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SPECIAL REPORT

Neuroprotection by caffeine and adenosine A_{2A} receptor blockade of β-amyloid neurotoxicity

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Adenosine is a neuromodulator in the nervous system and it has recently been observed that pharmacological blockade or gene disruption of adenosine A_{2A} receptors confers neuroprotection under different neurotoxic situations in the brain. We now observed that coapplication of either caffeine (1 – 25 μM) or the selective A_{2A} receptor antagonist, 4-(2-[7-amino-2(2-furyl)(1,2,4)triazolo (2,3-*a*)(1,3,5)triazin-5-ylamino]ethyl)phenol (ZM 241385, 50 nM), but not the A receptor antagonist, 8-cyclopentyltheophylline (200 nM), prevented the neuronal cell death caused by exposure of rat cultured cerebellar granule neurons to fragment 25 – 35 of β-amyloid protein (25 μM for 48 h), that by itself caused a near three-fold increase of propidium iodide-labeled cells. This constitutes the first *in vitro* evidence to suggest that adenosine A_{2A} receptors may be the molecular target responsible for the observed beneficial effects of caffeine consumption in the development of Alzheimer's disease.
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Abbreviations: $A\beta_{25-35}$, fragment with amino acids 25 – 35 of β-amyloid protein; CPT, 8-cyclopentyltheophylline; ZM 241385, 4-(2-[7-amino-2(2-furyl)(1,2,4)triazolo(2,3-*a*)(1,3,5)triazin-5-ylamino]ethyl)phenol

Introduction Adenosine is a neuromodulator in the nervous system that refrains neuronal excitability through activation of inhibitory A_1 receptors (Cunha, 2001). This has led to the exploitation of A_1 receptor ligands as potential neuroprotective agents, an effort that has been undermined by the cardiovascular side effects of these agents (de Mendonça *et al*., 2000). Adenosine can also activate facilitatory A_{2A} receptors, which are highly abundant in the basal ganglia and have low density elsewhere in the brain (Fredholm *et al*., 2002). The association of A_{2A} receptors with dopamine D_2 receptors in the basal ganglia has fostered the development of A_{2A} receptor antagonists as anti-Parkinsonian drugs (Fredholm *et al*., 2002). Interestingly, it was noted that A_{2A} receptor antagonists not only provided symptomatic relief but also arrested the progression of neurodegeneration in animal models of Parkinson's disease (Chen *et al*., 2001; Ikeda *et al*., 2002). Antagonists of A_{2A} receptors also confer neuroprotection in other neurodegenerative situations in the basal ganglia (e.g. Reggio *et al*., 1999; Popoli *et al*., 2002) and, surprisingly, also in the extra-striatal region of the brain, namely in cortical areas (e.g. Monopoli *et al*., 1998; Behan & Stone, 2002). A possible *in vivo* correlate of this neuroprotective role of A_{2A} receptor antagonists is the protection conferred by caffeine, an adenosine receptor antagonist, in Parkinson's disease (Schwarzschild *et al*., 2002). Interestingly, caffeine consumption has also recently been proposed to be a protective factor in the development of Alzheimer's disease (Maia & de Mendonça, 2002). This led us to test if caffeine and an A_{2A} receptor antagonist could protect neurons against the neurotoxicity caused by exposure to β-amyloid protein, a purported effector of neurodegeneration occurring in Alzheimer's disease (Vickers *et al*., 2000).

Methods Primary cultures of cerebellar granule cells were prepared from 8-day-old Wistar rats, as previously described (Porciúncula *et al*., 2001). Briefly, freshly dissected cerebella were incubated with 0.025% trypsin solution for 15 min at 37°C and disrupted mechanically in the presence of 0.08 mg ml^{-1} DNAse and 0.05% trypsin inhibitor. Cells were then seeded at a density of 1.5×10^5 cells cm^{-2} in a 96-well multiwell dish coated with 10 μg ml^{-1} poly-D-lysine and incubated in Eagle's basal medium supplemented with 10% fetal bovine serum, 50 μg gentamicin and 25 mM KCl. The growth of non-neuronal cells was inhibited by addition of 20 μM cytosine arabinofuranoside 18 – 24 h after seeding and the medium was not changed during the culture period. After 5 days of growth in a humidified atmosphere of 95% air and 5% CO_2 at 37°C, cells were deprived of serum and incubated in the absence or presence of fragment with amino acids 25 – 35 of β-amyloid protein ($A\beta_{25-35}$) (25 μM, from Sigma, Sintra, Portugal) without or with either caffeine (0.2 – 25 μM, from Sigma) or 8-cyclopentyltheophylline (CPT, 200 nM, from Sigma) or 4-(2-[7-amino-2-(2-furyl)(1,2,4)triazolo(2,3-*a*) (1,3,5)triazin-5-ylamino]ethyl)phenol (ZM 241385, 50 nM, from Tocris Cookson, Bristol, U.K.). Cellular viability was evaluated after 48 h by loading the cells for 3 min with Krebs buffer (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 1 mM $CaCl_2$, 6 mM glucose, 10 mM HEPES-Na, pH 7.4) containing 4 μg μl^{-1}

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propidium iodide (Calbiochem), and red-labeled cells were photographed using an inverted Nikon Diaphot Eclipse TE 300 with standard rhodamine filter set (excitation 540 nm; emission 617 nm). Pictures were then converted into black and white, and the number of white spots (cells labeled with propidium iodide) was quantified (Scion Image 4.02 software) and used as an index of cell damage. An evaluator blind to treatments performed all quantification procedures.

The values presented are mean ± s.e.m. of n experiments. To test the significance of the effect of a drug *versus* control, a one-way variance analysis (ANOVA) was used, followed by Duncan's test. $P \leqslant 0.05$ was considered to represent a significant difference.

Results The number of propidium iodide-labeled cells (i.e. nonviable cells) in cultures from which serum was withdrawn at day 5 was low (112 ± 15 cells per field, $n = 5$, see Figure 1a). When the cells were incubated without serum and with 25 μM $A\beta_{25-35}$ for 48 h from day 5 onwards, there was a 183 ± 43% ($n = 5$) increase in the number of nonviable cells ($P < 0.05$) compared to the control situation (withdrawal of serum) (Figure 1a, b). As illustrated in Figure 1b, the coadministration of caffeine (1 – 25 μM) at the time of application of $A\beta_{25-35}$ (25 μM) and serum withdrawal prevented, in a concentration-dependent manner, the $A\beta_{25-35}$-induced increase in the number of nonviable cells. Importantly, caffeine (25 μM), which fully prevented $A\beta_{25-35}$-induced neurotoxicity, did not significantly ($P > 0.05$) change the number of nonviable cells upon withdrawal of serum without adding $A\beta_{25-35}$ ($n = 5$, Figure 1b), indicating that endogenous extracellular adenosine selectively interferes with neuronal cell death caused by the β-amyloid fragment peptide rather than by serum withdrawal.

We then tried to determine which subtype of adenosine receptor was mainly responsible for the control of β-amyloid-induced neurotoxicity. As illustrated in Figure 1c, 8-cyclopentyltheophylline (CPT, 200 nM), which at this concentration is a selective A_1 receptor antagonist (e.g. Kessey & Mogul, 1998), did not significantly modify the β-amyloid-induced neurotoxicity. In contrast, the selective A_{2A} receptor antagonist, 4-(2-[7-amino-2(2-furyl) (1,2,4) triazolo (2,3-*a*) (1,3,5) triazin-5-ylamino)ethyl)phenol (ZM 241385) (50 nM) (see Cunha *et al*., 1997), almost completely prevented the $A\beta_{25-35}$-induced increase in the number of nonviable cells (Figure 1c). As observed for caffeine, both CPT (200 nM) and ZM 241385 (50 nM) failed to modify the number of nonviable cells upon withdrawal of serum without adding $A\beta_{25-35}$ ($n = 3$, Figure 1c).

Discussion The present results show that caffeine prevents the β-amyloid-induced neurotoxicity in cultured cerebellar neurons of the rat and that this neuroprotective effect is likely to be because of a blockade of adenosine A_{2A} rather than adenosine A_1 receptors. This finding adds to previous reports of neuroprotective effects resulting from adenosine A_{2A} receptor blockade in different stressful situations (Monopoli *et al*., 1998; Reggio *et al*., 1999; Chen *et al*., 2001; Ikeda *et al*., 2002; Behan & Stone, 2002; Popoli *et al*., 2002) suggesting that blockade of adenosine A_{2A} receptors may be a general neuroprotective mechanism.

However, neuroprotection afforded by blockade of A_{2A} receptors is, so far, phenomenological since the mechanism operated by A_{2A} receptors to confer robust neuroprotection is not known. Several mechanisms have been proposed, namely control of glutamate release, of glutamate clearance by

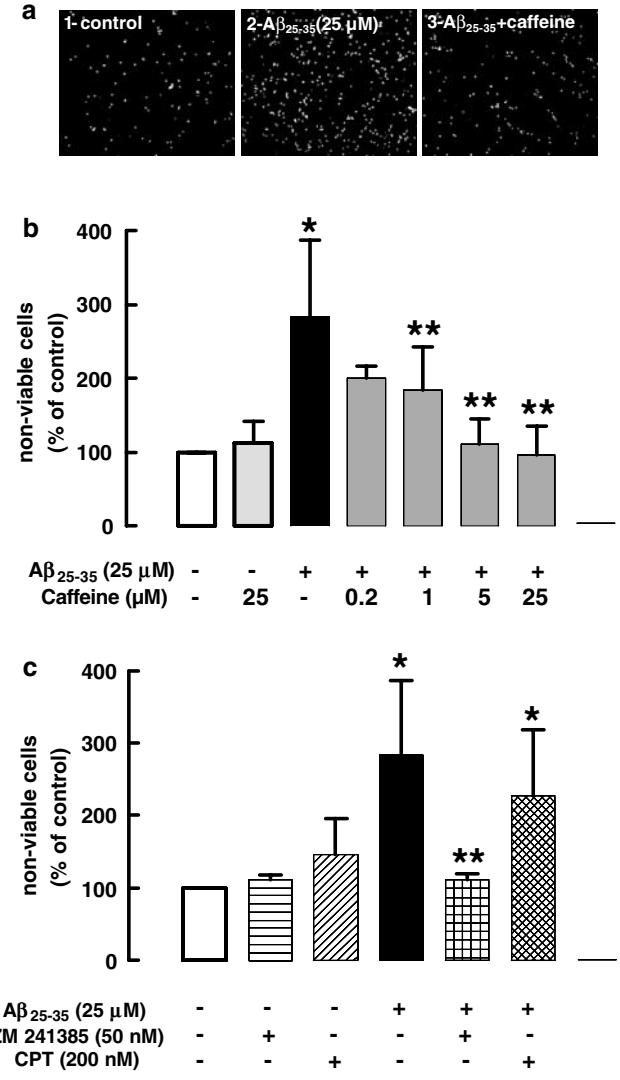

Figure 1 Caffeine and adenosine A_{2A} receptor antagonists are neuroprotective against β-amyloid-induced neurotoxicity. Rat cerebellar neurons were cultured for 5 days and serum was withdrawn at day 5 for 48 h, which led to a discrete pattern of propidium iodide-labeled cells, indicative of low number of nonviable cells (a-1) and first column from the left in (b) and (c). The exposure of cells to $A\beta_{25-35}$ (25 μM) in parallel with serum withdrawal increased the number of nonviable cells ((a-2), third and fourth columns from the left in (b) and (c), respectively) in comparison with the withdrawal of serum only (considered as control, 100% being the number of nonviable cells in this situation). (b) Administration of increasing concentrations of caffeine (1 – 25 μM) together with $A\beta_{25-35}$ (25 μM) attenuated and fully prevented the $A\beta_{25-35}$-induced neurotoxicity (see also (a-3)), but addition of caffeine (25 μM) failed to modify the number of nonviable cells upon serum withdrawal in the absence of $A\beta_{25-35}$ (second column in (b)). The data are mean ± s.e.m. of five experiments. (c) A selective A_{2A} receptor antagonist, ZM 241385 (50 nM, second column from the right), but not an A_1 receptor antagonist, CPT (200 nM, first column from the right), blocked the $A\beta_{25-35}$ (25 μM)-induced neurotoxicity, but failed to modify the number of nonviable cells upon serum withdrawal in the absence of $A\beta_{25-35}$ (second and third columns from the left). The data are mean ± s.e.m. of three experiments. *$P < 0.05$ *versus* control (first column from the left); **$P < 0.05$ *versus* effect of 25 μM $A\beta_{25-35}$ (third and fourth columns from the left in (b) and (c), respectively).

astrocytes, of inflammatory reactivity by microglia, of vascular resistance or a direct control of calcium entry or of cell cycling in neurons (discussed in Fredholm *et al*., 2002). The presently observed neuroprotection in cultured neurons is particularly instructive from a mechanistic point of view because it allows one to conclude that the blockade of adenosine A_{2A} receptors directly prevents neuronal death independent of astrocytes, microglia or vascular elements. This brings into stage recent evidence indicating that A_{2A} receptors are able to control both cell cycling in PC12 cells (Huang *et al*., 2001) and in cardiomyocytes (Zhao *et al*., 2001) and calcium entry into neurons (e.g. Gonçalves *et al*., 1997; Wirkner *et al*., 2000), thus being potentially able to control either apoptotic- and necrotic-like neuronal death. Certainly, further work needs to be carried out to test if β-amyloid-induced neurotoxicity in rat cultured cerebellar neurons mostly involves apoptotic- or necrotic-like features and to elucidate the molecular mechanisms operated by adenosine A_{2A} receptors to control this β-amyloid-induced neuronal cell death.

The present observation that adenosine A_{2A} receptor blockade mimics the neuroprotective effect of caffeine against β-amyloid-induced neurotoxicity strongly suggests that blockade of adenosine A_{2A} receptors might be the target for the observed protective effects of caffeine consumption in Alzheimer's disease (Maia & de Mendonça, 2002). It is worthwhile noting that the effects of adenosine A_{2A} receptor antagonists do not seem to desensitise over time (Xu *et al*., 2002) and that the extra-striatal density of adenosine A_{2A} receptors increases in aged animals (Cunha *et al*., 1995), making this receptor particularly interesting as a therapeutic target. It will be interesting to see if the long-term intake of adenosine A_{2A} receptor antagonists, now in clinical trials, will have the expected protective effect on cognitive status of the subjects enrolled in these studies.

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