Published in final edited form as:

Neurobiol Dis. 2011 January ; 41(1): 62–70. doi:10.1016/j.nbd.2010.08.020.

L-type voltage-gated calcium channel blockade with isradipine as a therapeutic strategy for Alzheimer's disease

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Abstract

There is strong evidence that intracellular calcium dysregulation plays an important pathological role in Alzheimer's disease, and specifically that beta amyloid may induce increases in intracellular calcium and lead to neuronal cell dysfunction and death. Here we investigated the feasibility of modifying Alzheimer's pathology with the L-type voltage-gated calcium channel blockers verapamil, diltiazem, isradipine and nimodipine. All four compounds protected MC65 neuroblastoma cells from amyloid beta protein precursor C-terminal fragment (APP CTF)-induced neurotoxicity. Isradipine was the most potent blocker, preventing APP CTF neurotoxicity at nanomolar concentrations. Intracellular beta amyloid expression was associated with increased expression of Cav 1.2 calcium channels and increased intracellular calcium influx from the extracellular space. Despite the cytoprotection afforded by calcium channel blockers, amyloid beta oligomer formation was not suppressed. The mechanism of cell death in MC65 cells is appeared to be caspase-3 independent. With the goal of determining if there is sufficient experimental support to move forward with animal trials of isradipine, we determined its bioavailability in the triple transgenic mouse model of AD. Subcutaneous implantation of carrier-bound isradipine (3 µg/g/day) for 60 days resulted in nanomolar concentrations in both the plasma and brain. Taken together, our *in vitro* results support the theory that calcium blockers exert protective effects downstream of the effects of beta amyloid. Isradipine's neuroprotective effect at concentrations that are clinically relevant and achievable *in vitro* and *in vivo* suggests that this particular calcium blocking agent may have therapeutic value in the treatment of Alzheimer's disease.

Keywords

Amyloid precursor protein; Beta amyloid oligomers; Calcium channel blocker; Neurotoxicity

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Introduction

Alzheimer's disease (AD) is a progressive, age-related neurodegenerative disease that impairs memory and learning by affecting selective neuronal populations predominantly in the hippocampus and cerebral cortex. Accumulation of extracellular beta-amyloid (A β) and intracellular neurofibrillary tangles composed of hyperphosphorylated tau are the pathologic hallmarks of AD. In both early-onset familial and late-onset sporadic forms of AD, a 4-kDa A β peptide cleavage product of two key enzymatic reactions mediated by β and γ secretases has been considered a causal factor of AD (Selkoe, 2000; Selkoe, 2001; Selkoe, 2004; Tanzi and Bertram, 2005). The fragment sizes of A β vary between 39–43 amino acid residues, with A β 40 being the most common species and A β 42, a longer, hydrophobic species with a tendency to be more fibrillogenic. Recent studies demonstrate that a soluble form of oligomeric A β is more toxic than the monomeric form, and consequently a conscious effort has been made to reconstitute oligomers from monomers in the laboratory setting to demonstrate toxicity in neuronal cells (Li *et al.*, 2009; Picone *et al.*, 2009; Pitt *et al.*, 2009; Poon *et al.*, 2009; Sondag *et al.*, 2009). Evidence from animals models of AD places a particular importance on the accumulation of intraneuronal A β oligomers and their role in early pathogenesis of AD (Billings *et al.*, 2005; Golde and Janus, 2005).

Most of our current knowledge of A β oligomer toxicity appears to stem from studies that determine the effects of external application of reconstituted, synthetic oligomers. Although these studies have contributed greatly to our knowledge, models of AD that explore the pathomechanisms of intracellular A β oligomers have generally been limited. *In vitro* studies utilizing human neuroblastoma (MC65) cells appear to overcome, at least partially, the limitations of previous methods because they eliminate the need to externally apply A β oligomers. MC65 cells are stably transfected with the amyloid precursor protein (APP)-C99 gene and conditionally express a fusion protein composed of the amino-17 and carboxyl-99 residue-containing fragment of APP (Sopher *et al.*, 1996; Sopher *et al.*, 1994). Expression is controlled by a tetracycline-responsive promoter whose activity is repressed in the presence of tetracycline (Tet+). Removal of tetracycline (Tet-) leads to expression of the C-terminal APP fragment and subsequent processing of this fragment into A β followed by accumulation of intracellular A β oligomers, and precipitous cell death by 3–4 days (Sopher *et al.*, 1996; Sopher *et al.*, 1994). Specific inhibition of γ -secretase in these cells can rescue MC65 cells from death, further confirming that A β production triggers the neurotoxicity (Maezawa *et al.*, 2006). Various antioxidants and chemical chaperones provide protection against A β -induced death of MC65 cells and these neuroprotective effects are associated with prevention of oligomer formation (Hong *et al.*, 2007; Jin *et al.*, 2002; Lin *et al.*, 2009; Woltjer *et al.*, 2007; Woltjer *et al.*, 2005b).

The precise mechanism of A β mediated cell death in MC65 cells or in AD, however, is still unclear. The Ca²⁺ hypothesis suggests that intracellular calcium increase in response to A β oligomer formation may lead to neuronal cell dysfunction and death in AD (Bezprozvanny and Mattson, 2008). Mechanisms that perturb normal neuronal Ca²⁺ homeostasis include aberrant Ca²⁺ influx through plasma membrane channels including the L-type voltage-gated Ca²⁺ channels (L-VGCC) and N-methyl-D-aspartic acid (NMDA) receptors (Arispe *et al.*, 1993; Pollard *et al.*, 1993). Memantine, an antagonist of the NMDA- Ca²⁺ channel receptor, improves cognition and reduces AD-like neuropathology in mouse models of AD and has beneficial effects in AD patients (Martinez-Coria *et al.*, 2010). Thus, treatments that interrupt aberrant Ca²⁺ influx may be promising therapeutic strategies for AD. L-VGCC blockers (CCBs) have demonstrated neuroprotection from A β in several paradigms utilizing external application of A β . In this study, we tested whether CCBs could inhibit neurotoxicity due to intracellular A β in MC65 cells. We compared the potency of four CCBs, and finally evaluated

whether target brain levels could be achieved with the most promising candidate, isradipine, administered after the onset of AD pathology in a triple transgenic mouse model of AD.

Material and Methods

Materials

Cell culture media, L-glutamine, and trypsin/EDTA were obtained from Invitrogen (Carlsbad, CA); fetal bovine serum was from Atlanta Biologicals (Lawrenceville, GA); and calcium channel blockers (verapamil, diltiazem, nimodipine, and isradipine) and thapsigargin (TPG) were from Sigma (St. Louis, MO). 6E10 monoclonal antibody was obtained from Covance Laboratories (Princeton, NJ), L-type Ca^{++} CP α 1C antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and caspase-3 polyclonal antibody was from Cell Signaling Technologies, Inc (Danvers, MA). Isradipine pellets were obtained from Innovative Research of America (Sarasota, FL). Unless otherwise mentioned all other chemicals were obtained from Sigma.

Cell culture

Human neuroblastoma/MC65 cells were cultured in 75-cm² TC flasks or 100×20 mm petri dishes containing MEMalpha growth medium supplemented with fetal bovine serum (10%), L-glutamine (2 mM) and tetracycline (1 µg/ml), and maintained in a tissue culture incubator at 37°C and in the presence of 5% CO₂ as described (Wadsworth et al., 2008; Woltjer et al., 2005a; Woltjer et al., 2007). We limited our assays to the early cell passages.

Cell survival assay

During the initial testing, specific concentrations of calcium channel blocker (CCB) optimal for cell survival was determined by the MTS assay following the manufacturer's protocol (Promega). All CCBs were dissolved in ethanol (vehicle). For the MTS assay, cells were harvested from TC flasks at 90% confluence using 0.05% trypsin/EDTA, washed twice with PBS, and plated into 96-well plates at a density of 15×10^3 cells/well in OPTIMEM medium in the presence (Tet+) or absence (Tet-) of tetracycline (1 µg/ml) and vehicle. Cells grown under Tet- condition usually die after approximately 3–4 days in culture in association with expression of endogenously produced A β oligomers, but the cells grown under Tet+ condition fully survive during this period (Woltjer *et al.*, 2005b). Initially, we established cell survival responses to a range of CCB concentrations for 72 h and chose the following specific concentrations that provided maximal protection against cell death: verapamil (25 µM), diltiazem (25 µM), nimodipine (10 µM), and isradipine (0.05 µM). Subsequently, Tet- cells were grown under these pre-determined concentrations of CCBs. The wells containing growth medium without cells served as background control, and Tet+ with vehicle served as a positive control. Each treatment was replicated in 6 wells and treatments were applied for periods of 24, 48, and 72 hours in each experiment. After each treatment period, a 20 µl mixture per well of a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine methosulfate, PMS), at a ratio of 20:1 was added, and the plate was incubated in TC chamber at 37°C for 3–4 hours before the absorbance spectrum was measured at wave length 490 nm using Spectra Max PLUS plate reader (Molecular Devices Inc). Percent cell survival in different treatments was determined relative to cell survival in Tet+ treatment. Experiments were repeated three to five times, and the experimental averages were used for statistical analyses.

To visualize the extent of cell survival, MC65 cells were stained with Coomassie Brilliant Blue R250 (BioRad; catalog number 161-0436) following 24, 48, and 72 h after culturing under Tet+ and Tet- conditions following the methods of (Mochizuki and Furukawa, 1987) with some

modifications. Briefly, medium was aspirated from the cells grown in 6-well plates, they were rinsed gently with 2 ml PBS, and then 2 ml of stain was applied for 1 minute. Stain was removed and cells were washed twice with PBS. Aquamount (Thermo Fisher) was applied to the wet cells and they were photographed with a 10× objective using an Olympus BS50 microscope.

Intracellular calcium assay

Intracellular Ca^{2+} levels were determined using the Fluo-4 NW Ca^{2+} Assay Kit following the manufacturer's protocol (Molecular Probes) and with slight modification to the published methods (Anekonda and Adamus, 2008). Briefly, MC65 cells at a density of 15×10^3 cells/well were grown in black, 96-well culture plates with (Tet+) and without (Tet-) tetracycline and vehicle. The same set of CCB treatments presented in MTS assay described above were included in the Ca^{2+} assay, and both assays were performed in parallel from the same batch of cells. After the treatment period, Fluo-4 NW dye (100 μl) containing 2.5 mM probenecid was added directly into each well to prevent the dye into the extracellular space, and the plate was incubated in a TC chamber at 37°C for 30 min, followed by another 30 minutes at the room temperature. Intracellular Ca^{2+} levels for each treatment and control were measured in terms of relative fluorescence units in live cells (RFU) for 10 min at room temperature using the Victor³ 1420 Multilabel Counter (Perkin Elmer) with specific excitation (485/20 nm) and emission (535/20 nm). To confirm if the intracellular Ca^{2+} levels originated from the intracellular store endoplasmic reticulum (ER) or extracellular space, Ca^{2+} assays were conducted in the presence or absence of 2 μM thapsigargin (TPG), a non-competitive inhibitor of sarco-endoplasmic reticulum Ca^{2+} ATPase channel (SERCA).

The fluorescence values of different cultures may be variable depending on the cell density, the time of exposure to the probe, cell loading with the fluorescent dye, and fluctuations in room temperatures. Therefore, we took special care to calibrate fluorescent values of all other treatments within each experiment relative to the Tet+ treatment; this strategy eliminated errors associated with absolute measurements of fluorescence and assigned relative values meaningful for comparing treatments across different experiments. Potential fluorescence contributed from Ca^{2+} leak from dead/dying cells was ruled out by confirming that fluorescence from conditioned medium was not significantly different than blank medium. Experiments were repeated three to five times for a given treatment, and the experimental averages were used for statistical analyses.

Quantitative real-time PCR (qRT-PCR)

The cells were grown in 100×20 mm petridishes under Tet- and Tet+ conditions for 24–72 hours as mentioned above. Total RNA was isolated from the cells using Ambion RNAqueous and reverse transcribed using the Superscript III First-strand Synthesis System for RT-PCR following manufacturer's protocol (Invitrogen). Relative mRNA β expression was determined using the Taqman Gene Expression Assay with commercially-available primer probe sets for human Cav 1.2, an alpha 1C subunit of L-VGCC encoded by the CACNA1C gene and glyceraldehydes-3-phosphate dehydrogenase (GAPDH), a housekeeping gene. Plates were analyzed on a StepOnePlus Real Time PCR system using the ddCt method (Livak and Schmittgen, 2001). All real time PCR reagents were from Applied Biosystems Inc (Foster City, CA).

Calpain activity assay

Cell lysates were analyzed for calpain activity using the InnoZyme Calpain ½ Activity Assay Kit (Calbiochem) according to the manufacturer's protocol. Briefly, MC65 cells (Tet- and Tet+) were grown in 100×20 mm petri dishes for 24 and 48 hrs. After the growth period, medium was removed, and the cells were washed with PBS. Cells were lysed in 200 μl of lysis buffer (Tris, 62.5 mM, pH 6.8; sodium dodecyl sulfate, 2%; glycerol, 10%) and then scraped and

collected into 1.5 ml eppendorf tubes. Cell lysates were sonicated on ice for 5 sec, then 50 μ l aliquots incubated with activation buffer, reducing agent and calpain substrate for 30 min at room temperature. Fluorescence was read at an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a Victor³ 1420 Multilabel Counter. Mean fluorescence for duplicate samples was corrected for total protein measured by the BCA assay (Pierce). Human calpain 1 was used as a positive control.

Thioflavin T (ThT) fluorescence

A β 1-42 (American Peptide) was dissolved in PBS at a final concentration 0.25 mg/ml and incubated for 24 h at 37°C in a 96 well fluorescent plate in the presence of vehicle (PBS), 0.05 μ M isradipine (total volume of 150 μ l). Following this incubation, ThT was dissolved in DMSO and 3 μ l added to each well (final concentration 5 μ M). Fluorescence was determined in triplicate samples using a Victor³ 1420 Multilabel Counter at excitation and emission wavelengths of 440 and 490 nm, respectively. Sample fluorescence was determined by averaging the three readings and subtracting the fluorescence of a ThT blank. The intrinsic fluorescence of each individual compound in the absence of ThT was negligible.

Cell harvesting, protein quantification and western blotting

MC65 cells were grown under Tet⁻ and Tet⁺ condition and CCBs (diltiazem, 25 μ M; verapamil, 25 μ M; nimodipine, 10 μ M; isradipine, 0.05 μ M) in 100 $\times$ 20 mm petri dishes in 10 ml OPTIMEM (Woltjer *et al.*, 2005b). After 72 h, the medium was removed, cells were washed with PBS, lysed in 300 μ l of lysis buffer (Tris, 62.5 mM, pH 6.8; sodium dodecyl sulfate, 2%; glycerol, 10%) containing protease inhibitor cocktail set VII (Calbiochem, San Diego, CA) without DTT or bromophenol blue and then scraped and collected into 1.5 ml eppendorf tubes. The cells were sonicated on ice for 10 sec, boiled at 95°C for 5–10 min, allowed to cool to room temperature, and centrifuged at 14,000 rpm for 5 min. The supernatant was transferred to a new tube, the protein quantity in each sample was determined using BCA Assay (Pierce), and then lysates were stored at –20°C until further use. Cell lysates from different treatments were normalized to the same final concentration of total protein using the lysis buffer, and then adjusted to a final concentration of 50 mM DTT and 0.01% bromophenol blue. A β 20 μ g aliquot of protein was loaded onto a 10–20% or 16% acrylamide gel containing tricine (Bio-Rad) for fractionation and the proteins transferred to nitrocellulose membranes (Millipore). The membrane was probed with 6E10 mouse monoclonal antibody, L-type Ca⁺⁺ CP α 1C rabbit polyclonal antibody, and caspase-3 rabbit polyclonal antibody, or the loading control β -tubulin antibody; then, treated with appropriate secondary antibody. Finally, the membrane was exposed to enhanced chemiluminescence solutions, and visualized using the Molecular Imager Gel Documentation System and protein bands were quantified using Quantity One Software (Bio-Rad).

Brain bioavailability of isradipine in mice

A small cohort of 17-month-old female triple transgenic AD mice (3 $\times$ Tg-AD) were implanted with carrier-bound isradipine pellets (n=4, 3 μ g/g/day, 60-day release, Innovative Research of America) or placebo control pellets (n=3). Following isradipine treatment, animals were euthanized by CO₂ inhalation and cervical dislocation, blood collected and brains quickly removed, dissected and prepared for analysis as described (Quinn *et al.*, 2007). Cerebella were collected from both isradipine and vehicle treated mice for determining bioavailability of isradipine in the brain and plasma. All animal procedures were performed in accordance with animal protocols approved by the Institutional Animal Care and Use Committee at the Portland VA Medical Center.

Analysis of isradipine in plasma and brain tissue was performed using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) as described (Park *et al.*, 2009). Briefly,

5 μ l of internal standard (amlodipine, 0.1 ng/ μ l, Sigma) and 100 μ l of 1 N NaOH were added to either 100 μ l of plasma or 100 mg of cerebellum homogenized in an equivalent volume of PBS. Samples were vortex mixed for 1 min. Five ml of methyl-t-butyl ether was added to all the tubes, which were extracted by agitating for 10 min. Subsequently, the tubes were frozen at -80°C after centrifugation at 4000 rpm for 5 min. The upper organic layer was transferred to another set of clean glass tubes and evaporated to dryness under nitrogen gas. The dry residue was dissolved with 0.1 ml of a methanol: 20 mM ammonium acetate mixture (90:10, v/v). Five μ l of the reconstituted upper layer was directly injected into the LC-MS/MS system. Isradipine concentrations were normalized to amlodipine internal standard and based on a standard curve ranging from 10 to 5000 pg/ml isradipine (Sigma).

The LC/MS/MS system consisted of an Accela HPLC interfaced to a TSQ Quantum Discovery (ThermoFisher) mass spectrometer. The analytical column was a Synergi HydroRP C18 (4 μ , 150 $\times$ 3 mm, Phenomenex). The mobile phase consisted of 20 mM ammonium acetate (solvent A) and 20 mM ammonium acetate in methanol (solvent B). Initial conditions were 80% solvent B that was increased to 98% over 2 min and held for 8 min. The system was returned to 80% solvent B and equilibrated for 5 min. The flow rate was 0.25 ml/min. The mass spectrometer was equipped with an electrospray interface (ESI) and operated in the positive ionization mode. The source parameters were: spray voltage 3000; sheath and aux gas flow rates, 40 and 15, respectively; tube lens voltage, 218 V; capillary voltage, 35 V; and capillary temperature, 275 $^{\circ}\text{C}$. This system was set up in selected reaction monitoring (SRM) mode, monitoring the transitions m/z 372.1 $\rightarrow$ m/z 312.2 and m/z 408.8 $\rightarrow$ m/z 237.9, for quantification of isradipine and IS, respectively. Scan event settings were scan width 1.5 m/z , scan time 0.5s, collision energy 15 and Q1/Q3 peak width 0.7. Data acquisition and analysis were performed using the Xcalibur software version 2.0 (ThermoFisher).

Statistical Analysis

Statistical significance of the treatments, treatment durations, and their interaction effects were determined for cell survival and intracellular calcium using one-way or two-way analysis of variance, and with appropriate t-tests. We also conducted the Bonferroni post-hoc tests, comparing all other treatments with Tet+. The treatment difference is considered significant if $P \leq 0.05$. All statistical analyses were performed using GraphPad Prism 5 software.

Results

Calcium channel blockers (CCBs) protect MC65 cells from death

Cell survival in Tet- treatment was significantly ($P \leq 0.05$) lower than Tet+ at 24, 48, and 72 h (Fig. 1A & B). In agreement with our previous findings, cell death increased progressively in response to tetracycline withdrawal and became severe after 72 h, as a direct result of increased expression of intracellular A β oligomer species (Woltjer et al., 2007; Woltjer et al., 2005b). The survival response to different CCB treatments was variable. Survival in verapamil was significantly ($p < 0.05$) higher than Tet+ at 72 h. Diltiazem, isradipine, and nimodipine treatments showed significantly lower ($P < 0.05$) survival than Tet+ at 24h, with a similar trend for the latter two at 48 and 72 h. Although some degree of statistical differences exists for survival in different CCB treatments, at 72 h treatment more than 80% of cells survived, suggesting that all of CCBs provided acceptable levels of protection against APP CTF-induced neurotoxicity.

To test whether cell survival was associated with changes in intracellular Ca^{2+} levels, Ca^{2+} levels were simultaneously determined in cells exposed to neuroprotective concentrations of CCBs for 24–72 h and expressed as relative fluorescence units per live cells (RFU/live cells) in Fig. 1C. Intracellular Ca^{2+} levels were statistically similar across all treatments at 24 and 48

hours. At 72 h, Ca^{2+} levels were significantly ($P \leq 0.01$) greater in Tet⁻ than Tet⁺ and all four CCBs attenuated Ca^{2+} levels to that of Tet⁺ control. These results strongly suggest that cell death in Tet⁻ treatment at 72 h is associated with increased intracellular Ca^{2+} .

Fig. 1D and 1E, respectively, show cell survival and Ca^{2+} levels measured at 72 h for varying concentrations of isradipine (0.001 to 5 μM), the CCB that showed potent protective effects at nanomolar concentrations. At isradipine concentrations 0.05 and 0.10 μM , survival levels were 84 and 77%, respectively; but for concentrations ≤ 0.10 μM ($P \leq 0.05$) or ≥ 0.50 μM ($P \leq 0.001$), survival levels declined significantly (Fig. 1D). Ca^{2+} levels at isradipine concentrations 0.05 & 0.10 μM were similar to Tet⁺ levels, but at concentrations ≥ 0.50 μM or at 0.001 μM calcium levels increased significantly ($P \leq 0.05$) (Fig. 1E). These results indicate that Ca^{2+} levels are tightly controlled and cells respond to small variations in intracellular Ca^{2+} .

To clarify whether the cytoplasmic Ca^{2+} originated from the extracellular space or intracellular stores in response to Tet withdrawal, MC65 cells grown under Tet⁺ and Tet⁻ conditions were treated with 2 μM thapsigargin (TPG), a non-competitive inhibitor of the sarco-endoplasmic reticulum Ca^{2+} ATPase channel, just before the measurement of Ca^{2+} at 24 and 72 h (Fig. 1F). Ca^{2+} levels increased significantly ($P \leq 0.01$) in responses to TPG treatment in both Tet⁺ and Tet⁻ treatments at 72 h, but not at 24 h. These results confirm our previous findings on typical cell response to TPG (Anekonda and Adamus, 2008), and mainly suggest that Ca^{2+} levels in the intracellular stores were maintained in both Tet⁺ and Tet⁻ conditions, implying that increased intracellular Ca^{2+} primarily originated from the extracellular space.

Expression of calcium channels increase in response to A β production in MC65 cells

Next, we tested the effect of A β accumulation on the expression of L-type voltage-gated calcium channels/L-VGCCs (Cav1.2 and Cav1.3) in MC65 cells following tetracycline withdrawal using qRT-PCR. Fig. 2A shows that the expression of Cav1.2 mRNA, one of the α -subunits expressed in neurons (Sinnegger-Brauns et al., 2009), is similar in both Tet⁺ and Tet⁻ treatments at 24 hour, but at 48 and 72-hour the expression is significantly ($p < 0.05$) upregulated following Tet removal compared to Tet⁺. Another predominant neuronal α -subunit Cav1.3 was not detectable in these cells (results not shown). Fig. 2B shows the expression of Cav1.2 at the protein level in Tet⁺ and Tet⁻ treatments assessed by western blot at 24, 48, and 72 hours. Although the results are statistically non-significant, the trend in protein expression shows a time-dependent up-regulation of Cav1.2 in MC65 cells with slightly greater expression occurring in Tet⁻ than Tet⁺ at each time point. These results suggest that A β oligomers in Tet⁻ cells may have triggered the upregulation of Cav1.2 which in turn may have resulted in Ca^{2+} influx from the extracellular space and increased cytoplasmic Ca^{2+} seen in Fig. 1C.

Calpain activity increased non-significantly in response to tetracycline withdrawal

A calpain activity assay was performed to determine the downstream response to increased intracellular Ca^{2+} at 24 and 48 hours following Tet withdrawal. Relative to activity in Tet⁺ treatment, calpain activity in Tet⁻ increased by 34% and 88%, respectively, at 24 and 48 hours, but the increase was statistically non-significant (Fig. 2C).

Expression of caspase-3 is not affected by CCB treatment

To determine if the increased intracellular Ca^{2+} affected calcium-calpain-caspase-3-mediated apoptotic pathway, we measured caspase-3 expression in MC65 cells treated with Tet⁺, Tet⁻, and four CCBs at 48 hours after Tet withdrawal (Fig. 2D & E). The expression of caspase-3 (35 kDa pro-caspase-3 band and the cleaved fragments of 19 & 17 kDa that indicate the activity) was similar in all treatments, strongly suggesting that the cell death occurring due to Tet withdrawal is independent of caspase-3 expression. In support of findings of this study, a

previous study has shown that Tet withdrawal is independent of caspase-3 activation (Sebastian et al., 2006).

Isradipine protects from A β neurotoxicity despite the persistence of oligomers

The effects of isradipine on A β oligomer expression was determined using western blot analysis 72 hours after Tet withdrawal using 6E10 antibody against human A β (Fig. 3A & 3B). Products of the transfected C99 construct are at the low-molecular weight portion of the gel image (Fig. 3A). Tet withdrawal results in expression of endogenous APP and its derivatives, which are present in all samples, including Tet+, in the higher-molecular weight range of the blot. A β oligomers of approximately 16 and 44 kDa, the C-terminal 99 amino acid fragment (C99), and an 8-kDa band previously defined as either a dimer (P8) (Woltjer *et al.*, 2005b) or a combination of unknown proteins with some A β species (Maezawa *et al.*, 2006), was not substantially affected by treatment with isradipine. The results are in contrast to those obtained with antioxidant and protein chaperone treatments of MC65 cells. These substances are also associated with preserved cell viability but lead to markedly less aggregation to higher-molecular weight complexes such as the 16- and 44 kDa bands observed in this blot (Woltjer *et al.*, 2007).

Thioflavin T (ThT) exhibits enhanced fluorescence upon binding to A β fibrils. Fig. 3C shows that ThT fluorescence is statistically similar in isradipine and vehicle treated samples, suggesting that isradipine did not interfere with A β fibril formation *in vitro* and the effects of isradipine is downstream of A β oligomer formation.

Isradipine is bioavailable to the brain

Finally, to determine the brain availability and tolerability, isradipine (3 μ g/g/day; 60-day release) was subcutaneously administered to 3 $\times$ Tg-AD mice. Isradipine was well-tolerated as evidenced by average weekly body weights (an indirect measurement of toxicity), which remained statistically similar for vehicle and isradipine-treated animals (Vehicle group, 32.5 $\pm$ 2.8 g; Isradipine group 30.5 $\pm$ 2.3 g). The LC-MS/MS chromatographs in Fig. 4 compare the plasma (A & B) and brain (C & D) concentrations of isradipine in vehicle (A & C) and isradipine (B & D) - treated mice. The average plasma concentration of isradipine in treated mice was 33 $\pm$ 7 ng/ml and brain concentration 47 $\pm$ 1 ng/mg compared to no trace of isradipine found in the vehicle treated tissues. These results show that isradipine is available to the brain at concentrations that are relevant to the therapeutic plasma and brain concentrations for treatment of hypertension (5–10 ng/ml).

Discussion

Our study results demonstrate: 1) neuroprotection against A β toxicity by CCBs in a cell culture model of intracellular A β oligomer production, 2) efficacy of isradipine at nanomolar concentrations, and 3) generation of nanomolar brain concentrations of systemically administered isradipine in the 3 $\times$ Tg mouse model of Alzheimer's disease. Our results in MC65 cells for the first time clearly show that after endogenous generation of A β oligomers, these cells up-regulate the expression of L-VGCCs. Although our results suggest that Ca²⁺ influx occurs predominantly from the extracellular space, contribution by intracellular stores cannot be fully discounted. Because these cells are stably transfected with the APP-C99 gene and the conditional expression of the 99 residue-containing C-terminal APP fragment (Jin et al., 2002; Sopher et al., 1996; Sopher et al., 1994) is controlled by a Tet responsive promoter, this *in vitro* system provides an excellent opportunity to track whether the time-course of intracellular Ca²⁺ influx is associated with cell death. We and others have previously demonstrated that MC65 cell death occurs only after the formation of A β oligomers (Maezawa et al., 2006; Woltjer et al., 2005b), but the mechanism of cell death has not been resolved. The

oligomerization process is detectable by western blotting within 24 h after tetracycline withdrawal with prominent intracellular A β oligomers (16–34 kDa) occurring after 72 h of Tet withdrawal (Hong et al., 2007; Jin et al., 2002; Lin et al., 2009; Maezawa et al., 2006; Woltjer et al., 2007; Woltjer et al., 2005b). However, the role of increased intracellular calcium in this cell death is unclear. Here we show that intracellular Ca²⁺ increased 7.5-fold after Tet withdrawal relative to that observed in Tet+ after 72 h of culture (Fig. 1C). Hence, neurotoxicity associated with the accumulation of soluble A β oligomers is accompanied by markedly increased intracellular Ca²⁺. While A β toxicity has been shown to be dependent on calcium influx in other model systems, this is the first demonstration of this mechanism in MC65 cells. Cav1.2 mRNA expression increases 2-fold at 48 h (Fig. 2A), but this does not translate into increased intracellular calcium until 72 h (Fig. 1C). This disparity may occur because at earlier time-points, increased intracellular calcium may be buffered by mitochondria and the ER; however, by 72 hours, intracellular control of calcium homeostasis is overwhelmed. In addition, there may be a time-lag between the mRNA expression peak at 48 h and the protein expression peak occurring mainly at 72 hours (Fig. 2B). Upregulation of Cav1.2 in MC65 cells is consistent with the notion that A β peptides enhance Ca²⁺ channel activity and promote α_{1C} subunit insertion into the plasma membrane. This has previously been demonstrated in HEK293 cells expressing human LTCC α_{1C} subunits (Scragg et al., 2005).

The mechanism of cell death in MC65 cells (or in AD) is still unclear. Earlier studies in MC65 cells have proposed that death in these cells is associated with intracellular accumulation of free radicals or oxidative stress in response to A β oligomer production (Sopher et al., 1996; Sopher et al., 1994). Although a recent study suggests that MC65 cell death is independent of the caspase-3 pathway (Sebastia *et al.*, 2006), none of the previous studies have shown the expression pattern of caspases-3 and its cleavage products coinciding with the time of A β oligomer production. To confirm whether the cell death in MC65 is independent of calcium-calpain- caspase-3-mediated pathway, we assessed calpain activity and caspase-3 expression levels in these cells. Our results appear to support the previous findings (Sebastia *et al.*, 2006). Although intracellular Ca²⁺ influx does not evoke caspases-3-dependent apoptosis, the precise mechanism of cell death remains to be determined. It is possible that caspase-3-independent mechanisms such as, paraptosis (cytoplasmic vacuolation and mitochondrial swelling in the absence of caspases), autophagy (cellular housekeeping), or mitotic catastrophe (failure of mitosis) may contribute to MC65 cell death (Broker *et al.*, 2005).

Based on our current findings on intracellular Ca²⁺, and the results from previous studies in MC65 cells, we propose the mechanism detailed in Figure 5. Withdrawal of Tet leads to the expression of the 99-residue C-terminal APP fragment, which in turn is sequentially processed into A β monomers by the action of β -secretase and γ -secretase. A β monomers are oligomerized within the cell and lead to increased Cav1.2 expression, intracellular calcium and finally cell death. Treatment of cells with antioxidants has shown to reduce the production of intracellular oligomeric species (Wadsworth et al., 2008; Woltjer et al., 2007; Woltjer et al., 2005b). The CCBs used in the current study including isradipine did not affect the formation of A β oligomers, C99, or the P8 fragment, indicating a protective mechanism that does not specifically require alterations in C99 metabolism; rather, CCBs provided partial protection against cell death by attenuating Ca²⁺ channel expression and the influx of Ca²⁺ into the cytoplasm, a later step in the cytotoxic mechanism (Figure 5).

Our study shows that all CCBs protect MC65 cells and prevent A β -induced Ca²⁺-influx despite variable cytoprotection achieved by these agents (Fig. 1). *In vivo* studies seem to indicate that the CNS penetration of dihydropyridine class of CCBs is better than verapamil or diltiazem (Pfeiffer, 2010). Although some epidemiologic studies suggest that CCBs may prevent (Forette *et al.*, 1998) or slow the rate of progression of AD (Fritze and Walden, 1995; Tollefson, 1990), large clinical trials with nimodipine have shown no benefit for the treatment of AD

(Lopez-Arrieta and Birks, 2002). Because CCBs exhibit high first-pass metabolism, it is possible that low brain bioavailability accounts for the failure of nimodipine in clinical trials. Alternatively, failure may have been due to the selective stimulation of $A\beta_{1-42}$. A cautionary note to the proposed use of nimodipine in AD shows that nimodipine selectively stimulates secretion of $A\beta_{1-42}$ *in vitro* and in the plasma of Tg2576 mice via a mechanism independent of its Ca^{2+} -blocking effects (Facchinetti *et al.*, 2006). These negative trials will continue to inhibit further examination of an L-VGCC blocking strategy for AD unless an alternative CCB can be shown to be superior to nimodipine and other CCBs for this purpose. If our observations are to be translated to clinical trials, it will be necessary to propose a CCB which is superior to nimodipine in terms of brain bioavailability or other relevant features.

Isradipine is a well-tolerated CCB that is approved for human use in the treatment of hypertension and which is currently in use in clinical trials for Parkinson's disease. Interestingly, increased [3H]isradipine binding in CA1 area of hippocampus is associated with significant cell loss in the Alzheimer's brain relative to controls, suggesting an inverse relationship between neuronal loss and L-VGCC expression in the memory region (Coon *et al.*, 1999). Isradipine has not yet been tested as a therapeutic strategy for AD. For the purpose of clinical trials, it is essential that CCBs be administered in a fashion that achieves and maintains therapeutically-effective concentrations. Animal studies suggest that subcutaneous administration of CCBs results in therapeutically-effective doses. Subcutaneous isradipine (2.5 mg/kg for 7 days) antagonizes hypoxia-induced hippocampal damage and memory impairments in rats (Barhwal *et al.*, 2009) and subcutaneous doses of 3 mg/kg for 7 days protects dopaminergic neurons in an *in vivo* mouse model of Parkinson's disease (Chan *et al.*, 2007). Subcutaneous nimodipine (5–15 mg/kg/day) maintains plasma levels at or above therapeutic concentrations for treating vasospasm in a rabbit model of subarachnoid hemorrhage, whereas oral administration fails to do so (Laslo *et al.*, 2004). Our results showed that isradipine concentrations in the range of 50–100 nM may be useful in protecting MC65 cells (Figure 1). We also showed that a subcutaneous isradipine (3 mg/kg, 60 day release) administered after the onset of AD pathology is well-tolerated and is bioavailable to the brain. Since nanomolar plasma levels of isradipine are feasible in human subjects, these data strengthen the argument for clinical evaluation of isradipine in AD. The concentrations of isradipine that are found in the plasma and brain are higher than therapeutic plasma concentrations for the management of hypertension (5–10 ng/ml) and inhibition of L-VGCC subunits (IC_{50} for Cav1.2 = 2.6 ng/ml at –60 mV). Levels are also higher than required for the prevention $A\beta$ -induced cytotoxicity in MC65 cells (18 ng/ml). Our future studies are poised to address: (1) appropriate non-toxic doses of isradipine, and (2) if isradipine can modulate the toxic effects of $A\beta$ and tau pathologies in the mouse models of AD.

Conclusion

Intracellular Ca^{2+} dysregulation in AD is emerging as a unifying mechanism of disease pathology. While the current study is focused on L-VGCC blockers, it is important to emphasize that Ca^{2+} trafficking is much more complex than described here, involving numerous organelles and mediators. This study shows that $A\beta$ oligomers are strongly associated with increased intracellular Ca^{2+} and CCBs, especially isradipine, can prevent such an influx at nanomolar concentrations and protect MC65 cells. Furthermore, our results suggest the possibility that isradipine with ready bioavailability in brain tissue may have value in clinical trials of patients with or at risk for AD.

Research highlights

- Intracellular amyloid β expression induces calcium influx in MC65 cells

- A β neurotoxicity parallels intracellular calcium influx
- L-type voltage-gated calcium channel blockers prevent A β -associated neurotoxicity
- Isradipine appears to be the most potent calcium channel blocker
- Isradipine is bioavailable to the brain in nanomolar concentrations

Acknowledgments

This work was supported by NIH/NEI 5R21 EY 018708-02 grant to TSA, financial support to TSA by NIH T32 AT002688-05 grant (P.I. Dr. Barry Oken), Department of Veterans Affairs Merit Review Grant to JFQ, and NIH P30AG008017 as well as the OHSU Dean's Award to RLW and JFQ. We thank Dr. Dennis Koop, Director of the OHSU Bioanalytical/Pharmacokinetics Core Facility, for help with LC-MS/MS analysis.

References

- Anekonda TS, Adamus G. Resveratrol prevents antibody-induced apoptotic death of retinal cells through upregulation of Sirt1 and Ku70. *BMC Res Notes* 2008;1:122. [PubMed: 19046449]
- Arispe N, et al. Alzheimer disease amyloid beta protein forms calcium channels in bilayer membranes: blockade by tromethamine and aluminum. *Proc Natl Acad Sci U S A* 1993;90:567–571. [PubMed: 8380642]
- Barhwal K, et al. Isradipine antagonizes hypobaric hypoxia induced CA1 damage and memory impairment: Complementary roles of L-type calcium channel and NMDA receptors. *Neurobiol Dis* 2009;34:230–244. [PubMed: 19385055]
- Bezprozvanny I, Mattson MP. Neuronal calcium mishandling and the pathogenesis of Alzheimer's disease. *Trends Neurosci* 2008;31:454–463. [PubMed: 18675468]
- Billings LM, et al. Intraneuronal A β causes the onset of early Alzheimer's disease-related cognitive deficits in transgenic mice. *Neuron* 2005;45:675–688. [PubMed: 15748844]
- Broker LE, et al. Cell death independent of caspases: a review. *Clin Cancer Res* 2005;11:3155–3162. [PubMed: 15867207]
- Chan CS, et al. 'Rejuvenation' protects neurons in mouse models of Parkinson's disease. *Nature* 2007;447:1081–1086. [PubMed: 17558391]
- Coon AL, et al. L-type calcium channels in the hippocampus and cerebellum of Alzheimer's disease brain tissue. *Neurobiol Aging* 1999;20:597–603. [PubMed: 10674425]
- Facchinetti F, et al. Nimodipine selectively stimulates beta-amyloid 1–42 secretion by a mechanism independent of calcium influx blockage. *Neurobiol Aging* 2006;27:218–227. [PubMed: 16399208]
- Forette F, et al. Prevention of dementia in randomised double-blind placebo-controlled Systolic Hypertension in Europe (Syst-Eur) trial. *Lancet* 1998;352:1347–1351. [PubMed: 9802273]
- Fritze J, Walden J, et al. Clinical findings with nimodipine in dementia: test of the calcium hypothesis. *J Neural Transm Suppl* 1995;46:439–453. [PubMed: 8821080]
- Golde TE, Janus C. Homing in on intracellular A β ? *Neuron* 2005;45:639–642. [PubMed: 15748837]
- Hong HS, et al. Combining the rapid MTT formazan exocytosis assay and the MC65 protection assay led to the discovery of carbazole analogs as small molecule inhibitors of A β oligomer-induced cytotoxicity. *Brain Res* 2007;1130:223–234. [PubMed: 17157826]
- Jin LW, et al. Novel tricyclic pyrone compounds prevent intracellular APP C99-induced cell death. *J Mol Neurosci* 2002;19:57–61. [PubMed: 12212794]
- Laslo AM, et al. Subcutaneous administration of nimodipine improves bioavailability in rabbits. *J Neurosci Methods* 2004;139:195–201. [PubMed: 15488232]
- Li S, et al. Soluble oligomers of amyloid Beta protein facilitate hippocampal long-term depression by disrupting neuronal glutamate uptake. *Neuron* 2009;62:788–801. [PubMed: 19555648]
- Lin CL, et al. Epigallocatechin gallate (EGCG) suppresses beta-amyloid-induced neurotoxicity through inhibiting c-Abl/FE65 nuclear translocation and GSK3 β activation. *Neurobiol Aging* 2009;30:81–92. [PubMed: 17590240]

- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402–408. [PubMed: 11846609]
- Lopez-Arrieta JM, Birks J. Nimodipine for primary degenerative, mixed and vascular dementia. *Cochrane Database Syst Rev*. 2002 CD000147.
- Maezawa I, et al. A novel tricyclic pyrone compound ameliorates cell death associated with intracellular amyloid-beta oligomeric complexes. *J Neurochem* 2006;98:57–67. [PubMed: 16805796]
- Martinez-Coria H, et al. Memantine improves cognition and reduces Alzheimer's-like neuropathology in transgenic mice. *Am J Pathol* 2010;176:870–880. [PubMed: 20042680]
- Mochizuki Y, Furukawa K. Application of coomassie brilliant blue staining to cultured hepatocytes. *Cell Biol Int Rep* 1987;11:367–371. [PubMed: 2440589]
- Park JH, et al. Quantification of isradipine in human plasma using LC-MS/MS for pharmacokinetic and bioequivalence study. *J Chromatogr B Analyt Technol Biomed Life Sci* 2009;877:59–64.
- Pfeiffer RF. Parkinson disease: calcium channel blockers and Parkinson disease. *Nat Rev Neurol* 2010;6:188–189. [PubMed: 20379202]
- Picone P, et al. Abeta oligomers and fibrillar aggregates induce different apoptotic pathways in LAN5 neuroblastoma cell cultures. *Biophys J* 2009;96:4200–4211. [PubMed: 19450490]
- Pitt J, et al. Alzheimer's-associated Abeta oligomers show altered structure, immunoreactivity and synaptotoxicity with low doses of oleocanthal. *Toxicol Appl Pharmacol* 2009;240:189–197. [PubMed: 19631677]
- Pollard HB, et al. A new hypothesis for the mechanism of amyloid toxicity, based on the calcium channel activity of amyloid beta protein (A beta P) in phospholipid bilayer membranes. *Ann N Y Acad Sci* 1993;695:165–168. [PubMed: 8239277]
- Poon WW, et al. beta-Amyloid impairs axonal BDNF retrograde trafficking. *Neurobiol Aging*. 2009
- Quinn JF, et al. Chronic dietary alpha-lipoic acid reduces deficits in hippocampal memory of aged Tg2576 mice. *Neurobiol Aging* 2007;28:213–225. [PubMed: 16448723]
- Scragg JL, et al. Alzheimer's amyloid peptides mediate hypoxic up-regulation of L-type Ca²⁺ channels. *FASEB J* 2005;19:150–152. [PubMed: 15494446]
- Sebastian J, et al. Carboxyl-terminal fragment of amyloid precursor protein and hydrogen peroxide induce neuronal cell death through different pathways. *J Neural Transm* 2006;113:1837–1845. [PubMed: 16752047]
- Selkoe DJ. Toward a comprehensive theory for Alzheimer's disease. Hypothesis: Alzheimer's disease is caused by the cerebral accumulation and cytotoxicity of amyloid beta-protein. *Ann N Y Acad Sci* 2000;924:17–25. [PubMed: 11193794]
- Selkoe DJ. Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev* 2001;81:741–766. [PubMed: 11274343]
- Selkoe DJ. Alzheimer disease: mechanistic understanding predicts novel therapies. *Ann Intern Med* 2004;140:627–638. [PubMed: 15096334]
- Sinnesger-Brauns MJ, et al. Expression and 1,4-dihydropyridine-binding properties of brain L-type calcium channel isoforms. *Mol Pharmacol* 2009;75:407–414. [PubMed: 19029287]
- Sondag CM, et al. Beta amyloid oligomers and fibrils stimulate differential activation of primary microglia. *J Neuroinflammation* 2009;6:1. [PubMed: 19123954]
- Sopher BL, et al. Neurodegenerative mechanisms in Alzheimer disease. A role for oxidative damage in amyloid beta protein precursor-mediated cell death. *Mol Chem Neuropathol* 1996;29:153–168. [PubMed: 8971693]
- Sopher BL, et al. Cytotoxicity mediated by conditional expression of a carboxylterminal derivative of the beta-amyloid precursor protein. *Brain Res Mol Brain Res* 1994;26:207–217. [PubMed: 7854049]
- Tanzi RE, Bertram L. Twenty years of the Alzheimer's disease amyloid hypothesis: a genetic perspective. *Cell* 2005;120:545–555. [PubMed: 15734686]
- Tollefson GD. Short-term effects of the calcium channel blocker nimodipine (Bay-e-9736) in the management of primary degenerative dementia. *Biol Psychiatry* 1990;27:1133–1142. [PubMed: 2187540]
- Wadsworth TL, et al. Evaluation of coenzyme Q as an antioxidant strategy for Alzheimer's disease. *J Alzheimers Dis* 2008;14:225–234. [PubMed: 18560133]

- Woltjer RL, et al. Proteomic determination of widespread detergent-insolubility including Abeta but not tau early in the pathogenesis of Alzheimer's disease. *FASEB J* 2005a;19:1923–1925. [PubMed: 16129700]
- Woltjer RL, et al. Effects of chemical chaperones on oxidative stress and detergent-insoluble species formation following conditional expression of amyloid precursor protein carboxy-terminal fragment. *Neurobiol Dis* 2007;25:427–437. [PubMed: 17141508]
- Woltjer RL. Role of glutathione in intracellular amyloid- α precursor protein/carboxy-terminal fragment aggregation and associated cytotoxicity. *J Neurochem* 2005b;93:1047–1056. [PubMed: 15857408]

Figure 1.

Effects of L-type voltage-gated Ca^{2+} channel blockers (CCBs) on survival and intracellular calcium levels in MC65 cells. Cells grown for 24 to 72 hours in the presence (Tet+) or absence (Tet-) of tetracycline and without CCBs served as positive and negative controls, respectively. (A) Effects of CCBs on cell survival. Cells were grown in Tet+, Tet-, and Tet- condition for 24, 48, and 72 h under the following CCB treatments: verapamil (25 μM), diltiazem (25 μM), nimodipine (10 μM), and isradipine (0.05 μM). Cell survival was assessed using the MTS assay after each treatment period. (B) MC65 cells were stained with Coomassie Blue at 24, 48, and 72 h after exposing to Tet+ and Tet- conditions. Live cells attached to the bottom of the slide are shown in the image. (C) Effects of CCBs on intracellular Ca^{2+} levels. Cells were cultured and treated as in (A). Intracellular Ca^{2+} levels in live cells were assessed as relative fluorescence units (RFU/Live cells) after each treatment period. (D) Effects of different concentrations of isradipine (0.001–5 μM) on survival levels in MC65 cells grown for 72 hours. Cell survival was assessed using the MTS assay. (E) Effects of different concentrations of isradipine (0.001–5 μM) on intracellular Ca^{2+} levels in MC65 cells grown for 72 hours. Intracellular Ca^{2+} in live cells was assessed as relative fluorescence units (RFU/Live cells). (F) Effects of thapsigargin (TPG; 2 μM) treatment on intracellular Ca^{2+} levels in MC65 cells cultured under Tet+ and Tet- conditions for 24 and 72 h. Each treatment was replicated on average 6 times in each of 3–5 experiments, and the error bars represent an average percentage of cell survival for the experiments. Pair-wise comparison with Bonferroni post-hoc tests were made on percent survival between Tet+ and other treatments. Statistical significance levels: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 2.

Expression of Ca^{2+} channels in MC65 cells and the downstream effects of increased Ca^{2+} . (A) mRNA expression of Ca^{2+} channel Cav1.2 was determined by qRT-PCR. (B) Cav1.2 protein expression was determined by western blot (representative blot shown for three extracts from each time point) and quantified by densitometry. MC65 cells were grown for 24, 48, or 72 hours under Tet+ and Tet- treatments. (C) Calpain activity was assessed in the cells grown for 24 and 48 hours under Tet+ and Tet- treatments. (D) Expression of caspase-3, an executioner protein diagnostic of mitochondria-mediated apoptosis, was determined by western blot. The cells were grown for 48 h under Tet+ and Tet- in the presence of vehicle (Veh) or the following CCBs (μM): diltiazem (Dil, 25), verapamil (Ver, 25), nimodipine (Nim, 10), and isradipine (Isr, 0.05). The blot was probed with anti-caspase-3 polyclonal antibody. (E) Densitometry analysis was performed on a 35 kDa pro-caspase-3 and the bands originating from cleaved fragments of 19 and 17 kDa. The bars represent average for each band relative to Tet+ band and adjusted for the loading control β -tubulin. Error bars are $\pm$ SEM.

Figure 3.

Effects of isradipine on the expression of Aβ oligomers and Aβ fibril formation. (A) Expression of Aβ oligomers in MC65 cells was determined by western blot. The cells were grown under Tet+ and Tet- in the presence of vehicle (Veh) or isradipine (Isr, 0.05 μM). The proteins were fractionated on 16% tricine gels and transferred to nitrocellulose membranes. The membrane was probed with 6E10 monoclonal antibody to Aβ oligomers. Tet+ and Tet-, respectively, indicate the presence and absence of tetracycline in the medium, and the cells were treated with CCBs for 72 h. APP= amyloid precursor protein. Aβ oligomers appear in the range of 16 and 44 kDa. C99 = C-terminal 99 amino acid-fragment, and P8 = an 8-kDa Aβ domain containing fragment. (B) Densitometry analysis of 16–32 kDa range Aβ oligomers. The bars represent average for each band relative to Tet+ Veh band and adjusted for the loading control β-tubulin. Error bars are ± SEM. (C) Thioflavin T (ThT) fluorescence assay to determine the effect of vehicle and isradipine (0.05 μM) on Aβ fibril formation.

Figure 4.

The bioavailability of isradipine in the plasma and brain homogenates of 3xTg-AD mice treated subcutaneously with isradipine (N=4) or vehicle pellets (N=3) for 60 days. (A) Vehicle plasma, (B) Treated plasma, (C) Vehicle brain, (D) Treated brain. The selective reaction monitoring (SRM) transitions shown as mass-to-charge ratios (m/z) are for isradipine 372-312 and internal standard (amlodipine) 409-238 for each set. The retention time for isradipine was 5.5 min and amlodipine 5.0.

Figure 5.

A proposed mechanism of cell death due to increased intracellular Ca^{2+} in MC65 cells. A C-terminal 99-amino acid-fragment (C99) is sequentially processed by β -secretase and γ -secretase enzymes to generate A β monomers, which in turn oligomerize rapidly inside the cell. A β oligomers increase intracellular Ca^{2+} levels and ultimately cause cell death. CCBs used in this study attenuated the intracellular Ca^{2+} rise without influencing A β formation or oligomerization. In stark contrast to the effects of CCBs, previously published studies have shown that antioxidants lead to inhibition of both A β oligomerization and subsequent cell death.