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Differential effects of angiotensin II receptor blockers on Aβ generation

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HIGHLIGHTS

- Telmisartan markedly increased Aβ generation.
- Losartan, valsartan and candesartan did not increase Aβ generation.
- Olmesartan significantly increased Aβ42 generation.
- Telmisartan increased the Aβ generation through angiotensin type 1a receptor-PI3K pathway.

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ABSTRACT

Angiotensin II receptor blockers (ARBs) are widely prescribed for the medication of systemic hypertension and congestive heart failure. It has been reported that ARBs can reduce the risk for the onset of Alzheimer's disease (AD) and have beneficial effects on dementia. Neurotoxic amyloid β-protein (Aβ) is believed to play a causative role in the development of AD. However, whether ARBs regulate Aβ generation remains largely unknown. Here, we studied the effect of ARBs on Aβ generation and found that telmisartan significantly increased Aβ40 and Aβ42 generation, but decreased the Aβ42/Aβ40 ratio. However, losartan, valsartan and candesartan did not increase Aβ generation, while olmesartan significantly increased Aβ42 generation. We also found that telmisartan increased the Aβ generation through angiotensin type 1a receptor (AT1a) and the receptor-related phosphotidylinositide 3-kinases (PI3K) pathway. Our findings revealed the different effects of ARBs on Aβ generation and provide new evidence for the relationship between antihypertensive treatment and AD pathogenesis.

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1. Introduction

Angiotensin II receptor blockers (ARBs) are used primarily for the treatment of hypertension where the patient has intolerance to angiotensin converting enzyme (ACE) inhibitor therapy since they are rarely associated with the persistent dry cough and/or angioedemia that limit ACE inhibitor therapy. The prevalence of hypertension increases to 51% in the group aged 60–74 years [1], and there is an almost 15-fold increase in the prevalence of dementia, predominantly Alzheimer's disease (AD), between the ages of 60 and 85 years [2]. AD is the most common form of dementia and is pathologically characterized by a reduction in brain volume, generally accompanied by brain shrinkage and cerebral amyloid plaques which are largely composed of neurotoxic amyloid β-protein (Aβ), which is generated from the cleavage of amyloid precursor protein (APP). Increasing evidence points to a link between hypertension and AD [3,4]. The epidemiological investigations have shown that AD rapidly progressed in elderly people who have a history of lifestyle-related diseases such as hypertension [5,6]. The clinical findings also indicate that cerebrovascular disease may play an important role in determining the presence and severity of the clinical symptoms of AD [7]. The incidence of AD is associated with the antihypertensive medications, including calcium-channel blockers [8], ACE inhibitors [9] and ARBs [10]. However, since the current

Abbreviations: ARBs, angiotensin II receptor blockers; AD, Azheimer's disease; Aβ, amyloid β-protein; AT1a, angiotensin type 1a receptor; ACE, angiotensin converting enzyme; MEFs, mouse embryonic fibroblasts; hAPP695, human 695-amino acid amyloid precursor protein; APP, amyloid precursor protein; ELISA, enzyme-linked immunosorbent assay; AT1, angiotensin II type 1 receptor; Ang II, angiotensin II; MAPK, mitogen activated protein kinase; PI3K, phosphotidylinositide 3-kinase; PKC, protein kinase C; AMPK, AMP-activated kinase; pAkt, phosphorylation of Akt; tAkt, total Akt.

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research on the association of ARBs and AD is limited to epidemiological research, there is no evidence concerning the direct effect of ARBs on Aβ generation. Here, we report the different effects of ARBs on Aβ generation among the ARBs examined. We found that telmisartan significantly increased Aβ40 and Aβ42 generation through the angiotensin type 1a receptor (AT1a) and the receptor related phosphotidylinositide 3-kinases (PI3K) pathway. Olmesartan significantly increased the Aβ42/Aβ40 ratio. However, losartan, valsartan and candesartan did not affect Aβ generation.

2. Materials and methods

2.1. Cell lines and cell culture

To generate *Agtr1a* deficient mouse embryonic fibroblasts (MEFs), we isolated MEFs from 13.5-day-old embryos of *Agtr1a* deficient mice (The Jackson Laboratory) with the C57BL/6 background, following the procedures as described previously [11]. All animal procedures were approved by the Iwate Medical University Committee for Animal Use. C57BL/6 MEFs and *Agtr1a* deficient MEFs were infected with human 695-amino acid amyloid precursor protein (hAPP695) cDNA by a retrovirus-mediated method according to published methods [12].

The two kinds of cells were cultured in DMEM (Wako Pure Chemical Industries) supplemented with 10% FBS (Sigma). Cells were maintained at 37 °C in an atmosphere of 5% CO_2 in a tissue culture incubator. The hAPP695 infected fibroblasts were passaged with the same cell concentration for each pathway inhibitor administration. After 1 h of incubation with the each inhibitor, the fibroblasts were treated with 20 μM telmisartan to examine which inhibitor reversed the increased Aβ generation by telmisartan.

2.2. Reagents

Telmisartan was purchased from Sigma and dissolved in DMSO. Losartan was purchased from Wako and dissolved in PBS. Valsartan, olmesartan and candesartan were purchased from TRC and were dissolved in DMSO. PD98059 and wortmannin were purchased from Cell Signaling. PD98059 was administered at the final concentration of 10 μM. Wortmannin was administered at the final concentration of 100 nM and 10 μM. Compound C and protein kinase C (PKC) inhibitor cocktail were purchased from Millipore. Compound C was administered at the final concentrations of 500 nM and the PKC inhibitor cocktail was administered at 5000 × dilution of the original solution. DAPT was purchased from Peptide Institute and was administered at the final concentration of 5 μM. PI3K activator is a 1732.8 Da peptide with the sequence KKHTDDGYMPMSPGVA and binds to the SH2 domain of the PI3K by the tyrosine phosphorylated version to activate the enzyme [13]. It was purchased from Santa Cruz Biotechnology. GW9662 was purchased from SIGMA and was administered at the final concentration of 10 μM.

2.3. Aβ measurement

Aβ40 and Aβ42 in conditioned media were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (Wako, Osaka, Japan), based on the manufacturer's instructions. Aβ40 and Aβ42 concentrations were normalized based on the amount of cell protein. All samples were measured in triplicate.

2.4. Cell lysate and cultured medium collection

Cells were lysed on ice in RIPA lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% sodium lauryl sulfate) containing a protease inhibitor mixture. The protein concentration of the collected supernatant was determined using a BCA protein assay kit (Thermo Fisher Scientific). Cell culture media was collected after centrifuging at 25,000 × *g* for 10 min at 4 °C and supplemented with a protease inhibitor.

2.5. Immunoblotting

APP fibroblasts were cultured to 70% confluence and starved overnight in serum-free medium prior to treatment. Starved cells were treated with 20 μM telmisartan for 5, 10, 15, 30 min and 1 h, and washed with 1 mM sodium orthovanadate before being lysed with 20 mM HEPES pH 7.0, 0.5% deoxycholic acid, 0.15 M NaCl, 0.1% SDS, 1% Nonidet P-40, 4 mM EDTA, 10 mM NaF, 10 mM $Na_4P_2O_7$, 2 mM sodium orthovanadate, containing a protease inhibitor cocktail (Roche). Wortmannin (100 nM) was administered prior to 20 μM telmisartan treatment. Equal amounts of protein from cell lysate were separated by SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon). The membranes were incubated with the primary antibodies overnight at 4 °C. Appropriate peroxidase-conjugated secondary antibodies were applied and the membranes were visualized by SuperSignal Chemiluminesence (Thermo Scientific). Total Akt (tAkt) was detected on the same membrane after stripping the anti-pAkt antibody. The rabbit anti-Akt and anti-pAkt (Ser-473) antibodies were purchased from Cell Signaling. To examine the expression of AT1a, the brain of C57BL/6 mice and the APP fibroblasts were lysed in RIPA buffer and the same amount of proteins were separated by SDS-PAGE. The AT1a antibody using for immunoblotting was purchased from Bioss.

2.6. Statistical analyses

We compared group differences by one-way ANOVAs followed a post hoc Bonferroni test. Statistical analyses were carried out using GraphPad Prism 5. A *P*-value < 0.05 was considered to represent a significant difference. Graphs are expressed as means ± s.e.m.

3. Results

3.1. Telmisartan increased Aβ generation markedly, but significantly decreased the Aβ42/Aβ40 ratio

To determine whether ARBs affect Aβ generation, we infected C57BL/6J fibroblasts with hAPP695 cDNA to generate constant human APP overexpression fibroblasts (APP fibroblasts), and then treated them separately with telmisartan, losartan, valsartan, olmesartan or candesartan for 72 h. Telmisartan increased Aβ40 and Aβ42 generation markedly, about 6- and 3.2-fold (Fig. 1a and b), respectively, compared to the controls, while olmesartan increased Aβ40 generation about 2-fold at the concentration of 5 μM (Fig. 1a) and Aβ42 generation about 3.2-fold (Fig. 1b) at the concentration of 10 μM. However, losartan, valsartan and candesartan did not show any clear increase in the Aβ generation (Fig. 1a and b). The Aβ42/Aβ40 ratio in serum increased in familial AD patients and is considered as a causal factor of AD [14]. Among the ARBs examined, telmisartan significantly decreased the Aβ42/Aβ40 ratio and had the lowest Aβ42/Aβ40 ratio, whereas, olmesartan had the highest Aβ42/Aβ40 ratio which was significantly higher than that of telmisartan (Fig. 1c). Losartan, valsartan and candesartan did not show any clear effect on Aβ42/Aβ40 ratio (Fig. 1c).

3.2. Telmisartan significantly increased Aβ generation via AT1a

Telmisartan has the highest affinity for angiotensin II type 1 receptor (AT1) among the examined ARBs [15]. Telmisartan also

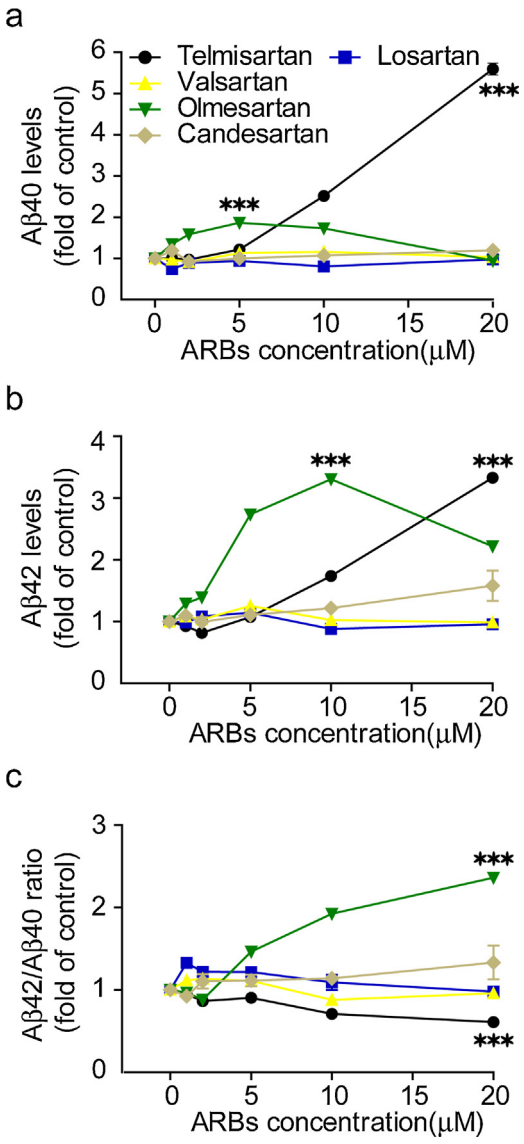

Fig. 1. Effects of ARBs on Aβ generation. (a) Aβ40 and (b) Aβ42 generation levels by administration of ARBs after 72 h in APP fibroblasts. (c) The effect of ARBs on the Aβ42/Aβ40 ratio. Error bars show means ± s.e.m., $^{***}P < 0.001$ by one-way ANOVA followed by a post hoc Bonferroni test.

can cross the blood–brain barrier and inhibit Ang II-mediated central effects, contributing to better blood pressure control [16]. As a subtype of AT1, AT1a has a crucial role for blood pressure regulation in mouse [17] and is expressed in mouse brain [18]. We first confirmed the expression of AT1a in mouse brain and APP fibroblasts (Fig. 2a). To investigate whether telmisartan increases Aβ generation via AT1a, we infected *Agtr1a*$^{-/-}$ fibroblasts with hAPP695 cDNA and confirmed the constant overexpression of human APP. Telmisartan did not increase the Aβ40 and Aβ42 generation in the AT1a deficient fibroblasts (Fig. 2b and c), suggesting that telmisartan increased Aβ generation through AT1a.

3.3. *Telmisartan increased Aβ generation through the AT1a-PI3K pathway*

Telmisartan achieves its effect by binding with AT1a, and AT1a correlates with the activation of mitogen activated protein kinase (MAPK), PI3K, PKC pathway and other pathways [19]. We next investigated which regulating pathways telmisartan depended on to increase Aβ generation. Administration of the PI3K pathway inhibitor, wortmannin, and γ-secretase inhibitor, DAPT, markedly blocked the increased Aβ generation by telmisartan (Fig. 3a), whereas, the MAPK pathway inhibitor, PD98059, the AMP-activated kinase (AMPK) inhibitor, compound C, and the PKC inhibitor did not block telmisartan-induced Aβ generation (Fig. 3a), suggesting that telmisartan increased Aβ generation through the PI3K pathway and this increased Aβ generation is regulated by γ-secretase. To examine the dose-response effect of wortmannin on reversing the increase in Aβ by telmisartan, we pretreated APP fibroblast with 10 μM wortmannin and then treated with telmisartan. Wortmannin 10 μM pre-treatment completely reversed the effect of telmisartan (Fig. 3a). We further confirmed the involvement of the PI3K pathway by determining the effect of PI3K activator on Aβ generation. We found that PI3K activator increased Aβ generation significantly (Fig. 3b). Furthermore, 30 min after the treatment of telmisartan significantly stimulated the phosphorylation of Akt (pAkt), the downstream signal of PI3K (Fig. 3c). This stimulation could be blocked by pre-treatment with wortmannin (Fig. 3d). These results suggest that telmisartan significantly increased Aβ generation by increasing PI3K activity. A previous report showed that telmisartan acts as a selective modulator of peroxisome proliferator-activated receptor gamma (PPARγ) and ameliorates cognitive deficit via PPARγ activation [20]. Thus, we investigated whether telmisartan could increase the Aβ generation through the PPARγ pathway. We treated APP fibroblasts with PPARγ inhibitor, GW9662, prior to telmisartan treatment and found that telmisartan still significantly increased Aβ generation, indicating that the increase in Aβ by telmisartan is independent of the PPARγ pathway (Fig. 3e). Thus, we conclude that telmisartan increased Aβ generation through the AT1a-PI3K pathway.

4. Discussion

In this study, we investigated the effects of ARBs on Aβ generation. We found that telmisartan increased the generation of Aβ40 and Aβ42 markedly, but had the lowest Aβ42/Aβ40 ratio among the ARBs examined and regulated Aβ generation through the AT1a-PI3K pathway. Olmesartan significantly increased Aβ42 generation and markedly increased the Aβ42/Aβ40 ratio. However, losartan, valsartan and candesartan did not show any clear effect of increasing Aβ generation or the Aβ42/Aβ40 ratio.

In our study, telmisartan, and valsartan are pharmacologically active molecules. Losartan is converted to its active metabolite–EXP3174. Candesartan and olmesartan are available as prodrugs [16]. However, losartan, candesartan and olmesartan prodrugs still have an affinity for AT1 [16]. It is possible that the prodrugs themselves can also be absorbed into the plasma. Moreover, losartan, candesartan and olmesartan are also prescribed for intravenous injection. Thus, the losartan, candesartan and olmesartan prodrugs will be in the plasma before being metabolized. Therefore, our results presented not only the effect of the active form of telmisartan and valsartan on Aβ generation, but also the effects of losartan, candesartan and olmesartan prodrugs on Aβ generation.

Telmisartan has the strongest binding affinity for AT1 among the various ARBs. The rank order of receptor binding affinity was telmisartan > olmesartan > candesartan > valsartan ≥ losartan [15]. Based on the increased Aβ generation levels by treatment of these ARBs (Fig. 1a), we deduce that the ARBs receptor binding affinity is closely correlated with Aβ generation. We hypothesize that AT1a receptor signal correlates with γ-secretase assembly or modulates γ-secretase activity. Our results suggest that the strong binding of telmisartan [15] to the receptor can block or stimulate AT1a receptor and then further affects the γ-secretase activity via PI3K pathway.

We also found that olmesartan significantly increased the Aβ42/Aβ40 ratio, suggesting that olmesartan also regulates

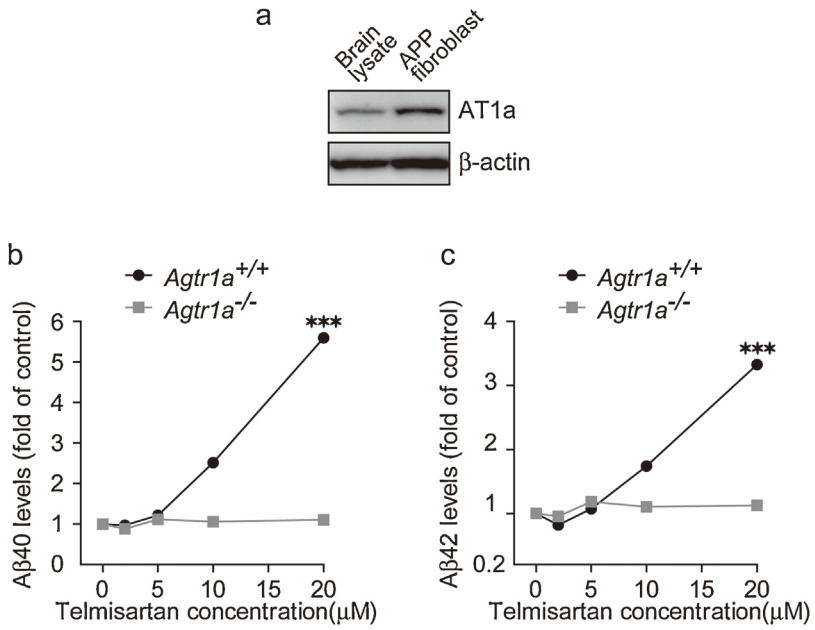

Fig. 2. The effect of telmisartan on Aβ generation in *Agtr1a*$^{-/-}$ APP infected fibroblasts. (a) The expression of AT1a in APP fibroblasts and mouse brain. After *Agtr1a* knockout, telmisartan did not increase (a) Aβ40 or (b) Aβ42 generation compared with *Agtr1a*$^{+/+}$ APP infected fibroblasts. Error bars show means ± s.e.m., ***$P < 0.001$ by one-way ANOVA followed by a post hoc Bonferroni test.

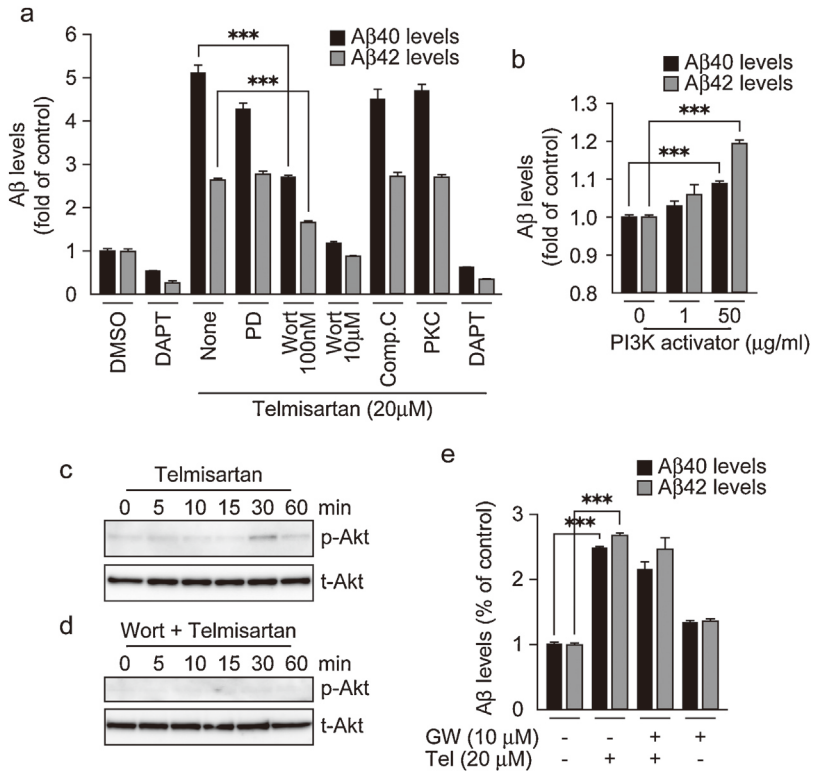

Fig. 3. The pathway that is related to the increased Aβ generation by telmisartan. (a) Wortmannin (Wort) decreased telmisartan increased Aβ generation markedly, whereas, PD98059 (PD), compound C (Comp. C) and PKC inhibitor cocktail (PKC) did not show any marked effect on inhibiting the telmisartan increased Aβ generation. DAPT thoroughly inhibited the telmisartan increased Aβ generation. (b) PI3K activator increased Aβ40 and Aβ42 generation significantly. (c) Telmisartan stimulated pAkt after 30 min treatment. (d) Wortmannin blocked telmisartan stimulated pAkt. (e) GW9662 (GW) did not block telmisartan increased Aβ generation. Error bars show means ± s.e.m., ***$P < 0.001$ by one-way ANOVA followed by a post hoc Bonferroni test.

Aβ generation and may increase the risk of cognitive decline and dementia. However, it has been reported that olmesartan administration inhibits cognitive decline by decreasing glucose and cholesterol levels and increasing neuroprotective factors [21]. It is possible that other effects, for example, decreasing glucose and cholesterol and increasing neuroprotective factors, can offset the neurotoxic effect of an increased Aβ42/Aβ40 ratio. The effect of olmesartan on cognitive deficit and dementia in APP transgenic mouse model needs to be examined.

Zhu et al. and Tian et al. demonstrated that Ang II had a significant role in increasing amyloidogenesis and tau pathology of AD [22,23]. These findings suggest that there is relation between AT1 and AD pathology and support our results. Once the AT1 receptor was activated, the Aβ generation could be increased. Although telmisartan increased Aβ40 and Aβ42 generation, it decreased the Aβ42/Aβ40 ratio, which is a causal factor for the onset of AD. Thus, the effect of telmisartan on AD pathogenesis needs to be determined.

AT1a is mainly activated by angiotensin II and its function can be carried out by the activation of MAPKs, PI3K, and other pathways [19]. We found that the PI3K pathway inhibitor, wortmannin, significantly reversed the increase in Aβ generation by telmisartan, whereas the other pathway inhibitors did not. These results suggest that telmisartan increase Aβ generation by stimulating the receptor related PI3K pathway. The intracellular amino acid of AT1 is near the carboxy terminal and the three intracellular loops of AT1. This provides an environment for the telmisartan to affect the intracellular signal transduction of AT1 after binding it [24]. There are substantial reports showing that telmisartan can affect the intracellular signal, for example, the PPARγ [25] and JNK pathway [26]. Therefore, after binding to the receptor, telmisartan is capable of affecting the downstream signal of AT1. AT1 is one kind of G protein-coupled receptors (GPCRs). It has been reported that the receptor blocker of GPCRs could activate the downstream signal of the receptor [27]. And our results also demonstrated that telmisartan could stimulate the downstream signal of AT1 and PI3K, pAkt, after 30 min treatment (Fig. 3c and d). Based on this evidence, it is possible that telmisartan affects AT1 as an agonist.

Based on the current reports, the ameliorative effect of ARBs on cognitive deficit is well-recognized. If one only considers that telmisartan increased Aβ generation, it is reasonable to conclude that telmisartan is related to the cognitive deficit and exacerbates the process of AD. In contrast, telmisartan decreased the Aβ42/Aβ40 ratio, which is considered as a causal factor of AD, and an increased Aβ42/Aβ40 ratio is sufficient to cause early onset AD [28]. The decreased Aβ42/Aβ40 ratio can be caused by the increased Aβ40 which is not the pathogenic form of Aβ and can inhibit the neurotoxicity and plaque formation of Aβ42 [29,30]. It is reasonable to deduce that the neuroprotective effect of telmisartan, which increased Aβ40, has a dominant role of offsetting the corresponding increased neurotoxic effect of Aβ42. Aβ is generated from the cleavage of APP by a β-secretase and γ-secretase-mediated cut, which is also called β-cleavage and γ-cleavage. γ-cleavage has a significant role for the Aβ40/Aβ42 ratio. We hypothesize that telmisartan regulates the γ-cleavage to modify the generation of Aβ40 and Aβ42 and further decreases the Aβ42/Aβ40 ratio, whereas, this effect was not found for the other ARBs examined. Thus, our results imply that ARBs play a significant role in the regulation of Aβ generation, and their relationship to the AD process needs to be determined.

Conflicts of interest

The authors declare no conflicts of interest.

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