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The carbonic anhydrase inhibitor methazolamide prevents amyloid beta-induced mitochondrial dysfunction and caspase activation protecting neuronal and glial cells in vitro and in the mouse brain

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ABSTRACT

Mitochondrial dysfunction has been recognized as an early event in Alzheimer's disease (AD) pathology, preceding and inducing neurodegeneration and memory loss. The presence of cytochrome c (CytC) released from the mitochondria into the cytoplasm is often detected after acute or chronic neurodegenerative insults, including AD. The carbonic anhydrase inhibitor (CAI) methazolamide (MTZ) was identified among a library of drugs as an inhibitor of CytC release and proved to be neuroprotective in Huntington's disease and stroke models. Here, using neuronal and glial cell cultures, in addition to an acute model of amyloid beta (Aβ) toxicity, which replicates by intra-hippocampal injection the consequences of interstitial and cellular accumulation of Aβ, we analyzed the effects of MTZ on neuronal and glial degeneration induced by the Alzheimer's amyloid. MTZ prevented DNA fragmentation, CytC release and activation of caspase 9 and caspase 3 induced by Aβ in neuronal and glial cells in culture through the inhibition of mitochondrial hydrogen peroxide production. Moreover, intraperitoneal administration of MTZ prevented neurodegeneration induced by intra-hippocampal Aβ injection in the mouse brain and was effective at reducing caspase 3 activation in neurons and microglia in the area surrounding the injection site. Our results, delineating the molecular mechanism of action of MTZ against Aβ-mediated mitochondrial dysfunction and caspase activation, and demonstrating its efficiency in a model of acute amyloid-mediated toxicity, provide the first combined in vitro and in vivo evidence supporting the potential of a new therapy employing FDA-approved CAIs in AD.

1. Introduction

Mitochondrial dysfunction has been associated with neurodegeneration and amyloid β (Aβ[3]) toxicity in many recent reports (Moreira et al., 2010b; Swerdlow et al., 2010), and constitutes a valuable therapeutic target for Alzheimer's disease (AD). Mitochondrial pathology and energy metabolism impairment are early events in AD patients and mouse models, preceding the formation of Aβ plaques and memory loss (Atamna and Frey, 2007; Beal, 2005; Moreira et al., 2010a; Santos et al., 2013). Recent findings in AD mice also revealed reduction in mitochondrial membrane potential (ΔΨ) and the emergence of dystrophic and fragmented mitochondria, as well as increased production of hydrogen peroxide (H_2O_2), with Aβ plaques identified as the source of toxicity (Calkins et al., 2011; Xie et al., 2013). Apoptotic cell death and caspase-3 activation have been implicated in the pathogenesis of AD. Up-regulation of pro-apoptotic proteins and DNA fragmentation were also found in the AD brain (Smale et al., 1995; Stadelmann et al., 1999). Our laboratory and others have recently shown that the release of cytochrome *c* (CytC) is one of the main events linking mitochondrial damage to caspase activation and oligomeric Aβ-mediated apoptotic cell death (Fossati et al., 2010, 2013; Kim et al., 2014). Mitochondrial deregulation and the presence of CytC in the cytoplasm is often detected after acute or chronic neurodegenerative insults (Friedlander, 2003; Solesio et al., 2013a; Wang et al., 2003; Zhu et al., 2002, 2004). CytC release, through activation of the "apoptosome", induces procaspase 9

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[3] Abbreviations:
Aβ = amyloid β; AD = Alzheimer's disease; Δψ = mitochondrial membrane potential; H_2O_2 = hydrogen peroxide; CytC = cytochrome C; CAI = carbonic anhydrase inhibitor; MTZ = methazolamide; HD = Huntington's disease; ROS = reactive oxygen species; IP = intraperitoneal; DR = death receptor.

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proteolysis and the formation of mature caspase 9. This enzyme in turn activates caspase 3, which plays important roles in neurodegeneration, particularly in AD (de Calignon et al., 2010; Eckert et al., 2003b; Marques et al., 2003). Hence, inhibiting the release of CytC could result in protection against Aβ challenge in AD models and eventually in AD patients. A number of drugs that are able to inhibit CytC release from isolated mitochondria have been identified (Wang et al., 2008); among them, the carbonic anhydrase inhibitor (CAI) methazolamide (MTZ), proved to be neuroprotective in models of Huntington's Disease (HD) and ischemic injury (Wang et al., 2008, 2009). A striking correlation was found between MTZ treatment and the preservation of $\Delta\Psi$ across the inner mitochondrial membrane, in HD models as well as in our previous studies on vascular and neuronal amyloid toxicity (Fossati et al., 2013; Wang et al., 2008). Of note is that this drug has been used in humans for many years for treatment of glaucoma and can easily cross the blood brain barrier. Moreover, CAIs are clinically used for the prevention of acute mountain sickness and related high-altitude cerebral edema, confirming the efficacy of the drugs in the brain and the safety of their systemic administration (Wright et al., 2008). Here, we studied the effects of MTZ on caspase activation and apoptosis induced by Aβ challenge in neuronal and glial cells through the modulation of CytC release and mitochondrial H_2O_2 production. Moreover, we analyzed the influence of MTZ on caspase 3 activation and neurodegeneration induced by Aβ after intra-hippocampal injection in the mouse brain. Our results provide *in vitro* and *in vivo* evidence highlighting the potential of pharmacological strategies employing MTZ as promising new therapeutic avenues to explore in AD.

2. Materials and methods

2.1. Aβ peptides

Synthetic homologs of the amyloid subunits Aβ42 and Aβ40-Q22 (the Dutch genetic variant, containing the E22Q substitution) were synthesized using *N-tert*-butyloxycarbonyl chemistry by James I. Elliott at Yale University. Aβ homologs were dissolved to 1 mM in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Sigma), incubated overnight to break down pre-existing β-sheet structures (Fossati et al., 2010), and lyophilized. Peptides were subsequently dissolved in DMSO to a 10 mM concentration, followed by the addition of deionized water to 1 mM concentration and by further dilution into culture media to the required concentrations for the different experiments.

2.2. Cell cultures

Human neuroblastoma cells (SH-SY5Y) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in DMEM/F12 medium (50:50, Mediatech, Manassas, VA) with 10% FBS. M059K Glioma cells were obtained from ATCC and maintained in DMEM with 10% FBS. Normal Human Astrocytes were purchased from Lonza (Walkersville, MD) and were cultured in Astrocyte Growth Medium (Lonza) containing the pertinent growth factors and FBS provided by the manufacturer.

2.3. Cell death ELISA

The extent of apoptosis caused by Aβ in presence or absence of MTZ was assessed by quantitation of nucleosome formation using Cell Death ELISA^{plus} (Roche Applied Science, Indianapolis, IN). Briefly, after incubation with the peptides, plates were centrifuged in a Beckman J-6B centrifuge (10 min; 1000 rpm), cells lysed, and DNA-histone complexes (nucleosomes) quantitated by Cell Death ELISA, as previously described (Fossati et al., 2010).

2.4. Thioflavin T binding assay

Binding of the different Aβ peptides to Thioflavin-T was monitored by fluorescence evaluation, as described (Fossati et al., 2010; Walsh et al., 1999). Briefly, after peptide aggregation at 50 μM concentration in culture media in presence or absence of MTZ, 6 μl aliquots from each of the time-points were added to 184 μl of 50 mM Tris–HCl buffer, pH 8.5, and 10 μl of 0.1 mM Thioflavin-T (Sigma). Fluorescence was recorded for 300 s in a LS-50B luminescence spectrometer (Perkin Elmer, Waltham, MA) with excitation and emission wavelengths of 435 and 490 nm (slit width 10 nm), respectively, as described (Solito et al., 2009; Viana et al., 2009).

2.5. Native gel electrophoresis and Western blot analysis

Electrophoretic analysis for assessment of peptide aggregation in presence or absence of MTZ was performed under native conditions using 5–30% gradient polyacrylamide gels, in absence of SDS, using 25 mM Tris/glycine, pH 8.8, as running buffer, as previously described (Fossati et al., 2010). Aβ oligomerization patterns were visualized by subsequent WB analysis. Briefly, proteins were electrotransferred to nitrocellulose membranes (0.45 μM pore size; Hybond-ECL, GE Healthcare Life Sciences, Piscataway, NJ) at 400 mA for 2.5 h, using 10 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS; Sigma) buffer, pH 11.0, containing 10% (*v*/*v*) methanol. After blocking with 5% non-fat milk in TBS containing 0.1% Tween 20 (TBST), membranes were immunoreacted with a combination of mouse monoclonal anti-Aβ antibodies 4G8 (epitope: residues Aβ18-22) and 6E10 (epitope: residues Aβ3-8), both from Covance (Princeton, NJ), at 1:3000 dilution each, followed by HRP-labeled anti-mouse IgG and ECL chemistry (Fossati et al., 2010).

2.6. Immunocytochemical evaluation of CytC release

Both SH-SY5Y cells and Glioma cells were plated on glass chamber slides (Thermo Fisher Scientific, Rochester, NY). Slides were pre-coated with poly-D-lysine for SH-SY5Y cells. After seeding, cells were allowed to attach for 1 day prior to treatment with Aβ in presence or absence of MTZ for 24 h. Cells were then washed with PBS, fixed with 4% paraformaldehyde (10 min, RT), washed again, and blocked for 1 h with 20 mg/ml BSA in PBS containing 0.3% Triton X-100 (PBST). Slides were further incubated with mouse monoclonal anti-CytC antibody (BD Biosciences; 1:200 in PBST containing 5 mg/ml BSA; 2 h, RT) followed by Alexa Fluor 488-conjugated anti-mouse IgG (Life Technologies, Grand Island, NY) 1:200 in PBST with 5 mg/ml BSA for 1 h at RT, as previously described (Fossati et al., 2013). Fluorescence signals were visualized in a Zeiss LSM 510 laser scanning confocal/Confocor2 microscope using a 40× DIC oil immersion objective and LSM 510 software; acquired images were processed and analyzed using ImageJ (National Institute of Health; http://rsbweb.nih.gov/ij/).

2.7. ELISA and Western blot assessment of CytC in mitochondrial and cytoplasmic subcellular fractions

Subcellular distribution of CytC in amyloid-challenged SH-SY5Y and glioma cells was determined in mitochondrial and cytoplasmic protein extracts prepared using Mitochondria Isolation Kit (MITOISO2, Sigma) following the manufacturer's specifications. Briefly, after amyloid challenge in presence and absence of Methazolamide, as above, cells were collected by trypsinization, resuspended in 10 mM HEPES, pH 7.5, containing 200 mM mannitol, 70 mM sucrose, 1 mM EGTA and Protease Inhibitor Cocktail, and homogenized with the aid of a Dounce glass homogenizer. Cell homogenates were centrifuged to remove unbroken cells and nuclei (600 ×*g*, 5 min, 4 °C) and supernatants were further centrifuged at 11,000 ×*g* (5 min, 4 °C) to subfractionate mitochondria.