Agonistic Autoantibodies to the $\alpha_1$-Adrenergic Receptor and the $\beta_2$-Adrenergic Receptor in Alzheimer’s and Vascular Dementia

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Introduction

With increasing life expectancy of humans in the industrialized countries, age-correlated dementia increasingly becomes a social and economic problem. Among the different forms of dementia, Alzheimer’s disease (AD) is the most common cause. Although recent research unravelled detailed molecular mechanisms of AD pathogenesis, the cause of the sporadic form is widely unknown, most likely because of its heterogeneous nature. Two proteins have been established as central pathomolecules, the plaque forming amyloid $\beta$ and the tangle composing hyperphosphorylated tau. Notably, the involvement of the $\beta_2$-adrenergic receptor ($\beta_2$-AR) in processing precursor proteins of amyloid-$\beta$ was recently reported [1]. Activation of $\beta_2$-AR resulted in enhanced plaque formation. The most obvious risk factor of AD is ageing. Other risk factors include hypercholesterolemia, hypertension, atherosclerosis, coronary heart disease, smoking, obesity and type 2 diabetes [2, 3]. There is evidence that the cognitive decline and central nervous system pathology may be secondary to pathologic alterations in the vasculature of AD patients’ brain [4]. Epidemiological, clinical pharmacological, neuroimaging and pathological studies support the notion that vascular disorders are the primary cause of sporadic, non-genetic AD.

The second most common form of dementia in older adults is vascular dementia. Often AD and vascular dementia coexist in older patients. The causes of this form of dementia are different, often diffuse disorders all leading to vascular lesions. People who suffered from ischaemic stroke developed dementia with a fourfold higher incidence than controls [5].

Abstract

Although primary causes of Alzheimer’s and vascular dementia are unknown, the importance of preceding vascular lesions is widely accepted. Furthermore, there is strong evidence for the involvement of autoimmune mechanisms. Here, we report the presence of agonistic autoantibodies directed at adrenergic receptors in the circulation of patients with mild to moderate Alzheimer’s and vascular dementia. In 59% of these patients, agonistic autoantibodies against the $\alpha_1$-adrenergic receptor and the $\beta_2$-adrenergic receptor were identified. The majority of positive patients (66%) contained both types of autoantibodies in combination. In a control group of patients with neurological impairments others than Alzheimer’s and vascular dementia, only 17% were found to harbour these autoantibodies. The autoantibodies to the $\alpha_1$-adrenergic receptor interacted preferably with the extracellular loop1 of the receptor. They were further studied in IgG preparations from the column regenerate of a patient who underwent immunoadsorption. The $\alpha_1$-adrenergic receptor autoantibodies specifically bound to the extracellular loop1 peptide of the receptor with an apparent $EC_{50}$ value of 30 nM. They mobilized intracellular calcium in a clonal cell line expressing the human form of the $\alpha_1$-adrenergic receptor. Our data support the notion that autoimmune mechanisms play a significant role in the pathogenesis of Alzheimer’s and vascular dementia. We suggest that agonistic autoantibodies to the $\alpha_1$-adrenergic and the $\beta_2$-adrenergic receptor may contribute to vascular lesions and increased plaque formation.
Adrenergic receptors (AR) belong to the superfamily of G protein-coupled receptors (GPCR), the most important cell surface receptors. AR mediate the action of catecholamines at the cellular level. They regulate numerous cellular functions such as muscle contraction including vascular tone. Circulating agonistic autoantibodies (agAAB) directed against AR have been found to be associated with different, mainly cardiovascular diseases in humans. It was shown that patients suffering from hypertension may contain agAAB predominantly to the $\alpha_1$-adrenergic receptor ($\alpha_1$-AR) or to the angiotensin II type 1 receptor $[6–8]$. There is growing evidence that these agAAB are of pathogenic relevance $[9, 10]$. In animal models, it was shown that agAAB to the $\alpha_1$-AR induce cellular remodelling and cause vascular damages $[11–13]$. Recently, we reported the presence of agAAB predominantly to the $\alpha_1$-AR in patients with type 2 diabetes $[14]$. Based on evidences of agAAB potency to cause vascular damages and of the crucial role of vascular disorders in AD and vascular dementia, we hypothesized the involvement of a GPCR-related autoimmune mechanisms in this disease. We show for the first time the presence of agAAB to the $\alpha_1$-AR and $\beta_2$-adrenergic receptor ($\beta_2$-AR) in patients with moderate to mild dementia of the AD and vascular type and studied properties of the $\alpha_1$-AR agAAB.

Materials and methods

Patients. Patient material was obtained from the Hospital of Internal Medicine/Geriatrics, Virchow-Clinics, Humboldt University Berlin. Serum samples from a collective of male and female older adults with mild to moderate dementia of the AD and vascular type (AD/vascular dementia) were investigated. Material from age-matched patients of both sexes with neurological impairments of other causes than AD/vascular dementia such as Parkinson’s disease, delirium and alcohol abuse syndromes served as controls. Patients included neither hypertension nor were under antihypertensive treatment. A third collective of patients investigated in the present study was from the German Heart Center, Berlin. These were outpatients with myocardial complications of different severity but no signs of neurological defects or cognitive impairments.

The column regenerate from immunoadsorption of a $\alpha_1$-AR agAAB positive patient with clinical signs of mild AD/vascular dementia was obtained from the Hospital of Internal Medicine/Geriatrics, Virchow-Clinics, Humboldt University Berlin. The regenerate contained the broad spectrum of patient’s immunoglobulins (IgG) at a concentration of about 2 mg protein per ml. For further use, aliquots of the regenerate were desalted and pre-buffered to phosphate-buffered saline (PBS) on Sepha-dex-G25 columns (PD-10 columns; GE Healthcare, Buckinghamshire, UK) and concentrated on Vivaspin 500 concentrators (Sartorius Stedim Biotech, Goettingen, Germany) to about the initial protein concentration. The IgG preparations were aliquoted and stored at $-20^\circ$ C.

Cell cultures. Cultures of neonatal cardiomyocytes were prepared from newborn, one to 3 days old rats essentially as described in $[15]$. Chinese hamster ovary cells stably transfected with the A-isofrom of the human $\alpha_1$-AR (CHO-$\alpha_1$) were kindly provided by Dr. Gerd Wallukat, Max Delbrueck Center for Molecular Medicine, Berlin. They were grown in F12 HAM medium supplemented with glutamine, 10% FCS and 1% penicillin streptomycin at 37 $^\circ$ C and 5% CO$_2$.

Detection of agonistic autoantibodies. Functional autoantibodies were detected utilizing the chronotropic responses of cultivated spontaneously contracting neonatal cardiomyocytes $[16]$. For monitoring the contraction rate of neonatal cardiomyocytes, a standardized bioassay based on computer-assisted recording of the contraction rate was employed. IgG fractions from serum samples were prepared according to a standard procedure as described in $[6]$. Patients IgGs were added to cultures of neonatal cardiomyocytes to a final dilution of 1:40. After incubation at 37 $^\circ$ C for 60 min, the contraction rate was measured. A total of up to 30 cells or cell clusters for each sample of a given immunoglobulin fraction were analysed. The chronotropic response to the exposition to patient IgGs compared to preceding control recordings is given as increases in the number of contractions per minute. Identification of the target GPCR type was performed using specific receptor antagonists, $10^{-7}$ M prazosin (Sigma-Aldrich, Deisenhofen, Germany) to block the $\alpha_1$-AR and $10^{-7}$ M ICI 188.551 (Sigma-Aldrich) for the $\beta_2$-AR.

Assessment of the receptor target site. For mapping the target site of agAAB at the $\alpha_1$-AR, the standardized, neonatal cardiomyocyte-based bioassay as described above was employed. IgG fractions were preincubated for 1 h at 4 $^\circ$ C with an excess of synthetic peptides (Bioysntan, Berlin, Germany) corresponding to amino acid sequences of the extracellular loop1 (LGYWAFLGRFVCN) or the extracellular loop2 (PAPEDEITCQINEE) of the human $\alpha_1$-AR (1 $\mu$g peptide per 1 $\mu$g IgG protein) to neutralize the respective agAAB population of the patient IgG preparation.

Binding experiments. The binding of patient IgG preparation to the extracellular loop1 peptide of the human $\alpha_1$-AR was characterized using ELISA techniques. Briefly, biotinylated peptides corresponding to either the first or the second extracellular loop of the human $\alpha_1$-AR were coupled to pre-blocked streptavidin-coated 96-well plates (Perbio Science, Bonn, Germany). All experimental steps were performed at room temperature. If not indicated otherwise, 5 $\mu$g of IgG protein was used and the
incubation time was 30 min. As secondary antibody, anti-human horseradish-conjugated anti-IgG was used. The antibody binding was visualized by the 1-Step Ultra TMB ELISA (Perbio Science). The optical density was quantified at 450 nm using a SLT Spectra multplate reader (TECAN, Crailsheim, Germany).

Analysis of intracellular calcium. The potential of patients IgG fractions to mobilize intracellular calcium (Ca²⁺) was assessed using Chinese hamster ovary cells stably transfected with the α1A-isofrom of the human 1-AR (CHO-α1). The cells were trypsinized and plated on black clear bottom 96-well plates suited for fluorescence measurements (Greiner, Frickenhausen, Germany) at a density of 30,000 cells per well. Next day, the medium was replaced by Hank’s balanced salt solution (HBSS; Sigma-Aldrich) buffered with 10 mM HEPES at pH 7.4 and incubated at 37 °C for 1 h. Then, cells were loaded with 5 μM FURA 2-AM or FURA PE3 (Merck Biosciences, Bad Soden, Germany) in the presence of 2.5 mM probenecide (Invitrogen, Karlsruhe, Germany) for 45 min at room temperature. After rinsing with HBSS, the cells were left for another 30 min to complete the enzymatic cleavage of the dye ester. Ca²⁺ was measured on a Victor X2 fluorescence multiplate reader (Perkin Elmer, Langenfeld, Germany) equipped with an injector unit. The cells were excited at wave lengths alternating between 340 and 380 nm, and the fluorescence signal emitted at 510 nm was recorded. Baseline Ca²⁺ of unstimulated cells was normalized to correct for variations between cell preparations. Data are given as relative units derived from the 340 nm/380 nm signal ratio.

Data analysis. Experimental data were analysed using GraphPad PRISM version 3.00 for Windows (GraphPad Software, San Diego, CA, USA). Statistical significant differences were determined with the unpaired Student’s t-test after data had been checked for normal distribution. Estimates for rate constants and EC₅₀ values were obtained by nonlinear regression.

Results

Analysis of serum samples from patients

A collective of 54 patients of both sexes with mild to moderate cognitive impairments because of AD/vascular dementia but without clinically manifested high blood pressure was recruited for the present study (Table 1). Bioassay analysis of IgG preparations from serum samples of these patients revealed that 59% (32 patients out of 54) reacted positively indicating the presence of agAAB. Experiments with specific receptor antagonists were performed to identify the targeted receptor type. The vast majority of bioassay-positive patients harboured agAAB specific to the 1-AR (91%). Eight positive patients carried agAAB to the 1-AR alone. Most of bioassay-positive patients (66%) were found to have two types of agAAB in combination, one directed against the 1-AR and one against the β₂-AR. There were only three patients who elicited agAAB activities solely towards the β₂-AR. Overall, β₂-AR agAAB were found in 75% of positive patients. In the age-related control group comprising patients with cognitive impairments others than AD/vascular dementia, only 2 out of 12 patients (17%) reacted positively in the bioassay (Table 1). One of them harboured both α1-AR and β₂-AR agAAB species, whereas one patient was positive solely for α1-AR. Thus, in patients with AD and vascular dementia, there was about 2.5-fold higher frequency of the occurrence of agAAB directed against the 1-AR and the β₂-AR.

For comparison, we analysed a collective of 36 patients of both sexes at the age of (mean ± SD) 67.8 ± 3.2 years who were under outpatient treatment with myocardial complications but had no signs of cognitive impairments. None of these patients was positive for agAAB against the 1-AR or the β₂-AR, but 11 (31%) harboured agAAB directed at the β₂-AR, which typically associate with dilated cardiomyopathy [16–18].

Identification agAAB target regions at the receptor

Hypertension-associated agAAB against the α1-AR described so far bind to the extracellular loop1 or loop2 of the receptor [11, 19]. To identify the target extracellular loops, we examined IgG preparations from three AD/vascular dementia patients positive for agAAB to the α1-AR in the cardiomyocyte contraction bioassay. In all three cases, the chronotropic response was abolished by preincubation of the IgG fraction with the loop1 peptide (Fig. 1). Preincubation with the loop2 peptide did not affect the chronotropic response to the application of patient IgG.

<table>
<thead>
<tr>
<th>Number</th>
<th>Age (mean ± SD)</th>
<th>MMSE (mean ± SD)</th>
<th>agAAB positive for AR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dementia</td>
<td>54</td>
<td>77 ± 10</td>
<td>23 ± 5</td>
</tr>
<tr>
<td>Control</td>
<td>12</td>
<td>83 ± 10</td>
<td>n.d.</td>
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</tbody>
</table>

*Mini-mental state examination.

This group includes patients with neurological impairments because of Parkinson’s disease, delirium, alcohol abuse and other heterogenous causes. These patients showed no signs of dementia and were not scored by MMSE.

n.d., not determined.
Data are given as means ± SEM. Measurements of each patient were performed in triplicates and repeated up to four times. IgG was abolished by preincubation with the L1 peptide. The measures of patient IgG preparations were studied and were found to be exclusively sensitive to the chronotropic response of spontaneously contracting neonatal cardiomyocytes to IgG preparations from AD/vascular dementia patients. Three patients were studied and were found to be exclusively sensitive to the L1 peptide. The chronotropic response to the application of patients’ IgG was abolished by preincubation with the L1 peptide. The measurements of each patient were performed in triplicates and repeated up to four times. Data are given as means ± SEM.

**Functional properties of agAAB to the α1-AR in patient IgG preparations**

Extensive studies of agAAB from patients are often hampered by the small volumina of serum samples routinely obtained for diagnostics. However, column regenerates from immunoadsorption provided sufficient quantities of IgG to study the agAAB to the α1-AR of the same patient in more detail. The patient who underwent immunoadsorption showed clinical signs of mild AD/vascular dementia with a mini-mental state examination (MMSE) score of 23. In the bioassay, the patient was positive for agAAB to the α1-AR, but negative for β2-AR agAAB.

**Binding studies**

Studies of the dependence of patient IgG binding on protein and time confirmed the presence of agAAB specific towards the first extracellular loop peptide of the α1-AR (Fig. 1A). There was no detectable binding to the second extracellular loop peptide. The binding to the first extracellular loop peptide of the α1-AR was linear up to 10 μg of IgG protein. The assessment of binding at 0.15 and 0.5 M sodium chloride revealed that the patient’s α1-AR agAAB pool consisted of antibodies with different avidity. In the presence of 0.5 M sodium chloride, binding was suppressed by about 40% compared to 0.15 M sodium chloride. The time course of binding typically showed a faster initial phase and slower second phase (Fig. 1B). It was best described by a two-phase exponential association with rate constants for the initial phase in the range of 0.5/minute. In competition experiments using increasing concentrations of the α1-AR loop1 peptide from 0.3 nM up to 5 μM, the IgG binding to the immobilized peptide was reduced dose-dependently (Fig. 1C). Nonlinear regression gave approximations for the EC_{50} value of about 30 nM.

**Measurement of intracellular calcium**

CHO-α1 cells stably expressing the human α1-AR were used to study the potential of patient IgG to affect Ca\textsuperscript{i}. Preliminary experiments demonstrated the efficient coupling of α1-AR activation to Ca\textsuperscript{i} mobilization in these cells. Figure 3A shows the effect of patient IgG on Ca\textsuperscript{i} in comparison with α1-AR activation by its agonist phenylephrine (PE). The application of patient IgG resulted in a rise of Ca\textsuperscript{i}. To exclude that the observed mobilization of Ca\textsuperscript{i} was because of a general IgG effect, the potency of a human control IgG preparation to rise Ca\textsuperscript{i} was compared to the action of patient IgG (Fig. 3B). The control IgG also mobilized Ca\textsuperscript{i} but to a lesser extent than the patient IgG. To test whether the increment in Ca\textsuperscript{i} caused by patient IgG requires the interaction with the α1-AR, the antibody preparation was preincubated with the α1-AR loop1 peptide. The peptide itself had no effect on Ca\textsuperscript{i} in CHO-α1. Preincubation with the peptide significantly reduced the elevation of Ca\textsuperscript{i} by patient IgG demonstrating the α1-AR-specific component of the patient IgG (Fig. 2C). However, there was a substantial remaining Ca\textsuperscript{i} mobilization obviously not depending on α1-AR activation. This was to be expected from data shown in Fig. 3B.

**Discussion**

Here, we report on the presence of a specific class of agAAB interacting with adrenergic GPCR in sera from patients with AD/vascular dementia. We identified these antibodies to be directed against the α1-AR and the β2-AR. AgAAB to the α1-AR were found in patients suffering from widespread diseases such as different types of hypertension [6, 7, 11, 19]. For the present investigations, patients with no clinically manifested hypertension were selected. Thus, the occurrence of agAAB associated with high blood pressure may be excluded. Therefore, the agAAB detected in the circulation of patients with AD/vascular dementia may be attributed to the disease.

Interestingly, agAAB to the α1-AR associated with AD/vascular dementia in patients investigated so far recognized loop1 of the α1-AR. This may suggest a preference of agAAB associated with AD/vascular dementia for the extracellular loop1 of the α1-AR. Further studies will be needed to elucidate whether α1-AR agAAB from these patients bind preferentially or exclusively to loop1 of the receptor. For comparison, in refractory hypertension, the specificity of α1-AR agAAB was with 24% and 27%
nearly equally distributed between the first and second extracellular loop, respectively [11].

AgAAB interacting with the $\alpha_1$-AR were shown to cause damages of the vasculature [13]. Considering the increasing evidences for the importance of vascular lesions in AD/vascular dementia, we therefore concentrated on agAAB to the $\alpha_1$-AR in the present study. The availability of larger quantities of IgG in the column effluent from immunoadsorption enabled us to study exemplarily properties of the agAAB population of the same patient. Notably, this patient proved positive for agAAB to $\alpha_1$-AR but negative for agAAB to the $\beta_2$-AR. The patient IgG elicited binding affinities towards the $\alpha_1$-AR loop1 peptide in the nanomolar range comparable to agAAB specific for the second extracellular loop of the receptor found in patients with refractory hypertension and to an antibody raised in rabbits against the putative receptor epitope [11, 20]. Our data from measurements of $\text{Ca}^2+$ gave evidence for the presence of a $\alpha_1$-AR-specific $\text{Ca}^2+$ raising component in the patient IgG preparation. The

Figure 2 Binding of patient IgG preparation to the immobilized $\alpha_1$-AR loop1 peptide. Shown are data from representative experiments run in triplicates. Values are corrected for unspecific binding. (A) Dependence of antibody binding on patient IgG protein. The given amount of IgG protein refers to 100 $\mu$l reaction volume ($\blacklozenge$) binding in the presence of 0.15 M NaCl, ($\blacktriangle$) binding in the presence of 0.5 M NaCl. (B) Time dependence of antibody binding. (C) Competition of binding by increasing concentrations of the free $\alpha_1$-AR loop1 peptide. Concentrations of the free peptide ranged from 0.3 nM to 5.0 $\mu$m. The patient IgG was preincubated with the free peptide in solution for 15 min.

Figure 3 Effect of patient IgG preparations (Pat IgG) on cytosolic calcium ($\text{Ca}^2+$) mobilization in CHO-$\alpha_1$ cells. The data represent mean values of independent measurements of three individual cell populations. (A) Time dependence of $\text{Ca}^2+$ mobilization by patient IgG compared to the $\alpha_1$-AR agonist phenylephrine (PE). The addition of patient IgG preparation and PE is indicated by the arrow. The additions were ($\blacklozenge$) 2.5 $\mu$g patient IgG; ($\blacktriangle$) 5 $\mu$g patient IgG; ($\blacklozenge$) 5 $\mu$M PE. (B) Effect of 5 $\mu$g human control IgG ($\bigtriangleup$) and 5 $\mu$g patient IgG ($\blacklozenge$) on $\text{Ca}^2+$ mobilization. The addition of IgG preparations is indicated by the arrow. (C) Inhibitory effect of preincubation of patient IgG with the free $\alpha_1$-AR loop1 peptide. Patient IgG was preincubated with 0.25 $\mu$M peptide for 15 min before addition to the cells. Peak values (five of each individual measurement) were taken for assessing the decrement in $\text{Ca}^2+$ mobilization. Data are given as means $\pm$ SEM (***P < 0.01).
$\alpha_1$-AR-specific agAAB apparently mobilized $Ca^{2+}$ from the same source as the receptor agonist PE. However, the utilized pathway is still obscure and remains to be elucidated. Our data obtained with preparations containing the broad spectrum of patient IgG suggest a complex action on $Ca_{\text{i}}$. Certain types of cell membrane Fc receptors couple to $Ca_{\text{i}}$ and may contribute to the effects observed with IgG preparations [21]. The potency of agAAB associated with AD/vascular dementia to elevate $Ca_{\text{i}}$ may be one pathogenic component of their action leading to cellular remodelling and dysfunction [12, 13]. Thus, the agAAB to the $\alpha_1$-AR may be considered candidate pathomolecules linked to vascular lesions, which are of crucial importance in the pathogenesis and progression of AD/vascular dementia. Furthermore, increased $Ca_{\text{i}}$ can facilitate amyloid toxicity in neurons by accelerating amyloid-$\beta$ formation which in turn leads to a progressive calcium overload finally resulting in cell death [22].

The second agAAB species identified in sera of AD/vascular dementia patients was directed at the $\beta_2$-AR. These agAAB stimulate the $\beta_2$-AR mimicking the physiological agonist. The $\beta_2$-AR was recently shown to regulate the protease $\gamma$-secretase that in sequential reactions with $\beta$-secretase cleaves the amyloid-$\beta$ precursor protein (APP) to the putative neurotoxic amyloid-$\beta$ [1]. The authors correlated chronic $\beta_2$-AR stimulation with increased plaque formation in an AD mouse model. Considering their longer biological half-life and their capability to disable cellular protective mechanisms, agAAB to GPCR may nearly permanently activate the target receptor, resulting in the case of the neuronal $\beta_2$-AR in enhanced plaque formation.

The contribution of autoimmunity to the pathology of dementia, both of the AD and the vascular type, has been an issue of controversies [23]. The brain belongs to the immune privileged organs of the body. A nearly ubiquitous occurrence of brain-reactive autoantibodies in human sera was reported, independent of any diseases [24]. Post-mortem immunostaining studies showed the penetration of IgG into the brain parenchyma of AD patients [24, 25]. This implicates a preceding impairment of the blood–brain barrier (BBB) that autoantibodies require to penetrate. Vascular impairments caused by agAAB to the $\alpha_1$-AR may contribute to compromise the BBB. This in turn would favour the penetration of agAAB to attack their respective target receptors at neurons. However, defective BBB is not an essential prerequisite of AD disease but can be attributed to an important subgroup of patients [26]. In these patients, agAAB may fully realize their pathogenic potential. Furthermore, vascular lesions are likely to decrease the clearance of $\beta$-amyloid from the central nervous system, thereby increasing the progression of the disease [27]. Future clinical studies on the removal of agAAB by immunoadsorption will unravel their significance for the progression and severity of AD/vascular dementia.

Investigations on postmortem brain tissue from AD patients led to the suggestion that IgG binding to the target neurons precedes morphological changes [25]. Thus, the detection of the potentially pathogenic agAAB may provide a valuable early marker to assess the risk for patients to develop AD/vascular dementia. This underscores the importance of adequate diagnostics of the individual patient. Consequently, personalized therapeutic strategies to specifically fight the action of agAAB may be employed. It was demonstrated that receptor antagonists are potent to abolish the action of agAAB thereby preventing end organ damages [10, 18]. Antagonists of the $\alpha_1$-AR are in therapeutic use, for example, in the treatment of benign prostate hyperplasia. However, they show diverse side effects. Clinically available antagonists specific to the $\beta_2$-AR are still lacking. A more costly but highly efficient strategy is immunoadsorption. It has been applied in patients with dilated cardiomyopathy and refractory hypertension with significant improvements of the clinical signs [11, 28, 29].

In summary, we demonstrated the presence of agAAB against the $\alpha_1$-AR and the $\beta_2$-AR in patients diagnosed to suffer from AD/vascular dementia. We characterized agAAB to the $\alpha_1$-AR to interact with the extracellular loop1. Their properties are comparable to agAAB associated with hypertension. We suggest these agAAB to be pathomolecules contributing to vascular lesions and increased plaque formation, two key factors in the pathogenesis and progression of AD/vascular dementia.

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