

Research Paper

Treadmill exercise decreases amyloid- β burden possibly via activation of SIRT-1 signaling in a mouse model of Alzheimer's diseaseJung-Hoon Koo^a, Eun-Bum Kang^a, Yoo-Sung Oh^b, Dae-Seung Yang^{c,*}, Joon-Yong Cho^{a,**}^a Department of Exercise Biochemistry, Korea National Sport University, Seoul 138-763, Republic of Korea^b Department of Exercise Prescription, University of Seoul, Seoul 163, Republic of Korea^c Department of Taekwondo Studies, University of Gachon, Gyeonggi-do, Republic of Korea

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ABSTRACT

Accumulation of amyloid- β (A β) correlates significantly with progressive cognitive deficits, a main symptom of Alzheimer's disease (AD). Although treadmill exercise reduces A β levels, the molecular mechanisms underlying the effects are not fully understood. We hypothesize that treadmill exercise decreases A β production and alleviates cognitive deficits by activating the non-amyloidogenic pathway via SIRT-1 signaling. Treadmill exercise improved cognitive deficits and alleviated neurotoxicity. Most importantly, treadmill exercise increased SIRT-1 level, which subsequently resulted in increased ADAM-10 level by down-regulation of ROCK-1 and upregulation of RAR β , ultimately facilitating the non-amyloidogenic pathway. Treadmill exercise-induced activation in SIRT-1 level also elevated PGC-1 α level and reduced BACE-1 and C-99 level, resulting in inhibition of the amyloidogenic pathway. Treadmill exercise may thus inhibit A β production via upregulation of SIRT-1, which biases amyloid precursor protein processing toward the non-amyloidogenic pathway. This study provides novel and valuable insight into the molecular mechanisms possibly by which treadmill exercise reduces A β production.

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1. Introduction

Alzheimer's disease (AD) is the most common form of dementia and progressive neurodegenerative disease, is characterized by extracellular amyloid- β (A β) and intracellular neurofibrillary tangles (Selkoe, 2001; Tanzi and Bertram, 2005). Although the pathogenic mechanisms of AD remain unclear, the A β cascade hypothesis is widely accepted to have an important role in the pathogenesis of AD; AD treatment has mainly focused on inhibiting A β production or increasing A β degradation.

Many authors have demonstrated that physical exercise reduces A β deposition, including treadmill exercise, voluntary wheel running, and environmental enrichment (Polito et al., 2014; Di Loreto et al., 2014; Maesako et al., 2012; Adlard et al., 2005; Yuede et al., 2009; Mainardi et al., 2014; Moore et al., 2016). Our previous studies also showed that treadmill exercise decreased A β accumulation, resulting in improved neuroprotection, inflammation, endoplasmic reticulum (ER) stress, insulin signaling, and cognitive function (Cho et al., 2003; Cho et al., 2010; Um et al., 2008; Um et al., 2011; Kang et al., 2013). This reduction of A β accumulation by physical exercise was associated with A β

degradation enzymes, synaptic plasticity, neurogenesis, and antioxidant factors (Moore et al., 2016; Um et al., 2011; Ke et al., 2011; Revilla et al., 2014), indicating that complex mechanisms may be involved in the degradation of A β levels by physical exercise. Simpler mechanisms of A β generation are regulated by sequential proteolysis of the amyloid precursor protein (APP) via the α , β , and γ secretase enzymes (Selkoe, 2001; Tanzi and Bertram, 2005). Generally, A β production, in the amyloidogenic pathway, occurs by sequential cleavage of APP via the β -site APP cleaving enzyme (BACE-1) and further cleavage by γ secretase (Cai et al., 2001). Inhibition of A β production, in the non-amyloidogenic pathway, results from the cleavage of APP by a disintegrin and metalloproteinase domain-containing protein 10 (ADAM-10) and γ secretase (Kuhn et al., 2010). Previous studies found that BACE-1 activity and ADAM-10 expression were increased and decreased in AD brains, respectively, resulting in greater A β production and neuronal cell death (Mao et al., 2012; Gong et al., 2013). These results suggest that regulation of APP processing by treadmill exercise, especially in the non-amyloidogenic pathway, may influence the amount of A β , implying a novel therapeutic strategy for inhibiting A β production. However, the precise molecular mechanisms underlying the effect of physical exercise on inhibition of A β production are not currently fully understood.

Sirtuin-1 (SIRT-1), a member of the sirtuin family, plays an essential role in regulating cellular homeostasis by influencing neuron survival, insulin sensitivity, glucose metabolism, and mitochondrial biogenesis

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(Lagouge et al., 2006; Milne et al., 2007; Guarente, 2013). SIRT-1 may regulate A β metabolism through modulation of APP processing in AD, and loss of SIRT-1 is closely associated with exacerbated A β production (Julien et al., 2009). The loss of SIRT-1 activity may reduce ADAM-10 expression and, as a result, increase A β production (Kumar et al., 2013; Luis et al., 2009; Haass and Selkoe, 2007). Conversely, resveratrol, a SIRT-1 activator, decreased BACE-1 activity and increased the resulting inhibition of A β production by promoting peroxisome proliferator-activated receptor- γ coactivator 1 α (PGC-1 α) activity (Wang et al., 2013). Similarly, SIRT-1 activation by caloric restriction may activate ADAM-10 via downregulation of Rho-associated kinase 1 (ROCK-1) and thus increase the non-amyloidogenic pathway in AD mice (Qin et al., 2006a; Qin et al., 2006b). Interestingly, ADAM-10 is positively regulated by retinoic acid receptor- β (RAR β), which is activated by SIRT-1 (Lichtenthaler, 2011; Tippmann et al., 2009; Lee et al., 2014). These results imply that activation of SIRT-1 may facilitate non-amyloidogenic processing of APP. However, the molecular mechanisms inhibiting A β production through SIRT-1 mediated-APP processing after treadmill exercise in AD are still unknown.

In this study, we hypothesize that treadmill exercise might decrease A β production, possibly by upregulating SIRT-1 signaling, which in turn increases the non-amyloidogenic pathway. Furthermore, we also examined whether treadmill exercise decreases BACE-1 and C-terminal fragment CTF 99 (C-99) levels by activating PGC-1 α expression via increased SIRT-1. These results provide a molecular mechanism and comprehensive understanding of treadmill exercise on inhibition of A β production in the NSE/APPsw-transgenic mouse model of AD.

2. Materials and methods

2.1. Transgenic animals

All animal experiments approved by the Institutional Animal Care and Use Committee at Korea National Sport University and were obtained from the center for laboratory animal resource in the Korea Food & Drug Administration (FDA). Male transgenic mice, NSE/APPsw, expressing human APP mutant under the control of neuron-specific enolase (NSE) were maintained in the genetic background of C57BL/6 \times DBA/2 mice. The mice were maintained under standard laboratory conditions (12:12 hour dark-light cycle, housed at 23 ± 1 °C with 50% relative humidity) with food and water (Purina Mills, Seoul, Korea) made available ad libitum. Mice were carried out in an accredited Korea FDA animal facility in accordance with the AAALAC international Animal Care Policies.

2.2. Treadmill exercise protocol

NSE/APPsw Tg mice and their control non-Tg mice at 12 months of age were divided into three groups: non-Tg sedentary mice (NTS, $n = 8$), Tg sedentary mice (TS, $n = 8$), and Tg-treadmill exercise mice (TE, $n = 8$). To perform the treadmill exercise, all mice were conducted at 10 m/min, 10 min/day for 5 days so that the mice became familiar with the treadmill exercise environment. After this period, treadmill exercise was performed (10 m/min, 30 min/day, 5 days/weeks) in the first 2 weeks and 50 min in the second 2 weeks. TE mice were then performed (12 m/min, 50 min/day, 5 days/weeks) in the third 3 weeks and last 5 weeks of exercise were performed (12 m/min, 60 min/day, 5 days/weeks), as described previously (Um et al., 2011).

2.3. Morris water maze test

The Morris water maze tests were performed as previously described (Um et al., 2011). Briefly, mice were tested in a circular pool filled with opaque water (diameter 1.0 m, height 0.4 m, $22 - 23$ °C). The mice learned to use visual cues in the room to navigate to an escape platform (diameter 10 cm) and located at a fixed position and hidden/submerged 1 cm below the surface of the water. The mice were released

into the pool from varying positions for a maximum of 60 s. If it did not find the platform within 60 s, experimenter gently guided the mice to the platform and let it sit on the platform for 20 s. On each of the five training days, we performed two trials with each animal and escape latency and, escape distance was recorded. The probe trial (60 s) was conducted on the morning of the sixth day with the platform removed from the pool and crossing time, dwelling time, and swimming pattern on the hidden platform zone previously placed was recorded. Escape latency, escape distance, and swimming pattern of all trial were recorded with a video camera connected to a computer equipped with the SMART-CS (Panlab, Barcelona, Spain) program.

2.4. Tissue preparation

For western blot experiments ($n = 5$ /group), the mice were euthanized by CO $_2$ asphyxiation, the brains were rapidly removed and the cortex was separated on ice after treadmill exercise. The samples were snap-frozen on dry ice and then stored at -80 °C. For immunohistochemistry experiments ($n = 3$ /group), the mice of each group were perfused transcardially with 50 mM phosphate buffered saline followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer at pH 7.4.

2.5. Isolation of brain mitochondria

Mitochondria fraction was isolated from brain cortex using a mitochondria extraction kit (IMGENIX, San Diego, USA) following the manufacturer's protocol. In brief, the cortex was rapidly minced and homogenized with 1 ml of homogenization buffer and centrifuged at 3000 rpm for 10 min. Transfer the supernatant to a clean and centrifuge at 12,000 rpm for 30 min, and then, transfer the supernatant (cytosolic fraction) into a micro-centrifuge tube and resuspend the pellet with 1 ml of suspension buffer and centrifuge at 12,000 rpm for 10 min. The obtain pellet were gently resuspend with 200 μ l of complete mitochondria lysis buffer and mixing end over end for 30 min. The final mitochondria fraction was obtained by centrifuged at 12,000 rpm for 5 min.

2.6. Isolation of brain nuclear

Nuclear fraction was prepared using a subcellular proteome extraction kit (Calbiochem, Darmstadt, Germany) following the manufacturer's instructions. In brief, the brain of each animal was homogenized with 1 ml of extraction buffer I and 5 μ l of protease inhibitor cocktail and centrifuged at $1000 \times g$ for 10 min. The obtained pellet were gently agitated with Extraction buffer II and 5 μ l of protease inhibitor cocktail for 30 min on the rotary shaker, and then, centrifuged at $6000 \times g$ for 10 min. The obtain pellet were gently agitated with 0.5 ml of Extraction buffer III, 5 μ l of protease inhibitor cocktail and 1.5 μ l of benzonase for 10 min on the rotary shaker. The final nuclear fraction was obtained by centrifuged at $7000 \times g$ for 10 min.

2.7. Immunohistochemistry

Immunohistochemical analyses were performed as previously described (Kang et al., 2013). The A β plaques in the sections were pre-treated with DAB (Invitrogen, CA, USA) for 40 min at room temperature (RT) using a blocking buffer containing 10% goat serum in phosphate buffer solution for 1 h, after which they were incubated at RT over night with primary unconjugated anti-A β (6E10; Covance, SIG-39320, USA) at 1:300 in blocking buffer. The A β in the sections were washed in washing buffer and incubated with secondary antibody, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Santa Cruz, sc-2005) at 1:200 for 2 h at RT. The peroxidase activity was visualized with diaminobenzidine (DAB) substrate kit (Vector Laboratory, Burlingame, CA, USA). The slides were observed under the microscope (Leica Microsystems, DM2500, Wetzlar, Germany).

2.8. Detection of apoptosis by TUNEL

TUNEL assay was used to detect in situ apoptotic cells with a commercial ApoTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA, USA). Briefly, the slides were incubated with sodium citrate for 15 min followed by several washings in distilled water. After quenching with 3% H₂O₂ and applying the equilibrium buffer for 20 min, the sections were incubated in TdT enzyme for 1 h at 37 °C and cleansed with Stop/Wash buffer for 10 min. The slides were rinsed in PBS (3 × 5 min), and then anti-digoxigenin peroxidase conjugate was applied on the tissues for 30 min at room temperature. After washing in PBS, the peroxidase activity was visualized with DAB substrate kit (Vector Laboratories, Burlingame, CA, USA), and nuclei were counter-stained with 0.5% methyl green for 6 min. The slides were examined using a fluorescent microscope (Leica Microsystems, DM 2500, Germany).

2.9. Histological scoring of the apoptosis

Apoptotic cells, TUNEL-positive cells in the cortex were identified when dark brown color is colocalized with nuclei. The number of apoptotic cells was counted using an image analyzer (Analysis Pro 3.2, Sis Co., Munster, Germany). The resultant TUNEL-positive cell numbers were considered as the total dead cell number in the cortex. Three mice per groups were assayed in triplicate by TUNEL assay.

2.10. Western blot analysis

The brain samples from the cortex of mice were homogenized in RIPA lysis buffer [1% triton X-100, 1% deoxycholate, 0.1% sodium dodecylsulphate (SDS)] and centrifuged at 13000 rpm at 4 °C for 30 min. Protein concentrations were measured by the protein assay kit (Bio-Rad, USA) and its protein (20 µg) were loaded by electrophoresis on a 7.5%–12% polyacrylamide gel for 90 min, after which they were transferred to a polyvinylidene fluoride membrane (Immuno-Blot, PVDF membrane, Bio-Rad, CA, USA) for 1 h at a constant voltage of 60–90 V and blocked with TTBS (TBS with 0.1% Tween-20) containing 5% skim milk for 90 min. Each membrane was then separately incubated overnight at 4 °C with specific antibodies: total APP and Aβ-40 (Genscript, dilution: 1:1000–1500); Aβ-42 (Kerafast, dilution: 1:1000); CTF β/α (Sigma, dilution: 1:1000); C-99, sAPPα, and sAPPβ (Covance, dilution: 1:1000); ADAM-10, ROCK-1, RARβ, PGC-1α, cytochrome c, cleaved caspase-3, SIRT-1, β-actin, GAPDH, Lamin B, and α-tubulin antibodies (Santa Cruz, dilution: 1:500–1000); BACE-1 antibody (Milipore, dilution: 1:800). The membranes were washed for 5 times for 8 min with washing buffer and incubated for 1 h with the secondary antibodies (HRP-conjugated goat anti-rabbit, HRP-conjugated goat anti-goat and HRP-conjugated goat anti-mouse from Invitrogen). The membrane blots were then developed using an ECL reagents (Santa Cruz Biotechnology, CA, USA). The density of the developed bands was determined using a ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA). To quantify changes in the all proteins level in the cortex, the expression of proteins in non-Tg sedentary mice (NTS) was normalized to 100% and all proteins level in other mice was present as a percentage of the NTS.

2.11. Graphs and statistical analysis

All graphs were performed in GraphPad Prism Software (GraphPad Prism Software, Inc., CA, USA) and the data were analyzed using SPSS (version 18.0, SPSS Inc., Chicago, IL, USA). All data were expressed as means ± SED. One-way analysis of variance was used to determine if there was a statistically significant interaction or main effect followed *Bonferroni post hoc tests* for multiple comparisons. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Treadmill exercise reduces Aβ levels in the NSE/APPSw-transgenic mice model of AD

To determine the effects of treadmill exercise, Aβ levels in the cortex of AD mice were analyzed by western blot and immunohistochemistry. Levels of Aβ-42 and Aβ-40 showed significant effects of group (Aβ-42; $F_{(2,12)} = 21.67, p < 0.001$; Aβ-40; $F_{(2,12)} = 19.17, p < 0.001$). The Aβ-42 and Aβ-40 levels in Tg-sedentary (TS) mice were significantly higher than in non-Tg-sedentary (NTS) mice, but this increase was significantly reduced in Tg-treadmill exercise (TE) mice after treadmill exercise (Fig. 1A–D). Furthermore, we analyzed Aβ immunoreactivity in the cortex at higher magnification (Fig. 1E). Immunostaining with an Aβ specific antibody (6E10) revealed widespread staining in the TS cortex, while TE mice showed markedly weaker immunoreactivity after treadmill exercise.

3.2. Treadmill exercise prevents Aβ-induced cell death in the NSE/APPSw-transgenic mice model of AD

Release of cytochrome c (cyto c) from the mitochondria to the cytosol is the initial step in an apoptotic pathway. Therefore, we analyzed Aβ-induced cell death proteins, including mitochondrial cyto c (m-cyto c) and cytosolic cyto c (c-cyto c), cleaved caspase-3, and m-cyto c:c-cyto c ratio (Fig. 2A). Levels of m-cyto c and c-cyto c and m-cyto c:c-cyto c ratio showed significant group effects (m-cyto c: $F_{(2,12)} = 70.00, p < 0.001$; c-cyto c: $F_{(2,12)} = 27.48, p < 0.001$; m-cyto c:c-cyto c: $F_{(2,12)} = 174.01, p < 0.001$). Significant decreases in m-cyto c levels and increases in c-cyto c levels were observed in TS mice compared with NTS mice, whereas treadmill exercise increased m-cyto c and decreased c-cyto c in TE mice (Fig. 2B, C). Moreover, the m-cyto c:c-cyto c ratio was downregulated in TS mice, and this was reversed after treadmill exercise (Fig. 2D). Similarly, cleaved caspase-3 showed significant effects of group ($F_{(2,12)} = 26.45, p < 0.001$). The level of cleaved caspase-3 was significantly increased in TS mice compared with NTS mice, but this increase was significantly reduced after treadmill exercise (Fig. 2E). Moreover, we evaluated correlations between Aβ-42 and cyto c levels in the mitochondria and cytosol. The m-cyto c level was negatively correlated with Aβ-42 ($r = -0.918, p < 0.0001$) (Fig. 2F), whereas the c-cyto c level was positively correlated with Aβ-42 ($r = 0.782, p < 0.0006$) (Fig. 2G). Finally, we examined apoptotic cell death using TUNEL assays. TUNEL-positive cells in the cortex were stained brown, and there was a significant effect of group ($F_{(2,6)} = 13.86, p < 0.01$). The TUNEL-positive cells in TS mice were significantly increased compared to NTS mice, whereas treadmill exercise significantly decreased the number of TUNEL-positive cells in TE mice (Fig. 2H, I). Collectively, these findings indicate that treadmill exercise repressed apoptotic cell death.

3.3. Treadmill exercise improves spatial learning and memory function in the NSE/APPSw-transgenic mice model of AD

To evaluate the effects of treadmill exercise on Aβ-induced behavioral deficits, we investigated spatial learning and memory using the Morris water maze test. Escape latencies revealed a significant effect of group on the five training days (D1: $F_{(2,21)} = 1.25, p < 0.31$; D2: $F_{(2,21)} = 5.33, p < 0.013$; D3: $F_{(2,21)} = 9.92, p < 0.001$; D4: $F_{(2,21)} = 14.80, p < 0.001$; and D5: $F_{(2,21)} = 22.78, p < 0.001$), and the times were much longer in TS mice than in NTS mice. However, on training days 2–5, TE mice showed shorter escape latencies than TS mice after treadmill exercise (Fig. 3A). Furthermore, escape distances showed a significant effect of group on five training days (D1: $F_{(2,21)} = 0.84, p < 0.45$; D2: $F_{(2,21)} = 2.06, p < 0.15$; D3: $F_{(2,21)} = 3.48, p < 0.05$; D4: $F_{(2,21)} = 11.98, p < 0.001$; D5: $F_{(2,21)} = 17.46, p < 0.001$) and were significantly

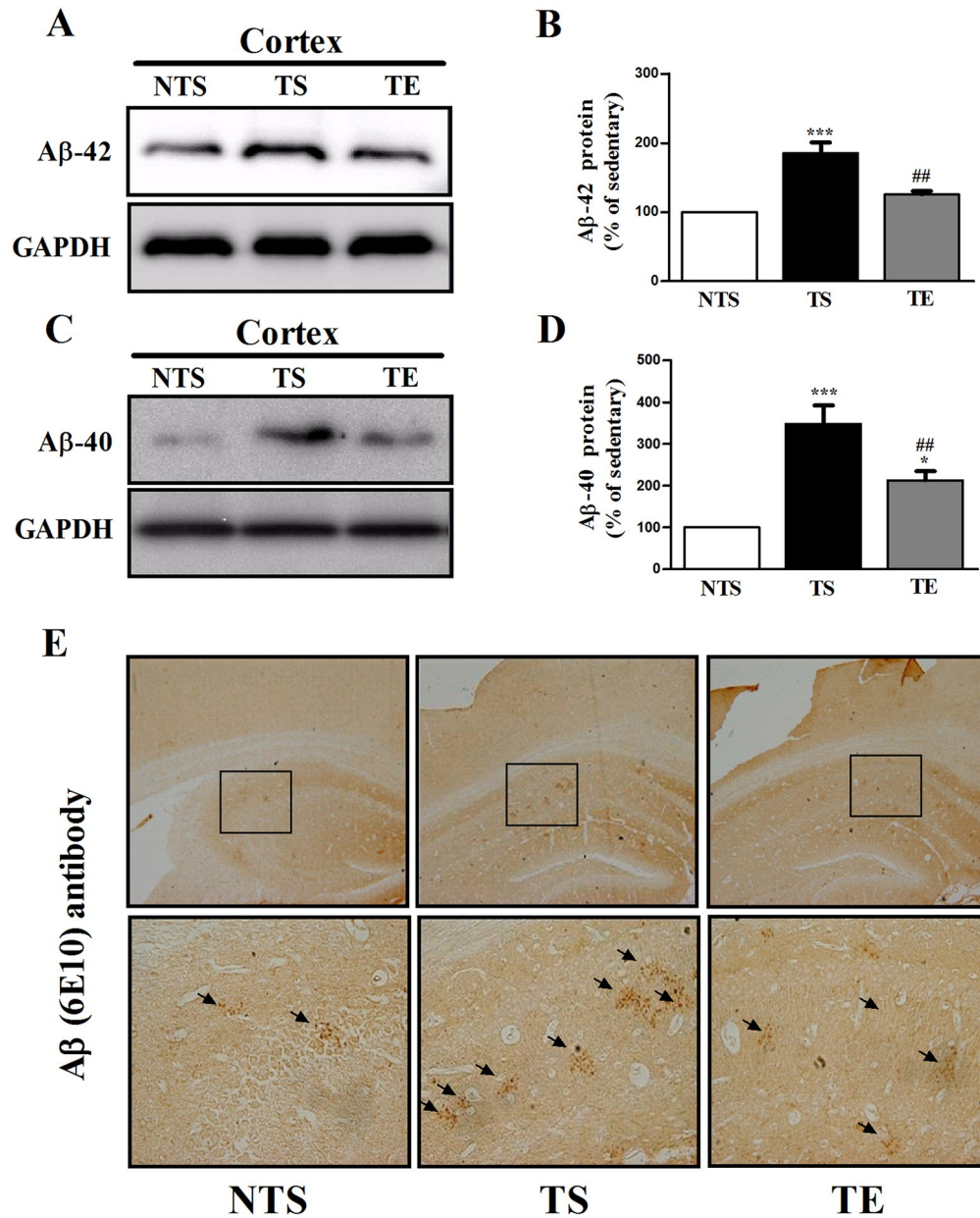


Fig. 1. Treadmill exercise reduces Aβ-42 and Aβ-40 expression in the cortex of NSE/APPsw transgenic mouse model of AD. A) Representative blot of western bands for Aβ-42 levels. B) Densitometry quantification of the Aβ-42 levels. C) Representative blot of western bands for Aβ-40 levels. D) Densitometry quantification of the Aβ-40 levels. E) Immunohistochemical analysis Aβ-42 and representative data of cortex sections (6 μm-thick) from each group are shown (n = 3/group). The resulting tissues were viewed using a microscope: Magnification × 100 (upper panel) × 400 (lower panel). Values are expressed to 100% for levels of NTS group. GAPDH was probed as housekeeping gene. All bars shown represent the means ± SEM (n = 5/group). A Bonferroni post-doc test: *p < 0.05, ***p < 0.001, compared to the NTS mice; ##p < 0.01, compared to the TS mice. Non Tg-sedentary (NTS), Tg-sedentary (TS), and Tg-treadmill exercise (TE).

increased in TS mice compared to NTS mice. After treadmill exercise, TE mice showed decreased escape distances on training days 3–5, compared to TS mice (Fig. 3B). In the probe trial test, we recorded the number of platform-crosses and the dwelling time in the target quadrant to evaluate long-term spatial learning and memory retention. The number of crossings and ratio of dwelling time data showed significant differences with respect to group (number of crossings: $F_{(2,21)} = 37.00$, $p < 0.001$; dwelling time: $F_{(2,21)} = 14.35$, $p < 0.001$). TS mice made significantly fewer crossings over the platform compared with NTS mice, while crossing times significantly increased after treadmill exercise (Fig. 3C). In addition, compared to NTS mice, TS mice had a shorter dwelling time in the target quadrant. However, TE mice spent significantly more time in the target quadrant after exercise (Fig. 3D). The three groups had similar swimming patterns, indicating that the observed differences in spatial learning

and memory retention were a result of cognitive dysfunction rather than swimming ability (Fig. 3E).

3.4. Treadmill exercise increases SIRT-1 expression in the NSE/APPsw-transgenic mice model of AD

Since SIRT-1 may regulate APP processing in AD, and the loss of SIRT-1 is closely associated with activated Aβ production (Julien et al., 2009), we investigated the effects of treadmill exercise on SIRT-1 activity. Levels of SIRT-1 showed significant effects of group ($F_{(2,12)} = 15.58$, $p < 0.001$). SIRT-1 activity was significantly decreased in TS mice compared to NTS mice, and it significantly increased after treadmill exercise (Fig. 4A, B). Moreover, to confirm the effect of treadmill activity on SIRT-1 activity and Aβ-42 levels, we performed a correlation analysis

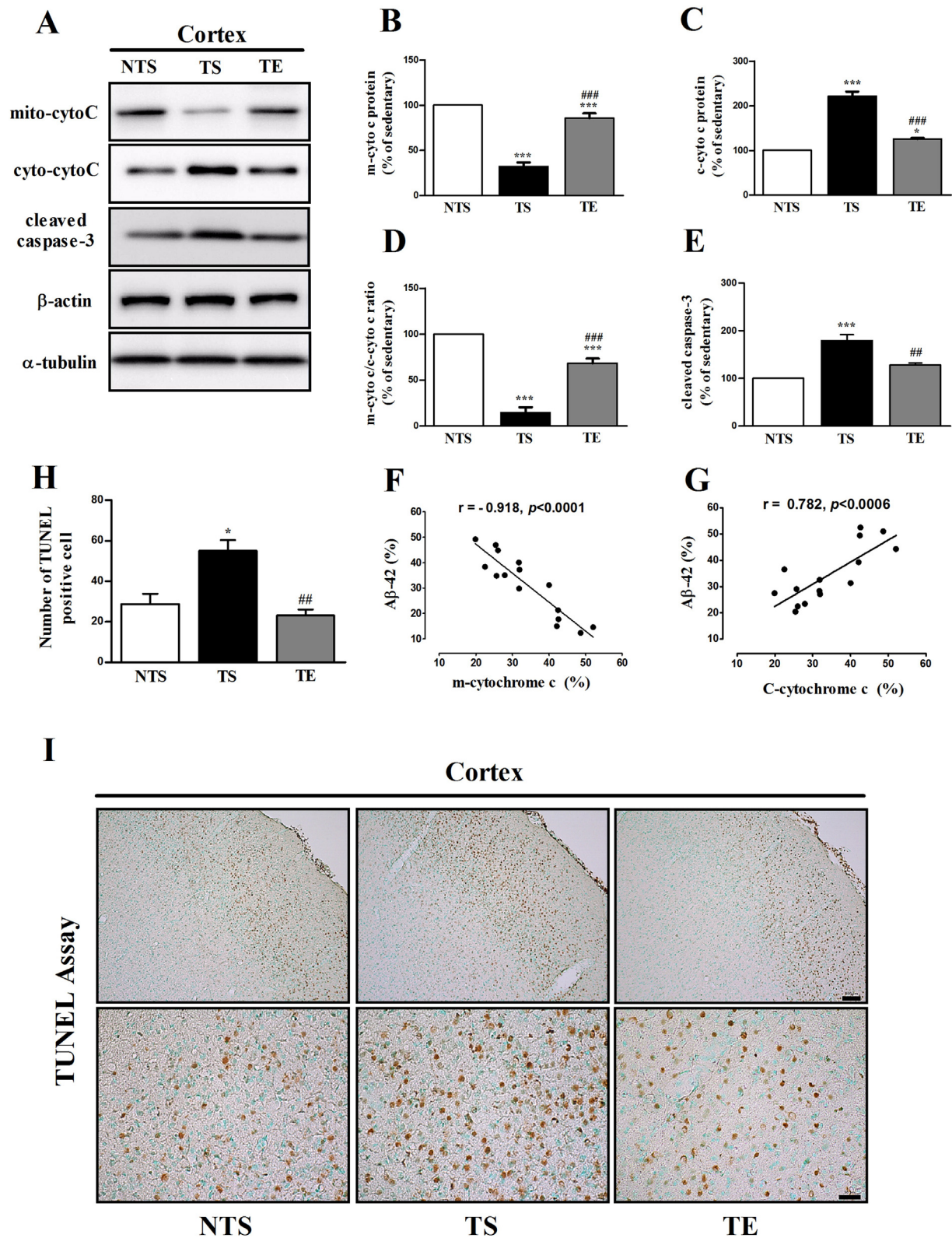


Fig. 2. Treadmill exercise alleviates A β -induced cell death in the cortex of NSE/APPsw transgenic mouse model of AD. A) Representative blot of Western bands for m-cyto c, c-cyto c, m-cyto c:c-cyto c ratio, and cleaved caspase-3 levels. B–E) The quantitative analysis of the m-cyto c, c-cyto c, m-cyto c:c-cyto c ratio, and cleaved caspase-3 levels. F) Correlation analysis of A β -42 and c-cyto c ($n = 5/\text{group}$). G) Correlation analysis of A β -42 and m-cyto c ($n = 5/\text{group}$). H) The number of TUNEL-positive cells: Magnification $\times 100$ (upper panel) $\times 400$ (lower panel). I) Photomicrograph of apoptotic cells in the cortex. Values are expressed to 100% for levels of NTS group. β -actin, and α -tubulin were probed as housekeeping gene. All bars shown represent the means \pm SEM ($n = 5/\text{group}$). A Bonferroni post-doc test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to the NTS mice; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to the TS mice. Non Tg-sedentary (NTS), Tg-sedentary (TS), and Tg-treadmill exercise (TE).

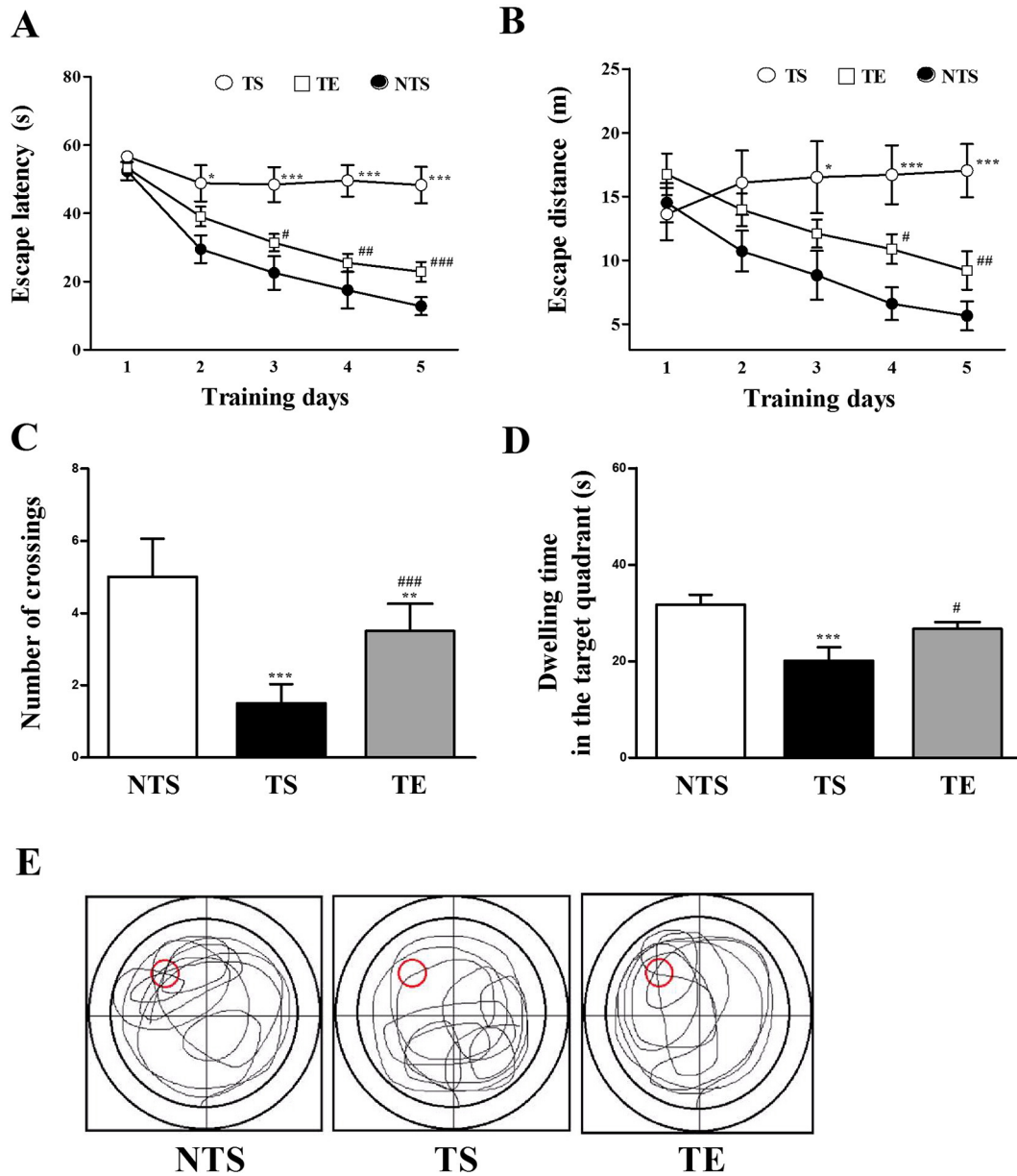


Fig. 3. Treadmill exercise ameliorates cognitive deficits in the cortex of NSE/APPsw transgenic mouse model of AD. A) Escape latency during 5 days of training. B) Escape distance during 5 days of training. C) In the probe trial, number of crossings on the hidden platform previously placed. D) In the probe trial, dwelling times on the hidden platform zone previously placed. E) Patterns of swimming in mice. All bars shown represent the means \pm SEM ($n = 8/\text{group}$). A Bonferroni post-doc test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to the NTS mice; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to the TS mice. Non Tg-sedentary (NTS), Tg-sedentary (TS), and Tg-treadmill exercise (TE).

between SIRT-1 and A β -42 levels (Fig. 4C). SIRT-1 was negatively correlated with A β -42 level ($r = -0.829$, $p < 0.0001$).

3.5. Treadmill exercise enhances the non-amyloidogenic pathway by increasing ADAM-10, possibly by activating SIRT-1 signaling in the NSE/APPsw-transgenic mice model of AD

Because α -secretase-induced cleavage of APP is the first process in inhibition of A β production, we investigated the effects of treadmill exercise on ROCK-1, RAR β , and ADAM-10 (Fig. 5A). The levels of ROCK-1, RAR β , and ADAM-10 showed significant effects of group (ROCK-1: $F_{(2,12)} = 44.91$, $p < 0.001$; RAR β : $F_{(2,12)} = 40.85$, $p < 0.001$; ADAM-10: $F_{(2,12)} = 53.84$, $p < 0.001$). In TS mice, RAR β levels significantly decreased, whereas ROCK-1 levels increased, compared to NTS mice (Fig. 5B, C). However, RAR β levels were upregulated and ROCK-1 levels downregulated after treadmill exercise. In addition, we evaluated correlations between SIRT-1 levels and RAR β and ROCK-1 levels. RAR β was

positively correlated with SIRT-1 ($r = 0.671$, $p < 0.0006$) (Fig. 5D), whereas ROCK-1 was negatively correlated with SIRT-1 ($r = 0.736$, $p < 0.0018$) (Fig. 5E). Finally, ADAM-10 levels were decreased in TS mice compared with NTS mice, but levels significantly increased after treadmill exercise (Fig. 5F).

3.6. Treadmill exercise reduces APP β -cleavage and increases APP α -cleavage in the NSE/APPsw-transgenic mice model of AD

Western blot analyses were performed to determine the effects of treadmill exercise on APP processing. Levels of total APP, α -secretase cleaved APP (sAPP α), β -secretase cleaved APP (sAPP β), carboxy-terminal fragments α (CTF α), and carboxy-terminal fragments β (CTF β) showed significant effects of group (total APP: $F_{(2,12)} = 0.93$, $p < 0.422$; sAPP α : $F_{(2,12)} = 48.50$, $p < 0.001$; sAPP β : $F_{(2,12)} = 19.09$, $p < 0.001$; CTF α : $F_{(2,12)} = 27.49$, $p < 0.001$; CTF β : $F_{(2,12)} = 12.15$, $p < 0.001$). Total APP levels were not significantly different among the

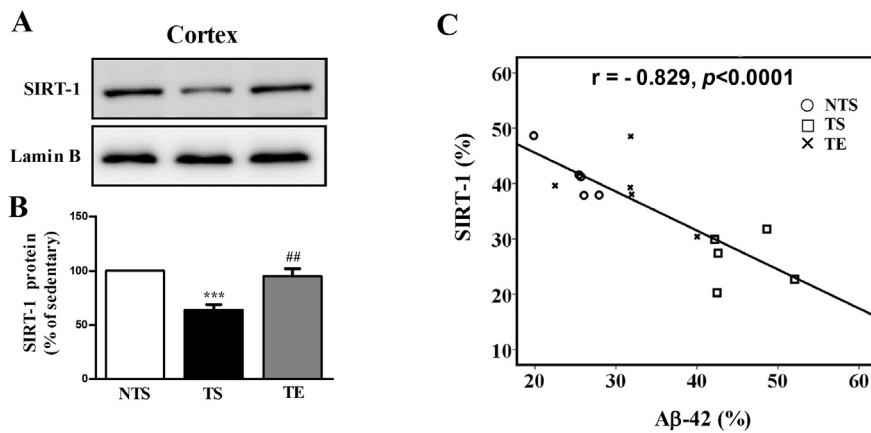


Fig. 4. Treadmill exercise increases SIRT-1 expression in the cortex of NSE/APPsw transgenic mouse model of AD. A) Representative blot of western bands for SIRT-1 levels. B) The quantitative analysis of the SIRT-1 levels. C) Correlation analysis of Aβ-42 and SIRT-1 ($n = 5/\text{group}$). Values are expressed to 100% for levels of NTS group. Lamin B was probed as housekeeping gene. All bars shown represent the means \pm SEM ($n = 5/\text{group}$). A Bonferroni post-doc test: *** $p < 0.001$, compared to the NTS mice; ## $p < 0.01$ compared to the TS mice. Non Tg-sedentary (NTS), Tg-sedentary (TS), and Tg-treadmill exercise (TE).

groups, indicating that treadmill exercise does not affect total APP levels (Fig. 6A, B). Compared to NTS mice, sAPPα and CTFα levels were significantly lower, whereas sAPPβ and CTFβ levels were significantly higher in TS mice. (Fig. 6C–G). Notably, treadmill exercise significantly increased sAPPα and CTFα levels and decreased sAPPβ and CTFβ levels in TE mice.

3.7. Treadmill exercise decreases amyloidogenic pathway possibly via inhibiting BACE-1 and C-99 by activating PGC-1α in the NSE/APPsw-transgenic mice model of AD

It has been demonstrated that PGC-1α reduces BACE-1 and its product C-99, which has been associated with activation of the amyloidogenic

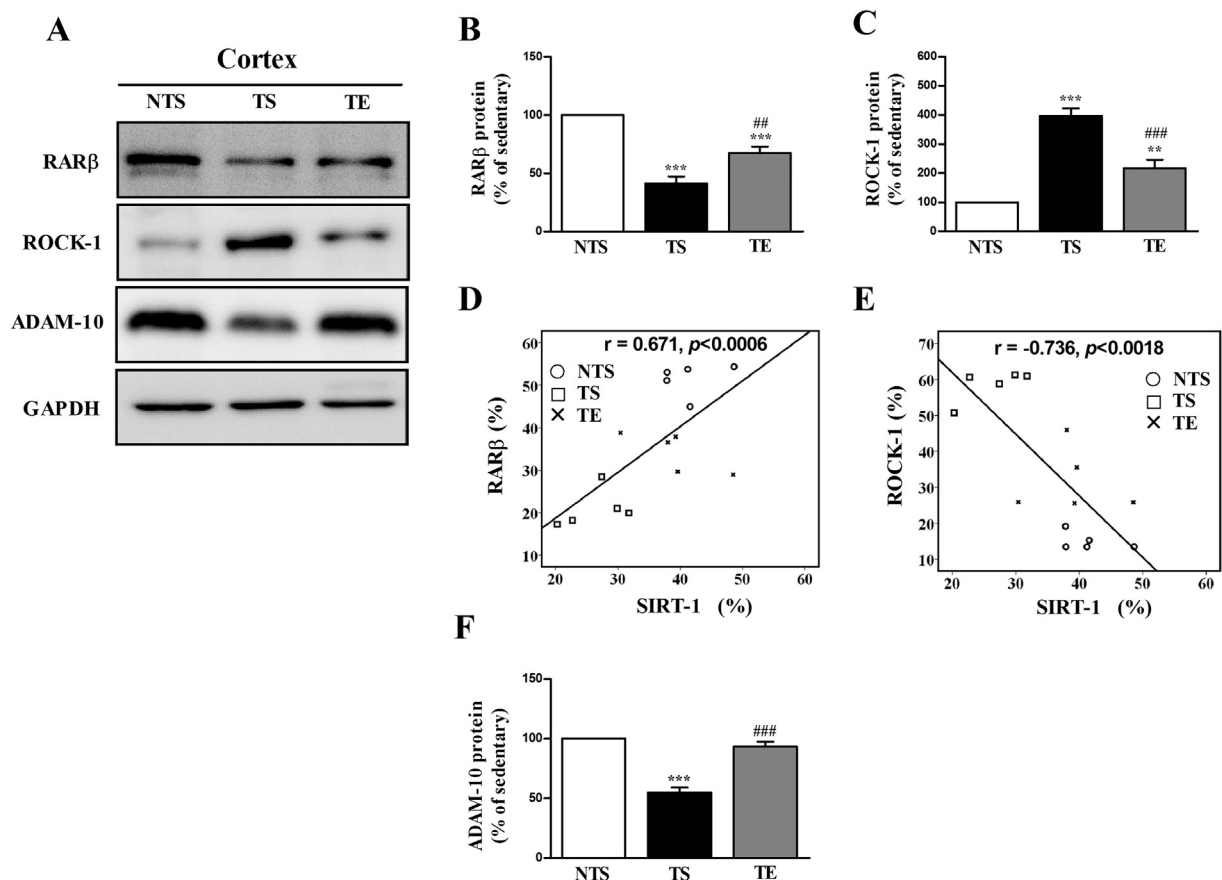


Fig. 5. Treadmill exercise increases ADAM-10 expression possibly via up-regulation of RARβ and down-regulation of ROCK-1 in the cortex of NSE/APPsw transgenic mouse model of AD. A) Representative blot of western bands for RARβ, ROCK-1, and ADAM-10 levels. B, C, F) The quantitative analysis of the RARβ, ROCK-1, and ADAM-10 levels. D) Correlation analysis of SIRT-1 and RARβ ($n = 5/\text{group}$). E) Correlation analysis of SIRT-1 and ROCK-1 ($n = 5/\text{group}$). Values are expressed to 100% for levels of NTS group. GAPDH was probed as housekeeping gene. All bars shown represent the means \pm SEM ($n = 5/\text{group}$). A Bonferroni post-doc test: ** $p < 0.01$, *** $p < 0.001$, compared to the NTS mice; ## $p < 0.01$, ### $p < 0.001$ compared to the TS mice. Non Tg-sedentary (NTS), Tg-sedentary (TS), and Tg-treadmill exercise (TE).

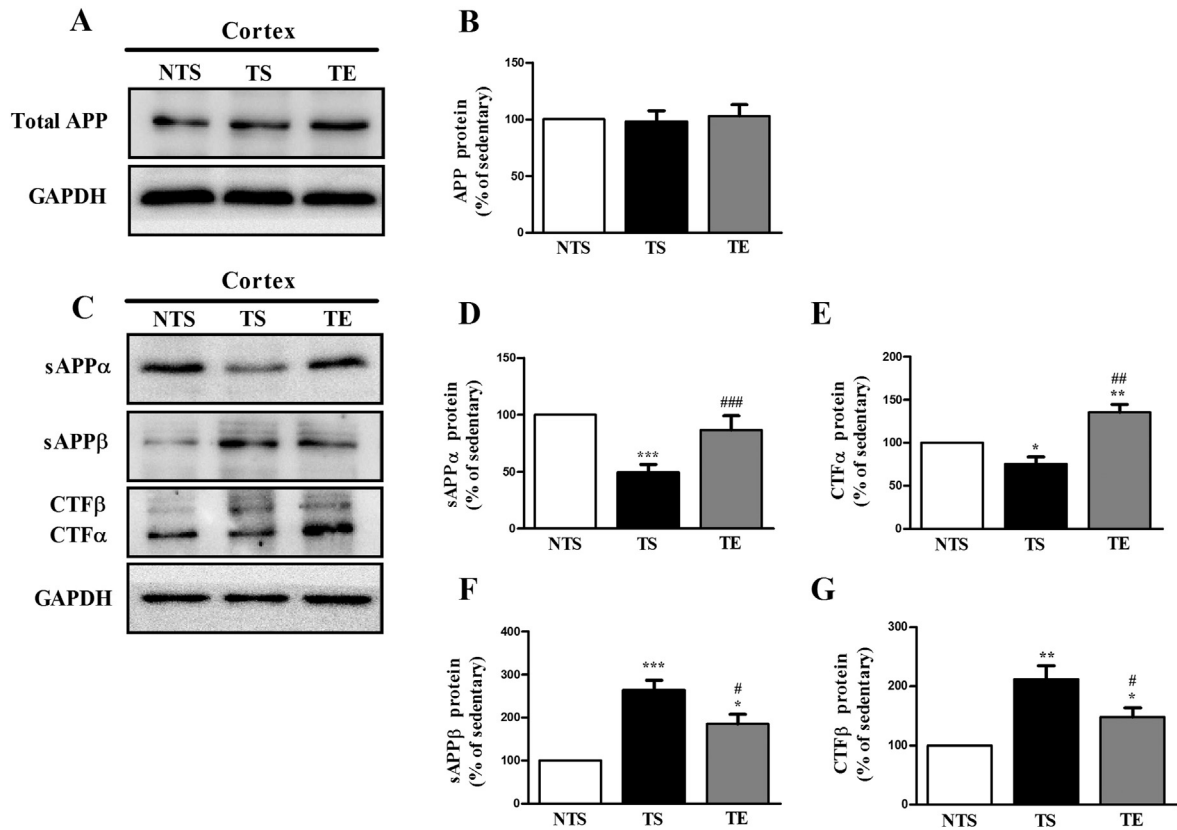


Fig. 6. Treadmill exercise reduces APP β -cleavage and increases APP α -cleavage in the NSE/APPsw-transgenic mice model of AD. (A) Representative blot of western bands for total APP levels. (B) The quantitative analysis of the total APP levels. (C) Representative blot of western bands for sAPP α , CTF α , sAPP β , and CTF β levels. (D–G) The quantitative analysis of the sAPP α , CTF α , sAPP β , and CTF β levels. Values are expressed to 100% for levels of NTS group. GAPDH was probed as housekeeping gene. All bars shown represent the means \pm SEM ($n = 5$ /group). A Bonferroni post-doc test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to the NTS mice; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to the TS mice. Non Tg-sedentary (NTS), Tg-sedentary (TS), and Tg-treadmill exercise (TE).

pathway (Marwarha et al., 2014). To determine the effects of treadmill exercise on PGC-1 α levels and further regulative effects on BACE-1 and C-99, we performed western blot analysis (Fig. 6A). Levels of PGC-1 α , BACE-1, and C-99 showed significant effects of group (PGC-1 α : $F_{(2,12)} = 80.25$, $p < 0.001$; BACE-1: $F_{(2,12)} = 25.80$, $p < 0.001$; C-99: $F_{(2,12)} = 36.88$, $p < 0.001$). PGC-1 α levels were significantly reduced in TS mice compared with NTS mice, but treadmill exercise significantly elevated PGC-1 α levels in TE mice (Fig. 6B). Moreover, to confirm the effect of treadmill exercise-induced SIRT-1 on PGC-1 α activity, we performed a correlation analysis of SIRT-1 activity and PGC-1 α level (Fig. 6C). SIRT-1 was positively correlated with PGC-1 α ($r = 0.802$, $p < 0.0003$). Consistent with increases in the PGC-1 α levels, TE mice with treadmill exercise showed significantly decreased BACE-1 and C-99 compared to TS mice (Fig. 6D, E).

4. Discussion

The present study demonstrated that treadmill exercise inhibited A β production, with subsequent mitigation in cell death and behavioral deficits in the cortex of an NSE/APPsw-transgenic mouse model of AD. In particular, we explored molecular mechanisms underlying inhibition of A β production and found that treadmill exercise increased SIRT-1 levels, subsequently resulting in increased ADAM-10 expression by downregulating ROCK-1 and upregulating RAR β . Furthermore, treadmill exercise increased PGC-1 α levels, which reduced BACE-1 and C-99 expression. Therefore, we demonstrated for the first time that treadmill exercise inhibits A β production possibly via upregulating SIRT-1/PGC-1 α signaling, which shifts APP processing toward the non-amyloidogenic pathway.

Although A β accumulation is generally viewed as a regulator of neurotoxicity in AD, the mechanism for this is still unknown and sometimes controversial. However, there is no doubt that deposition of A β is involved in the hallmark features of AD. As expected, our results showed A β levels were significantly increased in TS mice (Fig. 1), and these increase paralleled cell death. In this study, we found that cyto-c levels in mitochondria were significantly decreased and cleaved caspase-3, a substrate apoptotic factor of cyto-c, was increased in TS mice compared to NTS mice (Fig. 2). In addition, we found TS mice showed increased apoptotic cells (as evidenced by TUNEL assays, Fig. 2I). However, treadmill exercise appeared to reduce A β and alleviate cell death in TE mice. Furthermore, A β -induced neurotoxicity is associated with cognitive deficits in AD; we showed here that treadmill exercise reduced the cognitive dysfunction accompanying increased A β levels in AD (Fig. 3). TS mice exhibited significant deterioration of escape latencies and distances; these cognitive deficits were dramatically improved after treadmill exercise, indicating that treadmill exercise significantly ameliorated cognitive deficits by activating neuroprotective pathway and decreasing A β levels. These results are consistent with our previous studies and other reports (Um et al., 2011; Kang et al., 2013; Revilla et al., 2014; Garcia-Mesa et al., 2012). As mentioned above, previous studies demonstrated that cognitive deficit and neuronal cell death are alleviated by the reduction of A β plaques via physical exercise, suggesting that physical exercise may alleviate or delay AD. However, the molecular mechanism underlying the reduction of A β plaque induced by physical exercise is unknown. Based on this evidence, we continued to analyze the potential role of SIRT-1-mediated APP processing in mechanisms associated with A β production in AD, which may play a role in the early preventive effects on AD.

Loss of SIRT-1 expression has been observed in patients with AD, resulting in elevated A β production (Kumar et al., 2013; Julien et al., 2009), whereas SIRT-1 overexpression has been shown to decrease A β production (Marwarha et al., 2014; Porquet et al., 2013), indicating that activation of SIRT-1 by physical exercise may prevent A β production in AD brains. More recently, several studies demonstrated that physical exercise increases SIRT-1 expression (Bayod et al., 2015; Casuso et al., 2014). Voluntary exercise increased SIRT-1 activity and improved mitochondrial function in SAMP-8 model, which is aging-related disease mouse model (Bayod et al., 2015). In addition, oral quercetin supplementation and exercise also induce SIRT-1 expression and improved antioxidant defense in normal Wistar rats (Casuso et al., 2014). However, the molecular basis of how treadmill exercise might influence SIRT-1 in AD is still unknown. So far, only two studies have investigated the effects of physical exercise on the relationship between SIRT-1 and APP processing in AD. Revilla et al. (2014) demonstrated that long-term voluntary exercise increased SIRT-1 activity and improved synaptic function, while proteolysis of APP was not changed in 3xTg AD mice. Conversely, Polito et al. (2014) found that environmental enrichment counteracted memory deficits and reduced A β accumulation without affecting SIRT-1 expression and APP processing in AD mice. Here, we found that SIRT-1 levels significantly decreased, and this reduction paralleled the increasing A β accumulation in AD mice (Fig. 4). However, treadmill exercise remarkably increased SIRT-1 expression, which was associated with decreased A β accumulation. The differences between our results and previous studies may be due to the different type of physical exercise. In particular, previous studies of the effects of physical exercise on A β reduction, SIRT-1, and APP processing have utilized spontaneous physical exercise, such as wheel running and environmental enrichment (Polito et al., 2014; Revilla et al., 2014). These types of experiment give free access to voluntary exercise, but do not regulate the intensity, time, or distance of exercise. Conversely, the forced treadmill exercise used in this study allows for precise regulation of physical exercise demands, which maintain a constant intensity of physical exercise. However, forced treadmill exercise could activate negative physiological adaptations via the stress response (Arida et al., 2004; Yanagita et al., 2007). Interestingly, in our previous study, we found that level of corticosterone, a stress hormone, was not increased by treadmill exercise in AD mice (Um et al., 2011), indicating that low intensity treadmill exercise was not an intense stressor. Although we did not measure stress hormones in this study, we used

the same exercise protocol as in our previous study, indicating that low intensity treadmill exercise might be an effective way of upregulating treadmill exercise-induced SIRT-1 activity. However, some researchers demonstrated that voluntary running resulted in lower A β levels than treadmill exercise in AD mice (Yuede et al., 2009), and A β deposition was significantly decreased after the high intensity exercise compared with low intensity exercise (Moore et al., 2016). Therefore, further studies are necessary to determine all the precise molecular mechanisms through which treadmill exercise influences SIRT-1-mediated A β production in AD. Taken together, however, these results demonstrated that treadmill exercise reduced A β level by activating SIRT-1, strongly suggesting that treadmill exercise may prevent A β production through enhancement of the non-amyloidogenic pathway.

Based on these results, in this study, we continued to mechanistically investigate the potential role of treadmill exercise in SIRT-1-mediated APP processing in AD. On the basis of its ability to cleave APP, ADAM-10 was identified as the main α -secretase in neurons. Cleavage of APP by ADAM-10 implies higher levels of α -secretase cleaved APP (sAPP α) and carboxy-terminal fragments α (CTF α), and eventually activation of the non-amyloidogenic pathway (Kuhn et al., 2010; Ryan et al., 2013). Interestingly, SIRT-1 has been shown to increase ADAM-10 activity by upregulating RAR β and downregulating ROCK-1, thereby inhibiting A β production (Qin et al., 2006a, 2006b; Lee et al., 2014; Qin et al., 2008). RAR β is known to activate ADAM-10, resulting in increases in the non-amyloidogenic pathway (Tippmann et al., 2009). Conversely, ROCK-1 is a ubiquitous protein kinase that inhibits the non-amyloidogenic pathway by downregulating ADAM-10 (Tang et al., 2006). Previous studies showed that ROCK-1 activity increased in AD mouse and monkey brains, resulting in inactivation of ADAM-10, which was partially recovered by nutritional regimes such as calorie restriction and resveratrol supplements (Qin et al., 2006a; Qin et al., 2006b). Very interestingly, physical exercise has been suggested as a resveratrol and calorie restriction mimetic driven by SIRT-1 activity (Mercken et al., 2012; Handschin, 2016), supporting the hypothesis that treadmill exercise may regulate ADAM-10-mediated APP processing by inhibiting ROCK-1 and enhancing RAR β via activating SIRT-1. In the present study, we found that ADAM-10 expression was decreased by reducing RAR β and increasing ROCK-1, associated with decreased SIRT-1 levels in TS mice (Fig. 5). However, treadmill exercise dramatically increased ADAM-10 expression, which augmented sAPP α and CTF α expression (Fig. 6) by activating RAR β and inhibiting ROCK-1.

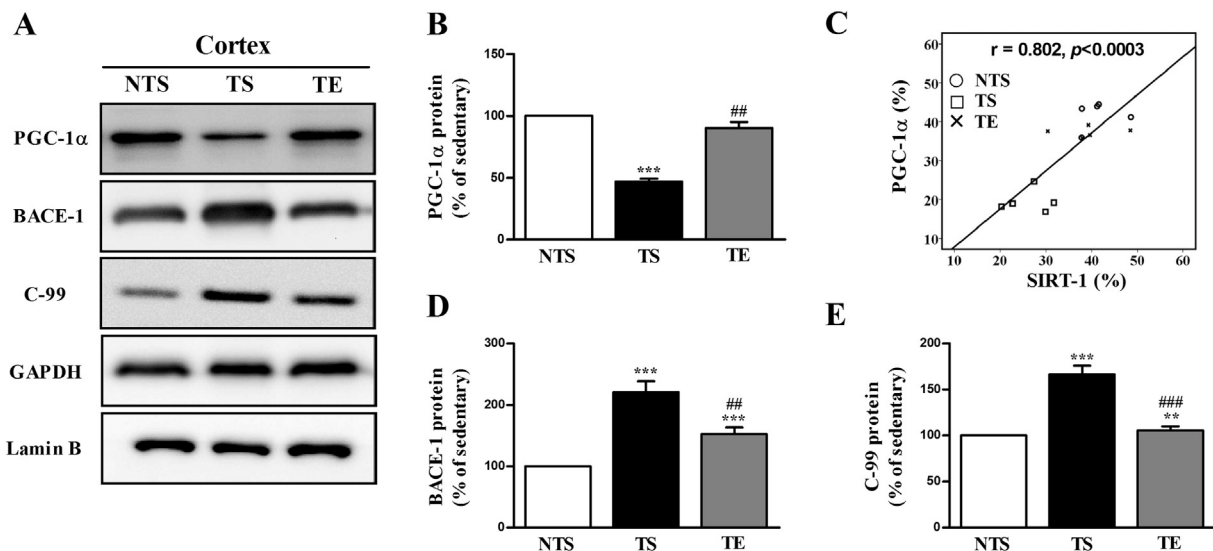


Fig. 7. Treadmill exercise decreases BACE-1 and C-99 expression possibly via activating PGC-1 α in the cortex of NSE/APPsw transgenic mouse model of AD. (A) Representative blot of western bands for PGC-1 α , BACE-1, and C-99 levels. (B, D, E) The quantitative analysis of the PGC-1 α , BACE-1, and C-99 levels. (C) Correlation analysis of SIRT-1 and PGC-1 α . Values are expressed to 100% for levels of NTS group. GAPDH and Lamin B were probed as housekeeping gene. All bars shown represent the means \pm SEM (n = 5/group). A Bonferroni post-doc test: **p < 0.01, ***p < 0.001, compared to the NTS mice; ##p < 0.01, ###p < 0.001 compared to the TS mice. Non Tg-sedentary (NTS), Tg-sedentary (TS), and Tg-treadmill exercise (TE).

These results demonstrate for the first time that treadmill exercise elevates ADAM-10 expression, which was associated with increased SIRT-1, which in turn downregulated ROCK-1 and upregulated RAR β , resulting in the activation of the non-amyloidogenic pathway.

SIRT-1 also activated PGC-1 α , a key regulator of glucose metabolism, mitochondrial biogenesis, and thermogenesis (Rodgers et al., 2008; Scarpulla, 2011). Several studies demonstrated that PGC-1 α expression was reduced in patients with AD and mouse model, coincident with activation of A β production (Pedros et al., 2014; Qin et al., 2009). In addition, reducing PGC-1 α levels increased A β production by activating BACE-1 expression (Katsouri et al., 2011; Gong et al., 2010). BACE-1 is the rate-limiting enzyme in APP processing and A β production (Vassar et al., 2009), so this finding suggests that treadmill exercise may inhibit A β production by inactivating BACE-1 expression via effects on PGC-1 α activity. Recently, two studies reported decreases in BACE-1 and its product, C-99, following physical exercise in transgenic mouse models of AD (Kang et al., 2013; Rao et al., 2015). Consistent with these studies, we found that BACE-1 levels increased (Fig. 7), accompanied by increased β -secretase cleaved APP (sAPP β) and carboxy-terminal fragments β (CTF β) in TS mice (Fig. 6). Importantly, treadmill exercise caused the reduction of BACE-1 and sAPP β and CTF β expression via activation of PGC-1 α , contingent on SIRT-1 signaling. These results strongly indicate that treadmill exercise also might reduce A β production by inhibiting the amyloidogenic pathway, possibly via the activation of SIRT-1/PGC-1 α signaling.

Although we suggest a connection among treadmill exercise, SIRT-1, APP processing, and A β production, the effect of treadmill exercise on A β reduction via the activation of the SIRT-1-mediated non-

amyloidogenic pathway remains unclear. Moreover, there are some controversial reports on the role of SIRT-1/PGC-1 α signaling in A β degradation in AD. For example, a recent study demonstrated that A β exposure increased PGC-1 α levels and decreased SIRT-1 expression in cultured astrocytes (Aguirre-Rueda et al., 2015). Another group indicated that PGC-1 α overexpression exacerbates A β accumulation and cognitive deficits in AD mice (Dumont et al., 2014). Further studies are thus necessary to clarify the specific mechanism through which treadmill exercise and the SIRT-1/PGC-1 α pathway are involved in the inhibition of A β production in AD. A limitation of our report is that we did not confirm any changes in the levels of AD-related proteins in NTS-exercise mice compared to NTS mice. Thus, the influence of genetic background in the effects of treadmill exercise could not be clarified. Another limitation is that we only measured the protein level of neuronal cell death-related factor. It is insufficient to prove that neuronal cell death was due to A β . Nevertheless, future investigations on influence of genetic background in the effect of treadmill exercise in NTS-exercise mice and confirmation of neuronal cell death in neuron should be performed in AD mice.

5. Conclusion

In summary, our study is the first to report that treadmill exercise might reduce A β production and, improve cognitive deficits possibly by increasing ADAM-10 and decreasing BACE-1 via activation of SIRT-1/PGC-1 α signaling, ultimately activating the non-amyloidogenic pathway and inhibiting the amyloidogenic pathway. This study provides new insight into the mechanisms of treadmill exercise-induced A β reduction, which represents a potential therapeutic strategy for AD (Fig. 8).

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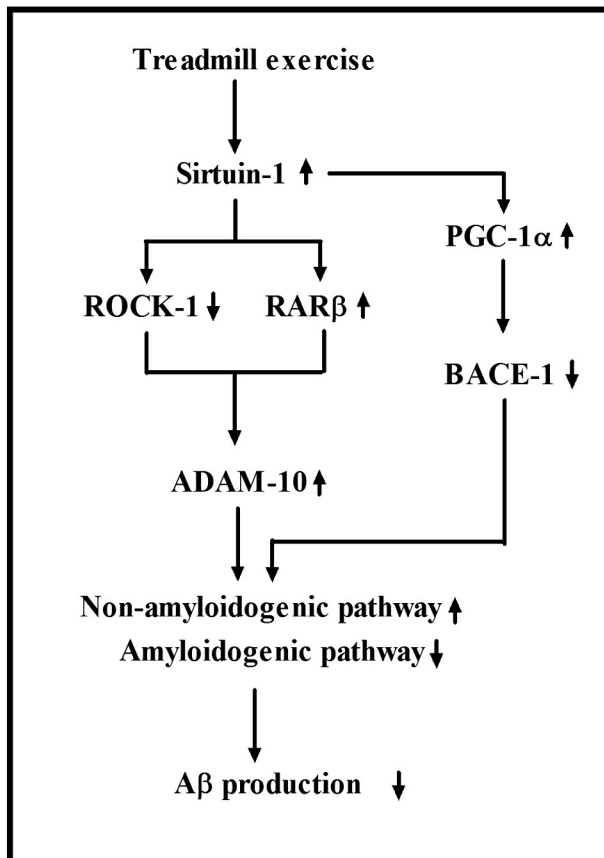


Fig. 8. The schematic summary of our study. The potential hypothesis relationships for treadmill exercise, SIRT-1 expression, non-amyloidogenic pathway and A β production. Treadmill exercise might reduce A β production and improve cognitive deficits possibly by increasing ADAM-10 and decreasing BACE-1 via activation of SIRT-1/PGC-1 α signaling, ultimately facilitating non-amyloidogenic pathway and inhibiting amyloidogenic pathway.

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