution containing 70% acetonitrile, 10% formic acid in presence of zeranol (10 μg/mL) as internal standard. After centrifugation at 15 000 *g* for 10 min, an aliquot of a 1 : 5 water dilution of the supernatant in presence of 0.05% acetic acid was injected into an HPLC-MS-MS system (Waters, Milliford, MA, USA). RES content was quantified as described by Wang *et al.* with minor modifications (Wang *et al.* 2005). The limit of detection was 0.04 μg/mL and the assay was linear up to 2 μg/mL; coefficients of variation (CVs) were between 10–20%.

RNA interference

SK-N-BE cells were seeded in a 24-well plate or a 8-well chamber slide (Nalgene, Rochester, NY, USA) at 60×10^3 cells/well and grown overnight. The next day, the medium was replaced with 250 μL of fresh complete medium and a pre-designed double-strand small-interfering RNA (siRNA) against SIRT1 mRNA sequence (sense: 5′-GCCUCACAUGCAAGCUCUAtt-3′, antisense: 5′-UAGAGCUUGCAUGUGAGGCtc-3′) (Ambion, Austin, TX, USA) was added in 50 μL serum-free Optimem (Invitrogen) (final

concentration: 300 nM) using SilentFect liposome formulation (siRNA lipid vehicle) (BioRad Laboratories, Hercules, CA, USA). The medium was not changed for 72 h from siRNA addition. To prove SIRT1 specific down-regulation, a siRNA negative control (CT-) with no match to any known human transcribed sequence was used.

Real-time PCR

1×10^6 SK-N-BE cells were seeded in a T25 flask. The next day, cells were silenced for SIRT1 expression as described above. After 72 h, total mRNA was extracted using a commercial kit (Promega Corp.); about 50 ng were reverse-transcribed using an oligo-dT and SIRT1 mRNA was quantified by a TaqMan real-time assay (Applied Biosystems, Foster City, CA, USA) using the following primers and probe: for 5′-ATAGAGTGGCAAAGGAGCAGATTAG-3′; rev 5′-TTGGTGGCAAAAACAGATACTGA-3′); probe: 5′-AGGCGG CTTGATGGT-3′.

Western blotting, immunocytochemistry and autophagy evaluation

Western blotting

1×10^6 cells were collected and incubated for 20 min in twice the pellet volume with ice-cold buffer A [1.5 mM $MgCl_2$, 10 mM KCl, 10 mM Tris–HCl pH 7.9, 3 mM dithiothreitol (DTT) and 0.1% NP-40 (Roche Diagnostics GmbH, Mannheim, Germany)], in presence of a protease inhibitor cocktail. Nuclei were collected at 4°C (500 *g* for 15 min) and resuspended 1 : 1 (vol/vol) in buffer B (20% glycerol, 1.5 mM $MgCl_2$, 10 mM KCl, 3 mM DTT in 20 mM Tris–HCl pH 7.9) and 1 : 3 (vol/vol) in buffer C (20% glycerol, 1.5 mM $MgCl_2$, 1.2 M KCl, 3 mM DTT in 20 mM Tris pH 7.9). After rolling at 4°C for 1 h, samples were centrifuged at 4°C (15 000 *g*, 1 h), the supernatant quantified by Bradford assay and stored at −80°C. 50 μg nuclear proteins (or 25 μg of whole cell lysate) were loaded on a 8% SDS-PAGE and transferred to a nitrocellulose filter that was incubated with SIRT1 antibody (Abcam Inc., Cambridge, MA, USA) diluted 1 : 200, with α-syn antibody (clone 42; BD Transduction Laboratories, Lexinton, KY, USA) diluted 1 : 2000 or with LC3 antibody (clone 4E12; MBL, Woburn, MA, USA) diluted 1 : 400, followed by a horseradish-conjugated antibody (Jackson Immuno Laboratories, West Grove, PA, USA) diluted 1 : 1000. SIRT1, α-syn or LC3 signal was visualized by ECL (Millipore, Billerica, MA, USA) and quantified by a gel documentation system (BioRad Laboratories).

Immunocytochemistry

50×10^3 SK-N-BE cells were cultured on a plastic chamber slide (Thermo, Rochester, NY, USA), fixed with 4% paraformaldehyde and permeabilized using 0.5% Triton-X 100, 0.2% fetal calf serum in PBS. Then, a SIRT1 antibody (Abcam Inc., Cambridge, MA, USA) diluted 1 : 25 in PBS + 1% HS was added, followed by a FITC-conjugated antibody (Jackson ImmunoResearch, Suffolk, UK) diluted 1 : 100 in PBS + 1% HS. Cells were then scanned by a fluorescence microscope coupled to a digital camera (Olympus Corporation, Tokyo, Japan).

Autophagy

Cells were cultured on plastic chamber slide (Thermo), fixed and permeabilized as above described. To assess autophagosomes formation, LC3-II immunoreactivity was detected by a primary antibody (clone 4E12; MBL, Woburn, MA, USA) diluted 1 : 50 followed by a FITC-conjugated secondary antibody 1 : 200. Cells were then scanned by a fluorescence microscope coupled to a digital camera (Olympus Corporation). Autophagy was inhibited by 3-methyladenine 10 mM.

Thioflavin-T and Hoechst 33258 staining

SK-N-BE cells were cultured on plastic chamber slides (Thermo) and treated with TAT-α-syn 3 μM for 24 h. After fixing with 2% paraformaldehyde, a 0.05% thioflavin-T solution in PBS was added and fluorescence emission was monitored as above described.

Apoptosis was assessed in SK-N-BE cells incubated with RES 7.5 or 15 μM alone or in combination with H_2O_2 75 μM for 48 h by Hoechst 33258 (1 mg/mL) staining. A total of 200 nuclei from three independent optical fields were counted.

Chemicals and reagents

All chemicals were of analytical grade. Unless specified, reagents were from Sigma (Sigma-Aldrich, St Louis, MO, USA) while disposable plasticware was from Becton Dickinson (BD, Franklin Lakes, NJ, USA).

Statistical analysis

Statistics was performed by StatView ver 5.0 program. One-way (or two-way) ANOVA was used, followed by Dunnett's or Tukey's *post-hoc* test. The significance limit was $p = 0.05$.

Results

Resveratrol prevents oxidative stress

To verify whether RES prevented cell death due to oxidative stress, we plotted a dose–response curve in SK-N-BE cells using H_2O_2 as toxic agent (Fig. 1a). The third dose reduced viability by about 50% and we decided to use this concentration for the following experiments. Similar results were obtained using 6-OHDA (Fig. 1b). At the final concentration of 75 μM cell viability was reduced by 45%. In subsequent protection experiments we used this 6-OHDA concentration.

We evaluated the intrinsic toxicity of RES and sirtinol, exposing cells to increasing doses of RES for 24 h and then measuring cell viability by MTT assay. Figure 1c shows that RES was slightly toxic but only from 10 μM. For the next experiments, we used the RES concentration of 7.5 μM. As we planned not only to activate SIRT1 by RES but also to inhibit it with sirtinol, we also checked sirtinol's toxicity (Fig. 1d). Cells were exposed for 24 h to increasing amounts of sirtinol and viability was assessed by MTT assay. We found an evident reduction of cell viability starting from 5 μM. We therefore decided to inhibit SIRT1 activity using the non-toxic sirtinol concentration of 2.5 μM.

To estimate RES bioavailability and confirm its uptake by SK-N-BE, cells were incubated at first with a single dose of RES 7.5 μM. After 24 h RES intracellular content was measured by quantitative mass-spectrometry but was hardly

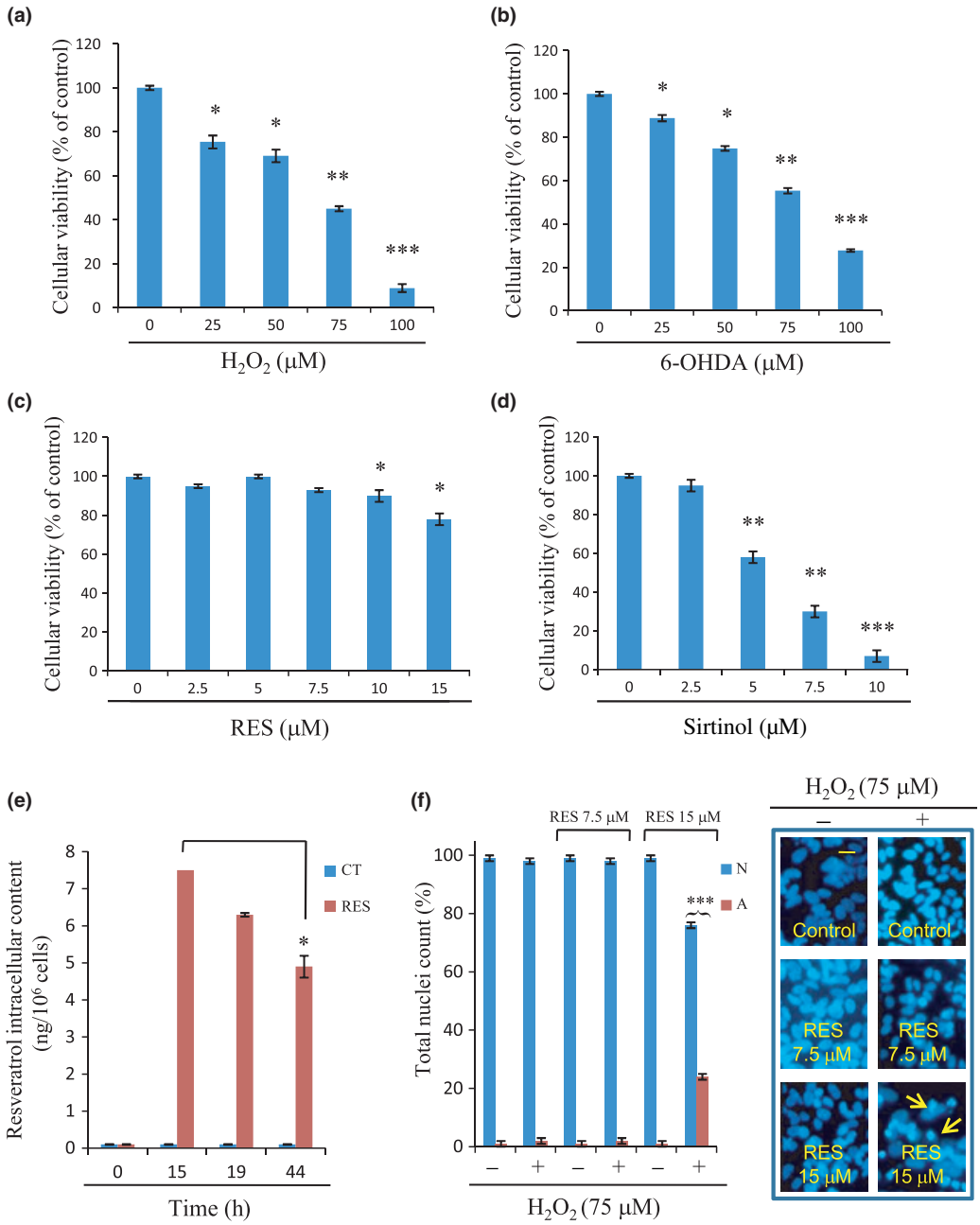


Fig. 1 Effect of oxidative stress and SIRT1 modulating compounds. SK-N-BE cells were exposed to H_2O_2 (a), 6-OHDA (b), RES (c) or sirtinol (d) for 24 h. Then, viability was assessed by MTT assay. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus control (untreated or vehicle-treated cells), one-way ANOVA and Dunnett's *post-hoc* test. (e) Evaluation of RES intracellular availability by mass spectrometry. RES 7.5 μM was added to medium and quantified by a HPLC-MS-MS apparatus. Each time-point was in triplicate and normalized to 10^6 cells. *$p < 0.05$, Tukey's test. (f) RES treatment did not trigger apoptosis. Cells were exposed to RES 7.5 or 15 μM alone or in combination with H_2O_2 75 μM for 48 h. After Hoechst 33258 staining, apoptotic nuclei were counted and plotted as percentage of total (N: normal; A: apoptotic; ***$p < 0.001$, vs. RES 15 μM alone, Tukey's test). The insert shows Hoechst 33258 stained nuclei under UV light. The arrows point to fragmented nuclei (magnification: 20×, scale bar: 15 μm).

detectable. To ensure a better RES bioavailability we incubated cells with RES 7.5 μM that was renewed after 15 h. Then, we measured RES intracellular content at different time-points (Fig. 1e). RES was uptaken by SK-N-BE cells and its concentration decreased over time. However, after 44 h from the experiment start it was still appreciable and around 60% in comparison to the second time-point (15 h). For subsequent experiments, we decided to keep the

two-time RES addition schedule and to perform a toxic challenge starting from 19 h from the first RES addition up to 43 h.

To exclude a RES pro-apoptotic effect we checked apoptosis in our model by Hoechst 33258 nuclear staining (Fig. 1f). RES 7.5 μM treatment for 48 h did not increase apoptotic nuclei percentage, even in presence of oxidative stress triggered by H_2O_2 75 μM. We were also unable to detect apoptosis with an increased RES dose (15 μM). However, apoptosis could be triggered in SK-N-BE cells, as a combined treatment with RES 15 μM and H_2O_2 75 μM significantly increased the number of fragmented nuclei (24%, $p < 0.001$, Tukey's test).

We moved on to assess RES protective effect against oxidative stress. We exposed SK-N-BE cells to H_2O_2 or 6-OHDA for 24 h, after double RES treatment (19 and 4 h before the toxic stimulus). As shown in Fig. 2a, RES pretreatment counteracted H_2O_2-induced damage, increasing cell viability by about 30%. The situation was similar for 6-OHDA (Fig. 2b). We have confirmed RES protective action by measuring intracellular reactive oxygen species (ROS) using a fluorescent ROS-sensitive probe (2′,7′-dichlorofluorescein diacetate [DCFH-DA]) (Wu *et al.* 2007). When cells were exposed to H_2O_2 for 24 h ROS increased in comparison to untreated cells, while RES pretreatment reduced ROS to the basal (Fig. S1a). A similar situation was reproduced using 6-OHDA as toxic agent (Fig. S1b).

In the same experiments shown in Fig. 2 we verified whether the antioxidant effect of RES was affected by the

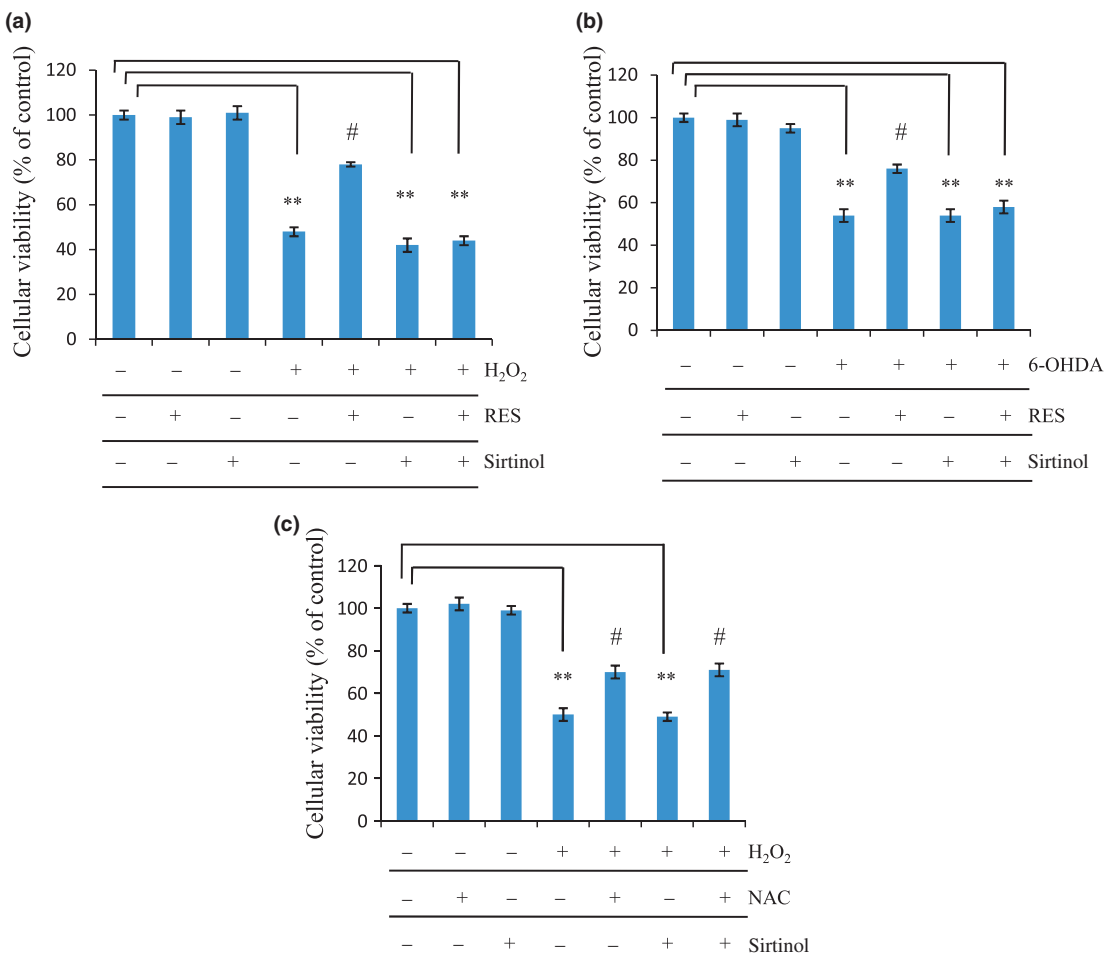


Fig. 2 RES protects from oxidative stress through SIRT1 activation. (a) SK-N-BE were incubated with RES 19 and 4 h before H_2O_2 75 μM addition. Two hours before each RES treatment, sirtinol 2.5 μM was added. $^{**}p < 0.01$ versus control (vehicle-treated cells); $\#p < 0.01$ for H_2O_2 (x) RES ($F_{int} = 41.8$), two-way ANOVA. (b) SK-N-BE were exposed to 75 μM 6-OHDA for 24 h and incubated with RES and/or sirtinol as above. $^{**}p < 0.01$ versus control (vehicle-treated cells); $\#p < 0.01$ for H_2O_2 (x) RES ($F_{int} = 18.7$), two-way ANOVA. (c) Cells were challenged with H_2O_2 75 μM for 24 h; NAC 0.6 mM was added 19 and 4 h before H_2O_2 addition, while sirtinol was added 2 h before each NAC treatment. $^{**}p < 0.01$ versus control (vehicle-treated cells); $\#p < 0.01$ for H_2O_2 (x) NAC ($F_{int} = 20.2$) and for H_2O_2 (x) NAC in presence of sirtinol ($F_{int} = 18.7$), two-way ANOVA.

SIRT1 inhibitor sirtinol (Denu 2005). When SK-N-BE cells exposed to H_2O_2 and pre-incubated with RES were treated by sirtinol, RES protective effect was no longer detectable (last column) and cell viability was comparable to the H_2O_2-treated group (Fig. 2a). A similar situation was found for 6-OHDA (Fig. 2b).

To verify whether SIRT1 was involved in a different antioxidant pathway we tested the effect of sirtinol on NAC, an antioxidant with no known interaction with sirtuins. The results are shown in Fig. 2c. NAC 0.6 mM alone had no toxic effect (cell viability in comparison to control around 100%). When cells were pre-incubated with NAC (19 h and 4 h before the oxidative stimulus) and then challenged by H_2O_2 for 24 h, cell viability increased of about 20% in comparison to H_2O_2 alone; however, when sirtinol was added 2 h before each NAC addition it did not prevent NAC's protective action.

SIRT1 silencing prevents the RES-mediated antioxidant effect

Our previous experiments suggested that SIRT1 activation by RES was able to counteract the deleterious effects of H_2O_2 and 6-OHDA. However, we wanted to demonstrate in more detail the selectivity of the RES-activating mechanism by inhibition of SIRT1 expression. We developed a siRNA approach to down-regulate SIRT1 mRNA. First, we verified SIRT1 expression and localization in our cell line by western blotting and immunocytochemistry (Fig. 3a). SIRT1 was detected in the nuclear protein fraction, but there was no reactivity in the cytosol extract. This nuclear localization was confirmed by immunocytochemistry, even if in this case a weak cytosolic signal was present (Fig. 3b). When we co-localized the false color fluorescence signal from nuclear staining (Hoechst 33258) and that from SIRT1 immunoreactivity we were able to demonstrate co-localization (Fig. 3b).

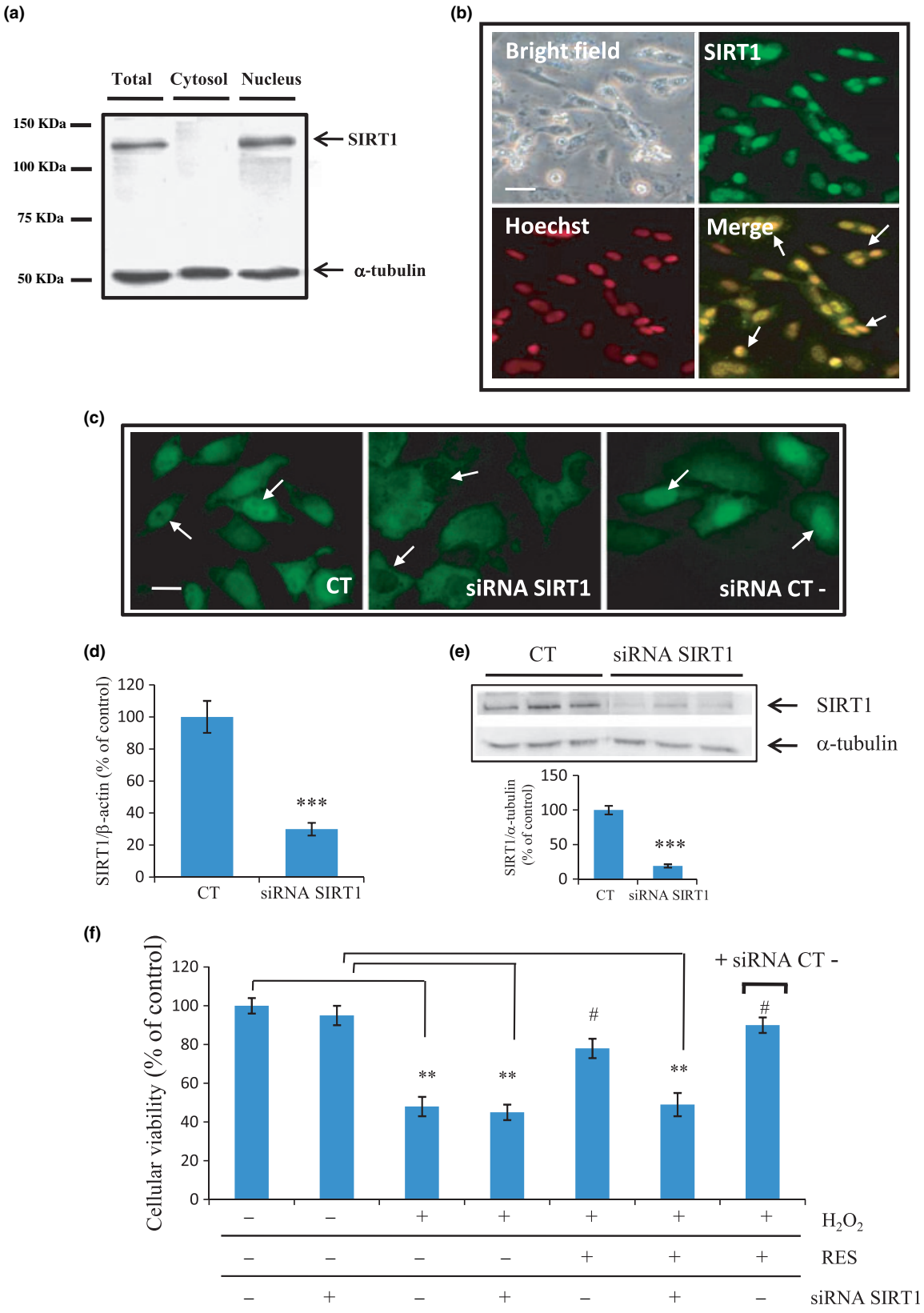
We transiently down-regulated SIRT1 expression by adding 300 nM siRNA for 72 h and checked SIRT1 down-regulation by immunocytochemistry (Fig. 3c). We detected a reduction of SIRT1 nuclear immunoreactivity, while the cytosol signal was unaffected. To check for the specificity of our silencing strategy, we incubated cells with a siRNA negative control (CT-). Using the same time/concentration conditions as for SIRT1 siRNA we found no reduction of SIRT1 immunoreactivity that was similar to the control condition (CT – cells incubated with siRNA lipid vehicle alone).

To measure SIRT1 mRNA reduction, we performed a real-time PCR experiment and a western blot analysis. A reduction of SIRT1 mRNA of about 70% was observed by real-time PCR using β-actin as internal standard (Fig. 3d); we confirmed this reduction by western blotting, after nuclei separation, using α-tubulin as internal standard (Fig. 3e). In the same experiments, we checked the effect of the siRNA (CT-) and no reduction of SIRT1 mRNA or protein level was registered (data not shown).

Under SIRT1 silencing, we set out to demonstrate the lack of RES-mediated protection against H_2O_2. This is summarized in Fig. 3f. As expected, the group challenged by oxidative stress but pre-incubated with RES was protected from cell damage. In contrast, when cells were silenced for SIRT1 and then exposed to H_2O_2 in the presence of RES, there was no difference from H_2O_2 alone. Further sustaining the specific involvement of SIRT1 in preventing oxidative stress, when SK-N-BE cells were exposed to RES but a siRNA negative control (CT-) was used the RES protective action was present (Fig. 3f). The same results were obtained using 6-OHDA as oxidative stimulus (data not shown).

We have also assessed the action of RES on ROS intracellular level (generated by H_2O_2 or 6-OHDA) under SIRT1 silencing. When SIRT1 was down-regulated, RES pretreatment was no more able to restore ROS basal level, even if a marginally significant reduction of ROS level in comparison to H_2O_2 or 6-OHDA alone was present (last column, $p = 0.08$ and $p = 0.07$ for H_2O_2 and 6-OHDA, respectively) (Fig. S1).

Fig. 3 SIRT1 silencing prevents RES-mediated protection against oxidative challenge. (a) SK-N-BE cells were harvested and nuclear proteins were separated; an aliquot (50 μg) was loaded on 8% SDS-PAGE and immunoblotted. Alpha-tubulin reactivity is also shown to confirm equal gel loading. (b) Cells were fixed, permeabilized and incubated with a SIRT1 primary antibody detected by a FITC-conjugated secondary antibody. Nuclei were visualized by Hoechst 33258 staining (red is a false color) (magnification: 10×, scale bar: 25 μm). (c) SIRT1 was silenced by a selective siRNA, 300 nM for 72 h. A siRNA negative control (CT-) was included. SIRT1 protein level was assessed by immunocytochemistry, as above. The arrows highlight nuclear SIRT1 immunoreactivity (magnification: 40×, scale bar: 10 μm). (d) Real-time PCR to evaluate SIRT1 mRNA reduction by siRNA. Cells were incubated with siRNA lipid vehicle alone (CT) or 300 nM siRNA against SIRT1 for 72 h, then total mRNA was extracted and SIRT1 transcript was quantified using β-actin as reference. ***$p < 0.001$, Dunnett's test versus CT (e) Western blotting and its quantification to confirm SIRT1 silencing. Cells were treated as just described, nuclear proteins were collected and subjected to SDS-PAGE. Alpha-tubulin was used as internal control. ***$p < 0.001$, Dunnett's test versus CT. (f) SK-N-BE cells were silenced for SIRT1 expression; 48 h after siRNA treatment cells were challenged with H_2O_2 75 μM and 24 h later cell viability was assessed by MTT assay. RES 7.5 μM was added 19 and 4 h before H_2O_2 addition. **$p < 0.01$ versus control (cells treated with RES vehicle alone or incubated with SIRT1 siRNA alone); #$p < 0.01$ for H_2O_2 (x) RES ($F_{int} = 19.0$) and for H_2O_2 (x) RES in presence of siRNA negative control (CT-) ($F_{int} = 18.0$), two-way ANOVA.



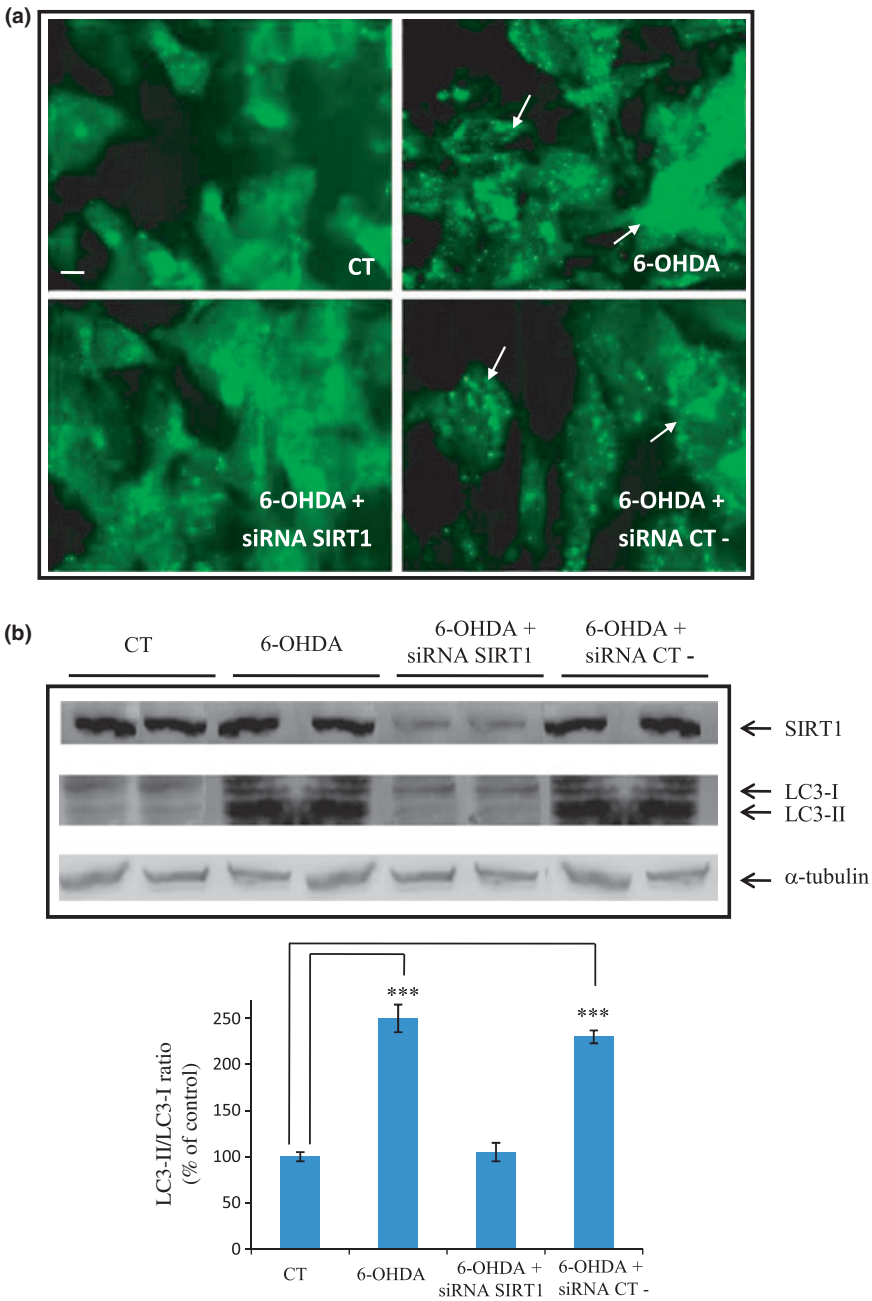


Fig. 4 SIRT1 silencing attenuates autophagy after oxidative stress. (a) SIRT1 silencing was done in SK-N-BE cells by the addition of a SIRT1-targeted siRNA, 300 nM for 72 h. A siRNA negative control (CT–) was included. At 48 h from silencing, 6-OHDA 75 μM was added for 24 h, while RES 7.5 μM was added twice 19 and 4 h before 6-OHDA. LC3-II immunostaining was performed to highlight autophagosome formation at the end of 6-OHDA treatment. The arrows highlight LC3-II bright spots reactivity under oxidative stress condition (magnification: 40×, scale bar: 10 μm). (b) Western blotting to assess LC3 reactivity in the same experimental conditions of (a). The graph shows the densitometric quantification of LC3-II/LC3-I ratio ($n = 4$ for group); $^{***}p < 0.001$ versus control (CT), one-way ANOVA followed by Tukey's *post-hoc* test.

RES protection against 6-OHDA (and H_2O_2, data not shown) was also reproduced when SK-N-BE cells were differentiated by retinoic acid to increase their neuronal-like phenotype (Fig. S2b).

SIRT1 mediates autophagosomes formation after oxidative stress

Starting from the literature (Lee *et al.* 2008; Pyo *et al.* 2008) we set out to verify SIRT1 involvement in autophagy in our model. Cells were pre-incubated with siRNA for SIRT1 and then challenged by 6-OHDA for 24 h. At the end of the oxidative stimulus, autophagosomes formation was assessed by LC3-II immunoreactivity (Pyo *et al.* 2008) (Fig. 4a). LC3-II reactivity was increased after the oxidative stress, indicating that SK-N-BE cells triggered autophagy as protective mechanism. When SIRT1 was down-regulated by siRNA, LC3-II reactivity after oxidative stress decreased, while it kept unchanged in presence of a siRNA negative control (CT-). RES treatment did not further increase the autophagosomes formation detected with this technique. A similar scenario was observed using H_2O_2 as oxidative agent (data not shown). We confirmed LC3-II activation by western blotting (Fig. 4b). In presence of 6-OHDA, the LC3-II/LC3-I ratio increased in comparison to control. When SIRT1

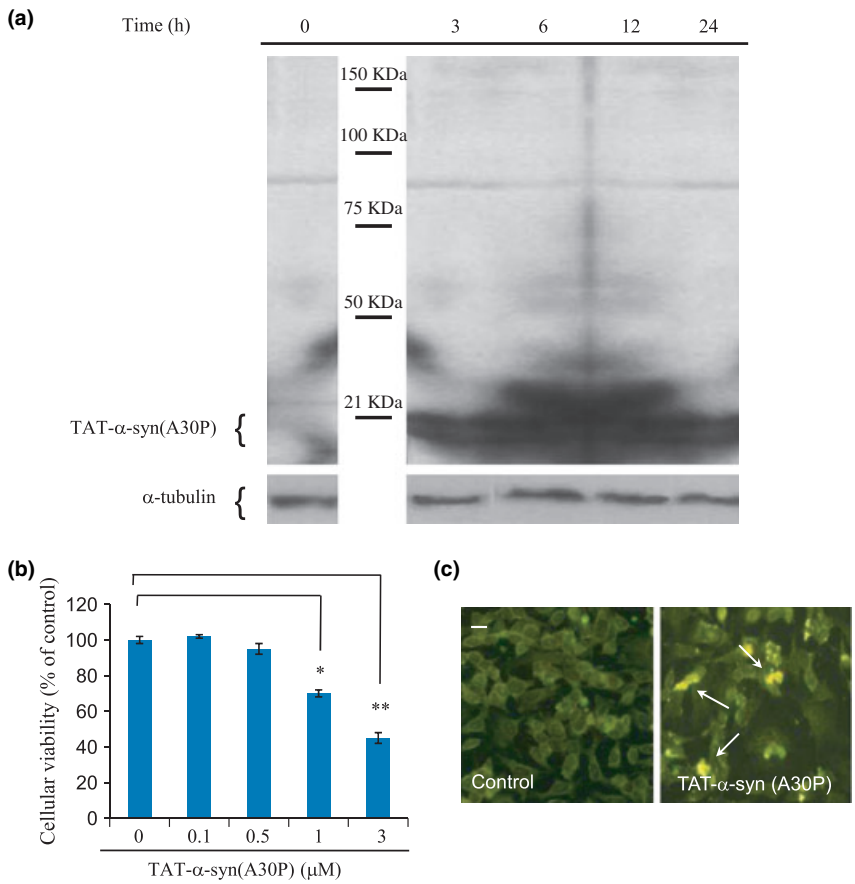


Fig. 5 TAT-α-syn(A30P) toxicity. (a) Western blot for TAT-α-syn availability inside SK-N-BE cells. Cells were incubated with TAT-α-syn(A30P) 1 μM for increasing time intervals. (b) Dose–response graph depicting TAT-α-syn(A30P) toxic effect. Cells were exposed to TAT-α-syn(A30P) 3 μM for 24 h, then viability was measured by MTT assay. $^{*}p < 0.05$; $^{**}p < 0.01$ versus control (untreated cells), one-way ANOVA and Dunnett's *post-hoc* test. (c) Thioflavin-T staining showing amyloid aggregation of TAT-α-syn(A30P) 3 μM. The arrows indicate intracellular thioflavin-T-positive inclusions (magnification: 20×, scale bar: 15 μm).

expression was down-regulated, the LC3-II/LC3-I ratio was not different from basal condition, while a siRNA negative control was comparable to 6-OHDA (Fig. 4b).

To investigate whether RES protection involved autophagy, we pre-incubated cells with RES and then we inhibited autophagy by 3-methyladenine (3-MA) one hour prior of the oxidative challenge. We found no evidence of a reduced RES protective effect under 3-MA (data not shown).

RES treatment prevents cell death triggered by TAT-α-syn(A30P) or Aβ42

We decided to check whether RES could prevent cell death triggered by aggregation-prone proteins relevant for neurodegenerative diseases, as α-syn(A30P). To let the protein freely cross the plasma membrane, we expressed a fused variant having at the N-terminal the TAT sequence [TAT-α-syn(A30P)] (Albani *et al.* 2004). We decided to use the A30P mutant as it gave more toxicity in our model than the wild type form. First, we demonstrated that TAT-α-syn(A30P) was detectable inside cells by western blotting (Fig. 5a). The basal SK-N-BE α-syn level was negligible (first lane, 0 h), while the fusion protein TAT-α-syn(A30P) 1 μM was detectable inside cells starting from 3 h after its addition to cell medium and migrated around 21 kDa. TAT-α-syn(A30P) was still detectable after 24 h from cell medium addition. Then, we incubated cells with increasing amounts of the fusion protein (Fig. 5b). There was a dose-dependent reduction of cell viability and at 3 μM it was comparable to the toxic effect produced by H_2O_2 and 6-OHDA. We were also able to detect the formation of thioflavin-T positive intracellular aggregates when cells were incubated with TAT-α-syn(A30P) 3 μM for 24 h (Fig. 5c).

Figure 6a shows the protective effect of RES against TAT-α-syn(A30P) 3.0 μM toxicity. RES pre-incubation (19 and 4 h before the protein addition) counteracted TAT-α-syn(A30P)'s noxious effect. The recovery in cell viability was about 30%. To confirm the importance of SIRT1 activation in this protective mechanism, we also incubated cells with sirtinol and in this case RES protective effect was no longer detectable (Fig. 6a). To find out how RES protected against TAT-α-syn(A30P) toxicity we checked autophagosomes formation but we did not notice any increase of LC3-II reactivity (data not shown). Then, we measured intracellular ROS and we found that TAT-α-syn(A30P) increased ROS level and this effect was prevented by RES, while sirtinol addition counteracted RES positive action (Fig. S3a).

Finally, we evaluated RES's protective effect against Aβ42, the fibrillogenic fragment of beta-amyloid precursor

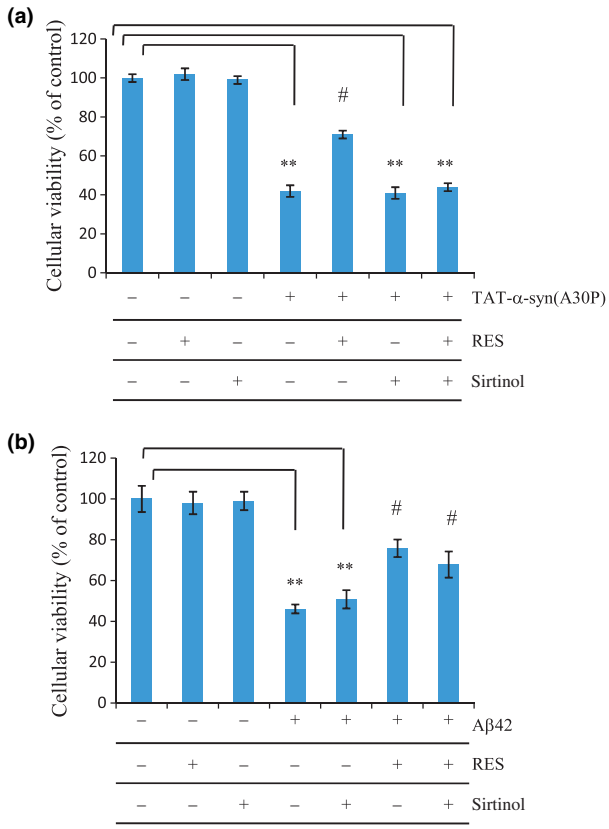


Fig. 6 RES is able to prevent cell toxicity arising from aggregation-prone protein. (a) SK-N-BE cells were pre-incubated with RES 7.5 μM, 19 and 4 h before the addition of TAT-α-syn(A30P) 3 μM. After 24 h, cell viability was measured by MTT assay. Sirtinol 2.5 μM was added 2 h before RES. $^{**}p < 0.01$ versus control (RES-vehicle-treated cells); $\#p < 0.01$ for TAT-α-syn (x) RES ($F_{int} = 17.0$), two-way ANOVA. (b) Cells were exposed to Aβ42 10 μM for 24 h and viability was assessed by MTT assay. RES 7.5 μM was added 19 and 4 h before Aβ42 and sirtinol 2.5 μM was added 2 h before RES. $^{**}p < 0.01$ versus control (RES-vehicle-treated cells); $\#p < 0.05$ for Aβ42 (x) RES ($F_{int} = 6.0$) and for Aβ42 (x) RES ($F_{int} = 5.8$) in presence of sirtinol, two-way ANOVA.

protein (βAPP) that is considered a major player in AD and is of interest in Down syndrome (DS) (Isacson *et al.* 2002). We ran the experiment summarized in Fig. 6b. A 24 h incubation with Aβ42 10 μM was toxic to cells, with a significant reduction of cell viability (about 45%). When RES 7.5 μM was added (19 and 4 h before the toxic challenge), Aβ42 toxicity was largely prevented. Unexpectedly, when cells were exposed to sirtinol before each RES addition the protective effect was still present (though slightly less). To find out how RES protected against Aβ42 toxicity we measured at first intracellular ROS. Aβ42 incubation increased ROS production, this effect was counteracted by RES addition but was insensitive to sirtinol (Fig. S3b). As this sirtinol-independent protection suggested a RES direct destabilization effect on Aβ42 fibrils, we performed a fluorimetric binding assay as described by Ahn *et al.* (Ahn *et al.* 2007) with minor modifications. We found a weak dissociating effect of RES on Aβ42 fibrils after 24 h (around 20% dissociation, $p = 0.045$) (Fig. S4). Moreover, we verified by atomic force microscopy (AFM) whether RES was able to counteract Aβ42 fibril formation. RES presence delayed Aβ42 oligomers and protofibrils formation up to 6 h, while at 24 h no evident difference in comparison to Aβ42 alone was found (Fig. S5).

Discussion

High levels of protein and DNA oxidation have been reported in post-mortem brains of AD, PD or DS affected patients (Markesbery and Lovell 2007; Zana *et al.* 2007; Zhu *et al.* 2007). However, several basic investigations and clinical trials have found no real benefit of an antioxidant strategy in AD, PD or DS (Iannello *et al.* 1999; Pham and Plakogiannis 2005; Yang *et al.* 2008).

The natural compound RES is a polyphenolic molecule with antioxidant properties that has been intensively investigated as enzymatic activator of SIRT1, a protein involved in longevity and calorie restriction. SIRT1 activation or overexpression was reported as neuroprotective (by a still unknown mechanism) in models of AD, amyotrophic lateral sclerosis (ALS) or polyglutamine disease (Chen *et al.* 2005; Parker *et al.* 2005; Qin *et al.* 2006; Kim *et al.* 2007).

Our work addressed first the question whether RES was beneficial in counteracting the oxidative stress triggered by H_2O_2 and 6-OHDA, and whether this protective action was dependent on SIRT1. We developed an *in vitro* model using the human neuroblastoma cell line SK-N-BE that is sensitive to 6-OHDA toxicity, as it can take up dopamine (Agrati *et al.* 1997). Our cells expressed SIRT1 at nuclear level, as SIRT1 cytosol reactivity detected in immunocytochemistry experiments was probably an unspecific signal as it was unaffected by siRNA treatment. RES had a similar protective action against H_2O_2 and 6-OHDA, suggesting it produces a robust general antioxidant response. This neuroprotective mechanism was sensitive to sirtinol, supporting an involvement of SIRT1 enzymatic activation. However, it is also possible that RES direct antioxidant action contributed to cell protection, as even in presence of sirtinol ROS levels after oxidative challenge were not as high as in absence of RES. Another element that indicates SIRT1 activation underlying RES's antioxidant effect came from the siRNA experiment, where SIRT1 transient down-regulation was sufficient to prevent the RES antioxidant mechanism evaluated in terms of cell viability. We confirmed in differentiated SK-N-BE cells RES's antioxidant effect and its SIRT1-dependent mechanism, further sustaining the importance of this pathway. On the contrary, SIRT1 catalytic activity was not involved in NAC's protection that was insensitive to sirtinol.

An interesting point was the effect of oxidative stress and SIRT1 silencing on autophagy. An involvement of SIRT1 in autophagy has already been reported (Lee *et al.* 2008) as well as autophagosome formation after oxidative stress challenge (Pyo *et al.* 2008). Our data confirm that SIRT1 is important for autophagosome formation, but as RES did not increase LC3-II and its protection was insensitive to 3-MA we found no evidence that SIRT1-dependent autophagy is the protective mechanism in our model.

The second step was to assess RES's protective action against the toxicity from aggregation-prone proteins. RES positive mechanism was still detectable when SK-N-BE cells were challenged with the plasma membrane-permeable fusion protein TAT-α-syn (A30P) (Albani *et al.* 2004). In this situation too, SIRT1 was likely involved, as sirtinol treatment before addition of RES prevented the latter's positive action against TAT-α-syn(A30P) toxicity. Our data pertaining TAT-α-syn are, to our knowledge, the first indication of a protective role of SIRT1 against α-syn toxicity and they depict an intriguing scenario where different sirtuins have different effect. In fact, Outeiro *et al.* (Outeiro *et al.* 2007) found that SIRT2 down-regulation was effective in counteracting α-syn toxicity. It will be of interest to clarify a possible functional interaction between SIRT1 and SIRT2 in controlling α-syn aggregation and related toxicity. Interestingly, a role of the *C. elegans* SIRT1 homologue Sir2.1 as a suppressor of human α-syn inclusion formation during aging has been reported (van Ham *et al.* 2008). Our data do not support an involvement of autophagy in SIRT1 mediated protection against TAT-α-syn(A30P) toxicity. However, TAT-α-syn (A30P) addition increased intracellular ROS level that was reduced by RES in a sirtinol-dependent way.

Finally, we checked RES neuroprotection against Aβ42, the fibrillogenic fragment of the βAPP. We were able to reproduce a protective effect, but sirtinol did not totally reverse it. This suggests that RES can prevent Aβ42 toxicity by direct interaction with Aβ42 fibrils, as already reported (Ahn *et al.* 2007; Rivière *et al.* 2007). We found a weak RES destabilization effect on Aβ42 fibrils but also an antifibrillogenic RES action that delayed Aβ42 oligomers and fibrils formation. This double effect can partly account for RES protection against Aβ42 toxicity. In addition, we measured an increase of ROS level after Aβ42 treatment that was reversed by RES, confirming the reported data in PC12 cells (Jang *et al.* 2007). In short, in our cell model RES protected from Aβ42 toxicity independently from SIRT1 and acting on Aβ42 fibril formation and stability as well as by reducing intracellular Aβ42-dependent ROS generation. However, SIRT1 protection against Aβ42 might be present independently from its catalytic activity. This scenario is supported by the literature, where an *in vitro* protective effect of SIRT1 in neuronal cells that was not reversed by sirtinol has been reported (Pfister *et al.* 2008). Finally, as RES is able to trigger several cell-protective mechanisms (for instance, the proteasome) (Marambaud *et al.* 2005) further investigation is required to assess a RES-dependent proteasome activation also in our cell line after Aβ42 challenge.

In summary, the SIRT1 activator RES prevented oxidative stress triggered by H_2O_2 or 6-OHDA in our cellular model and this protective pathway involved SIRT1. Similarly, the toxicity arising from TAT-α-syn(A30P) protein aggregation can be counteracted by RES and was sirtinol-sensitive, while a SIRT1-independent RES action against Aβ42 fibrils was probably present.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. (a) RES treatment prevents ROS production by H_2O_2 in SK-N-BE cells. (b) RES treatment counteracts ROS generation by 6-OHDA in SK-N-BE cells.

Figure S2. RES treatment prevents 6-OHDA toxicity in differentiated cells.

Figure S3. (a) RES treatment prevents ROS production by TAT-α-syn(A30P) in SK-N-BE cells. (b) RES treatment counteract s ROS generation by Aβ42 in a sirtinol-independent way.

Figure S4. RES binds and weakly dissociates Aβ42 fibrils after 24 h.

Figure S5. RES can delay Aβ42 fibril formation.

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