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Dual blockade of the A_1 and A_{2A} adenosine receptor prevents amyloid beta toxicity in neuroblastoma cells exposed to aluminum chloride

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ABSTRACT

In a previous work we have shown that exposure to aluminum (Al) chloride ($AlCl_3$) enhanced the neurotoxicity of the amyloid beta$_{25-35}$ fragment (Abeta$_{25-35}$) in neuroblastoma cells and affected the expression of Alzheimer's disease (AD)-related genes. Caffein, a compound endowed with beneficial effects against AD, exerts neuroprotection primarily through its antagonist activity on A_{2A} adenosine receptors (A_{2A}R), although it also inhibits A_1Rs with similar potency. Still, studies on the specific involvement of these receptors in neuroprotection in a model of combined neurotoxicity (Abeta$_{25-35}$ + $AlCl_3$) are missing. To address this issue, cultured SH-SY5Y cells exposed to Abeta$_{25-35}$ + $AlCl_3$ were assessed for cell viability, morphology, intracellular ROS activity and expression of apoptosis-, stress- and AD-related proteins. To define the role of A_1R and A_{2A}Rs, pretreatment with caffein, specific receptor antagonists (DPCPX or SCH58261) or siRNA-mediated gene knockdown were delivered. Results indicate that $AlCl_3$ treatment exacerbated Abeta$_{25-35}$ toxicity, increased ROS production, lipid peroxidation, β-secretase-1 (BACE1) and amyloid precursor protein (APP). Interestingly, SCH58261 successfully prevented toxicity associated to Abeta$_{25-35}$ only, whereas pretreatment with both DPCPX and SCH58261 was required to fully avert Abeta$_{25-35}$ + $AlCl_3$-induced damage, suggesting that A_1Rs might also be critically involved in protection during combined toxicity. The effects of caffein were mimicked by both N-acetyl cysteine, an antioxidant, and desferrioxamine, likely acting through distinct mechanisms. Altogether, our data establish a novel protective function associated with A_1R inhibition in the setting of combined Abeta$_{25-35}$ + $AlCl_3$ neurotoxicity, and expand our current knowledge on the potential beneficial role of caffein to prevent AD progression in subjects environmentally exposed to aluminum.

1. Introduction

Aluminum (Al) has been suggested as a potential neurotoxic metal implicated in the progression of a number of neurodegenerative diseases, including Alzheimer's disease (AD) (Gorell et al., 1999; Nakamura et al., 2000; Rondeau et al., 2000). Its causative relationship with AD remains to be fully outlined, although numerous evidences have proposed that Al might modify some of the pathogenetic mechanisms that contribute to the development of this neurodegenerative disease (Chen et al., 2011; Cuajungco et al., 2000; Flaten, 2001; Lovell et al., 1993; Lukiw and Pogue, 2007; Rondeau et al., 2009).

One of the main mechanisms of Al toxicity is the disruption of the intracellular redox environment and involves alterations in metal homeostasis (Becaria et al., 2006; Harris et al., 1996; Kawahara and Kato-Negishi, 2011). Imbalance in metal homeostasis seems to be caused by the similarity of the physical and chemical properties of Al with other metals, which enables Al to aberrantly mimic their biological functions to drive biochemical abnormalities. Specifically, Al-mediated alterations in iron (Fe) homeostasis have been indicated as pivotal factors that render this metal toxic (Peto, 2010; Ward et al., 2001; Wu et al., 2012). Indeed, the interaction between Al and Fe contributes to the formation of reactive oxygen species (ROS). To support this, elevated levels of ROS have been shown in various models of Al toxicity (Bhasin et al., 2012; Lemire et al., 2011; Nayak et al., 2010; Yuan et al., 2012) and oxidative damage has been systematically observed in the brain and other organs of animals exposed to the metal (Li et al., 2012; Nehru and Anand, 2005; Praticò et al., 2002). Other evidences have suggested that Al can induce toxicity in primary human neural

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cells by promoting the up-regulation of pro-inflammatory and pro-apoptotic genes (Lukiw et al., 2005).

In relationship to AD pathogenesis, Al has been detected in senile plaques and tangle-bearing neurons (Miu et al., 2003), suggesting that this metal could play an active role as a potential cofactor in AD. Such hypothesis is further strengthened by our previous report showing that treatment with Al chloride ($AlCl_3$) in neuroblastoma cells exposed to low concentrations of the amyloid β fragment 25-35 ($A\beta_{25-35}$) altered the early mRNA expression profile of molecules involved in the processing of Aβ, including β-secretases 1–2 and the amyloid precursor protein (APP) (Castorina et al., 2010).

APP is a glycoprotein involved in the maintenance of neuronal viability and neurite outgrowth (Milward et al., 1992). In physiological conditions, APP undergoes proteolytic cleavage through α-secretase and two β-secretases known as the β-site APP-cleaving enzyme 1 (BACE1) and the β-site APP-cleaving enzyme 2 (BACE2) to give rise to C-terminal fragments (CTFα or CTFβ) that are finally cleaved by γ-secretases to produce either the nonamyloidogenic peptide (p3) or the amyloidogenic peptide Aβ. According to the "amyloid hypothesis", dysregulated generation or degradation of Aβ represents the triggering event in the pathogenic cascade of AD (Satoh and Kuroda, 2000; Wilquet and De Strooper, 2004).

ROS and/or supraphysiological Aβ concentrations are both key elements in the activation of the stress-responsive nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB), the major transcriptional regulator of both APP, β- and γ-secretases (Buggia-Prevot et al., 2008; Chami et al., 2012; Guglielmotto et al., 2012). Hence, it is possible that coexposure to Al in the presence of Aβ creates an intracellular oxidative environment that exacerbates Aβ-induced NF-κB transcriptional activity, thereby giving rise to an aberrant vicious circle that leads to increased expression of pro-inflammatory, pro-apoptotic and AD-related molecules, augmented Aβ levels and consequently, neuronal cell death. Based on this theory, it is reasonable that compounds endowed with antioxidant properties may counteract the detrimental effects of combined exposure to Al and Aβ.

Caffein, the most widely consumed psychoactive drug, is a nonselective competitive A_1R and $A_{2A}R$ antagonist (Fredholm and Lindström, 1999). Most of the neuroprotective and memory enhancing effects of caffein have been attributed to its ability to antagonize $A_{2A}R$ (Dall'Igna et al., 2003; Prediger et al., 2005). Blockade of $A_{2A}R$ has consistently been found to afford neuroprotection against different brain insults, an effect that involves antioxidant activity (Cunha, 2005; Endesfelder et al., 2013). It has also been postulated that caffein consumption is inversely correlated with the incidence of AD (Maia and de Mendonça, 2002), an evidence substantiated by data obtained in Aβ toxicity models (Dall'Igna et al., 2003). However, direct evidence of the involvement of A_1R inhibition in neuroprotection by caffein has also been provided using a model of methylmercury poisoning (Björklund et al., 2007), inferring on the possibility that epigenetic factors such as environmental exposure to pollutants or metals, including Al, might contribute to modify the cellular mechanisms triggered by caffein to afford protection against Aβ.

To address these issues, we tested the hypothesis that exposure of neuroblastoma cells to $AlCl_3$ (10 μM) in the presence of low concentrations of $Abeta_{25-35}$ (2 μM) could affect the cellular mechanisms of response to caffein. We discovered that during combined toxicity, full protective activity was retained only when both A_1R and $A_{2A}Rs$ were mutually antagonized. These findings, together with other biochemical evidences provided in the present work, suggested that $AlCl_3$ increases $A\beta_{25-35}$-induced oxidative damage and that caffein protection through the activation of the intracellular antioxidant machinery requires dual blockade of A_1R and $A_{2A}R$ to work at its full potential.

2. Materials and methods

2.1. Cell cultures

Human neuroblastoma SH-SY5Y cells were purchased from the ATCC (CRL-2266, Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and penicillin/streptomycin (Sigma). Cells were used at early passages (P10–P20) and left undifferentiated. The reason why we did not perform our studies on differentiated neuroblastoma cells was based on previous evidences indicating that, upon differentiation, profound changes in mitochondrial metabolism, antioxidant defences and survival pathways occur (Schneider et al., 2011; Wang et al., 2006).

2.2. Amyloid $beta_{25-35}$ fragment preparation

The $Abeta_{25-35}$ fragment (Sigma) was dissolved in double-distilled deionized water at a concentration of 400 μM (stock solution). To obtain the neurotoxic form of $Abeta_{25-35}$, the peptide solution was placed in an incubator at 37 °C for 7 days and stored at −80 °C until use. During the experiments, aliquots from the peptide stock solution were added directly to the culture medium: the final concentration used was 2 μM.

2.3. Assessment of cell viability (MTT assay)

Cell viability was assessed using the cell proliferation kit I (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Roche)) following a protocol recently described (Castorina et al., 2014). SH-SY5Y cells were seeded into 96-well plates at 10^4 cells/well. DMEM (Sigma–Aldrich) containing 0.5 mg/ml MTT was added in each well. Following incubation for 4 h at 37 °C, medium was collected and DMSO (100 μl) was added. Formazan formed by the cleavage of the yellow tetrazolium salt MTT was then measured at 550–600 nm using a microplate reader (BioRad Biophotometer).

2.4. Morphological appearance and Hoechst 33258 staining

Morphological appearance and Hoechst staining were evaluated using bright field or immunofluoresce microscopy, respectively. 1×10^5 cells were seeded in 35 mm plastic dishes and treated as indicated in the related Section 3. To study morphology, cells were examined using an Axioplan Zeiss light microscope (Jena, Germany) and photographed with a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany). Dead cells were determined by three different observers blind to the experiments based on rounded cell morphology, presence of apoptotic bodies, pyknotic irregular cell shape and subsequent detachment and fragmentation of cells. Cell death was estimated by dividing the mean no. of round-shaped cells *per* field/mean total no. of cells *per* field (values obtained from at least five randomly selected fields in three independent experiments). To confirm apoptosis, Cells were fixed with a solution of methanol/acetic acid (3:1, v/v) for 30 min, washed three times in PBS and incubated for 15 min at 37 °C with 0.4 μg/ml Hoechst 33258 dye as previously described (Castorina et al., 2008).

2.5. Measurement of intracellular reactive oxygen species (ROS)

To measure ROS produced within cells we used the cell-permeable H_2O_2-sensitive probe DCFDA. The extent of H_2O_2 generation was defined as ROS generation for our convenience. The dye 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) was purchased from Sigma. Fluorescence