Resveratrol via sirtuin-1 downregulates RE1-silencing transcription factor (REST) expression preventing PCB-95-induced neuronal cell death

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Resveratrol (3,5,4′-trihydroxystilbene) (RSV), a polyphenol widely present in plants, exerts a neuroprotective function in several neurological conditions; it is an activator of class III histone deacetylase sirtuin 1 (SIRT1), a crucial regulator in the pathophysiology of neurodegenerative diseases. By contrast, the RE1-silencing transcription factor (REST) is involved in the neurotoxic effects following exposure to polychlorinated biphenyl (PCB) mixture A1254. The present study investigated the effects of RSV-induced activation of SIRT1 on REST expression in SH-SYSY cells. Further, we investigated the possible relationship between the non-dioxin-like (NDL) PCB-95 and REST through SIRT1 to regulate neuronal death in rat cortical neurons. Our results revealed that RSV significantly decreased REST gene and protein levels in a dose- and time-dependent manner. Interestingly, overexpression of SIRT1 reduced REST expression, whereas EX-527, an inhibitor of SIRT1, increased REST expression and blocked RSV-induced REST downregulation. These results suggest that RSV downregulates REST through SIRT1. In addition, RSV enhanced activator protein 1 (AP-1) transcription factor c-Jun expression and its binding to the REST promoter gene. Indeed, c-Jun knockdown reverted RSV-induced REST downregulation. Intriguingly, in SH-SYSY cells and rat cortical neurons the NDL PCB-95 induced necrotic cell death in a concentration-dependent manner by increasing REST mRNA and protein expression. In addition, SIRT1 knockdown blocked RSV-induced neuroprotection in rat cortical neurons treated with PCB-95. Collectively, these results indicate that RSV via SIRT1 activates c-Jun, thereby reducing REST expression in SH-SYSY cells under physiological conditions and blocks PCB-95-induced neuronal cell death by activating the same SIRT1/c-Jun/REST pathway.

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Introduction

Polychlorinated biphenyls (PCBs) are a large family of human developmental neurotoxictants comprising 209 possible congeners (McFarland and Clarke, 1989). They are generally divided into two groups: PCB dioxin-like compounds and PCB non-dioxin-like compounds (NDL). The former compounds bind to the aryl hydrocarbon receptor (AhR), thereby exerting a marked toxicity similar to that of polychlorinated dioxins and furans (Weintraub and Birnbaum, 2008). The latter compounds, instead, despite showing a very low affinity for AhR, also induce neurotoxic effects in vitro and in vivo (Giesy and Kannan, 1998). We have previously shown that the PCB mixture A1254 increases the repressor element 1 (RE1)-silencing transcription factor (REST) mRNA and protein expression (Formisano et al., 2011) through ERK2/Sp1/Sp3 pathway (Formisano et al., 2015a), which, in turn, by recruiting HDAC3 on the Synapsin-1 promoter causes neuronal death in cortical neurons (Formisano et al., 2015b). In mature neurons, REST is quiescent, but it can be reactivated during normal postnatal development, driving the switch from immature to mature NMDA receptors (Rodenas-Ruano et al., 2012). Accordingly, REST has been closely investigated in brain ischemia where it plays an important role in determining neuronal death in hippocampal and cortical neurons (Formisano et al., 2007, 2013, 2015; Noh et al., 2012). One study, for instance, reports that in insulted hippocampal neurons, casein kinase 1 suppresses activation of REST protein expression, thereby promoting neuroprotection (Kaneko et al., 2014). Recently, there has been a growing interest in resveratrol (RSV) (3,5,4′-trihydroxystilbene), a polyphenol contained in red wine and known to exhibit beneficial effects in...
several neurological disorders such as Alzheimer Disease (Li et al., 2012), Parkinson Disease (Wu et al., 2011), stroke (Morris et al., 2011) and Amyotrophic Lateral Sclerosis (Mancuso et al., 2014). The most commonly cited mechanism of action of RSV is the activation of the longevity factor silent mating type information regulation 2 homolog 1 (SIRT1) protein (Tang, 2010), a histone deacetylase (HDAC) that is basally expressed in the adult mammalian brain, predominantly in neurons (Donmez, 2012). SIRT1 utilizes nicotinamide (NAD⁺) (Donmez and Outeiro, 2013) as a substrate to catalyze deacetylation of various substrates involved in a broad range of physiological functions, including control of gene expression, metabolism, and aging (Rahman and Islam, 2011). Considering the role played by REST in PCB-induced neurotoxicity, here we explored the mechanism by which resveratrol-induced activation of SIRT1 blocks neurotoxicity after PCB exposure. For this study, we evaluated how RSV via SIRT1 regulates REST mRNA and protein expression in human neuroblastoma SH-SYSY cells. Furthermore, we studied the effect of NDL PCB-95 alone or in combination with RSV on the survival of cortical neurons and its correlation with REST.

Material and methods

Drug and chemicals

The compound 2,3,6-2′,5′-pentachlorinated biphenyl (PCB-95) (cod: RPC-130AS) (stock solution 305 μM) was purchased from Ultra Scientific (North Kingstown, RI, USA). Culture media and sera were obtained from Invitrogen (Milan, Italy). The SIRT1 activator 3,5,4′-trihydroxy-trans-stilbene resveratrol (RSV) (cod. R5010; stock solution 10 mM), H2O2 (cod. H1009; stock solution 10 mM) and the 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Molecular Probes, Irvine, CA, USA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The SIRT1 inhibitor 6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide EX-527 (cod. sc-203044; stock solution 20 mM) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For those requiring dilution in DMSO, the final DMSO concentration was 0.1% (vehicle). Under these conditions, DMSO did not induce cellular toxicity.

Cell lines and culture conditions

Human neuroblastoma SH-SYSY cells between the 10th and 30th passage (IRCCS Azienda Ospedaliera Universitaria San Martino-ISTITIuto Nazionale per la Ricerca sul Cancro, Genoa, Italy) were grown as previously published (Cocco et al., 2015). Cells were incubated for 24 h with RSV and EX-527 at 1, 2, 5, and 10 μM and with PCB-95 at 1, 2, 4, 8, and 16 μM. For time-dependent experiments, cells were treated with 8 μM RSV and 5 μM EX-527 for 6, 12, 24, and 36 h. Furthermore, RSV and EX-527 (both at 5 μM for 24 h) were simultaneously added to the medium to study the effect on REST and C-Jun mRNA and protein expression and the binding of c-Jun, RNA Pol II, and histone H4 acetylation on REST gene promoter. To evaluate the effects of PCB-95-induced cellular toxicity, SH-SYSY cells were seeded and treated for 24 h with PCB-95 at 1, 2, 4, 8, and 16 μM. Then, the effects of RSV on PCB-95-induced neurotoxicity were explored by seeding and treating cells for 24 h with RSV at 0.25, 0.5, 1, 5, and 10 μM, alone or in combination with 8 μM PCB-95. All the above mentioned experiments were performed in DMEM medium containing 1% FBS to avoid loss of the PCB-95 effect through binding to serum component. The experiments on cortical neurons (DIV7) were approved by the Animal Care Committee of “Federico II”, University of Naples, Italy and prepared as previously described (Formisano et al., 2015b). In brief, cells were treated for 24 h with PCB-95 at 1, 2, 4, 8, and 16 μM, and with RSV at 6.25, 12.5, and 25 μM. To study the effects of RSV on PCB-95-induced neurotoxicity, neurons were seeded and treated for 24 h with vehicle, or with RSV 25 μM alone or in combination with 8 μM PCB-95. All these experiments were performed in serum-free neurobasal medium. SH-SYSY cells were plated in 24-multiwell plates at a density of 2 × 10⁴ for the LDH assay, in 6-multiwell plates at a density of 2.5 × 10⁴ for the determination of free radical production and in 100-mm well plates at a density of 10 × 10⁴ for qRT-PCR, Western Blot, and ChIP analyses. Cortical neurons were plated in 24-well plates at a density of 1 × 10⁴ for LDH, and in 100-mm well plates at a density of 15 × 10⁴ for qRT-PCR and Western Blot analyses.

Cell transfection

Anti-sense (AS) and missense (MS) phosphorothioate oligonucleotides (ODNs) against human SIRT1, p65 (AS SIRT1, AS p65, MS SIRT1, and MS p65), and human and rat c-Jun (AS c-Jun and MS c-jun) were transfected into SH-SYSY cells and cortical neurons at a concentration of 1 μM (Guida et al., 2014). Afterward, Opti-MEM was removed and cells were incubated in fresh medium for 24 h. Western blot was subsequently carried out to test the knockdown efficiency. After ODN transfection, experiments with PCB-95 alone or in combination with RSV were carried out. In brief, cells were incubated for 24 h with 8 μM PCB-95 alone or in combination with 5 μM RSV in SH-SYSY cells or with 25 μM RSV in cortical neurons. MS and AS oligonucleotides against human SIRT1, c-Jun, p65 mRNA, and rat c-Jun mRNA have already been published (Ohkawa et al., 1999; Peterson et al., 2002; Hsieh et al., 2008; Busch et al., 2012). SIRT1 silencing in cortical neurons by small interfering RNA (siRNA) was carried out as previously reported (Formisano et al., 2015b). In brief, cells were transfected with scrambled control (siCTL; sc-37007) and siRNAs against rat SIRT1 (siSIRT1; sc-108043) at 400 nM (Santa Cruz Biotechnology, Santa Cruz, CA).

After siRNA transfection, cells were incubated in a fresh medium for 24 h. Next, they were collected and subjected to western blotting to assess knockdown efficiency. Alternatively, neurons were exposed to PCB-95 (8 μM) alone or in combination with RSV (25 μM) for 24 h. For SIRT1 overexpression experiments in SH-SYSY cells, cells were transfected with the empty vector pECE; the plasmid carrying SIRT1 cDNA was purchased from Addgene (cod: 1791) (Brunet et al., 2004).

For Western Blot and qRT-PCR analyses, cells were plated in 100-mm dishes and transiently transfected for six hours with 15 μg of DNA using Lipofectamine suspended in Opti-MEM. Next, Opti-MEM was removed and replaced with fresh medium and cells were left to incubate for 24 h. To evaluate cell transfection efficiency, siRNAs and ODNs were mixed with a plasmid encoding the enhanced green fluorescent protein marker; a fluorescence density of 50% for SH-SYSY cells and 30%–50% for cortical neurons, respectively, was detected (data not shown).

Lactate dehydrogenase (LDH) assay

LDH Cytotoxicity Kit (1000882) (Cayman, DBA, Milan, IT) was used to assess cell death. LDH efflux into the medium was measured after cortical neurons and SH-SYSY cells were exposed to PCB-95 alone or in combination with RSV for 24 h, respectively (Formisano et al., 2015b). Triton X-100 Sigma-Aldrich (St. Louis, MO, USA) was used as a positive control for cytotoxicity and its value was considered 100%.

Determination of reactive oxygen species (ROS) production

SH-SYSY cells were treated with PCB 95 (8 μM) and RSV (5 μM), alone or in combination, for 6 h and H2O2 (600 μM) for 30 min. After that ROS production was measured by 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) (Molecular Probes, Irvine, CA, USA) as previously described (Amoroso et al., 1999; Pannaccione et al., 2005). Specifically, cells were loaded with 10 μM DCF-DA for 30 min at 37 °C, in a medium whose composition was in mM: NaCl 138, KCl 2.7, CaCl₂ 1.2, MgCl₂ 1.2, PBS 10, glucose 10, pH 7.4 (standard medium). After the loading period, cells were washed thrice and the reaction was stopped by addition of 2,6-di-tert-butyl-4-methylphenol (0.2% in ethanol) and EDTA (2 mM). Images were acquired by a fluorescence microscope (Nikon Eclipse
Reverse transcription-real time PCR

Real-time RT-PCR was carried out with cDNAs reverse-transcribed from total RNA by using Light Cycler-FastStart DNA Master SYBR Green I (Roche: 03030230001 Foster City, CA) and ABI PRISM 7000 software (Applied Biosystems) (Formisano et al., 2015b). The primer pairs used for Synapsin-1, NCX1, and β-Actin were the same as those reported elsewhere (Formisano et al., 2011; Vinciguerra et al., 2014). All reactions were performed in triplicate in one assay with a non-template blank for each primer pair as a control for contamination or primer-dimer formation; the cycle threshold (CT) value for each experimental group was determined. The results were normalized to the CT values using the 2−ΔΔCT formula. β-Actin in the same sample. Differences in mRNA content between groups were expressed as 2−ΔΔCT (Formisano et al., 2015b).

Statistical analysis

Data are expressed as mean ± SEM. Statistical comparisons between the experimental groups were performed using one way ANOVA followed by Newman Keuls test. P value <0.05 was considered statistically significant.

Results

RSV reduces REST mRNA and protein expression in a time- and dose-dependent way in SH-SYSY cells

SH-SYSY cells were incubated with a non toxic concentration of RSV (5 μM) (Lee et al., 2007) at different time points (6 to 36 h), and expression levels of REST mRNA were determined by q-RT-PCR. REST mRNA expression was significantly reduced by RSV at 6 h as compared to vehicle, reaching a maximum reduction at 24 h of incubation (Fig. 1a). At 24 h, different concentrations of RSV reduced REST mRNA in a dose-dependent manner (Fig. 1b). Western blot analysis showed that after 12 h of RSV incubation, REST protein expression was significantly reduced, reaching a maximum reduction at 24 h as compared to vehicle (Fig. 1c) in SH-SYSY cells. In addition, RSV-induced REST reduction was dose dependent (Fig. 1d). These experiments identified 5 μM of RSV as the concentration that produced an intermediate degree of reduction of REST gene and protein product.

SIRT1 reduces REST mRNA and protein expression in SH-SYSY cells

It is known that RSV regulates SIRT1 protein expression and deacetylase activity (Sea et al., 2012). q-RT-PCR and Western Blot analysis showed that RSV (5 μM/24 h) did not modify SIRT1 mRNA and protein expression (Suppl. Fig. 1a and b). Interestingly, when SH-SYSY cells were treated with the SIRT1 inhibitor EX-527 (1 to 10 μM), REST gene expression and protein levels significantly increased in a dose and time-dependent manner compared with vehicle (Fig. 2a–d). These results suggest that RSV modulates SIRT1 activity, but it has any effects on SIRT1 mRNA and protein. Likewise, when SH-SYSY cells were treated with RSV in the presence of EX-527, RSV-induced reduction of REST mRNA and protein expression was completely blocked (Fig. 2e,f). To further confirm that RSV through SIRT1 decreases REST protein expression, RSV-treated cells were transfected with specific antisense and sense oligonucleotides for SIRT1 (AS SIRT1 and MS SIRT1, respectively). Notably, AS SIRT1 transfection was able to decrease SIRT1 protein by almost 75% (Suppl. Fig. 2a). As well, AS SIRT1 significantly blocked REST mRNA and protein reduction as compared to cells transfected with MS SIRT1. Interestingly, when cells were transfected with AS SIRT1, REST mRNA and protein expression levels increased (Fig. 2f, n), whereas when they were treated with a construct overexpressing SIRT1 (Brunet et al., 2004) by almost 60% (Suppl. Fig. 2b), REST mRNA and protein levels decreased (Fig. 2f, n). We then asked whether REST reduction is functional. To answer this question, we performed q-RT-PCR to examine the gene expression of two REST target genes, viz, the sodium/calcium exchanger 1 (NCX1) (Formisano et al., 2013) and Synapsin-1 (Formisano et al., 2011) in SIRT1 silenced or overexpressing
SIRT1 knock-down significantly reduced NCX1 and Synapsin-1 mRNA expression as compared to MS SIRT1 (Suppl. Fig. 2c,e), whereas SIRT1 overexpression significantly increased NCX1 and Synapsin-1 mRNA as compared to the empty vector (Suppl. Fig. 2d,f).

SIRT1 does not bind to human REST promoter sequence in vivo

Since RSV regulates gene expression by increasing SIRT1 binding to the promoter sequence (Pezzolla et al., 2015), we investigated whether SIRT1 binds to the REST human promoter (GenBank accession no. AB024498). The rationale for this experiment was that the REST promoter sequence comprises three alternative promoters A, B, and C that regulate the exons a, b and c (Fig. 2o). Interestingly, each exon can splice directly into exon d, which contains the ATG translation initiation site of REST sequence (Koenigsberger et al., 2000; Ravache et al., 2010). Accordingly, we divided the two alternative REST promoter sequences A and B into two different fragments A1, A2 and B1, B2, respectively, whereas the C promoter sequence, termed C1, was left intact (Fig. 2o). ChIP experiments with SIRT1 antibody were performed using specific primers for these five fragments of the REST promoter. In RSV-treated cells, when chromatin was precipitated with SIRT1 antibody, no signal was detected in all REST promoter fragments. However, in SH-SY5Y cells, SIRT1 was able to bind only to the promoter sequence of its known target gene, erythropoietin (EPO) (Dioum et al., 2009), as compared to control IgG (Fig. 2q). This result suggests that SIRT1 does not bind to the REST human promoter sequence in SH-SY5Y cells.

RSV through c-Jun reduces REST mRNA and protein expression

REST human gene promoters have multiple binding sites for the following transcription factors: activator protein-1 (AP-1), nuclear factor-kappa B (NF-κB), and specificity protein 1 (Sp1) (Ravache et al.,...
To identify the transcription factors responsible for RSV-induced REST reduction, we treated cells with RSV for 6, 12, and 24 h. Western Blot analysis showed that phospho p65 (p-p65) and p-c-Jun subunits of the NF-κB and AP-1 complexes were increased time dependently at 6, 12 and 24 h (Fig. 3a,b). Interestingly, RSV did not modify Sp1 and Sp3 protein expression (Fig. 3c,d).

Then, to evaluate the role of p65 and c-Jun in RSV-induced REST mRNA and protein reduction, p65 and c-Jun were knocked down in SH-SY5Y cells with antisense oligonucleotides (AS ODNs) (Fig. 3e,f). Notably, AS ODNs for c-Jun (AS c-Jun) and p65 (AS p65) significantly reduced c-Jun and p65 expression by 60% and 70%, respectively, as compared to MS c-Jun and MS p65 (Fig. 3e,f). Interestingly, only AS ODN against c-Jun, but not against p65, reverted the reduction of RSV-induced REST gene and protein expression as compared to vehicle (Fig. 3g,h). Notably, AS c-Jun did not modify REST protein expression in non RSV-treated cells (Suppl. Fig. 3c).

qRT-PCR revealed that c-Jun mRNA was significantly increased after 6, 12, and 24 h of RSV (5 μM) treatment, as compared to vehicle (Fig. 4a). Importantly, qRT-PCR and Western blotting at 12 h showed that EX-527 reduced RSV-induced mRNA and protein expression (Fig. 4b,c). Notably, EX-527 did not modify P-c-Jun protein expression in non RSV-treated cells (Suppl. Fig. 3a). Two putative AP-1 binding sites have previously been identified on the REST gene human promoter sequence at −2168/−2162 and −1990/−1984 from the transcription start site (+1) (GenBank accession no. AB024498) (Ravache et al., 2010). Thus, to test whether c-Jun could bind directly to these sites, which we named AP-1 (a) (−2168/−2162) and AP-1 (b) (−1990/−1984) (Fig. 4d), after RSV treatment, we performed ChIP. Interestingly, when chromatin from cells treated with vehicle...
was precipitated with the c-Jun antibody, the fragment containing the AP-1 (b) site, but not that including the AP-1 (a) site, was amplified as compared with control IgG. Moreover, the binding activity of c-Jun to AP-1 site (b), but not to AP-1 site (a), was markedly increased after RSV treatment (Fig. 4d, e), in parallel with an increase in RNA Pol II binding and histone H4 acetylation, as compared to vehicle (Fig. 4f-g). Interestingly, RSV-increased binding activity of c-Jun or the decreased binding of RNA Pol II and histone H4 acetylation to AP-1 (b) site was reverted by co-application of EX-527 (Fig. 4d-g). These data suggest that SIRT1 increases c-Jun binding to the REST gene promoter at the AP 1 (b) site, thereby reducing REST gene transcription.

Resveratrol reduced PCB-95-induced cell death by blocking the increase in REST mRNA and protein levels in SH-SY5Y cells

Exposure of PCB mixture A-1254 for 24 h at 30.6 μM reduces SH-SY5Y cell survival by about 50% through an increase in REST mRNA and protein expression (Formisano et al., 2015a, 2015b). Since the A1254 mixture consists primarily of NDL PCBs (>99%) (Inglefield et al., 2001), we tested the effect of NDL congener PCB-95 on neuronal death. Interestingly, we found that 24 h exposure to PCB-95 (1–16 μM) induced neuronal cell death in a concentration-dependent manner, as evaluated by LDH assay (Fig. 5a). Since 8 μM PCB-95 induced a 50% reduction in cell survival, this concentration was used for further experiments. To investigate
the role of RSV in PCB-95-induced toxicity, cells were treated with RSV at concentrations of 0.25, 0.5, 1, 5 and 10 μM. LDH assays showed that cell survival significantly improved in a dose-dependent manner when cells were co-treated with RSV, as compared to cells exposed to PCB-95 alone (Fig. 5b) and that RSV afforded maximum protection at 5 μM. In addition, at 24 h, PCB-95-induced increase in REST mRNA and protein expression was reverted after RSV (5 μM) treatment (Fig. 5c,d).

Importantly, since REST overexpression diminished H2O2-induced cell death reducing production of reactive oxygen species (ROS) and consequent apoptosis activation (Lu et al., 2014) we performed experiments to evaluate whether PCB-95-induced neurotoxicity was associated with oxidative stress and inflammation. In particular, free radical production was monitored by DCF-DA in neuroblastoma cells exposed to PCB 95 (8 μM), RSV (5 μM) alone or in combination. As shown in Supplemental Fig. 4a, after 6 h PCB 95 (8 μM) alone or in combination with RSV (5 μM) failed to modify intracellular ROS levels. On the other hand, ROS levels significantly increased when cells were exposed to H2O2, that is a known oxidative stress stimulus (Lu et al., 2014). Furthermore PCB 95 exposure did not cause any change of P-p65 expression (Suppl. Fig. 4b), suggesting a lack of involvement of the inflammation marker in the neurotoxic effect of the pollutant. Collectively these results indicate that oxidative stress and inflammation are not associated to PCB-95-induced neurotoxicity.
When cortical neurons were exposed to different concentrations of PCB-95 (1–16 μM; Fig. 6a) for 24 h, a dose-related increase in cell death was observed, as evidenced by LDH assay. As in SH-SY5Y cells, PCB-95 at 8 μM was able to reduce cell viability by about 50% and was therefore chosen for our experiments in cortical neurons. It is noteworthy that cell death induced by PCB-95 (8 μM) at 24 h was inhibited in a concentration-related manner when RSV was added to the culture medium at different concentrations (6.25–50 μM) (Fig. 6b); notably, maximum neuroprotection was obtained with 25 μM RSV. Similarly, the increase in REST mRNA and protein expression induced by PCB-95 at 24 h was blocked upon addition of RSV (25 μM) (Fig. 6c,d). Notably, PCB-95-induced increase in calpain expression was blocked by RSV co-application; in contrast, the apoptotic marker procaspase-3 was unmodified (Fig. 6e,f). Moreover, PCB-95-induced decrease in P-c-Jun was reverted by RSV, as compared to the vehicle (Fig. 6g).

We next investigated the function of c-Jun and SIRT1 in RSV-blocked PCB-95-induced REST gene and gene product increase. To this aim, we knocked down c-Jun and SIRT1 with ODN for c-Jun (AS c-Jun) and with siRNA for SIRT1 (siSIRT1). Notably, siSIRT1 and AS ODNs for c-Jun significantly reduced SIRT1 and c-Jun expression by 55% and 65%, respectively (Suppl. Fig. 3b,d).

Intriguingly, transfection of AS c-Jun and siSIRT1 blocked RSV capability to reduce PCB-95-induced REST mRNA and protein expression as...
compared to RSV-treated cells and transfected with MS c-Jun and siCTL, respectively (Fig. 6h,i). Then, to evaluate the role of REST in PCB-95-induced toxicity, we knocked down its expression. Particularly, siRNA for REST was recently published (Formisano et al., 2013, 2015b). Unsurprisingly, siREST transfection reduced PCB-95-induced neurotoxicity (Fig. 6l). Interestingly, co-incubation with RSV significantly reduced the detrimental effect of PCB-95, as compared to vehicle (Fig. 6l).

Discussion

In the present study, we showed that resveratrol (RSV) reduces REST expression at the transcriptional level via activation of SIRT1. As a result,
our results are in accordance with previous reports demonstrating that RSV causes an increase in c-Jun mRNA (Shih et al., 2002). Considering the key role played by SIRT1 in this process, we could hypothesize that RSV was able to increase SIRT1 binding to c-Jun gene promoter sequence. Such increased SIRT1 binding then determined an increase in its binding activity to the REST human gene promoter. This effect. Interestingly, exposure to NDL-PCB-95 at 8 μM damaged approximately 50% of SH-SY5Y cells and cortical neurons. However, RSV counteracted PCB-induced cell death at 5 μM in SH-SY5Y and at 25 μM in cortical neurons. These results confirm earlier findings demonstrating that RSV induces neuroprotection at 25 μM in neuronal primary cultures (Sakata et al., 2010) and at 5 μM in SH-SY5Y cells (Lee et al., 2007), and that primary neurons are more resistant to RSV neuroprotection.

Intriguingly, we found that SIRT1 knockdown blocked RSV-induced neuroprotection in neurons, thereby corroborating the hypothesis that RSV via SIRT1 activation determines neuronal survival. In fact, specific siRNAs against SIRT1 are known to abrogate RSV-induced neuroprotection in in vitro models of brain ischemia (Wang et al., 2013) and in retinal ganglion cells after H2O2 treatment (Khan et al., 2012).

Moreover our results are also in line with the neuroprotective role played by c-Jun. In fact, c-Jun over-expressing cells are more resistant to okadaic acid-induced apoptosis (Dragunow et al., 2000) and its induction in ischemic conditioning is able to rescue CA1 hippocampal from neuronal death (Sommer et al., 1995).

It has been shown that REST protects against oxidative stress down-regulating apoptotic gene expression whereas in this paper we found that REST up-regulation induced by PCB-95 determines cell-death. However, the toxic pathways triggered by oxidative stress and PCB-95 seem to be different. In fact oxidative stress, inflammation and apoptosis do not occur in our experimental conditions. Indeed PCB-95 exposure failed to increase ROS production and the expression of P-p65 protein, a marker of inflammation. Furthermore in PCB-95 treated cells a necrotic and not an apoptotic cell death occur. Our findings suggest that oxidative stress, apoptosis and inflammation are not associated to PCB-95 induced neurotoxicity in our experimental model, and that RSV-induced neuroprotection is mediated by SIRT1/c-Jun pathway, that counteracts REST increase. Consistently, we have previously shown that PCB mixture A1254 through REST induces necrotic cell death in neurons (Formisano et al., 2011, 2015a, 2015b). Regarding the PCB concentrations tested in the present study, they have already been used in previous reports and are within the range of PCBs detected in wildlife animals (Mariussen et al., 2002). Specifically, PCB-95, but not other PCBs or polybrominated diphenyl ethers (PBDEs), is significantly higher in post-mortem brains of children with a syndromic form of autism, compared with neurotypical controls (Mitchell et al., 2012). Furthermore, PCB-95 crosses the blood–brain barrier and can interact with targets (Kania-Korwel et al., 2012).

Collectively, these results indicate that resveratrol through SIRT1/c-Jun pathway regulates REST expression at the basal level in SH-SY5Y neuronal cells and is involved in the neurotoxic effect of NDL PCB-95. Furthermore, these results indicate that resveratrol exerts a neuroprotective effect against the toxic effect of PCBs by increasing SIRT1 activity with neurotypical controls (Mitchell et al., 2012). Furthermore, PCB-95 crosses the blood–brain barrier and can interact with targets (Kania-Korwel et al., 2012).

Collectively, these results indicate that resveratrol through SIRT1/c-Jun pathway regulates REST expression at the basal level in SH-SY5Y neuronal cells and is involved in the neurotoxic effect of NDL PCB-95. Furthermore, these results indicate that resveratrol exerts a neuroprotective effect against the toxic effect of PCBs by increasing SIRT1 activity in turn causes a reduction in c-Jun binding to the REST promoter sequence. Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2015.08.010.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Authorship contributions

Conceived and designed the experiments: NG, GL, SA, AS, GDR and LF. Performed the experiments: NG, GL, PM, GDR, and LF. Analyzed the data: NG, GL, AS, GDR, and LF. Contributed reagents/materials/analysis tools NG, GL, GDR, IMT and LF. Wrote the paper: LMTC GDR and LF.

Transparency document

The Transparency document associated with this article can be found, in online version.
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References


Toxicol. 28, 511–518.


Toxicol. 28, 511–518.


