Neuroprotective Role of Trans-Resveratrol in a Murine Model of Familial Alzheimer’s Disease

David Porqueta, Christian Griñán-Ferréa, Isidre Fererre, Antoni Caminsa, Coral Sanfeliub, Jaume del Valle and Mercè Pallàs

Abstract. The amyloid-β protein precursor/presenilin 1 (AβPP/PS1) mouse model of Alzheimer’s disease (AD) has provided robust neuropathological hallmarks of familial AD-like pattern. AD is a neurodegenerative process that causes severe cognitive impairment; it is characterized by the accumulation of amyloid-β (Aβ) and hyperphosphorylated tau forms and by oxidative and inflammatory processes in brain. Currently, efforts are made to understand biochemical pathways because there is no effective therapy for AD. Resveratrol is a polyphenol that induces expression and activation of several neuroprotective pathways involving Sirtuin1 and AMPK. The objective of this work was to assess the effect of oral resveratrol administration on AβPP/PS1 mice. Long-term resveratrol treatment significantly prevented memory loss as measured by the object recognition test. Moreover, resveratrol reduced the amyloid burden and increased mitochondrial complex IV protein levels in mouse brain. These protective effects of resveratrol were mainly mediated by increased activation of Sirtuin 1 and AMPK pathways in mice. However, an increase has been observed in IL1β and TNFα gene expression, indicating that resveratrol promoted changes in inflammatory processes, although no changes were detected in other key actors of the oxidative stress pathway. Taken together, our findings suggest that resveratrol is able to reduce the harmful process that occurs in AβPP/PS1 mouse hippocampus, preventing memory loss.

Keywords: AMPK, inflammation, mitochondria, resveratrol, sirtuin 1

INTRODUCTION

A central issue in cognitive neuroscience of research on aging is pinpointing precise neural mechanisms that determine cognitive outcome in late adulthood, as well as identifying early markers of neurodegeneration and preventive strategies to impede cognitive impairment in aging. Alzheimer’s disease (AD) is the most prevalent neurodegenerative disorder associated with age and characterized by senile plaques and neurofibrillary tangles. Senile plaques are formed by the accumulation of amyloid-β (Aβ1–40) and Aβ1–42 peptides, which result from the sequential cleavage of amyloid-β protein precursor (AβPP) by β-secretase (BACE) and γ-secretase. The production of Aβ peptides is prevented by alternative cleavage of AβPP by α-secretase followed by γ-secretase. Donmez et al.
have reported that production of Aβ and plaques in the 3xTg-AD transgenic mouse model of AD is reduced by the overexpression of Sirtuin 1 (SIRT1) and is increased by knocking out SIRT1 in brain. SIRT1 directly activates transcription of the gene encoding α-secretase, the ADAM10 gene. Additionally, SIRT1 deacetylates and coactivates the Retinoic acid receptor β, a known regulator of ADAM10 transcription. It is noteworthy that in AD, mitochondrial function is affected. Reduction of mitochondrial complex IV (MCIV) activity [2, 3] and increased reactive oxygen species production [4, 5] was reported elsewhere. Accumulation of mitochondrial DNA changes might increase reactive oxygen species production and reduce mitochondrial adenosine triphosphate (ATP) in an age-dependent manner. Increases of somatic mitochondrial DNA in aging might contribute to AD development [6].

An early-onset form of AD, familial AD (defined for individuals <65 years of age), accounts for 5% of all cases and is directly linked with highly penetrant autosomal dominant mutations in one of three different genes: the presenilin 1 (PS1) gene; the PS2 gene, or the PP gene.

Resveratrol, a natural polyphenolic compound found in grapes and red wine, increases metabolic rate, insulin sensitivity, mitochondrial biogenesis, and physical endurance, and also reduces fat accumulation in mice and, at the brain level, is postulated as an option to prevent AD [7–9]. Although it is thought that resveratrol targets SIRT1, this remains controversial because resveratrol also activates 5'-Adenosine monophosphate (AMP)-activated protein kinase (AMPK), which also regulates insulin sensitivity and mitochondrial biogenesis. Um et al. [10] demonstrated that the metabolic effects of resveratrol are dependent on AMPK using knockout mice. In addition, resveratrol, during food deprivation (caloric restriction), causes an increase in the cellular AMP/ATP ratio, resulting in the activation of AMPK, which initiates a signaling process that recruits mediators of oxidative metabolism and mitochondrial biogenesis including PGC1-α, the peroxisome proliferator-activated receptor-gamma (PPAR-γ), and others [11].

Double-transgenic AβPP/PS1 mice express a chimeric mouse/human AβPP bearing the Swedish mutation (MoHu AβPP695sw) and a mutant human PS1-dE9, both causative of familial AD. Transgenic mice show senile plaques in cortical and hippocampal areas starting at 4 months [12] and impaired memory and learning performance between 6 and 15 months [13–15]. However, these mice have no alterations in motor function or anxiety-related behavior [16]. Moreover, these animals have increased BACE activity [17], decreased ADAM10 expression [18], and decreased synaptosomal synaptophysin [19]. In reference to inflammatory markers AβPP/PS1 mice present elevated levels of IL-1β [20] and TNF [21].

The aim of this work was to elucidate the interplay between the amyloidogenic pathway, SIRT1 and AMPK signaling, and resveratrol as a preventative agent in the mouse familial AD model AβPP/PS1. The generation of this knowledge would afford us new clues to confront neurodegenerative disorders with abnormal AβPP processing, such as familial AD, and to help in preventing the processes linked with this illness.

**METHODS**

**Animals and resveratrol feeding**

All animal protocols were conducted and approved by the Ethics Committee for Animal Use from the University of Barcelona in accordance with the Generalitat de Catalunya guidelines for the Care and Use of Laboratory Animals. All efforts were made to minimize animal numbers and distress. AβPPsw/ePS1dE9 male mice (2 months of age) that were treated and age-matched control littersmates were both fed with mouse chow (Harlan Diet) containing 1% resveratrol [22] or the same chow without resveratrol (n = 10 in each group) for 10 months. Daily resveratrol consumption was calculated to be 4 mg/kg/day (mean of food intake of 4 g/animal/day).

**Novel object recognition test (NORT)**

Changes in cognition were tested with an object-recognition task as described elsewhere [23]. Animals were placed in a 90°, two-arm, 25-cm-long, 20-cm-high, and 5-cm-wide black maze. Light intensity in the middle of the field was 30 lux. The objects to be discriminated were made of plastic (object A, 5.25 cm in height, and object B, 4.75 cm in height). For the first 3 days, the mice were individually acclimatized to the apparatus for 10 min. On day 4, the animals were submitted to a 10-min acquisition trial (first trial), during which they were placed in the maze in the presence of two identical novel objects (A+A or B+B), which were localized at the end of each arm. A 10-min retention trial (second trial) occurred 2 h later. During this second trial, objects A and B were placed in the maze, and the time that the animal explored the new object (tn) and the old object (to) were recorded. A Discrim-
D. Porquet et al. / Neuroprotective Role of Trans-Resveratrol in a Murine Model of Familial Alzheimer’s Disease

In order to avoid object preference biases, objects A and B were counterbalanced so that one half of the animals in each experimental group were first exposed to object A and then to object B, whereas the remaining one half first saw object B and then object A. The maze and the objects were cleaned with 96% ethanol between experiments to eliminate olfactory cues.

**Brain processing**

Animals were anesthetized with 80 mg/kg of sodium pentobarbital and intracardially perfused with saline serum. Afterward, brains were dissected and separated sagitally into two hemispheres: one for histological staining, and the other, for protein and RNA extraction. Hemispheres for histological staining were frozen by immersion in isopentane, chilled on dry ice, and stored at −80°C until sectioning. Thereafter, the frozen brains were embedded in Optimal cutting temperature (OCT) cryostat-embedding compound (Tissue-Tek; Torrance, CA, USA), cut into 20-μm-thick sections on a cryostat (Leyca Microsystems, Germany) at −18°C, and placed on slides. Slides containing brain sections were fixed with acetone for 10 min at 4°C, allowed to dry at room temperature, and then frozen at −20°C until further staining. The remaining hemispheres were dissected and stored at −80°C until protein or RNA extraction.

**Thioflavin S staining**

Slides were allowed to defrost at room temperature and then were rehydrated with Phosphate-buffered saline (PBS) for 5 min. Later, the brain sections were incubated with 0.3% Thioflavin S (Sigma-Aldrich) for 20 min at room temperature in the dark. Subsequently, these were submitted to washes in 3-min series, specifically with 80% ethanol (2 washes), 90% ethanol (1 wash), and 3 washes with PBS. Finally, the slides were mounted using Fluoromount (EMS), allowed to dry overnight at room temperature, and then frozen at −20°C until further staining. The remaining hemispheres were dissected and stored at −80°C until protein or RNA extraction.

**Protein extraction**

Brains were micronized by freezing with liquid nitrogen and grinding with a mortar. For total protein extraction, lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, pH 7.4), EDTA-free Protease inhibitor cocktail (Roche, Mannheim, Germany), and Phosphatase inhibitor cocktail 1 (Sigma-Aldrich, St. Louis, MO, USA) were added to microwaved tissue and left on ice for 30 min. Then, the samples were centrifuged at 10,000×g for 10 min and a supernatant with total protein content was collected. All of the protein extraction steps were carried out at 4°C. Protein concentration was determined by the Bradford protein assay.

**Western blot**

For western blot analysis, 20 μg of protein were denatured at 95°C for 5 min in sample buffer (0.5 M Tris-HCl, pH 6.8, 10% glycerol, 2% Sodium dodecyl sulfate [SDS], 0.05% bromophenol blue), separated by Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) onto 8–12% polyacrylamide gels and transferred onto Immobilon polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated overnight at 4°C with the following primary antibodies: anti-β-actin (Sigma; 1:10,000); anti-Sirt1 (Abcam, 1:1,000); anti-p53 (Abcam, 1:500); anti-acetyl p53 (L382) (Millipore, 1:500); anti-AMPK (Cell Signaling, 1:1,000); anti-p-AMPK (Cell Signaling, 1:1,000); OXPHOS cocktail and porin (1:500; MitoSciences); catalase (1:2,000; Calbiochem), and superoxide dismutase (SOD) (1:3000; Calbiochem) diluted with Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 5% Bovine serum albumin (BSA). Membranes were then washed and incubated with secondary antibodies and diluted with TBS-T for 1 h at room temperature. Protein bands were visualized using a Chemiluminescent horseradish peroxidase substrate (Millipore) and ChemiDoc XRS + (Bio-rad). Band intensities were quantified by densitometric analysis using Image Lab software and values were normalized to β-actin.

**RNA extraction and gene expression determination**

Total RNA isolation was carried out by means of Tri-zol reagent following the manufacturer’s instructions. RNA content in the samples was measured at 260 nm, and the purity of the samples was determined by the A260/A280 ratio in a NanoDrop ND-1000 (Thermo Scientific). Samples were also tested in a Bioanalyzer 2100B (Agilent Technologies) to determine the RNA integrity number.
Reverse transcription-polymerase chain reaction (RT-PCR) was performed as follows: 2 μg of messenger RNA (mRNA) was reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Then, qPCR was performed using TaqMan gene expression assays (Applied Biosystems), doing triplicates for each gene and cDNA sample in 96-well optical plates. The TaqMan probes used were Interleukin-1β (Il1b) (Mm00434228_m1), Interleukin-6 (Il6) (Mm00446190_m1), Tumor necrosis factor (Tnf) (Mm00443260_g1), Nuclear factor erythroid 2-related factor 2 (Nfe2l2) (Mm00477784_m1), Heme oxygenase-1 (Hmox) (Mm00516005_m1), and Jun (Jun) (Mm00495062), with TATA-binding protein (Tbp) (Mm00446971_m1) as housekeeping. For each 20 μl of TaqMan reaction, 9 μl cDNA (25 ng) was mixed with 1 μl 20x probe of TaqMan Gene Expression Assays and 10 μl of 2x TaqMan Universal PCR Master Mix. The reactions were carried out using the following parameters: 50 °C for 2 min; 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and at 60 °C for 1 min using the StepOnePlus Real-Time PCR System (Applied Biosystems). Finally, all TaqMan PCR data were normalized to TBP using the delta-delta Ct method.

Statistical analysis

Results were analyzed statistically by GraphPad PRISM (GraphPad Software, Inc.) software. Data are presented as mean ± Standard error of the mean (SEM), and means were compared with two-tailed, unpaired Student t-test. In the Object recognition test (ORT), a one-sample t-test was used to examine whether single columns were different from zero. Statistical significance was reached when p values were <0.05. All the experiments and its statistics were conducted with 7 to 10 animals for each experimental group.

RESULTS

Resveratrol ameliorated short-term memory in AβPP/PS1 mice

To date, it has been found that transgenic mice that overexpress AβPP/PS1 show loss of memory [13–15]. ORT demonstrated that at 12 months, AβPP/PS1 mice fed with resveratrol chow exhibited significantly improved memory capabilities (Fig. 1A) in comparison to AβPP/PS1 control mice.

The previously mentioned beneficial effects of resveratrol were supported by an increase in synaptophysin, a presynaptic protein that reflects an improvement in synaptic activity in resveratrol-fed mice (Fig. 1B).

Resveratrol treatment reduced plaque pathology but did not alter AβPP and its carboxy terminal fragments (CTF) in AβPP/PS1 mice

To test the effect of resveratrol on amyloid plaque pathology, thioflavin S staining was used. Resveratrol markedly reduced thioflavin S-positive compact plaques compared with control mice (Fig. 2). Quantification of plaques revealed a significant reduction in plaque counts and plaque burden in hippocampus and
Fig. 2. A) Thioflavin S staining of Aβ plaques in mouse brains. Representative images of histopathological brain state of AβPP/PS1 control and resveratrol-fed mice. White arrows are representative indicators of the presence of Aβ plaques in the studied areas, cortex (C), and hippocampus (H). Bar chart showing quantification of the number of amyloid plaques in the cerebral cortex (B) and hippocampus (C) of AβPP/PS1 control and resveratrol-treated animals. For quantification parameters, see Materials and Methods. Bars represent mean ± Standard error of the mean (SEM). Student t-test *p<0.05; **p<0.01 versus control.

medial cortex of resveratrol-fed mice compared with controls (Fig. 2A-C).

To investigate the mechanism responsible for the resveratrol-induced reduction in plaque counts, enzymes that mediate AβPP cleavage were determined. We found no changes in PS1, but a significant reduction in BACE and ADAM 10 protein levels were observed (Fig. 3A–C).

Moreover, full-length AβPP and AβPP cleavage products β-CTF (C99) and α-CTF (C83) levels were determined by western blot using AβPP G369 antibody (against the AβPP cytoplasmic tail). Resveratrol
Fig. 3. Levels of presenilin 1 (PS1) (A), ADAM10 (B), β-secretase (BACE) (C), AβPP (D), C99 (E), and C83 (F) in AβPP/PS1 control and resveratrol-fed mice. Bars represent mean ± Standard error of the mean (SEM). Student t-test: *p < 0.05; **p < 0.01 versus control.
Fig. 4. Western blot analysis for Sirtuin 1 (SIRT1) (A), acetylated-p53 (B), phospho-5’-Adenosine monophosphate (AMP)-activated protein kinase (p-AMPK) (C), mitochondrial complex IV (MCIV) (D), and serine-threonine kinase liver kinase B (LKB) (E). Bars represent mean ± Standard error of the mean (SEM). Student t-test: *p<0.05 versus control.
Resveratrol treatment increases the phospho-AMPK (p-AMPK)/LKB pathway in AβPP/PS1 although SIRT1 levels are decreased

Because previous studies demonstrated that resveratrol activates the nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylase SIRT1, we tested whether the SIRT1 protein level was upregulated in AβPP/PS1 mice fed with resveratrol. As demonstrated in Fig. 4, resveratrol treatment did not increase SIRT1, but a significant diminution in acetyl-p53, one of the main substrates of SIRT1, was observed (Fig. 4A, B). Moreover, p-AMPK and also p-LKB were significantly increased in mice fed resveratrol (Fig. 4C–E), indicating modulation by this polyphenol on phosphorylation, thus on the activity of these two kinases. In reference to mitochondria and according to stimulation of AMPK, an increase in MCIV expression, but not in other complexes, was determined after resveratrol treatment (Fig. 4D).

Effect of resveratrol treatment on cytokine gene expression and catalase/superoxide dismutase (CAT/SOD) protein levels in hippocampus

To test the effect of resveratrol on inflammation and oxidative stress, different markers for these were determined. Resveratrol feeding increased Il1b and Tnf mRNA expression (Fig. 5A, B). In contrast, no changes were determined in Il6, Nfe2l2, Hmox, and Jun expression (data not shown). Protein levels of CAT and SOD were also unchanged (Fig. 5C, D).

DISCUSSION

AβPP/PS1 mice express a chimeric mouse/human amyloid-β protein precursor (MoHu AβPP695swe) and a mutant human presenilin 1 (PS1-dE9) that lead to
altered AβPP and PS1 proteins. Both cause Aβ plaques from 3 months onward, and they increase in number and distribution with disease progression in parallel with increased levels of brain-soluble Aβ1–42 and Aβ1–40, but also with a reduced Aβ1–42/1–40 ratio with age. Amyloid deposition in plaques is accompanied by altered mitochondria and increased oxidative damage, post-translational modifications, and the accumulation of altered proteins at the dystrophic neurites surrounding plaques [24]. This model is taken as a familial AD murine model. Resveratrol has been described as a natural compound with pleiotropic neuroprotective activities, linked with antioxidant properties and the modification of activity of different enzymatic pathways, such as the SIRT1 pathway, AMPK activation, or cellular protein degradation machinery [25].

AβPP/PS1 mice fed with resveratrol showed a significant decrease in the number and intensity of amyloid plaques, when measured by specific S-thioflavine staining. Reduction in plaque pathology did not appear to be due to altered AβPP processing toward the non-amyloidogenic pathway, because high-molecular-weight AβPP (holo AβPP), C99 (Carboxy terminal fragment beta, CTFβ), and C83 (Carboxy terminal fragment alpha, CTFα) were unaltered; this is in agreement with a study conducted by Karuppagounder and coworkers [26] on Tg19959 fed with resveratrol. However, the significant decrease in BACE levels would ultimately have altered the amyloidogenic pathway, leading to a reduction in amyloid burden found in AβPP/PS1 fed for a lengthy period with resveratrol. Evidence is compelling that a decrease in proteasome activity occurs in AD brains [27, 28] and in AD murine models, including AβPP/PS1, a familial AD model [24, 29], and in vitro, resveratrol promotes Aβ clearance by increasing intracellular proteosomal activity without affecting Aβ- producing enzyme activities (β-secretase and γ-secretase) [30]. This mechanism on the proteasomal system exerted by resveratrol could be synergistic to BACE reduction, explaining the decrease in plaques observed with resveratrol treatment, albeit than a decrease in ADAM10 was also determined.

Improvement of short-term memory in these mice was evaluated by the novel object recognition test (NORT), a test widely used to evaluate learning and memory [24, 31]. The brain areas involved in this test are mainly hippocampus and perirhinal cortex. Moreover, NORT requires no external motivation, reward or punishment, thus avoiding the amyogenic role in cognition impairment, although AβPP/PS1 mice do not present anxiety behavior [32, 33]. Synaptophysin, an integral membrane glycoprotein present in presynaptic vesicles, was used to quantify synapse number. In our case, AβPP/PS1 mice resveratrol fed increased synaptophysin levels, correlating with short-term memory improvement and reduction in amyloid plaques.

Several reports suggest that resveratrol possesses antioxidant properties [34]; thus, a tantalizing alternative speculation is that resveratrol-induced reduction in plaques may be occurring through its effect on the cellular antioxidant machinery. We measured protein levels of CAT and SOD, but there were no changes observed. In relation to the expression of several genes related with oxidative stress and inflammation, there were no changes found in the NfκB2 and Il10 genes, which are implicated in neuroprotection mechanisms [35, 36], whereas cytokines linked with inflammation processes, such as IL-1β and TNFα, were increased. In AD, abnormal accumulation of Aβ is thought to be intimately linked with the immune system [37]. The majority of efforts have been carried out in order to understand the role of activated astroglia and microglia, which secrete cytokines that act in favor of phagocytosis and AβPP and Aβ peptide clearance, giving rise to the hypothesis that slight increases in cytokines will be protective by removing Aβ and reducing the plaque burden in brain. Our results indicate that at this time of treatment, there is no effective action of resveratrol on the inflammation and oxidative machinery of AβPP/PS1 mice; the antioxidant effect could have occurred at an earlier treatment time, as described for another AD murine model by Karuppagounder and coworkers [26]. However, long-term resveratrol treatment allows fine control of antioxidant machinery by increasing IL-1β and TNFα, leading to a hormetic process that ultimately yielded a reduction in plaque number and the beneficial effect on memory, as we observed clearly in this familial AD mouse model at a late age. This fact cannot rule out the possibility that resveratrol exerts an earlier antioxidant effect at younger ages, acting as a preventive more than as a curative agent.

In addition, resveratrol has been postulated to have its beneficial effects on the lifespan, neurodegeneration, and memory improvement by activation of SIRT1 [38, 39]. Because SIRT1 activation is one of the main targets defined for the pharmacological effects of resveratrol, the levels of this deacetylase were determined. Here we observed a diminution in SIRT1 protein levels, but a decrease in acetyl-p53 was determined concomitantly. These results are in agreement...
with the literature in other transgenic models and in in vitro experiments where a reduction in plaque pathology without increase in sirtuin levels [26] but decrease in PGCIα acetylation was found [39]. The decline in SIRT1, together with p53 deacetylation status, could reflect decreased synthesis of this deacetylase, but increased activity mediated by resveratrol [40–42] or by crossregulation with the AMPK/LKB pathway [34, 43, 44].

Cantó and Auwerx [45] demonstrated that activation of AMPK stimulated the functional activity of SIRT1 by increasing the intracellular concentration of NAD+. Interestingly, SIRT1 was able to deacetylate LKB1 kinase, which subsequently increased its activity [46]. Because LKB1 is an upstream activator of AMPK, this signaling pathway stimulates the activation of AMPK. This positive feedback loop between SIRT1 and AMPK can also potentiate the function of the other AMPK-activated signaling pathways [47].

In the current study, the surprising resveratrol-induced decline in SIRT1 levels is consistent with AMPK/LKB activation.

The AMPK and SIRT1 signaling pathways are highly conserved energy sensors of increased levels of AMP and NAD+, respectively, and AMPK signaling is involved in the regulation of energy metabolic homeostasis [48]. In line with this, resveratrol also normalized mitochondrial function and enhanced mitochondrial biogenesis in the spinal cord of SOD1G93A amyotrophic lateral sclerosis mice [49].

The majority of the pathways mentioned previously are involved in mitochondrial function, and in this particular organelle, the AβPP/PS1 life-long treatment with resveratrol induced an increase in complex IV, MCIV, because this action can be taken in account for delineating the neuroprotective role of resveratrol in AβPP/PS1 mice.

In conclusion, this study supports some unexplored pathways responsible for the neuroprotective effects of resveratrol in AβPP/PS1 mice, namely, the equilibrium among SIRT1 and AMPK signaling, mitochondrial status, and inflammatory changes. In addition to other reports [26, 50], our findings indicate that the onset of this neurodegenerative disease may be delayed or mitigated employing dietary resveratrol, which is able to protect against Aβ plaque formation and cognitive loss. Further studies need to be conducted to elucidate the precise regulatory mechanisms that can be modulated by resveratrol in the AβPP/PS1 mouse model.

ACKNOWLEDGMENTS

We thank Maggie Brunner, M.A., for revising the language and style of the manuscript. This study was supported by grants SAF-2011-23631 and SAF-2012-39852 from the “Ministerio de Educació y Ciencia” and 2014SGR/525 from the “Generalitat de Catalunya”.


REFERENCES


