

Effect of Resveratrol on Reactive Oxygen Species-Induced Cognitive Impairment in Rats with Angiotensin II-Induced Early Alzheimer's Disease

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Abbreviations:

AD, Alzheimer's disease; Ang II, Angiotensin II; A β 42, Fbrillar β -amyloid; BP, Blood pressure. BDNF, Brain-derived neurotroph factor; CNS, Central nervous system. ICV, intracerebroventricular. NTS, Nucleus tractus solitarii. NADPH oxidase, nicotinamide adenine dinucleotide phosphate-oxidase; MCI, mild cognitive impairment; ROS, Reactive oxygen species; SHRs, Spontaneously hypertensive rats.

Abstract

Recent studies have indicated that several anti-hypertensive drugs may delay the development and progression of Alzheimer's disease (AD). However, the relationships among AD, hypertension, and oxidative stress remain to be elucidated. In the present study, we aimed to determine whether treatment with resveratrol reduces reactive oxygen species (ROS) generation in the brain, thereby reducing cognitive impairment in rats with angiotensin II (Ang-II)-induced early AD. Male WKY rats with Ang-II-induced AD were treated with losartan or resveratrol for 2 weeks. Our results revealed that treatment with resveratrol (10 mg/kg/day) decreased blood pressure, increased levels of brain-derived neurotrophic factor (BDNF) in the hippocampus, and decreased ROS production in the nucleus tractus solitarius (NTS) in the Ang-II groups. In addition, inhibition of Tau^{T231} phosphorylation in the hippocampus using resveratrol significantly abolished Ang-II-induced expression of A β precursors, active caspase 3, and glycogen synthase kinase 3 β (GSK-3 β)^{Y216} while increasing Akt^{S473} phosphorylation. Notably, resveratrol reversed impairments in hippocampal-dependent contextual memory induced by deleting NADPH oxidase and NOX2. Overall, our results suggest that resveratrol exerts neuroprotective effects against memory impairment and hippocampal damage in a rat model of early stage AD by reducing oxidative stress. These novel findings indicate that resveratrol may

represent a pharmacological option for patients with hypertension at a risk of AD during old age.

Introduction

Alzheimer's disease (AD) is an age-dependent neurodegenerative disorder associated with abnormal energy metabolism, representing the most common neurodegenerative disorder worldwide [1, 2]. AD is characterised neuropathologically by the formation of senile plaques and neurofibrillary tangles, and clinically by the progressive deterioration of memory and other cognitive functions [3], which cannot be prevented using currently available treatments [4]. The pathogenesis of AD is considered a multifactorial neurodegenerative process, in which several pathways are damaged due to oxidative stress injury, abnormal energy processing, mitochondrial dysfunction, and inflammation [5, 6]. Moreover, recent studies have reported that hypertension is a major risk factor for the development of AD in old age [7]. The nucleus tractus solitarius (NTS) is located in the dorsal medulla of the brainstem, which is the primary integrating centre for cardiovascular regulation and other autonomic functions of the CNS. Specifically, increases in circulating and brain levels of angiotensin II (Ang-II), a primary effector peptide of the renin–angiotensin–aldosterone system, not only play important roles in the genesis of arterial hypertension, but also in the pathophysiology of AD [8]. Therefore, a relationship appears to exist among hypertension, cerebrovascular disease, and decreases in cognitive function—an index of hippocampal dysfunction [9]. However,

it remains to be determined whether hippocampal changes result from pre-existing hypertension, or whether hypertension and brain pathology reflect a central defect associated with AD [10].

Excessive binding of Ang-II to the AT1 receptor in the brain negatively affects cognition by blocking hippocampal long-term potentiation (LTP) and enhancing β -amyloid production [11, 12]. In particular, such binding may lead to widespread neuronal injury, including increases in reactive oxygen species (ROS), activation of NADPH oxidases, blood-brain barrier (BBB) breakdown, decreased access to nutrients, and β -amyloid toxicity in the cerebral vasculature [11, 13, 14].

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a stilbenoid, a type of natural phenol that may exert beneficial effects in a variety of human diseases such as type 2 diabetes, obesity, and cancer [15]. Resveratrol has attracted increasing attention due to its neuroprotective effects, which include facilitating anti-amyloidogenic cleavage of β -amyloid precursor protein, promoting clearance of neurotoxic A β peptides, and reducing oxidative stress [16, 17]. In addition, accumulating evidence indicates that resveratrol attenuates oxidative imbalances by simultaneously enhancing ROS production and downregulating key antioxidant enzymes such as copper-zinc superoxide dismutase (SOD1) and manganese superoxide dismutase (SOD2) [18].

Previously, we demonstrated that resveratrol treatment abolishes ROS generation

and enhances SOD2 expression, thereby negatively regulating Rac1-induced NADPH oxidase levels in the NTS during oxidative stress-associated hypertension[19]. Previous studies have further suggested that oxidative imbalances and the resultant neuronal damage play a critical role in the initiation and progression of AD [20]. NAD(P)H oxidase plays a role in multiple central autonomic networks associated with Ang-II-induced ROS in neurons [21]. In our previous study, spontaneously hypertensive rats (SHRs) were treated with losartan or tempol for 2 weeks, following which we observed significant decreases in systolic blood pressure (SBP) and ROS generation in the NTS [22]. Therefore, in the present study, we aimed to determine whether treatment with resveratrol improves ROS generation in the brain, thereby reducing cognitive impairment in rats with Ang-II-induced early AD.

Materials and Methods

Experimental chemicals

All experimental chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated.

Animals

Ten-week-old male WKY rats were obtained from the National Science Council

Animal Facility (NSCAF; Taipei, Taiwan) and housed in the animal room of Kaohsiung Veterans General Hospital (VGHKS; Kaohsiung, Taiwan). Both the NSCAF and VGHKS are internationally certified by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALACi). Rats housed in the animal room of VGHKS were fed in a specific pathogen-free (SPF) room. SPF facilities are designed to maintain rodents in an environment that is free of certain infectious organisms that are pathogenic and/or capable of interfering with research objectives. The rats were kept in individual cages in a light-controlled room (12-hour light/12-hour dark cycle), the temperature of which was maintained between 23°C and 24°C. The rats were given normal rat chow (Purina; St. Louis, MO) and tap water *ad libitum*. All animal research protocols were approved by the Animal Research Committee, and the institutional review board at VGHKS approved all study procedures (VGHKS-2018-A002). The study was performed in accordance with approved guidelines and conducted in compliance with the Declaration of Helsinki.

The rats were housed in an animal room at VGHKS and randomly divided into the following four groups, with six rats in each group: (1) the sham group, in which each rat received an intracerebroventricular (ICV) injection of artificial cerebrospinal fluid (aCSF) and an oral dose (1 ml/kg body weight (BW)) of distilled water once every 24 hours (vehicle control); (2) the WKY + Ang-II group, in which each rat

received an ICV injection of angiotensin II (14.4 $\mu\text{g}/\mu\text{l}$) and an oral dose of distilled water; (3) the WKY + Ang-II + losartan group, in which rats treated with losartan (10 mg/kg BW, Chunghwa Yuming Healthcare, Taiwan) received an ICV injection of angiotensin II; (4) the WKY + Ang-II + resveratrol group, in which rats treated with resveratrol (10 mg/kg BW) received an ICV injection of angiotensin II. All surgeries were performed in an effort to minimise animal suffering.

After 14 days, rats were euthanised using CO_2 in accordance with the 2013 AVMA Guidelines for the Euthanasia of Animals. All rats were killed using 100% CO_2 , with death occurring within 2 to 5 minutes. Brains were immediately dissected on ice, frozen, and maintained at -20°C until assayed.

ICV injection procedure

ICV infusion experiments were performed following a stabilisation period of at least 30 minutes after the insertion of the microinjector into the ventricular-guided cannula. Blood pressure was monitored for 3 days after drug infusion. As a vehicle control, we analysed the effect of an ICV injection of aCSF (142 mmol/L NaCl, 5 mmol/L KCl, 10 mmol/L glucose, and 10 mmol/L HEPES, pH 7.4). Ang-II (14.4 $\mu\text{g}/\mu\text{l}$ /per day) was dissolved in aCSF. Resveratrol was initially dissolved in DMSO and then diluted in ddH₂O at a final concentration of 1% DMSO. Basal blood pressure was examined

prior to injection. The daily ICV drug infusions were performed over a 2-min period and delivered as a single bolus of a final volume of 5 μ L from day 0 to day 14.

BP measurement

Using a previously described tail-cuff method (CODA, Kent Scientific Corporation, USA), we measured SBP and heart rate prior to losartan or resveratrol treatment (day 0). Measurements were also obtained 3, 7, 11, and 14 days after surgery, as well as prior to sacrifice. All measurements were performed between 08:00 am and 12:00 am. In this method, the reappearance of pulsation on the digital display of the BP cuff was detected using a pressure transducer and was amplified and recorded as the SBP. During measurement, we obtained a rapid series of 10 individual readings, the highest and lowest of which were discarded, while the remaining eight were averaged.

Morris water maze (MWM)

Cognitive and behavioural functions were assessed using the MWM task. Briefly, a circular pool (diameter: 180 cm) was filled with tap water maintained at $24 \pm 1^\circ\text{C}$. The escape platform (diameter: 12 cm) was submerged 2 cm below the surface of the water. Each rat underwent four trials per day over four consecutive days (day 3 to day 6). For each trial, the rat was placed in the pool (facing pool wall) at one of four

selected starting points (north, south, east, or west pole). Upon locating the platform, the rat was allowed to remain there for 20 s before being returned to its cage. If the rat did not find the platform within 90 s, the time was recorded as 90 s, following which the rat was guided to the platform and allowed to remain there for 20 s. The escape latency and swim speed were measured using a video tracking system (EthoVision XT, Noldus, USA). After the last training trial, rats were subjected to a probe trial in which the platform was removed. Animals were placed in the pool at the same pole and allowed to swim for 2 min. The time that each animal spent in the quadrant that had previously contained the hidden platform was recorded.

Measurement of A β 40 and A β 42 in the hippocampus and post-hypothalamus

We measured levels of soluble A β 40 and A β 42 in the hippocampus and post-hypothalamus using Ultrasensitive Rat A β 40 and A β 42 ELISA kits (Arigo Biolaboratories Corp, Taiman), in accordance with manufacturer instructions. Expression levels were detected using a Biochrom Anthos Zenyth 200rt Microplate Reader (Cambridge, UK).

Immunoblotting analysis

Total protein was prepared by homogenising hippocampal and post-hypothalamic

tissues in lysis buffer containing a protease inhibitor cocktail and a phosphatase inhibitor cocktail. The sample was subsequently incubated for 1 h at 4°C. The protein extracts (20 µg/sample based on the BCA protein assay, Pierce Chemical Co., Rockford, IL, USA) were resolved on a 6% polyacrylamide gel and transferred to a PVDF membrane (GE Healthcare, Buckinghamshire, UK). The membranes were incubated in the appropriate anti-P-Tau^{T231} (ab151559), anti-P-Akt^{S473} (4060, Cell Signaling Technology, Beverly, MA), anti-P-GSK-3βS9 (05-643, EMD Millipore, Billerica, MA), anti-P-GSK-3β^{Y216} (ab75745), anti-amyloid precursor protein (ab12266), anti-gp91-phox (sc5827, Santa Cruz Biotechnology, Dallas, TX), anti-Tau (ab80579), anti-Akt (9272), anti-p67-phox (sc7663), anti-GSK-3β (07-389), anti-p47-phox (sc14015), anti-p22-phox (sc11712), or anti-caspase 3 (9662) antibodies. The membranes were then incubated in an HRP-labelled goat anti-rabbit secondary antibody at 1:10,000. The membranes were developed using an ECL-Plus detection kit (GE Healthcare).

Immunohistochemistry analysis

For immunohistochemistry experiments, initial specimen processing and staining were performed as described earlier [22]. Sections were first stained with avidin-biotin peroxidase (goat anti-rabbit biotin), followed by double-indirect staining

with horse anti-mouse AP. Briefly, sections were incubated with both primary antibodies (Anti-P-TauT231, anti-P-TauT181, or anti-BDNF) overnight at 4°C. Slides were then washed three times with PBS, following which they were incubated for 1 h at room temperature (RT) with a combination of biotinylated HRP-conjugated goat anti-rabbit (1:200) secondary antibodies. After three washes with PBS, the sections were incubated with ABC complex (1:50) for 30 min at RT. After three washes with PBS, sections were visualised using the DAB substrate kit (Vector Laboratories, Burlingame, CA, USA) and counterstained with haematoxylin. After rinsing in PBS, the sections were dehydrated using a graded series of ethanol and xylene. The sections were then photographed using an Olympus microscope equipped with a Nikon Cool Scan 995 digital camera (Nikon, Tokyo, Japan).

Statistical analyses

All data are expressed as the mean \pm standard error of the mean (SEM). Paired *t*-tests were used to compare baseline and post-treatment BP measurements, while a one-way analysis of variance (ANOVA) with Scheffe's *post hoc* testing was employed to evaluated differences among the groups. The level of statistical significance was set at $P < 0.05$.

Results

BDNF levels and superoxide imbalances contributed to hypertension and early AD

Previous studies have suggested that BDNF-deficient mice are more susceptible to stress-induced oxidative damage, as indicated by the direct association between indicators of oxidative stress and BDNF levels in the brain [23]. Oxidative stress is an important pathogenic factor in the development of hypertension. Angiotensin receptors (AT1R) play a pivotal role in the development and maintenance of hypertension during oxidative stress [24]. Accumulating evidence indicates that BDNF and its interaction with ROS may be crucial for several symptoms of neurodegenerative and neuropsychiatric conditions. Thus, we aimed to determine whether ROS levels were significantly increased in the NTS of mice with Ang-II-induced AD, and whether such increases led to down-regulation of BDNF release in the hippocampus. In addition to increased SBP, rats with Ang-II-induced AD exhibited significantly higher levels of superoxide in the NTS and hippocampal areas CA1, CA3, and DG than those in the control group. Furthermore, treatment with losartan or resveratrol reversed these effects (Fig. 1A, B and Fig 2 A, B). Interestingly, treatment with losartan or resveratrol markedly increased BDNF levels in the hippocampus and NTS of rats with Ang-II-induced AD (Fig. 1C and Supplementary

Fig. 1).

To investigate whether resveratrol limits ROS production via inhibition of NADPH oxidases during Ang-II-induced early AD, we examined the expression of NADPH oxidase subunits and SOD when both Ang-II and resveratrol were administered. Our results indicated that resveratrol abolished increases in the expression of NOX2, the NADPH oxidase subunit p67-phox, and reduced levels of SOD2 in the hippocampus (Fig. 2C and D). These results indicate that the elimination of ROS may be required to increase BDNF levels and activate the depressor response. Taken together, these findings suggest that resveratrol mitigates oxidative stress, normalises BDNF levels, and reduces BP in Ang-II-induced early AD.

Resveratrol impaired the activity of A β precursors, active caspase 3, and GSK-3-Tau by normalising renal AT1R signalling in the hippocampus of rats with Ang-II-induced early AD

Accumulating evidence has indicated that patients with AD exhibit significantly higher levels of anti-AT1R and tau than healthy controls [25]. However, the angiotensin system increases the permeability of the BBB, induces oxidative stress in the microcirculation of the brain, and leads to an increase in A β and tau pathologies [26]. Therefore, we investigated whether resveratrol affects AT1R signalling in the

hippocampus of rats with Ang-II-induced early AD. ICH experiments demonstrated that treatment with losartan or resveratrol influenced the expression of phosphorylated Tau^{T231} in hippocampal areas CA1, CA3, and DG in rats with Ang-II-induced AD (Fig. 3A and B). Immunoblot analyses of proteins extracted from the hippocampus demonstrated that treatment with losartan or resveratrol decreased the expression of AT1R, A β precursors, and active caspase 3 in Ang-II-induced groups. Similarly, GSK-3 β ^{Y216} expression and Tau^{T231} phosphorylation in the hippocampus were significantly attenuated by treatment with losartan or resveratrol. The addition of losartan or resveratrol increased Akt activation, as well as hyperphosphorylation of critical Akt substrates, in Ang-II-induced groups (Fig. 3C and D). Resveratrol treatment resulted in favourable shifts in Ang-II-induced expression of AT1R, A β precursors, active caspase 3, GSK-3, and tau activation in the hippocampus. These results suggest that resveratrol attenuated Ang-II-induced tau pathologies, thereby improving Akt activation and attenuating down-regulation of the AT1R–A β –caspase 3–GSK-3 β signalling pathway.

Resveratrol treatment improved spatial learning and memory in rats with Ang-II-induced early AD

Fibrillar A β is the major constituent of senile plaques in the brains of patients with

AD. Some patients with early-onset familial AD exhibit elevated levels of A β 42/A β 40 [27]. To investigate whether resveratrol limits AT1R signalling to contribute to improvements in spatial learning and memory during Ang-II-induced early AD, we examined the expression of A β 42/A β 40 and MWM results in rats treated with losartan or resveratrol. Rats with Ang-II-induced AD exhibited significantly higher levels of A β 42/A β 40 in the hippocampus than controls (Fig. 4A, histograms 1 and 2). Interestingly, treatment with losartan or resveratrol markedly inhibited A β 42/A β 40 expression in the hippocampus of rats with Ang-II-induced AD (Fig. 4A, histograms 3 and 4). While all rats exhibited progressive decreases in escape latency during the MWM task (Figure 4B), Ang-II-treated rats took significantly longer to reach the platform than controls ($P < 0.01$), suggestive of notable cognitive damage. Furthermore, this increase in escape latency was significantly ameliorated by losartan and resveratrol treatment (day 5, day 6, and 7, $P < 0.05$ vs. Ang-II groups). While rats with Ang-II-induced AD spent significantly less time in the central area than control groups, rats treated with losartan spent significantly more time in this area than other rats with Ang-II-induced AD. No significant differences were observed between losartan and resveratrol treatment in the Ang-II groups (Fig. 4C). The rats exhibited similar motor capabilities, as swim speed did not differ between the groups (Fig. 4D), suggesting that differences in latency were not due to differential swim speed. These

results suggest that resveratrol and losartan attenuated Ang-II-induced impairments in hippocampal-dependent and contextual memory.

Discussion

In the present study, we aimed to determine whether treatment with resveratrol improves ROS generation and cognitive function in rats with Ang-II-induced early AD. Our results revealed that treatment with resveratrol for 2 weeks decreased BP, increased levels of BDNF in the hippocampus, and decreased ROS production in the NTS in the Ang-II groups. Overall, our results suggest that resveratrol exerts neuroprotective effects against memory impairment and hippocampal damage in a rat model of early stage AD by reducing oxidative stress.

Alzheimer's Disease International (ADI) has determined that, in the next 30 years, the number of patients with AD will more than quadruple in India, China, other countries in Asia, Australasia, and Oceania, from approximately 16 million in 2010 to approximately 61 million by 2050. The total worldwide costs of dementia in 2010 were estimated to be \$604 billion (USD) (70% of which occur in Europe and North America), representing approximately 1% of the global gross domestic product [28].

A previous population-based study revealed that hippocampal atrophy (HA) is usually attributed to the neurofibrillary tangles and neuritic plaques associated with

AD [29]. Several studies have indicated that the clinical end points of AD are strongest in those who have never been treated for hypertension. Additional studies have demonstrated that treatment with antihypertensive medication reduces the risk associated with high BP [30, 31]. In the present study, we observed that central BP is regulated by ROS levels in the NTS, which are thought to contribute to down-regulation of BDNF expression in both the NTS and hippocampus of rats with Ang-II-induced AD. In addition, our results suggest that resveratrol not only attenuated increases in superoxide levels in the NTS and increased BDNF expression, but also increased the antioxidant capacity of the NTS in rats with hypertension. Our findings further demonstrated that Ang-II increased the generation of superoxide and the activity of the A β -caspase 3-Akt-GSK-3 β -Tau pathway by positively regulating NADPH oxidase levels. Such changes were also accompanied by decreases in SOD2 and BDNF expression in the hippocampus. However, treatment with resveratrol improved cognitive function in rats with Ang-II-induced early AD by abolishing ROS generation and reducing activity of the A β -caspase 3-Akt-GSK-3 β -Tau pathway activity by negatively regulating NADPH oxidase and NOX2 levels. Therefore, our findings suggest that early treatment with resveratrol lowers oxidative stress, preserves SOD function, and attenuates the development of hypertension (Fig. 5).

Studies involving patients with hypertension have revealed that high BP is

associated with atrophy of the hippocampus and temporal lobe, as well as an increased risk of cognitive decline, suggesting that such patients are at increased risk of developing AD [29]. Indeed, neurofibrillary tangles, senile plaques, and neuronal lesions have been observed in patients with hypertension [32]. While such findings suggest a relationship among hypertension, cerebrovascular disease, and decreased cognitive function [9], it remains to be determined whether hippocampal changes are the consequences of pre-existing hypertension, or whether hypertension and brain pathology reflect a central defect in patients with AD [10].

Some studies have suggested that SHR and rats with DOCA-salt-induced hypertension exhibit low BDNF expression and deficient neurogenesis in the hippocampus. However, treatment with oestrogens may normalise brain parameters (i.e., BDNF levels) by decreasing peripheral BP in both rat groups [33, 34].

In the present study, treatment with losartan or resveratrol not only normalised BP and superoxide levels in the NTS, but also markedly increased BDNF levels in the hippocampus and NTS of rats with Ang-II-induced AD (Fig. 1D and E and Supplementary Fig. 1). These results indicate that the elimination of ROS may be required to increase BDNF levels and activate the depressor response. To our knowledge, our study provides the most conclusive evidence that resveratrol improves cognitive function by attenuating AT1R-induced ROS generation, thereby decreasing

A β and tau pathologies in rats with Ang-II-induced early AD. ROS in the brain are thought to contribute to the neuropathogenesis of hypertension by enhancing sympathetic nervous system activity. The key mechanism for reduced nitric oxide (NO) bioavailability is oxidative stress [35, 36]. Oxidative stress can be defined as increased bioactivity of ROS relative to antioxidant defences [37]. In accordance with our present findings, our previous studies demonstrated that resveratrol decreases BP better than rosuvastatin, abolishes ROS generation, and enhances activity of the ERK1/2-RSK-nNOS pathway by activating AMPK to negatively regulate Rac1-induced NADPH oxidase levels in the NTS during oxidative stress-associated hypertension[38].

While current pharmacological approaches simply provide symptomatic improvement rather than prevent or delay cognitive decline, many commonplace medications are being re-evaluated for their potential benefits among patients with AD. Treatment of vascular risk factors has been associated with a reduced incidence of AD and slower cognitive decline in patients with AD. Angiotensin converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs) are widely prescribed as antihypertensive drugs, acting on the renin–angiotensin system (RAS). Some research indicates that they may be superior to other antihypertensive drugs because the RAS is thought to participate in the neuropathogenesis of AD via both

vascular and amyloid pathways [26]. Consequently, several high-quality longitudinal studies have explored the precise influence of hypertensive drugs targeting the RAS among patients with AD. However, our results suggested that resveratrol exerts effects similar to those of the Ang-II receptor antagonist losartan by attenuating Ang-II-induced tau pathologies, improving Akt activation, and attenuating down-regulation of the AT1R–A β –caspase 3–GSK-3 β signalling pathway (Fig. 3). Resveratrol targets the CNS, can cross the BBB, and induces neuroprotective effects [39]. Notably, Vingtdeux et al. observed that resveratrol is present in the brain following oral administration, indicating that it may exert direct effects in patients with neurological disorders [40].

Our results demonstrated that treatment with resveratrol for 2 weeks decreased BP, attenuated ROS production in the NTS, and increased BDNF levels in the hippocampus of rats with Ang-II-induced early AD. In addition, inhibition of Tau^{T231} phosphorylation in the hippocampus using resveratrol significantly abolished Ang-II-induced expression of A β precursors, active caspase 3, and glycogen synthase kinase 3 β (GSK-3 β)^{Y216} while increasing Akt^{S473} phosphorylation. Interestingly, resveratrol reversed impairments in hippocampal-dependent and contextual memory induced by deletion of NADPH oxidase and NOX2.

Overall, our results suggest that resveratrol exerts neuroprotective effects against

memory impairment and hippocampal damage in a rat model of early stage AD by reducing oxidative stress. These novel findings indicate that resveratrol may represent a pharmacological option for patients with hypertension at risk for the development of AD during old age. Furthermore, our findings may aid in the identification of molecular targets for recovering memory pathways, potentially leading to the development of new therapeutic strategies.

Author contributions

The study was conceived and designed by Pei-Wen Cheng, Yu-Te Lin, Yi-Chung Wu, Ching-Jiunn Tseng. Pei-Wen Cheng conducted most of the experiments with assistance from Hsin-Hung Chen, Chia-Jung Li, and Chi-Cheng Lai. The paper was written by Pei-Wen Cheng, with contributions from Gwo-Ching Sun, Tung-Chen Yeh, and Ching-Huang Lin.

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Figure legends

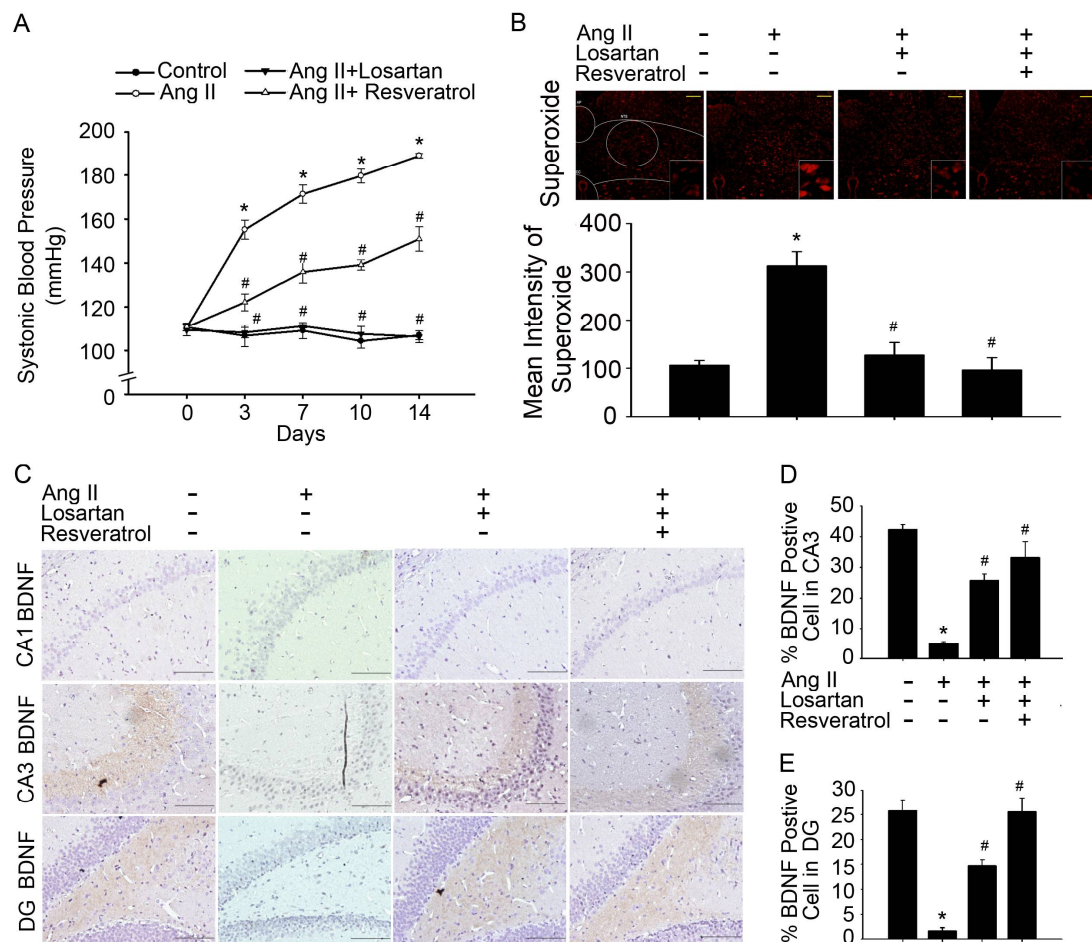


Fig. 1. Downregulation of BDNF levels was associated with increased superoxide expression in rats with Ang-II-induced early Alzheimer's disease (AD). (A) Time course of systolic blood pressure (SBP) after intracerebroventricular administration of angiotensin II (Ang-II) for 2 weeks. The filled circles (●) represent the WKY group, the open circles (○) represent the Ang-II group, the inverted filled triangles (▼) represent the Ang-II + losartan group, and the open triangles (△) represent the Ang-II + resveratrol group. SBP was measured on days 0, 4, 7, 11, and 14. The data are presented as the mean \pm SEM; $n = 6$. * $P < 0.05$ vs. the WKY and # $P < 0.05$ vs. the

Ang-II group. **(B)** Confocal microscopy analysis of DHE-treated brain sections in the nucleus tractus solitarius (NTS) after treatment with losartan or resveratrol. Bar graph showing the superoxide production ratio after treatment with Ang-II and/or losartan or resveratrol. Note the significant decrease in Ang-II-induced superoxide production after the administration of losartan or resveratrol. **(C)** *In situ* qualitative analysis of BDNF-immunopositive cells in the hippocampus of AD model rats. Scale bar, 200 μ m. **(D-E)** Bar graph showing BDNF-expressing cells after treatment with Ang-II and/or losartan or resveratrol. Note the significant increase in Ang-II-induced BDNF production after the administration of losartan or resveratrol. The percentage of BDNF-positive cells was determined by counting the BDNF-expressing cells in each hemisphere of the hippocampus (CA1 and CA3) at $\times 200$ magnification. These values were divided by the total number of cells in the same paraffin section. BDNF: brain-derived neurotrophic factor.

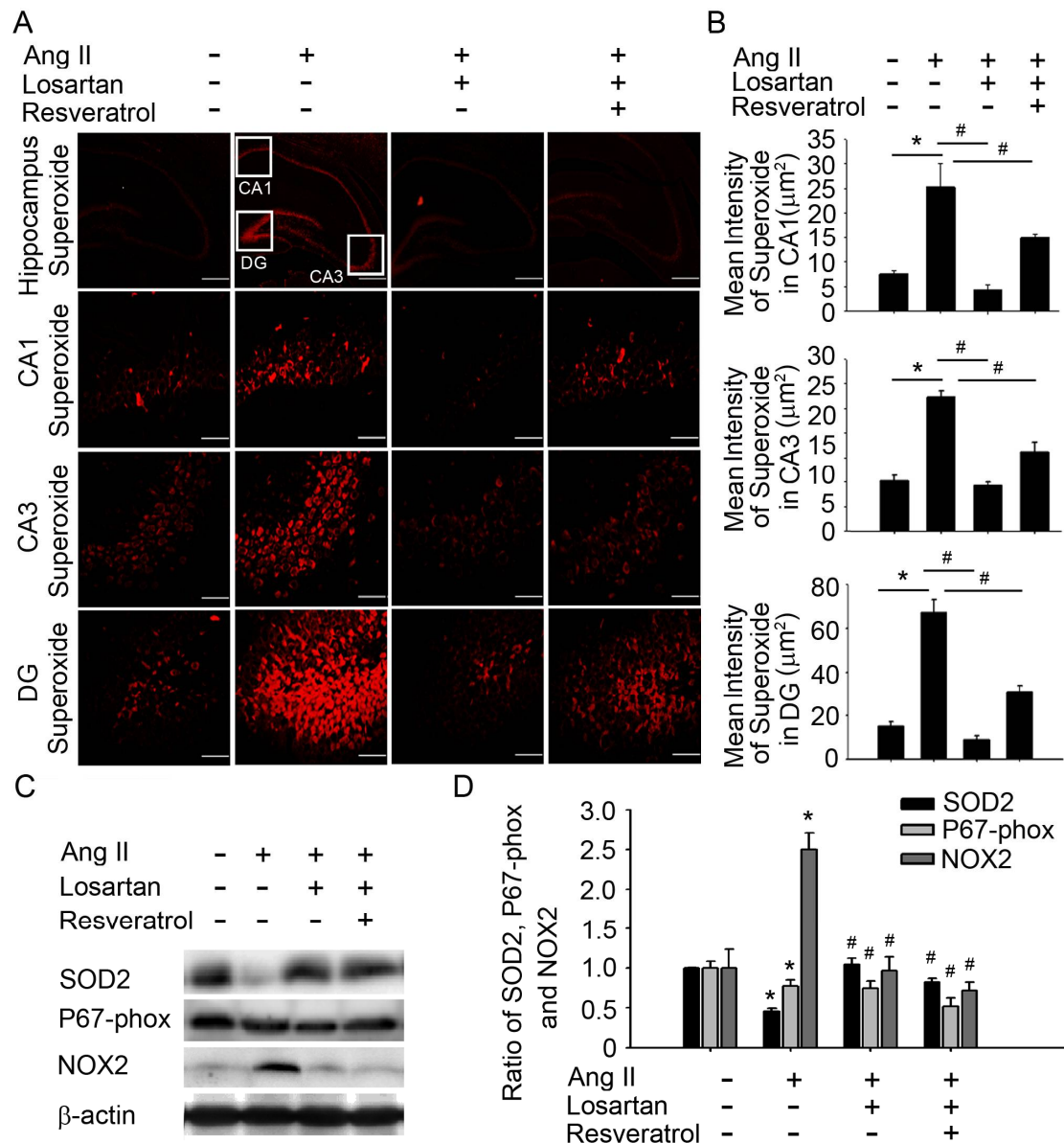


Fig. 2. Resveratrol abolished Ang-II-induced increases in superoxide and NADPH oxidase activity and reduced SOD2 activity in the hippocampus of AD model rats. (A) Confocal microscopy analysis of DHE-treated brain sections in the hippocampus after treatment with losartan or resveratrol. **(B)** Bar graph showing the superoxide production ratio after treatment with Ang-II and/or losartan or resveratrol. Note the significant decrease in Ang-II-induced superoxide production after the administration of losartan or resveratrol. **(C-D)** Quantitative immunoblot analysis

demonstrating decreased expression of the NADPH oxidase subunit p67-phox and decreases in the NOX2 ratio in the hippocampus of Ang-II-treated rats following treatment with losartan or resveratrol. The level of SOD2 protein in the hippocampus was significantly increased following treatment with losartan or resveratrol. (The values are presented as the mean \pm SEM; n = 6. *P < 0.05 vs. the WKY group. #P < 0.05 vs. the Ang-II group. Ang-II: angiotensin II; AD: Alzheimer's disease.

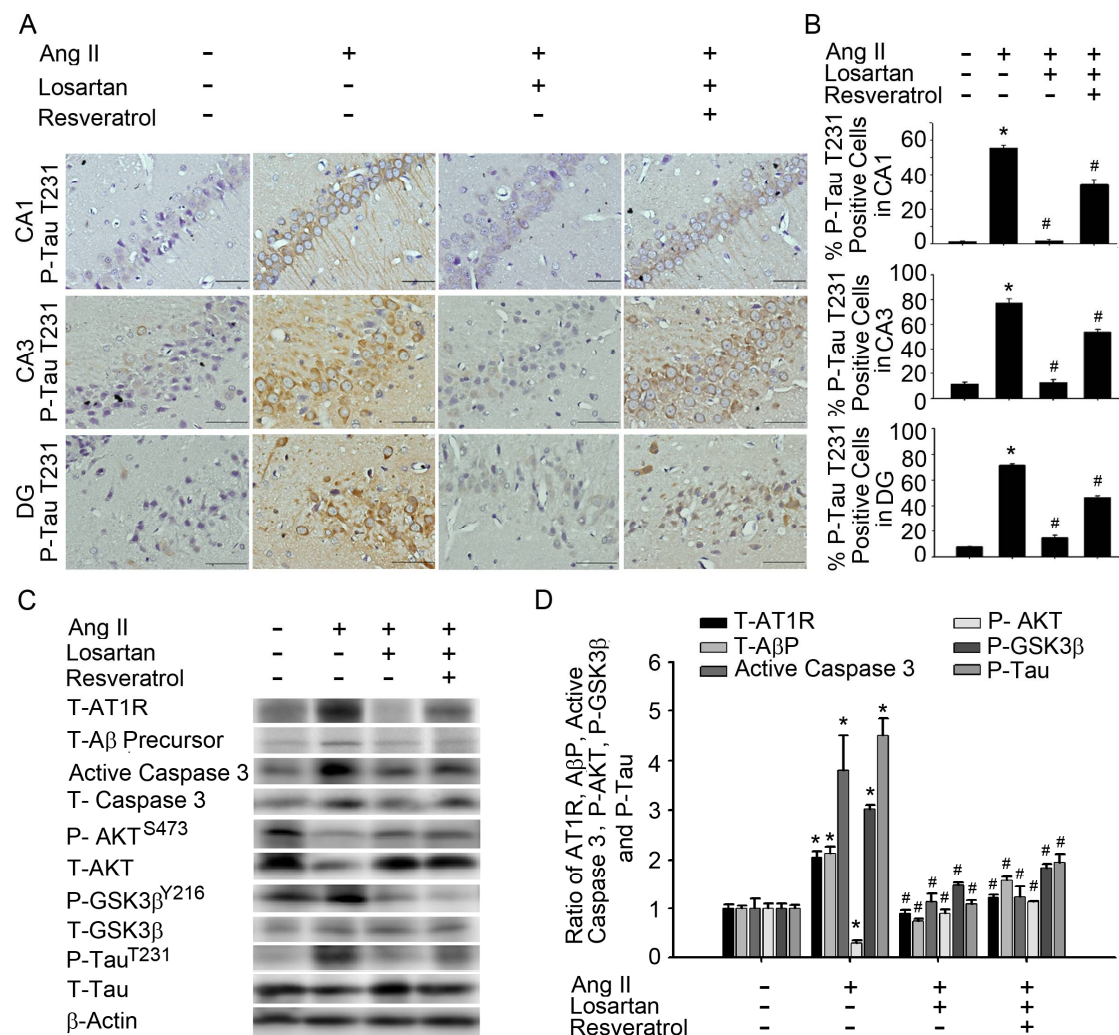


Fig. 3. Resveratrol attenuated Ang-II-induced A β precursor and caspase 3-Akt-GSK-3 β -Tau pathways in the hippocampus of AD model rats. (A) *In situ* qualitative analysis of P-Tau^{T231}-immunopositive cells in the hippocampus of AD model rats. Scale bar, 200 mm. (B) Bar graph showing P-Tau^{T231}-expressing cells after treatment with Ang-II and/or losartan or resveratrol. Note the significant decrease in Ang-II-induced P-Tau^{T231} production after the administration of losartan or resveratrol. The percentage of P-Tau^{T231}-positive cells was determined by counting

the P-Tau^{T231}-expressing cells in each hemisphere of the hippocampus (CA1, CA3, and DG) at $\times 200$ magnification. These values were divided by the total number of cells in the same paraffin section. **(C)** Immunoblot demonstrating decreased levels of the proteins T-AT1R, T-A β precursor, T-active-caspase 3, P-GSK-3 β ^{Y216}, and P-Tau^{T231} in the hippocampus after treatment with Ang-II and/or losartan or resveratrol. **(D)** Quantitative immunoblot analysis demonstrating reductions in T-AT1R, T-A β precursor, T-active-caspase 3, P-GSK-3 β ^{Y216}, and P-Tau^{T231} expression in the hippocampus of rats with Ang-II-induced AD following treatment with losartan or resveratrol. The values are presented as the mean \pm SEM; n = 6. *P < 0.05 vs. the WKY group. #P < 0.05 vs. the Ang-II group. AD: Alzheimer's disease; Ang-II: angiotensin II.

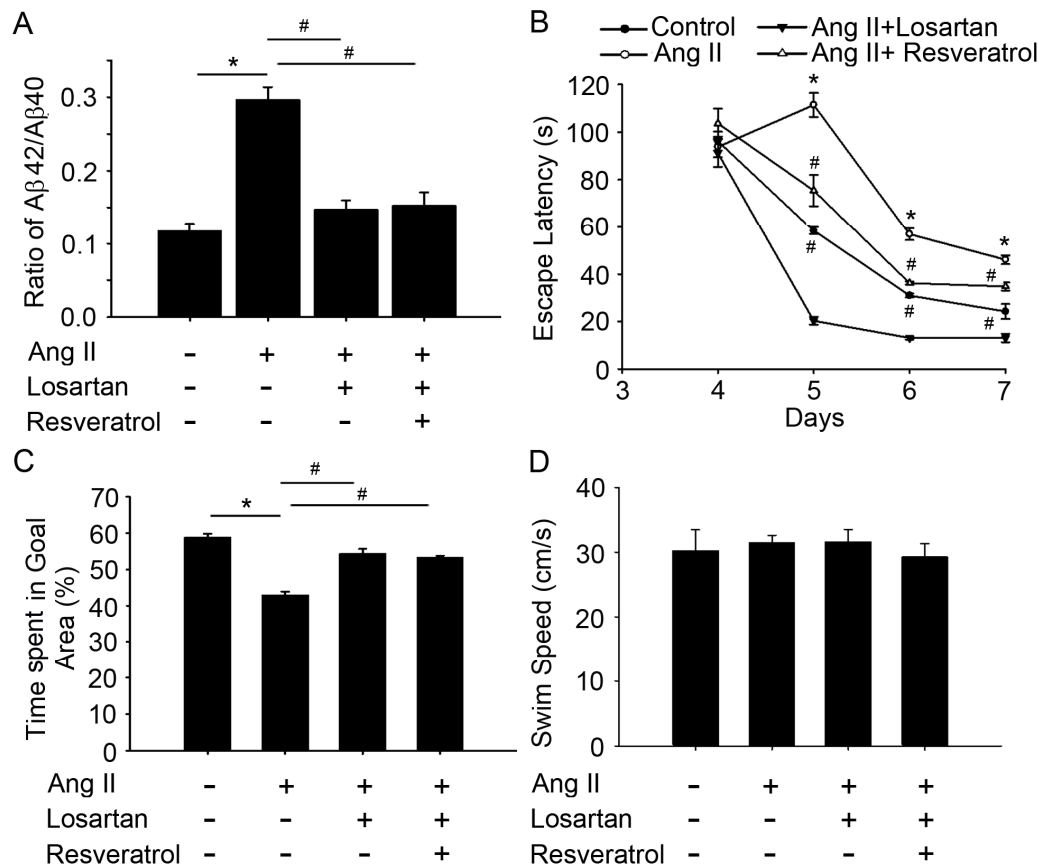


Fig. 4. Resveratrol reversed impairments in hippocampal-dependent and contextual memory in rats with Ang-II-induced early Alzheimer's disease (AD).

(A) Bar graph showing the A β 42 production ratio after treatment with Ang-II and/or losartan or resveratrol. Note the significant decrease in Ang-II-induced A β 42 production after the administration of losartan or resveratrol. (B) Bar graph showing the latency to find the hidden platform. (C) Bar graph showing the time spent in the central area. (D) Bar graph showing the swim speed. Note that learning and memory deficits were also reversed in Ang-II-treated rats after losartan or resveratrol administration. The values are presented as the mean \pm SEM; $n=6$. * $P<0.05$ vs. the WKY rats and # $P<0.05$ vs. the Ang-II-treated rats. Ang-II: angiotensin II.

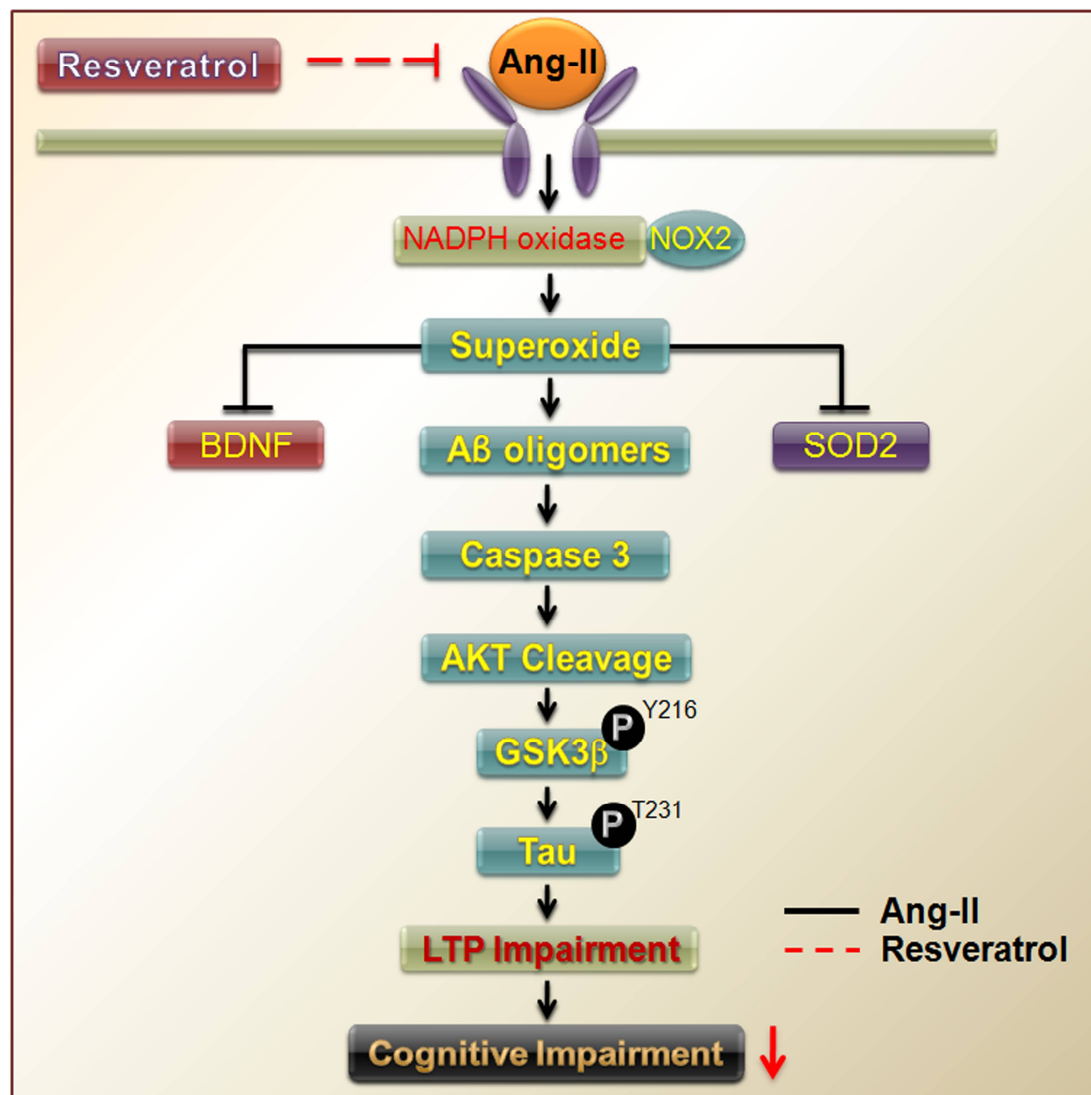


Fig. 5. Resveratrol attenuated ROS-induced cognitive impairments in rats with Ang-II-induced early Alzheimer's disease (AD).

Ang-II not only increased the generation of superoxide and the activity of the Aβ–caspase 3–Akt–GSK-3β–Tau pathway by positively regulating NADPH oxidase levels, but also attenuated SOD2 and BDNF expression in the hippocampus (black line). However, treatment with resveratrol improved cognitive impairments in rats with Ang-II-induced early AD by abolishing ROS generation and reducing activity of the Aβ–caspase 3–Akt–GSK-3β–Tau pathway by negatively regulating NADPH

oxidase and NOX2 levels (red line). ROS: reactive oxygen species; BDNF: brain-derived neurotrophic factor.