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The K_{ATP} Channel Activator Diazoxide Ameliorates $A\beta$ and Tau Pathologies and Improves Memory in the 3xTgAD Mouse Model of Alzheimer's Disease

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Abstract

Compromised cellular energy metabolism, cerebral hypoperfusion and neuronal calcium dysregulation are involved in the pathological process of Alzheimer's disease (AD). ATP-sensitive potassium (K_{ATP}) channels in plasma membrane and inner mitochondrial membrane play important roles in modulating neuronal excitability, cell survival, and cerebral vascular tone. To investigate the therapeutic potential of drugs that activate K_{ATP} channels in AD we first characterized the effects of the K_{ATP} channel opener diazoxide on cultured neurons, and then determined its ability to modify the disease process in the 3xTgAD mouse model of AD. Plasma and mitochondrial membrane potentials, cell excitability, intracellular Ca^{2+} levels and bioenergetics were measured in cultured cerebral cortical neurons exposed to diazoxide. Diazoxide hyperpolarized neurons, reduced the frequency of action potentials, attenuated Ca^{2+} influx through NMDA receptor channels, and reduced oxidative stress. 3xTgAD mice treated with diazoxide for 8 months exhibited improved performance in a learning and memory test, reduced levels of anxiety, decreased accumulation of $A\beta$ oligomers and hyperphosphorylated tau in the cortex and hippocampus, and increased cerebral blood flow. Our findings show that diazoxide can ameliorate molecular, cytopathological and behavioral alterations in a mouse model of AD suggesting a therapeutic potential for drugs that activate K_{ATP} channels in the treatment of AD.

Keywords

K_{ATP} channels; diazoxide; hyperpolarization; calcium; excitotoxicity; cerebral blood flow; 3xTgAD; hippocampus; learning and memory

Introduction

Although the initiating factors and sequence of molecular and cellular events leading to the dysfunction and death of neurons in Alzheimer's disease (AD) are not fully understood, the accumulation of amyloid β -peptide ($A\beta$) and hyperphosphorylated tau, oxidative stress, impaired energy metabolism and a disruption of neuronal Ca^{2+} regulation are implicated [1-4]. In addition, cerebral neurovascular alterations that result in reduced cerebral blood flow (CBF), impaired blood brain barrier (BBB) function and reduced glucose utilization have been shown to occur early in the disease process [5,6]. The reduced energy availability and increased oxidative stress that occur in AD may promote neuronal membrane depolarization and overactivation of glutamate receptors resulting in excitotoxic damage to the neurons [2,4]. Membrane depolarization promotes release of the excitatory neurotransmitter glutamate which then activates receptors resulting in the influx of Ca^{2+} through the N-methyl-D-aspartate (NMDA) receptor channel [7]. Excessive and sustained increases in the intracellular Ca^{2+} concentration in neurons may impair synaptic function, and damage and kill neurons in AD [4]. The role of NMDA receptor overactivation in excitotoxic damage to neurons led to the development the NMDA receptor channel blocker memantine as a therapeutic that can slow cognitive decline in some patients with moderate to severe AD [8]. However, the benefit of memantine is limited, and there is a need for other agents that protect neurons and improve cognitive function.

ATP-sensitive potassium (K_{ATP}) channels are multi-protein complexes consisting of inwardly rectifying K^+ channel subunits (Kir6.1 or Kir6.2) and sulfonylurea receptors subunits (SUR1, SUR2A or SUR2B) [9]. K_{ATP} channels in the plasma membrane play important roles in reducing neuronal excitability in cardiac cells, neurons and other excitable cells [10,11]. K_{ATP} channels are also present in the mitochondrial inner membrane, although the molecular composition of such mito- K_{ATP} channels remains to be established [12,13]. Data suggest that mito- K_{ATP} channels participate in the regulation of mitochondrial volume and mitochondrial membrane potential ($\Delta\Psi_m$) thereby affecting the production of ATP and reactive oxygen species (ROS) [14,15]. K_{ATP} channel subunits are present in high amounts in the brain where they are expressed in neurons, astrocytes and vascular endothelial cells [11,16]. A distinct feature of K_{ATP} channels is that they couple cellular energy status to electrical activity [17], and so modulate neuronal excitability during different physiological and pathological conditions [18]. K_{ATP} channels are activated when cellular ATP level falls below a critical value thereby reducing excitability to maintain ion (Na^+ and Ca^{2+}) homeostasis and conserve ATP levels [19,20].

Pharmacological activation of mitochondrial K_{ATP} channels using drugs such as diazoxide has been shown to protect cardiac cells and neurons in the brain against ischemic damage and death in experimental models of myocardial infarction [21,22] and stroke [20,23]. We previously reported that diazoxide can protect cultured hippocampal and cortical neurons against hypoxic and oxidative injury [15] and $A\beta$ toxicity [24]. Diazoxide has been used to treat severe hypertension for more than 30 years [25]. Diazoxide also modulates vascular tone and increases CBF [15,26], suggesting an additional potential benefit for neurological disorders such as AD in which cerebral blood flow is reduced. In the present study we show that long-term administration of K_{ATP} channel opener diazoxide suppresses the $A\beta$ and tau pathologies, and ameliorates memory deficits, in the 3xTgAD mouse model of AD. Diazoxide also improved neuronal bioenergetics and increased CBF. These findings suggest a therapeutic potential for drugs that activate K_{ATP} channels in the treatment of AD.

Materials and Methods

Cerebral cortical cell cultures

Procedures for preparation and maintenance of dissociated neuronal cell cultures from embryonic day 18 rat (Sprague Dawley) embryos have been described previously [27]. Briefly, cortical tissues were removed and subjected to mild trypsinization and trituration. Dissociated neurons were seeded onto polyethylenimine-coated glass coverslips and maintained at 37°C in Neurobasal medium containing B-27 supplements, 2 mM L-glutamine, 1 mM HEPES and 0.001% gentamycin sulfate. All experiments were performed using 7 to 8 day-old cultures.

Time lapse confocal imaging of $\Delta\Psi_p$, $\Delta\Psi_m$ and $[K^+]_i$

Plasma membrane potential ($\Delta\Psi_p$) and mitochondrial membrane potential ($\Delta\Psi_m$) were measured by confocal imaging of neurons loaded with the fluorescent probes DiBAC₄ ($\Delta\Psi_p$) and TMRE ($\Delta\Psi_m$) (Molecular Probes, Eugene Oregon) using methods similar to those described previously [28,29]. Fluorescence images of cells were acquired by sequential scanning for each probe at its excitation and emission wavelengths. There was no interference between signals from each probe as determined by selective closing of individual wavelength channels. Average pixel intensity per cell was obtained from 10-20 neurons in 3-5 cultures for each experimental treatment protocol. Cells were incubated for 30 min in medium containing both fluorescent probes and then washed with fresh medium; cells were incubated in the presence of 50 nM TMRE (50 nM) during the imaging process [29]. The intracellular K⁺ level was assessed with the K⁺-selective fluorescence indicator 1,3-benzenedicarboxylic acid, 4,4'-[1,4,10,13-tetraoxa-7,16-diazacyclooctadecane-7,16-diylbis(5-methoxy-6,2-benzofurandiyl)]bis-, tetrakis [(acetyloxy) methyl] ester (PBFI-AM, Molecular Probes) using methods described previously [15,30]. Cells were incubated for 45 min in the presence of 5 μ M PBFI-AM in HEPES-buffer consisting of (in mM): 145 NaCl, 5 KCl, 1.8 CaCl₂, 0.8 MgCl₂, 10 D-glucose, 10 HEPES (295 mOsm, pH 7.2). Images of PBFI fluorescence in neuronal cell bodies were acquired at excitation and emission wavelengths of 340 and 510 nm, respectively.

Imaging of intracellular Ca²⁺ and NAD(P)H

For monitoring intracellular Ca²⁺ levels, cells were loaded with the probe Fluo-4 and images of fluorescence in neuronal cell bodies were acquired before and during exposure to treatments using excitation and emission wavelengths of 488 and 520 nm, respectively. Data are presented as the average pixel intensity per neuron relative to baseline intensity in recordings from cells in 3-5 different cultures (20-40 cells per culture). Autofluorescence of NAD(P)H, the reduced form of nicotinamide adenine dinucleotide, was monitored by exposing the cells to an excitation wavelength of 360 nm and acquiring the fluorescence emission at 450 nm. Nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) (together abbreviated NAD(P)H) are fluorescent in their reduced forms and non-fluorescent in their oxidized forms.

Glibenclamide BODIPY FL ligand binding

Glibenclamide BODIPY FL, a cell permeable fluorescence ligand of SUR K_{ATP} channel proteins (Molecular Probes) was added to the culture medium (100 nM) and incubated for 20-30 minutes at 35°C, then washed with fresh probe-free medium. Live cell images were acquired by confocal microscopy (Zeiss 510) with excitation at 360 nm and emission at 450 nm. Cells were also loaded with the $\Delta\Psi_m$ indicator TMRE for mitochondrial co-localization.

RT-PCR analysis

Relative levels of K_{ATP} channel subunit mRNAs in neurons were quantified using RT-PCR with superscript III kit (Invitrogen). Primers for subunits of K_{ATP} were: Kir6.1, forward primer, 5'- GAGTGAAGTGTGCGACACCAGA -3'; reverse primer, 5'- CGATCACCAGAACTCAGCAA -3'. Kir6.2, forward primer, 5'- TCCAACAGCCCCGCTCTAC -3'; reverse primer, 5'- GATGGGGACAAAACGCTG-3'. SUR1, forward primer, 5'- GGAGCAATCCAGACCAAGAT-3'; reverse primer, 5'- AGCCAGACGAATGATGACAG-3'. SUR2A, forward primer, 5'- GTTCTGCCTGGCCAGGGCC-3'; reverse primer, 5'- GTCTACTTGTGGTCATCACCAA-3'. SUR2B, forward primer, 5'- GTTCTGCCTGGCCAGGGCC-3'; reverse primer, 5'- CCTACAGGGAGTGTCCCTCAGACC-3'. The PCR conditions used were: 94°C 1 min and 26 cycles of 94°C for 30 s, 55°C 1 min and 68°C 1 min.

Patch-clamp whole cell recording

The membrane potential was measured using a patch-clamp amplifier (Axopatch-200B) and responses were recorded using a nystatin-perforated recording configuration using methods described previously [31]. The ionic composition of the external solution was (in mM) 150 NaCl, 5 KCl, 1 MgCl₂, 10 glucose, and 10 HEPES. The internal solution for the nystatin-perforated patch was 150 KCl and 10 HEPES; nystatin was dissolved in the internal solution at 50-100 mg ml⁻¹ just before use. The solution containing diazoxide was applied to the cells using a rapid perfusion valve control system (Warner Instruments).

Measurements of O₂ consumption

The O₂ consumption rates were measured using a thermo-jacketed Clark type oxygen electrode with mini-stirring bar at 37°C in digitonin-permeabilized cells as described before (Liu et al., 2006). Signals were collected through a standard 2-channel oxymetric recorder. Cells were suspended in culture medium at a density of 1 × 10⁷ cells/mL. After centrifugation, cells were resuspended in a Hepes-buffer containing (in mM): 145 NaCl, 5 KCl, 1.8 CaCl₂, 0.8 MgCl₂, 10 D-glucose, 10 Hepes, 5 succinate, 0.3 ADP, with/without rotenone (2 μM) (pH 7.0 and 295 mOsm) in the chamber under constant stirring at 37°C. The data were represented as ng atom O/min/1 × 10⁷ cells at 37°C. Cell viability was 95-100% as assessed by trypan blue exclusion assay before and after the oxygen consumption assay.

3xTgAD mice, drug administration and behavioral tests

3xTgAD mice were generated from a presenilin-1 mutant (PS1_{M146V}) mouse embryo that was transfected with two expression plasmids, one containing a cDNA encoding an AD-linked Swedish double mutation of amyloid precursor protein (APP_{KM670/671NL}) and the other a cDNA encoding a tau mutation (tau_{p301L}) that causes frontotemporal lobe dementia, both under the control of the Thy-1 promoter [36]. While 3xTgAD mice consistently develop Aβ and tau pathology, and behavioral deficits, there has been considerable variability among laboratories with regards to the time course of appearance and progression of the pathological processes. The 3xTgAD mice used in the present study had been backcrossed for 8 generations onto a C57BL/6 background, and developed the Aβ and tau pathologies, and behavioral deficits, over a protracted time course compared to the original 3xTgAD mice [36] which were on a mixed strain genetic background. 3xTgAD mice were administered diazoxide provided in the drinking water (10 mg/kg/day), or water alone (untreated controls) ad libitum beginning at 4 months of age. The pH of the diazoxide-containing drinking water was normalized. There were 10 animals in each group. At 13 months of age the behavioral tests of spatial learning and memory (Morris Water Maze) and spontaneous locomotor activity (open field) were performed using methods described previously [33]. For the Morris water maze test, a circular tank was filled with water

made opaque with non-toxic white paint ($23 \pm 1^\circ\text{C}$) to a depth of 100 cm. Spatial visual cues (each was a different shape) were applied to the walls of each quadrant. A clear circular platform (10 cm in diameter) was submerged about 1 cm below the water surface. A video camera was mounted on the ceiling in the center of the pool and connected to a computerized tracking/image analyzer system. The swimming path was monitored with a Videomex tracking system and data was collected using Videomex Water Maze Software (Columbus Instruments, Ohio, USA). On each test day the mice went through four trials of the hidden platform training from each of the four starting positions (North, South, East and West). A trial lasted until the mouse found the platform or until 60 seconds had elapsed. If the mouse did not find the platform before 60 seconds it was gently guided back to the platform and placed on it for 30 seconds. A probe trial was performed after 6th day of training; the platform and visual cues were removed and the animals were allowed to swim freely for 60 seconds and the amount of time spent in the target quadrant were measured. Spontaneous locomotor activity in the open field was assessed using an apparatus equipped with infrared light-sensitive photo cells. The apparatus was placed in a lit, ventilated and quiet testing room. Four animals were placed in four identical apparatuses, and distance traveled, ambulatory counts, vertical counts and stereotypic counts were recorded for a 30 minute period.

Assessments of rCBF

Regional CBF was determined in the territory of the middle cerebral artery (MCA) using laser-doppler blood flowmetry monitor (Perimed, Stockholm, Sweden) with a flexible 0.5 mm fiberoptic probe. Briefly, mice were kept anesthetized using isoflurane through a vapor mask with probes fixed on the surface of thinner skull, and maintained at 37°C pad. Dynamic changes of regional CBF were continuously monitored and recorded using computer flowmetry software [15]. Diazoxide was administered intravenously in a single dose of 5 mg/kg body weight.

Immunoblots

One week after behavioral testing, the mice were euthanized and their brains were rapidly removed and cut in half sagittally. One half of the brain was fixed for 24 hours in 4% paraformaldehyde in PBS for immunohistochemical analysis, and the hippocampus and cerebral cortex from the other half were dissected and flash frozen and stored at -80°C for biochemical analyses. For immunoblots, hippocampal tissue was homogenized with cell lysis buffer and protein was quantified using BCR reagent. Proteins were separated on a 10% bis-Tris gel (Invitrogen). The membranes were blotted 1:1000 with anti-APP/anti-A β (Signet Laboratories; clone 6E10), anti-total tau (AT180) and anti-phosphorylated tau (AT8) antibodies. The membranes were then incubated in a solution containing HRP-conjugated secondary antibody (Vector Laboratories) and visualized by enhanced chemiluminescence (Amersham). To quantify levels of synapse-associated proteins, holo-APP and A β oligomers we added 400 μl of M-PER buffer supplemented with 2x protease inhibitor (EMD Bioscience) to frozen brain tissue samples, and the tissue was homogenized using a sonicator. After homogenization, the samples were centrifuged at $13,000\times g$ for 15 minutes at 4°C . The supernatant soluble fraction from each sample was subjected to protein assay and then divided into three equal volumes. For determination of SNAP25 and synaptophysin levels, equal amounts of protein (as determined by Bradford protein assay) of the soluble fraction from each sample was loaded in a 'Criterion' (BioRad) gel and electrophoresed for 1 hour at 200 V. Proteins in the gel were electrophoretically transferred onto a PVDF membrane, and checked by the Ponceau stain. After blocking the membrane with 5% milk dissolved in TBST, specific areas of the membrane were probed with anti-SNAP25 (Millipore) and anti-synaptophysin (Santa Cruz Biotechnology) antibodies. A specific strip of the membrane was also probed with anti- β actin (Sigma) antibody for normalization purposes. Each strip of the membrane was probed with secondary antibodies specific for the respective primary antibodies and

chemoluminescence buffer (GE) was added. The specific protein band signals were obtained on X-ray film and band intensities were quantified using 'Image-J' software (NIH). Quantified band intensities of SNAP25 and synaptophysin were normalized to β actin band densities and the results were plotted by 'Graph Pad' software. For quantification of A β oligomers equal amounts of protein from the soluble fraction of the brain lysate of each sample were run in a 4-12% Bis-Tris gradient gel. Lysate from one vehicle-treated animal was not included in the analysis because of very low protein content. After transfer, the membrane was probed with monoclonal 6E10 antibody (Covance) and the band signals were obtained as described above.

Immunohistochemistry

Fixed brains were cryoprotected in 30% sucrose solution in PBS, and 15- μ m-thick cryostat sections were cut sagittally through the entire hippocampus and adhered to a coated slide. Endogenous peroxidase was quenched by incubation with 0.3% peroxide in methanol for 30 minutes. A Citrate buffer (pH 6.0) was applied to the tissue sections for antigen retrieval before primary antibody incubations. The slides were incubated with a rabbit polyclonal anti-human A β (dilution 1:200; Cell Signaling, clone#2454) or a mouse monoclonal anti-human PHF-Tau (dilution 1:200; Pierce Endogen, clone# AT180) and incubated overnight at 4°C. A standard streptavidin-biotin-peroxidase complex labeling kit (Vector ABC kit; USA) was used to visualize A β and p-Tau immunoreactions. Tissues were stained for 5 minutes with 3,3'-diaminobenzidine as the chromogen (Vector, USA), dehydrated through a graded ethanol series, cleared in xylene, and cover-slipped. Primary antibodies were omitted in negative controls.

Statistical Analysis

Two way ANOVA was used for comparisons among the different treatment groups, and multiple post-hoc comparison *t*-tests with equal variances were performed for comparisons between pairs of animal or treatment groups.

Results

Evidence that diazoxide activates plasma membrane and mitochondrial K_{ATP} channels in cerebral cortical neurons

RT-PCR analysis showed that pore forming inwardly rectifying K⁺ channel subunits (Kir6.1 or Kir6.2) and sulfonylurea receptor subunits (SUR1, SUR2A or SUR2B) are expressed in cultured cortical neurons (Fig. 1A). To determine the subcellular distribution of K_{ATP} channels in neurons we employed confocal microscopy to visualize the location of fluorescently tagged glibenclamide (glibenclamide BODIPY FL; green fluorescence), a K⁺ channel ligand that binds specifically to sulfonylurea receptor (SUR) subunits. Neurons were co-stained with the mitochondrial dye TMRE (red fluorescence). Images of the cells reveal the presence of K⁺ channels in neurons with a staining pattern suggesting localization of K⁺ channels to both the plasma membrane and mitochondria (Fig. 1B). The intensity of the glibenclamide-associated signal that co-localized with mitochondria was particularly strong.

We next directly determined the effect of diazoxide on neuronal excitability by recording membrane potential in single neurons prior to, during and after exposure of the neurons to diazoxide. Exposure of neurons to 200 μ M diazoxide resulted in rapid membrane hyperpolarization accompanied by cessation of spontaneous action potentials (Fig. 1C). Following washout of diazoxide the membrane potential slowly recovered and spontaneous action potentials resumed after a period of 10-15 minutes. Exposure of neurons to a lower concentration of diazoxide (30 μ M) resulted in a modest membrane hyperpolarization and cessation of action potentials; following washout of diazoxide the firing of action potentials resumed within 3-4 minutes (Fig. 1C, right). Thus, diazoxide reduces neuronal excitability.

We next determined the effects of diazoxide on cell plasma membrane potential ($\Delta\psi_p$), the mitochondrial membrane potential ($\Delta\psi_m$), and intracellular K^+ levels in cultured neurons. Cells were loaded with the fluorescent probes DiBAC₄ and TMRE to measure $\Delta\psi_p$ and $\Delta\psi_m$, respectively, and the probe PBFI to measure the intracellular K^+ concentration ($[K^+]_i$) (Supplemental Figure 1A; Fig. S1A). Time-lapse images of cells were acquired with sequential multiple channel scanning to simultaneously monitor fluorescence signals from all three probes. To confirm the reliability of the method we first determined the responses to plasma membrane depolarization. Upon exposure to KCl the DiBAC₄ signal increased rapidly and remained elevated for several minutes (Fig. S1B). The $\Delta\psi_p$ depolarization was accompanied by a decrease of the TMRE signal indicating depolarization of the $\Delta\psi_m$, and a transient increase in the PBFI signal indicating K^+ influx. Exposure of neurons to 30 μ M diazoxide resulted in a transient hyperpolarization of the plasma membrane which was accompanied by a transient decrease in the $[K^+]_i$ (Fig. S1C). Exposure to a higher diazoxide concentration (200 μ M) resulted in a larger and more prolonged plasma membrane hyperpolarization and reduction in the $[K^+]_i$; subsequent addition of the K^+ channel blocker TEA accelerated recovery of the $\Delta\psi_p$ and $[K^+]_i$. Diazoxide depolarized the mitochondrial membrane (Fig. S1C, D and E). The effects of diazoxide on $\Delta\psi_p$, $\Delta\psi_m$ and $[K^+]_i$ were markedly attenuated in neurons that were pretreated with TEA (Fig. S1D) demonstrating the involvement of K_{ATP} channels. In contrast, the Na^+ channel blocker tetrodotoxin (TTX; 500 nM) did not modify the effects of diazoxide on $\Delta\psi_p$, $\Delta\psi_m$ and $[K^+]_i$ (Fig. S1E).

Activation of K_{ATP} channels modifies neuronal bioenergetics and inhibits NMDA-induced elevation of intracellular Ca^{2+} levels

During metabolic stress, excessive release of the excitatory neurotransmitter glutamate can overactivate postsynaptic glutamate receptors resulting in sustained influx of Ca^{2+} through NMDA receptor channels, a process implicated in the pathogenesis of AD [4,34]. We therefore determined the effects of diazoxide on the resting $[Ca^{2+}]_i$, cellular NAD(P)H levels (an indicator of cellular energy status) and $\Delta\psi_m$ in cultured neurons. Diazoxide (30 μ M) induced a small transient decrease of the $[Ca^{2+}]_i$ which was associated with a transient elevation of NAD(P)H levels and a small decrease of the $\Delta\psi_m$ (Fig. 2A). A higher concentration of diazoxide (200 μ M) resulted in a transient decrease of the $[Ca^{2+}]_i$ and a transient elevation of NAD(P)H levels, followed by sustained reductions in levels of $[Ca^{2+}]_i$, NAD(P)H and $\Delta\psi_m$ (Fig. 2B). Exposure of neurons to ATP, an inhibitor of K_{ATP} channels, resulted in transient increases of $[Ca^{2+}]_i$ and NAD(P)H, but no change of the $\Delta\psi_m$ (Fig. 2C). A subsequent exposure to ATP (5 minutes after the first application of ATP) resulted in sustained increases of $[Ca^{2+}]_i$ and NAD(P)H levels without affecting the $\Delta\psi_m$ (Fig. 2C). Exposure of neurons to 100 mM NMDA resulted in a progressive increase of the $[Ca^{2+}]_i$ which was largely abolished when neurons were pretreated with diazoxide (200 μ M) demonstrating the ability of diazoxide to protect neurons against NMDA receptor-mediated Ca^{2+} overload (Fig. 2D, E). When neurons were treated with diazoxide 2 min after exposure to NMDA, the increase of the $[Ca^{2+}]_i$ was halted (Fig. 2F).

Treatment with diazoxide improves spatial learning and memory in 3xTgAD mice

3xTgAD mice were generated from a presenilin-1 mutant mouse embryo that was transfected with two expression plasmids, one containing a cDNA encoding an AD-linked (Swedish) mutant form of amyloid precursor protein and the other a cDNA encoding a tau mutation that causes frontotemporal lobe dementia, both under the control of the Thy-1 promoter [36]. 3xTgAD mice develop progressive accumulation of A β and hyperphosphorylated tau in the hippocampus and cerebral cortex, and learning and memory deficits, as they age [37-39]. To determine whether diazoxide might modify the disease process and functional deficits of AD, diazoxide was provided in the drinking water beginning at 4 months of age in 3xTgAD mice (approximately 10 mg/kg/day diazoxide). At 12 months of age, the water maze test was

performed to evaluate learning and memory ability in untreated and diazoxide-treated 3xTgAD mice. Our previous study showed that 3xTgAD mice exhibited greater goal latencies and swimming path lengths beginning on the second maze training trial day compared to non-transgenic control mice [39]. Compared with untreated 3xTgAD mice, 3xTgAD mice treated with diazoxide exhibited reduced goal latencies (Fig. 3A) and path lengths (Fig. 3B) to find the hidden platform in the water maze suggesting improved learning and memory ability. The swimming speeds and floating times did not differ significantly in vehicle and diazoxide-treated 3xTgAD mice (Fig. 3C, D). Diazoxide-treated 3xTgAD mice did not differ significantly from untreated 3xTgAD mice in distance traveled, ambulatory counts and stereotypic counts in the open field test; however, the diazoxide-treated 3xTgAD mice did exhibit significantly greater vertical counts, suggesting an anxiolytic effect of the drug (Supplemental Figure 2).

Levels of pre-synaptic proteins are elevated by diazoxide treatment in 3xTgAD mice

We next sought a biochemical correlate of the beneficial effect of diazoxide on learning and memory in the 3xTgAD mice. To this end, we first measured levels of SNAP-25 and synaptophysin, two presynaptic proteins that play important roles in neurotransmitter release. Levels of SNAP-25 protein were significantly greater in cerebral cortical samples from diazoxide-treated 3xTgAD mice compared to untreated 3xTgAD mice ($p = 0.0081$) (Fig. 4A). Similarly, levels of synaptophysin were significantly greater in cerebral cortical samples from diazoxide-treated 3xTgAD mice compared to untreated 3xTgAD mice ($p = 0.03$) (Fig. 4B).

Diazoxide treatment reduces the accumulation of A β and hyperphosphorylated tau in the hippocampus and cerebral cortex of 3xTgAD mice

To determine whether the beneficial effect of diazoxide treatment on cognitive function is associated with lessening of the underlying A β and tau pathologies, we killed the mice 1 week after behavioral testing and performed immunoblot analysis of APP, A β and phospho-tau levels in tissue samples from the hippocampus and cerebral cortex. Levels of holo-APP and A β oligomers were significantly greater in both the cerebral cortex (Fig. 5A) and hippocampus (Supplemental Fig. 3). To elucidate the nature and relative amounts of A β oligomers, we performed additional analyses 4-12% Bis-Tris gradient gel method that allows detection of A β trimers and tetramers. The results show that levels of A β trimers and tetramers were significantly lower in cerebral cortex of diazoxide-treated 3xTgAD mice compared to untreated 3xTgAD mice (Fig. 5B, C).

To further evaluate the effects of diazoxide on A β deposition, we immunostained brain sections from control and diazoxide-treated 3xTgAD mice with antibodies against A β and phospho-tau (p-tau) (AT180). In the brains of untreated 3xTgAD mice, dense extracellular A β oligomer aggregations with plaques of variable sizes were present in the cerebral cortex and hippocampus (Fig. 6A). Levels of extracellular A β deposits were markedly reduced in the hippocampus and cerebral cortex of diazoxide-treated 3xTgAD mice (Fig. 6A, d-f) compared to untreated 3xTgAD mice (Fig. 6A, a-c). Interestingly, however, levels of intracellular A β immunoreactivity in diazoxide-treated 3xTgAD mice were high in CA1 neurons and in some neurons in the subiculum and cerebral cortex.

3xTgAD mice exhibit hyperphosphorylated tau, which accumulates in greatest amounts in hippocampal CA1 neurons and in pyramidal neurons in layer IV of the cerebral cortex [36, 38]. Levels of p-tau immunoreactivity were greater in neurons in the subiculum, CA1 and cortical neurons in untreated 3xTgAD mice compared to diazoxide-treated 3xTgAD mice (Fig. 6Ba-f), suggesting that diazoxide suppresses the cellular and molecular alterations that result in accumulation of hyper-phosphorylated tau. We next performed immunoblot analysis of cerebral cortical samples from untreated and diazoxide-treated mice using antibodies against total tau and p-tau. Total tau and p-tau levels were significantly greater in untreated 3xTgAD

mice compared to diazoxide-treated 3xTgAD mice, whereas total tau levels were unaffected by diazoxide treatment (Fig. 7).

Diazoxide treatment increases cerebral blood flow

Because diazoxide has an arterial vasodilating effect and reduces blood pressure in humans [25,32], we next determined whether diazoxide treatment affects cerebral blood flow (CBF) in 3xTgAD mice. Regional CBF was determined in the territory of the middle cerebral artery using laser doppler flowometry with a flexible 0.5 mm fiberoptic probe fixed on the exposed brain surface. CBF was recorded continuously beginning 5-10 minutes before and ending 20-30 minutes after diazoxide administration (5 mg/kg intravenous bolus of diazoxide). CBF increased within 1-2 min of administration and remained elevated for at least 30 minutes (Supplemental Fig. 4).

Discussion

Treatment of 3xTgAD mice for 8 months with the K_{ATP} channel opener diazoxide ameliorated cognitive impairment and lessened the pathological accumulation of A β and hyperphosphorylated tau in the hippocampus and cerebral cortex. The increased activity of K_{ATP} channels in the plasma membrane and/or mitochondria in neurons, and vasodilation of cerebral vessels resulting in increased CBF, may each contribute to the beneficial effects of diazoxide in this mouse model of AD (Figure 8). We found that diazoxide hyperpolarized the plasma membrane and reduced excitability of hippocampal neurons, which was associated with reduced Ca^{2+} influx through NMDA receptor channels. Previous studies have shown that activation of glutamate receptors and Ca^{2+} influx can promote tau hyperphosphorylation in hippocampal neurons in culture [40] and in vivo [41,42]. Our findings therefore suggest that diazoxide decreases tau hyperphosphorylation by suppressing glutamate receptor-mediated Ca^{2+} influx. Diazoxide may also protect synapses against A β -induced oxidative stress and dysfunction because we found that levels of the synaptic proteins SNAP-25 and synaptophysin were elevated in brain tissue samples from diazoxide-treated 3xTgAD mice compared to control 3xTgAD mice. The latter finding is consistent with the results of a previous study showing that anticonvulsants such as valproic acid can protect hippocampal neurons from being damaged and killed by A β [43]. Tau hyperphosphorylation and aggregation is believed to play a role in cognitive impairment in AD and, indeed, mutations that cause tau hyperphosphorylation and accumulation are sufficient to cause dementia in patients with frontotemporal lobe dementia [44]. Moreover, data from tau and A β immunization studies suggest an important contribution of tau pathology to memory impairment in 3xTgAD mice [45].

Diazoxide-treated 3xTgAD mice exhibited lower amounts of A β oligomers in the hippocampus and cerebral cortex compared to untreated 3xTgAD mice. Although we did not establish the specific mechanism by which diazoxide treatment lowers A β levels, we did observe that diazoxide treatment decreases the amount of full-length APP suggesting that diazoxide may reduce the amount of A β produced by cells. Other possibilities are that diazoxide inhibits amyloidogenic processing of APP by β - and γ -secretases, or diazoxide may increase the clearance of A β from the brain. Previous studies have shown that synaptic activity [46], activation of NMDA receptors [47] and Ca^{2+} influx [48] can increase the production of A β . Diazoxide may therefore reduce A β production by decreasing neuronal excitability and activation of NMDA receptors (Figure 8). Impaired mitochondrial energy metabolism and increased oxidative stress are implicated in the pathogenesis of AD [2,49], and experimental reductions in neuronal energy availability and increases in oxidative stress have been shown to increase production of A β [50,51]. Consistent with the latter possibility, previous studies have shown that treatments that increase cellular energy levels [52] or decrease mitochondrial

free radical levels [53] can protect neurons against A β toxicity. It should be noted, however, that it is possible that the mechanism responsible for the beneficial effects of diazoxide in 3xTgAD during an 8 month treatment may not be identical to the mechanism by which it affects neuronal excitability and vulnerability to acute excitotoxic insults.

Diazoxide was previously shown to protect cultured neurons against excitotoxicity and A β toxicity [23,24]. The latter actions may contribute to the preservation of cognitive function and synaptic integrity in diazoxide-treated 3xTgAD mice in the present study. Indeed, the only drug thus far shown to slow the progression of AD in some patients is memantine, an NMDA receptor blocker [54]. Moreover, recent studies have shown that memantine, which is a channel-blocking drug with a fast off rate and voltage-dependent binding properties [54], lowers A β levels in neuronal cultures and in APP/PS1 double transgenic mice [55], and reduces AD-like neuropathology in 3xTgAD mice [56]. Memantine's ability to protect neuronal cells against degeneration, to increase cell viability and/or metabolic activity, and to lower amyloidogenic pathway is noteworthy. Since some of these attributes are shared by diazoxide, it would be interesting to test the ability of diazoxide to modify the disease process in AD patients. Alternatively, a combinatorial approach with diazoxide and cholinesterase inhibitors could also be tested.

We found that diazoxide increased CBF suggesting that increasing cerebral perfusion and preservation of cellular energy reserves and reduced production of free radicals could be the mechanism by which diazoxide protects neurons. Hypertension is a risk factor for AD [57, 58] and reduced CBF occurs early in the disease process before cognitive deficits are evident [59]. Cerebral hypoperfusion could accelerate amyloidogenesis and impair A β clearance, and decreased delivery of glucose and oxygen to neurons may lead to neuronal degeneration and decline of cognitive function. Age-related hypertension and atherosclerosis may contribute to cerebral hypoperfusion in AD. Epidemiological data suggest that anti-hypertensive drugs reduce the risk of AD [60]. We found that diazoxide, which was originally developed and marketed as an anti-hypertensive drug [25], increased CBF in mice, presumably by activating K_{ATP} channels in vascular smooth muscle cells [61]. Others have shown that K⁺ channel openers can protect cerebrovascular arteries against A β -induced endothelial cell dysfunction [62]. Therefore, the beneficial effects of diazoxide on cognitive function in 3xTgAD mice may be due, in part, to improved cerebrovascular function.

Interestingly, we found that whereas overall levels of A β oligomers, and extracellular accumulations of A β , were reduced in diazoxide-treated 3xTgAD mice, the levels of intracellular A β immunoreactivity were greater in some neurons (particularly CA1 and subicular neurons) of diazoxide-treated 3xTgAD mice. Although previous findings suggested that intracellular A β can damage neurons [63], we had reported in our original study of 3xTgAD mice that A β immunoreactivity first appears intracellularly during the early stages of the disease process, and then accumulates progressively outside of cells as the disease worsens [36]. In the present study the diazoxide-treated 3xTgAD mice performed better than age-matched control 3xTgAD mice in the water maze tests. Moreover, tau pathology was reduced in diazoxide-treated compared to control 3xTgAD mice in the same neuronal populations in which intracellular A β immunoreactivity was elevated. Our findings therefore suggest that, with regards to the evolution of A β pathology, diazoxide slows but does not prevent the disease process. Our data further suggest that the ability of diazoxide to reduce neuronal oxidative and metabolic stress may be more important to the pathogenesis of the neuronal dysfunction and behavioral impairment than is A β , per se.

Our findings demonstrate that long-term diazoxide treatment reduces A β and tau pathologies and improves cognitive function in a mouse model of AD. Diazoxide activates K⁺ channels in both the plasma membrane and mitochondrial inner membrane, and both of these sites of action

are likely to contribute to its ability to suppress the AD-like disease process in the 3xTgAD mice. By hyperpolarizing the plasma membrane, diazoxide can reduce A β production and NMDA receptor-mediated cellular Ca²⁺ overload. Simultaneously, activation of mito-KATP channels may preserve cellular energy substrates and reduce mitochondrial free radical production. Collectively, these actions of diazoxide likely contribute to the mechanism by which it retards the AD process and improves cognitive function in the 3xTgAD mice. These preclinical findings prompt consideration of clinical trials of K_{ATP} channel openers in patients with mild cognitive impairment and AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

K_{ATP} channel subunits are present and functional in cortical neurons. **A.** RT-PCR analysis of mRNAs encoding K_{ATP} channel components, including inwardly rectifying K^+ channel subunits (Kir6.1 or Kir6.2) and sulfonylurea receptor subunits (SUR1, SUR2A or SUR2B), in cultured cerebral cortical neurons. **B.** Confocal images showing cultured cortical neurons stained with TMRE to label mitochondria (red) and fluorescently tagged glibenclamide to label sulfonylurea receptor K_{ATP} channels (green). **C.** Whole-cell patch clamp recordings of membrane potential in culture hippocampal neurons before, during and after exposure to the indicated concentrations of diazoxide (DZ). Note that diazoxide hyperpolarized the cell membrane and abolished spontaneous action potentials. The membrane potential returned to the resting potential and action potentials resumed following washout of diazoxide. Similar results were obtained in recordings from 5-6 neurons.

Figure 2.

Diazoxide inhibits $[Ca^{2+}]_i$ responses to NMDA receptor activation in cortical neurons. **A - C.** The $[Ca^{2+}]_i$ (Fluo-4, green), NAD(P)H levels (autofluorescence; blue) and mitochondrial membrane potential (TMRE; red) were measured prior to and during exposures to diazoxide (DZ; 30 or 200 μ M) or ATP (5 μ M; an inhibitor of K_{ATP} channels). **D.** Representative time-lapse images (5 second interval between images) of $[Ca^{2+}]_i$ in four hippocampal neurons prior to and after exposure to 100 μ M NMDA (the asterisk indicates the time of addition of NMDA). **E and F.** The $[Ca^{2+}]_i$ in hippocampal neurons exposure to diazoxide (200 μ M) or vehicle and then exposed to 100 μ M NMDA at the indicated time points. Note that diazoxide significantly suppressed the NMDA-induced increase of $[Ca^{2+}]_i$ ($p < 0.01$). Values are the mean and SD of measurements made in 3-5 separate cultures (10-15 neurons per culture).

Figure 3.

Diazoxide treatment ameliorates spatial memory deficits in 3xTgAD mice. 3xTgAD mice were treated with diazoxide or vehicle (control) for 8 months beginning at 4 months of age (see Methods) and were then tested in the water maze **A – D**. 3xTgAD mice were tested in the hidden platform version of the water maze on 6 consecutive days and goal latencies (**A**), path length (**B**), percentage of time floating (**C**) and swim speed (**D**) were measured. Values are the mean and SD ($n = 10$ mice per group). $*p < 0.05$ compared to corresponding value for diazoxide treated 3xTgAD mice. **E**. Results of probe trial test. Diazoxide treated 3xTgAD spent significantly more time in the target quadrant compared to untreated 3xTgAD mice. $*p < 0.05$ compared to corresponding value for untreated 3xTgAD mice.

Figure 4.

Levels of synaptic proteins are elevated in diazoxide-treated 3xTgAD mice. Immunoblots and densitometric analysis of the blots (graphs) of the presynaptic proteins SNAP-25 and synaptophysin in cortical tissue samples from untreated and diazoxide-treated 3xTgAD mice. Values were normalized to β -actin bands are the mean and SD.

Figure 5.

Levels of full-length APP and Aβ oligomers are reduced in diazoxide-treated 3xTgAD mice compared to untreated 3xTgAD mice. **A.** Immunoblot and densitometric analysis (graphs) of holo-APP in cortical tissue samples from untreated and diazoxide-treated 3xTgAD mice. **B and C.** Immunoblot and densitometric analysis (graphs) of 15 kDa Aβ trimers (B) and 20 kDa Aβ tetramers (C) in cortical tissue samples from untreated and diazoxide-treated 3xTgAD mice. Values were normalized to β-actin bands and are the mean and SD.

Figure 6.

Diazoxide treatment reduces extracellular accumulations of A β and intracellular accumulations of hyperphosphorylated tau in the hippocampal formation and cerebral cortex of 3xTgAD mice. A. Brain sections from untreated (left) and diazoxide-treated (right) 3xTgAD mice showing A β immunoreactivity (see Methods). The upper panels are high magnification images of the regions of the subiculum (a and d), CA1 region (b and e) and cerebral cortex (c and f), respectively, of the regions marked with the same letters in the subjacent lower magnification images. Note that levels of extracellular A β deposits are reduced in the diazoxide-treated mouse, while levels of intracellular A β appear to be increased in CA1 neurons and scattered cortical neurons. B. Images of hyperphosphorylated tau (p-tau) immunoreactivity in neurons in the subiculum (a and d), CA1 region (b and e) and cerebral cortex (c and f) of brain sections from untreated (left) and diazoxide-treated (right) 3xTgAD mice. Note that levels of p-tau are reduced in all three brain regions of diazoxide-treated mice compared to untreated mice.

Figure 7. Diazoxide treatment decreases levels of hyper-phosphorylated tau in the brains of 3xTgAD mice. **A.** Immunoblots of samples from the cerebral cortex of 12 month-old untreated control 3xTgAD mice and 12 month-old 3xTgAD mice that had been treated for 8 months with diazoxide. The blots were probed with antibodies against total tau (T46), p-tau (AT180) and β-actin (see Methods). **B.** Results of densitometric analysis of immunoblots showing levels of total tau and p-tau (normalized to β-actin level). Values are the mean and SD (n = 6-8 mice per group). *p < 0.05.

Figure 8.

Model for the mechanism underlying the beneficial effects of K_{ATP} channel activation in a mouse model of Alzheimer's disease. Diazoxide activates K_{ATP} channels in the plasma membrane and mitochondria. Activation of plasma membrane K_{ATP} channels in neurons results in membrane hyperpolarization and decreased calcium influx which suppresses glutamate release and prevents excitotoxic calcium overload. Activation of mitochondrial K_{ATP} channels results in reduced production of reactive oxygen species (ROS). The reduction of intracellular calcium and ROS levels results in decreased production of Aβ and reduced tau pathology. Activation of K_{ATP} channels in cerebral blood vessels results in increased cerebral blood flow (CBF) and increased clearance of Aβ from the brain. In these ways, diazoxide can promote synaptic plasticity and preserve cognitive function.