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Pyruvate prevents the development of age-dependent cognitive deficits in a mouse model of Alzheimer's disease without reducing amyloid and tau pathology

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ABSTRACT

Amyloid-β (Aβ) deposition and tau-dependent pathology are key features of Alzheimer's disease (AD). However, to date, approaches aimed at counteracting these two pathogenic factors have produced only modest therapeutic outcomes. More effective therapies should therefore consider additional pathogenic factors like energy production failure, hyperexcitability and excitotoxicity, oxidative stress, deregulation of metal ion homeostasis, and neuroinflammation.

Pyruvate is an energy substrate associated with neuroprotective properties. In this study, we evaluated protective effects of long-term administration of pyruvate in 3xTg-AD mice, a preclinical AD model that develops amyloid-β- and tau-dependent pathology.

Chronic (9 months) treatment with pyruvate inhibited short and long-term memory deficits in 6 and 12 months old 3xTg-AD mice as assessed with the Morris water maze test. Pyruvate had no effects on intraneuronal amyloid-β accumulation and, surprisingly, the molecule increased deposition of phosphorylated tau. Pyruvate did not change aerobic or anaerobic metabolisms but decreased lipid peroxidation, counteracted neuronal hyperexcitability, decreased baseline levels of oxidative stress, and also reduced reactive oxygen species-driven elevations of intraneuronal Zn^{2+} as well as glutamate receptor-mediated deregulation of intraneuronal Ca^{2+}.

Thus, pyruvate promotes beneficial cognitive effects without affecting Aβ and tau pathology. The molecule mainly promotes a reduction of hyperexcitability, oxidative stress while favors the regulation of intraneuronal Ca^{2+} and Zn^{2+} homeostasis rather than acting as energy substrate.

Pyruvate can be therefore a valuable, safe, and affordable pharmacological tool to be associated with classical anti-Aβ and tau drugs to counteract the development and progression of AD-related cognitive deficits and neuronal loss.

Introduction

Amyloid-β (Aβ) oligomers and the deposition of amyloid plaques, along with the formation of neurofibrillary tangles (NFTs) made of hyperphosphorylated tau (p-tau), are considered key pathogenic determinants of Alzheimer's disease (AD; Hardy and Higgins, 1992; Hardy and Selkoe, 2002). However, to date, therapeutic approaches aimed at counteracting Aβ dysmetabolism and tau-related pathology have only produced very modest therapeutic results. A more comprehensive line of intervention aimed at effectively addressing additional and complementary pathogenic factors is therefore greatly needed.

AD is a clinically heterogeneous and multi-factorial disorder. On the permissible background offered by the aging brain, energy

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-β; NFTs, neurofibrillary tangles; p-tau, hyperphosphorylated tau; ROS, reactive oxygen species; $[Zn^{2+}]_i$, intraneuronal Zn^{2+}; $[Ca^{2+}]_i$, intraneuronal Ca^{2+}; m.o, month old; m.o.a, months of age; i.p, intraperitoneal injection; MWM, Morris water maze; CI, CII, CIV and CV, complex I, II, IV, and V; LDH, lactate dehydrogenase; DTDP, 2,2′-dithiodipyridine; NMDA, N-Methyl-D-aspartate; BN-PAGE, blue-native polyacrylamide gel electrophoresis; GC-MS, gas chromatography coupled to mass spectrometry; TBARS, thiobarbituric reactive substances; NMDAR, N-methyl-D-aspartate receptor; HEt, Hydroethidine.

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production failure, mitochondrial dysfunction, neuronal hyperexcitability, excitotoxicity, oxidative stress, alteration of brain levels of metal ions (Ca^{2+}, Zn^{2+}, Cu^{2+}, Fe^{3+}), neuroinflammation, deregulation of neurotrophic factors, all synergistically act to promote AD-related pathology and cognitive decline (Ittner and Götz, 2011).

Hippocampal hyperactivation, a phenomenon found in preclinical AD models and patients (Bakker et al., 2012; Vossel et al., 2013) leads to network instability, cognitive dysfunction and can trigger an excitotoxic overdrive that is instrumental to promote the neuronal loss found in the disease (Palop and Mucke, 2009). Excitotoxicity is central for the injurious deregulation of intraneuronal Ca^{2+} ($[Ca^{2+}]_i$) and Zn^{2+} ($[Zn^{2+}]_i$) (Sensi et al., 2009). Deregulation of brain Zn^{2+} homeostasis, in particular, exacerbates Aβ oligomerization, NFT formation, mitochondrial dysfunction and also furthers the generation of oxidative stress (Bush, 2013; Sensi et al., 2009).

Pyruvate, the end product of glycolysis possesses antioxidant properties and protects against excitotoxic insults in vitro and in vivo (Gramsbergen et al., 2000; Izumi and Zorumski, 2010; Maus et al., 1999). Interestingly, pyruvate strongly attenuates neuronal death resulting from exposure to extracellular Zn^{2+} (Sheline et al., 2000) and is neuroprotective against the Zn^{2+}-dependent neuronal loss observed in animal models of transient cerebral and retinal ischemia (Lee et al., 2001; Yoo et al., 2004). As for AD, oxidative stress dependent Zn^{2+} deregulation has been found in cultured neurons obtained from the 3xTg-AD mouse, a widely employed preclinical AD model (Sensi et al., 2008). In cultured 3xTg-AD cortical neurons, $[Zn^{2+}]_i$ rises triggered by oxidation were in fact found to be significantly increased compared to non-Tg cultures, thereby indicating the presence of a distinct intracellular milieu that is likely making the cells more susceptible to Zn^{2+}-dependent neuronal injury (Sensi et al., 2008).

In light of these observations, we explored whether chronic administration of pyruvate is beneficial in the 3xTg-AD model. The model seems particularly suitable for preclinical studies as this AD mouse develops both amyloid- and tau-dependent pathology along with oxidative stress, Ca^{2+} and Zn^{2+} deregulation, hyperexcitability and also shows an acceleration in the expression of genes that are actively associated with aging (Chakroborty et al., 2012; Gatta et al., 2014).

Materials and methods

Chemicals

FluoZin-3AM, Fluo-4FF AM, Fluo-4AM, Hydroethidine (Het) and pluronic acid were purchased from Molecular Probes (Life Technologies). Sodium pyruvate, N-Methyl-D-aspartate (NMDA), 2,2′-dithiodipyridine (DTDP), glycine as well as chemicals employed to prepare HEPES-buffered control salt solution (see below) and perform blue-native polyacrylamide gel electrophoresis (BN-PAGE), were purchased from Sigma-Aldrich. Cell cultures media and sera were purchased from Gibco (Life Technologies).

Animal model

All experiments employing animal models were approved by the local institutional Ethical Committee (CeSI protocol #: AD-301) and in compliance with national (D.L. n. 116, G.U., suppl. 40, 18 February 1992) and international laws and policies. 3xTg-AD mice were generously provided by Frank LaFerla. Mice were grouped-housed, given access to food and water ad libitum and maintained in a 12:12 light:dark cycle. Behavioral tests were performed during light phase of the cycle.

3 month old (m.o.) male and female 3xTg-AD mice were treated with an intraperitoneal injection (i.p.) of 500 mg/kg pyruvate for a period of 9 months. The goal of the study was to evaluate effects of chronic pyruvate treatment and, given the plasmatic half-life of the molecule (Choi et al., 2013), we set to treat mice three times per week to achieve and maintain high pyruvate levels o in the brain. The 3xTg-AD control group was treated with a saline solution. 12 m.o. 3xTg-AD mice were killed and brains sampled as previously described (Corona et al., 2010).

Cell cultures

Near-pure neuronal cultures were prepared from embryonic (E15 or E16) 3xTg-AD mice. Cerebral cortices were removed and dissected in ice-cold dissecting medium and then placed in trypsin (0.25%) for 10 min at 37 °C. Tissue was centrifuged, supernatant discarded, and pellet mechanically dissociated with a glass Pasteur pipette. Cells were then re-suspended in plating medium containing Neurobasal Medium supplemented with L-Glutamine (0.5 mM), 5% fetal bovine serum, 5% horse serum, 1× B27, and 0.2% penicillin/streptomycin. Cell suspensions were plated onto 25 mm glass bottom dishes pretreated with poly-DL-lysine and laminin (Sigma-Aldrich) and incubated in a controlled atmosphere (5% CO_2 and 90% humidity). 3 days after plating, non-neuronal cell growth was inhibited by adding 10 μM of cytosine arabinofuranoside. Twice a week, 25% of the medium was replaced with equal amounts of fresh Neurobasal medium. Neurons were used after 12 to 17 days (D.I.V.) in vitro. The age-range of the cultures was chosen as full maturation of NMDA and AMPA receptors occurs by the 12 D.IV. (Jones and Baughman, 1991; Li et al., 1998; Stanika et al., 2009). We have previously and extensively employed neuronal cultures within this age bracket and found, in this and previous studies, comparable results in terms of NMDAR-evoked responses when investigating cation deregulation and subsequent mitochondrial dysfunction (Canzoniero et al., 2013; Dugan et al., 1995; Sensi et al., 1997). In agreement with our previous studies, no differences were found for these endpoints (cation deregulation and mitochondrial effects) in present neuronal cultures assayed at the two opposite sides of the 12–17 D.I.V. spectrum.

Morris water maze (MWM) test

MWM tests were performed according to what previously described (Masciopinto et al., 2012). Briefly, the same mice were tested at 6 and 12 m.o.a., however, upon statistical analysis, Grubb's test was performed to detect outliers and led us to evaluate, in the final statistical analysis, a slightly reduced number of mice per condition. Both 6 m.o. (treated, n = 27; untreated n = 18) and 12 m.o. (treated, n = 22; untreated, n = 19) 3xTg-AD mice were trained for 3 consecutive days with four trials per day and an inter-trial time of 20 min. 1.5 and 24 h after the end of the last training trial, probe tests were conducted to assess retention of the spatial memory. During both probe trials, the platform was removed from the pool (Panlab/Harvard apparatus) and mice were allowed to swim freely for 60 s. Parameters employed to evaluate memory skills were time spent to reach the platform location (latency) and number of crosses over the platform location (crosses).

Immunohistochemistry

Immunohistochemistry analysis was carried out in accordance with a previously employed protocol (Corona et al., 2010). Mice to be assayed with immunohistochemistry analysis were randomly chosen from pool of mice that had been behaviorally tested before the assay. Briefly, carnoy-fixed and paraffin-embedded brains of treated (p-tau and Aβ, n = 12) and untreated (p-tau, n = 10; Aβ, n = 11) 3xTg-AD mice were sagittally sectioned. Antigen retrieval was performed by microwave treatment at 750 W for 10 min for p-tau and by water bath at 100 °C for 20 min for Aβ, both in 10 mmol/L sodium citrate buffer (pH 6.0). Tissue sections were incubated overnight with the primary antibody, after the

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