Introduction

One of the key pathological hallmarks of Alzheimer’s disease (AD) is the accumulation of amyloid β peptide (Aβ) which can be detected in the extracellular space, vasculature and within neurons. Intraneuronal accumulation of Aβ is suggested to be one of the earliest pathological events and has been shown to be associated with cognitive deficits and neurodegeneration (see [1] for review). Vascular risk factors are implicated in the aetiology of AD and hypertension in mid-life, in particular, they are associated with AD and cognitive decline (reviews [2-5]). In addition to epidemiological studies, neuropathological studies have also indicated that
there is a link between hypertension and risk of AD, via the demonstration that hypertension increases accumulation of Aβ.

However, whilst several studies have indicated that hypertension may play an important role in development of cognitive decline or AD there is conflicting evidence from the study of anti-hypertensives. In some [6-12, but not all [13-14]], observational studies to date, anti-hypertensive drugs slowed the incidence or rate of cognitive decline in patients with mild cognitive impairment (MCI) or AD. The disparities in these studies may reflect methodological limitations such as small sample size and low statistical power [15-16]. Of the most promising findings, have been those associated with the use of the anti-hypertensives, angiotensin converting enzyme inhibitors (ACE-Is) or angiotensin receptor blockers (ARBs). ARBs are associated with a lower incidence and rate of progression of AD and dementia than that seen in patients given ACE-Is or other anti-hypertensive medication [8]. Moreover, ARBs have also been reported to enhance cognitive function in normal people [17]. ARBs are newer, more expensive and less widely used than ACE-Is and as a result, preclinical and epidemiological data on their use and relevance in experimental models of dementia and in human subjects is currently limited. Nonetheless a recent trial involving 25,475 patients demonstrated a significant improvement in the mini-mental state examination score over a 6-month period of treatment with eprosartan [18]. Losartan also improved performance of immediate and delayed memory tasks in very elderly hypertensive patients [19]. These findings have prompted calls for further study of the utility of these drugs in preserving cognition or at least slowing the progression of dementia [8, 12, 20-22].

The mechanism by which anti-hypertensives may be beneficial is unclear and may be related to direct effects on lowering blood pressure and by improving blood flow, and there is evidence that they might have direct actions on Aβ accumulation. However these latter links are confounded by several in vitro, in vivo and ex vivo laboratory studies that demonstrate ACE promotes the degradation of Aβ [23-28] whereas ACE-I can result in an increase or accumulation of Aβ [24, 27]. These findings raise concern that long term use of ACE-Is may exacerbate accumulation of Aβ and perhaps accelerate cognitive decline [21, 27-29]. To date, there has been a lack of investigation of the long-term effects of ACE-I although one in vivo study has indicated that the ACE-I, captopril, has no effect on Aβ deposition in a mutant mouse model of AD [26].

The major vasotonic product of ACE activity, angiotensin II (AngII), is prevented from binding to its receptors by ARBs without altering ACE activity. ARB treatment has been shown to improve cognitive performance and reduce Aβ pathology in in vivo models of AD [27, 30-31] and while in animal studies valsartan reduced Aβ accumulation and improved cognitive performance in Tg2576 mice [27], telmisartan improved cognitive function in mice given intracerebral injections of Aβ1-40 [30], and olmesartan improved cognitive and cerebrovascular function in mice that were given intracerebral injections of Aβ1-40 (actions independent of the antihypertensive activity of the drug) and reduced cerebrovascular dysfunction in young APP23 mice [32]. Thus, ARBs may be more beneficial as a treatment strategy, acting to reduce Aβ load as compared to ACE-I that may increase accumulation of Aβ.

In view of the links between anti-hypertensives and Aβ accumulation and potential disparities between their modes of action, the present study sought to compare the effects of an ACE-I (captopril) and ARBs (valsartan, eprosartan) on Aβ accumulation. Since recent evidence emphasises the importance of intraneuronal and oligomeric Aβ as a precursor of degeneration and cognitive decline, we examined the effects of these compounds in a 3xTGAD mouse model of AD at an age in which intraneuronal Aβ is present but extracellular amyloid deposition absent. The accumulation of intraneuronal Aβ is associated with cognitive deficits in this model [34] and thus alterations in intraneuronal Aβ as a result of ACE-I or ARBs may be indicative of cognitive alterations.

Methods

Animals

Adult (3-4 months old), male triple transgenic (3xTGAD; PS1m146,KI; Thy1.2-APP swe; Thy1.2-tauP301L) mice [33] were used in this study. The animals were group-housed under a 12-hour light/dark cycle with access to food and water
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ad libitum until the day of the experiment. Room temperature in the animal house was maintained at approximately 21 ± 2°C. Mice (n=39 total; all groups n=10, except Valsartan n=9) were randomly assigned to a treatment group prior to study commencement. Researchers were blinded to drug treatment at all times. All animal procedures were performed according to the Animal Scientific Procedures Act of 1986 under license from the United Kingdom Home Office.

Drug treatment

The drugs were administered in drinking water to avoid the stress of repeated injections over a 2-month period and to mimic the human situation. The ARBs used were eprosartan (0.8g/l) and valsartan (0.17g/l) and the ACE-I was captopril (5g/l) and a control group received drinking water only. The doses selected were informed by pilot studies on C57Bl/6J mice in which each of the drugs was administered in several different concentrations (captopril 2, 5 and 10g/l; eprosartan 0.3, 0.6 and 0.9g/l, valsartan 0.05 and 0.2g/l) and mean arterial blood pressure (MABP) monitored over 28 days. None of the final doses selected had any significant effect upon MABP (Figure 1) and so hypotension and vascular insufficiency were removed as potential confounding factors. The dose of eprosartan (eprosartan monosodium salt, Solvay pharmaceuticals) equates to ~13mg/kg in humans, which is the maximum that may be prescribed in human patients (www.bnf.org). That of valsartan (valsartan monosodium salt, LGM Pharma) equates to approximately twice the average dose administered to human patients, and half the maximum prescribable dose and is comparable to previous studies in which doses of 10–40mg/kg inhibited Aβ accumulation in vitro and in vivo [27]. The dose of captopril (LGM Pharma) was approximately 30 times greater than the maximum dose normally prescribed for human patients.

The drugs used in this study are all weak acids, and are thus well absorbed in their non-ionised form from the stomach. To prepare the ARBs for administration in drinking water, they were first dissolved in 3-4ml sterile distilled water (dH2O) plus a small amount of 5M sodium hydroxide (NaOH) titrated to the amount of drug being used. This solution was vigorously vortexed until the drug was completely dissolved, then added to the appropriate volume of sterile dH2O with continuous stirring. The final solution was further neutralized to pH 7.0 by addition of 5M NaOH. Captopril was prepared by adding the drug to the appropriate volume of sterile dH2O and neutralizing to pH 7.0 by the addition of 5M NaOH with continuous stirring. All drinking solutions were freshly prepared twice each week, the containers wrapped in aluminium foil to avoid potential photochemical changes and kept at room temperature to avoid potential precipitation from solution. Fluid consumption was monitored twice weekly throughout the study.

Blood pressure measurement

MABP was monitored non-invasively with a tail sphygmomanometer (Panlab, L5002). MABP was measured at baseline, prior to the start of drug delivery, 2wk after drug delivery had begun and immediately before termination of the experiment (at 2mo). Ten measurements were taken each day and the average for each mouse determined from the last 5 measurements.
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Mice were habituated to the procedure for 5d before each measurement day to minimise stress and anxiety. Visual inspection of the pulse trace was carried out to ensure that movement artefacts had not occurred during the measurement; if these were detected the measurement was discarded. Any measurement that coincided with a heart rate of over 700 beats per minute (bpm) was discarded as this is indicative of excessive stress in the animal [34].

Tissue preparation

After 2mo the animals were anaesthetised with isoflurane and sacrificed by transcardial perfusion with cold saline. Brains were harvested and hemisected midsagittally. One hemisphere was snap frozen in liquid nitrogen for biochemical analyses, the other fixed in 4% paraformaldehyde for 48h for histology and immunohistochemistry. From each mouse, a kidney was isolated and snap-frozen for subsequent analysis of ACE activity.

ACE activity assay

ACE activity was determined using the fluorogenic substrate (Abz-FRK(Dnp)-P; BIOMOL). Brain tissue or kidney was homogenised in lysis buffer with Biospec 2.3mm Zirconia/Silica beads using a PrecellyS automatic homogeniser for 2 x 15s at 6000 rpm. The homogenates were then spun for 15min at 13000 rpm in a centrifuge refrigerated to 4°C. The protein concentration of each sample was measured using Total Protein kit (Sigma). This value was used to calculate the volume of each sample to be used subsequently in the ACE activity assay, in which the protein concentration in each sample was standardised to 50µg/ml total protein. Samples were diluted in HEPES buffer (595mg in 50ml dH2O; pH 6.5) and 50µl of each sample was incubated for 18h at 26°C with Abz-FRK(Dnp)-P. Triplicates of each sample were incubated with 10ug (100mM) captopril (Biomol International, Exeter, UK) which inhibits the reaction by over 90% [35]. The fluorescence of Abz-FRK(Dnp)-P was measured with excitation at 320nm and emission at 405nm in a 96-well plate using a FLUOstar OPTIMA plate reader, and the difference between captopril-inhibited and non-inhibited samples used to calculate ACE activity. ACE activity was expressed in arbitrary units. Treatment groups were compared by 1-way ANOVA, with significance set at P<0.05.

Immunohistochemistry

The fixed brain tissue was sliced coronally at 3mm intervals and the slices embedded in paraffin wax. Sections 6µm in thickness were collected onto Superfrost slides and dried on a hotplate. The sections were dewaxed and hydrated through a series of graded alcohols and washed in phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide in methanol for 30min. For labelling of Aβ, antigen retrieval was carried out by covering the sections in 80% formic acid for 10min. Antigen retrieval for APP was carried out by boiling the sections in citric acid buffer twice for 5min. Sections were blocked in normal horse serum for 1h at room temperature then incubated overnight with the anti-Aβ antibody 4G8 (Millipore) or anti-APP (Chemicon Clone 22C11), both diluted 1:1000, at 4°C. Sections were washed in PBS, incubated with anti-mouse biotinylated secondary antibody (1:100) for 1h at room temperature, washed and bound antibody visualised by the avidin/biotin method (ABC Elite kit, Vector laboratories) with diaminobenzidine (DAB) as the chromogen.

In 3xTGAD mice, aged 5-6mo, Aβ is detectable intraneuronally but at this age there is no extracellular deposition of Aβ. To quantify intraneuronal Aβ and APP, three non-overlapping images of the CA1 field of the hippocampus and three from somatosensory cortex were captured from each immunostained section [x40 magnification] and the percentage area of section labelled for Aβ or APP was determined using ImageJ software. Four separate sets of measurements were made for each animal in each region of interest and the mean for each region calculated. Treatment groups were compared by 1-way ANOVA with significance set at P<0.05.

Tissue homogenisation

Brains were homogenized in 10 volumes (wt:vol) of tissue homogenization buffer (THB; 250mM sucrose, 20mM Tris base, 1mM EDTA, 1mM EGTA). The homogenate was mixed 1:1 with 0.4% diethanolamine buffer (DEA; 200µl DEA, 1ml 5M NaCl, ddH2O to 50ml) and centrifuged at 135,000xg at 4°C for 1h. The supernatant was saved as the soluble fraction. The insoluble pellet was re-suspended in cold formic acid, briefly sonicated and spun at 135,000xg at 4°C for 1h. The resulting supernatant was
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Quantification of full length APP

Soluble proteins were extracted from brain tissue as described. 10µg was loaded onto a Tris-bis 4-12% gel and the proteins separated by electrophoresis at 150V for 1.5h. Each of 3 gels contained samples from the control group and samples from an individual drug treatment group, to avoid inter-gel comparisons. Proteins were transferred to a PVDF membrane and probed with MAB348 (Millipore) to detect full length APP and GAPDH antibody (Abcam) to assess protein loading. Bands were visualised with the appropriate Odyssey infrared secondary antibodies (IRDye; LiCor) and band intensity measured using Odyssey Infrared Imaging System (LiCor). APP measurements were adjusted for (the slight) variations in GAPDH band intensity. Each treatment group was compared to control using Student’s t test with significance set at P>0.05 and the mean for control and treatment group was calculated for each membrane and visualised in a histogram.

Levels of oligomeric Aβ

The levels of oligomeric Aβ were measured in the soluble fraction by dot blot analysis with the A11 antibody. 2µg of soluble protein was loaded in duplicate into the 96-well manifold of the bio-dot microfiltration apparatus (Bio-rad) below which a pre-wetted nitrocellulose membrane had been placed. Samples were rapidly filtered through the membrane, which was removed and placed into blocking buffer (Odyssey) for 1h at room temperature with constant agitation. Membranes were incubated overnight with A11 antibody at 4°C with constant agitation. After they had been washed, membranes were incubated with infrared secondary antibodies (IRDye; LiCor) for 45min at room temperature and scanned using Odyssey infrared Imaging System (LiCor). Relative optical density was measured and the mean for each sample calculated. Treatment groups were compared by 1-way ANOVA with Dunn’s post hoc multiple comparison test and significance set at P<0.05.

Statistical analysis

Statistical tests were performed with GraphPad Prism, version 5 (GraphPad Software, La Jolla, CA). Changes in MABP were analysed by repeated measures ANOVA. ACE activity and Aβ and APP immunolabelling were analysed by one-way ANOVA with Dunn’s test for post hoc comparisons between experimental and control groups. Statistical significance was set at P<0.05 for all analyses.
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Results

Mean arterial blood pressure

Mean arterial blood pressure (MABP) measurements at 2wk and 2mo after starting treatment were compared with that at baseline (Figure 2). There was no significant change in MABP in controls, mice given eprosartan (0.8g/litre) or valsartan (0.17g/litre). However captopril (5g/litre) induced a significant reduction in MABP in 3xTGAD mice at 2wk and 2mo after the commencement of treatment (p=0.0006, Figure 2).

ACE activity

ACE activity was assessed in homogenates of both brain and kidney

Figure 3. ACE inhibitor and ARB treatment does not affect ACE activity in the 3xTGAD mouse. 3xTGAD mice were treated with captopril (5g/litre), eprosartan (0.8g/litre), valsartan (0.17g/litre) and drinking water for 2 months. ACE activity was determined in brain (a) and kidney (b) tissue using a fluorogenic substrate for Angiotensin converting enzyme; ACE activity is expressed in arbitrary units.

Figure 4. ACE inhibitor and ARB treatment does not affect intraneuronal levels of amyloid or amyloid precursor protein (APP) in the 3xTGAD mouse. 3xTGAD mice were treated with captopril (5g/litre), eprosartan (0.8g/litre), valsartan (0.17g/litre) and drinking water for 2 months. Brain sections were stained for amyloid (a,c) and APP (b,d) and visualised in hippocampus CA1 (a, b) and somatosensory cortex (c, d). The percentage area stained was determined using computer-assisted image analysis (ImageJ).
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in all groups to determine the effects of treatment on central and peripheral ACE activity (Figure 3). ACE activity tended to be higher in the brain than the kidney. No significant effect of drug treatment on ACE activity was detected in either brain or kidney, this despite the clear physiological effect of captopril on blood pressure.

**Immunohistochemistry**

Intraneuronal Aβ and APP were detected in the hippocampus and cortex in the control treated group and drug-treated groups (Figure 4). No extracellular Aβ deposition was seen in any of the groups. Quantification of intraneuronal Aβ immunolabelling revealed only slight variation between the groups in the area of the CA1 field of the hippocampus which was immunopositive for Aβ, and the differences were not significant. There was greater variation in the cortex, where the labelling between groups varied by as much as 50%, being lowest in captopril-treated mice and highest in those given valsartan, but once again these differences did not reach statistical significance. APP labelling varied only slightly (and not significantly) between the groups in both the hippocampus and the cortex.

**Levels of APP and oligomeric Aβ**

The total protein levels of full-length APP were determined in Western blots prepared from brain tissue homogenates of all control and drug-treated mice. The protein levels of APP did not differ significantly between any of the drug exposure groups and controls (Figure 5).

Oligomeric Aβ could be detected by the antibody A11 as shown in Western blots of the soluble
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and not insoluble fraction in both 3xTGAD mice and AD patients (Figure 6). Subsequently the levels of total oligomeric Aβ species were compared between the control and drug-treated groups by dot blot analysis. There were no significant differences in the levels of oligomeric Aβ between the controls and any of the treatment groups.

Discussion

Reduction in blood pressure and improvements in cerebral blood flow have been the most commonly suggested mechanisms to explain observations that anti-hypertensive treatments may be beneficial in AD, although there has been some suggestion of a more direct action on Aβ accumulation. However, conflicting indications from pre-clinical studies suggest that ACE-Is, by inhibiting ACE, may have an adverse effect on Aβ [23, 25-26, 36-37]. This study investigated the potential impact of ACE-Is and ARBs on intraneuronal Aβ and oligomeric levels of Aβ in the 3xTGAD mouse model of AD.

Present findings in relation to previous research

Intraneuronal Aβ and APP were detected in all groups of mice, consistent with other reports on animals of the same age (5-6mo) in this transgenic model [33] and at this age there was no evidence of extracellular Aβ deposition in any of the groups at 6mo. Thus we were able to address whether anti-hypertensives had an effect on early intraneuronal and oligomeric Aβ. Our data robustly demonstrate that treatment over 2 months with an ACE-I, captopril, or two ARBs, valsartan and eprosartan; have no significant effect on the levels of soluble APP (sAPP), intraneuronal oligomeric Aβ or oligomeric Aβ levels. These observations partly agree with those of Hemming and colleagues [38] who also found no differences in levels of soluble and insoluble Aβ (total), Aβ40 and Aβ42 in captopril or losartan treated 3xTGAD mice as well as in an older captopril treated J20 transgenic mice. In contrast, perindopril, another reported brain-penetrating ACE-I, improved cognitive deficits in Aβ25-35-injected mice [39] and Aβ1-40-injected Sprague Dawley rats [24]. In both of these studies reductions in brain ACE activity were also reported after short treatment periods but the reductions did not approach the abolition of ACE activity reported by Hemming and colleagues [38].

In other studies using ARBs, Wang and colleagues found diminished Aβ pathology in Tg2576 mice treated with valsartan for 5mo which coincided with better cognitive performance [27]. Takeda and colleagues also showed that a short course of olmesartan (4-5 weeks) ameliorated cerebrovascular dysfunction and oxidative stress in young APP23 mice but did not coincide with any changes to soluble or insoluble levels of Aβ1-40 and Aβ1-42. Similarly

Figure 6. ACE-inhibitor and ARB treatment does not affect oligomeric Aβ species (A11) in young 3xTGAD mice treated with captopril (5g/litre), eprosartan (0.8g/litre), valsartan (0.17g/litre) and drinking water for 2 months. Levels of oligomeric Aβ were assessed by dot blot but there was no statistical difference in the levels of oligomeric Aβ determined between the drug-treated and control groups (a). Western blot analysis of Aβ as detected by A11 antibody showing presence of multiple oligomeric species in soluble (S) fractions, but not insoluble (IN) fractions, prepared from brain homogenates of Alzheimer disease (AD) cases and 3xTGAD mice (b).
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effects were also found by the same group in an Aβ1-40-injected mouse model [32].

Levels of brain and kidney ACE activity were comparable in this study in contrast to the findings of Hemming and colleagues [38] who used slightly younger (3mo) 3xTGAD mice which were treated for 28d with 2g/l of captopril and a different ARB (losartan at 0.6g/l). While the brain ACE activity data were similar between the studies, in that no differences were found across groups of animals, our kidney measurements differed in not showing a detectable reduction in ACE activity in captopril treated mice, despite a clear hypotensive effect. [38]. The ARBs used in both studies had no effects on ACE activity, although the losartan used by Hemming et al showed a modest reduction in ACE activity which we did not find for either valsartan or eprosartan.

In contrast to other studies we attempted to disentangle possible anti-hypertensive and other unknown drug specific effects on the disease phenotype by selecting from pilot studies the maximal drug doses that were well tolerated but did not alter mean arterial blood pressure (MABP) in otherwise normotensive wild-type mice. However the same doses applied to 3xTGAD mice evoked a significant progressive and sustained reduction (19%) in MABP (p=0.0006) in the captopril treated group although not in the valsartan or eprosartan groups. No blood pressure measurements were reported by Hemming and colleagues for the 3xTGAD mice [38]. The absence of any MABP-lowering effects from valsartan in our 3xTGADs is consistent with the observations of Wang et al on Tg2576 mice, in which valsartan (10 or 40mg/kg/d) ameliorated Aβ pathology and improved spatial memory deficits [27].

Possible explanations for the differences

Noteworthy in the current study was the lack of extracellular Aβ deposition in contrast to some reports in 3xTGAD mice in the age-range studied [33], and the lack of captopril-mediated reductions in ACE activity in the kidney [38]. Studies of other ACE-Is in other murine [39] and rodent [24] models documented reductions in brain ACE activity which neither this nor another study [38] of 3xTGAD found. The studies on ACE in 3xTGAD mice differed in the methods used. Hemming and colleagues [38] administered 2g/
and colleagues in 3xTGAD [38]. Although in our pilot studies this dose did not affect blood pressure in C57Bl6/J mice, it is clear that optimisation in 3xTGAD would have been advisable. Another limitation of the present study was that we did not assess cognitive function; Takeda et al [32] found that the ARB olmesartan improved spatial learning in APP23 mice despite a lack of change in Aβ level. In addition, the lack of change in brain ACE activity after systemic captopril administration does raise questions regarding the alleged BBB penetrability of captopril [10, 12]. The brain penetrating ACE-I perindopril was shown to reduce brain ACE activity in another study [39]. Finally it is worth considering that we focussed our study on the measurement of intraneuronal oligomeric Aβ and levels but arguably anti-hypertensives may exert beneficial effects via other mechanisms such as by improving cerebral blood flow and haemodynamics which would impact positively on vascular integrity.

Implications of the present findings

We found no evidence that ACE-Is had a detrimental effect on neuronal or total oligomeric Aβ or precipitated extracellular Aβ pathology in the 3xTGAD mice studied. This would suggest that if any of these compounds did exert an action on Aβ that at this age the Aβ was able to be metabolised or cleared efficiently. Data from other similar studies provide reassurance that ACE-Is may not increase AD pathology [38] and may improve cognitive and cerebrovascular function in people[27, 30-32].

Conclusions, including plans for future studies

The present study was limited to a short-term treatment strategy with different anti-hypertensives in a 3xTGAD model but longer-term treatments should be assessed to define in a number of models both familial and sporadic. Future studies should assess blood pressure, cerebrovascular function, cognitive performance and ACE activity in different cellular and tissue compartments, as well as a range of markers of AD pathology.

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