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Adenosine A_1 and A_3 receptors protect astrocytes from hypoxic damage

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ABSTRACT

Brain levels of adenosine are elevated during hypoxia. Through effects on adenosine receptors (A_1, A_{2A}, A_{2B} and A_3) on astrocytes, adenosine can influence functions such as glutamate uptake, reactive gliosis, swelling, as well as release of neurotrophic and neurotoxic factors having an impact on the outcome of metabolic stress. We have studied the roles of these receptors in astrocytes by evaluating their susceptibility to damage induced by oxygen deprivation or exposure to the hypoxia mimic cobalt chloride ($CoCl_2$). Hypoxia caused ATP breakdown and purine release, whereas $CoCl_2$ (0.8 mM) mainly reduced ATP by causing cell death in human D384 astrocytoma cells. Further experiments were conducted in primary astrocytes prepared from specific adenosine receptor knock-out (KO) and wild type (WT) mice. In WT cells purine release following $CoCl_2$ exposure was mainly due to nucleotide release, whereas hypoxia-induced intracellular ATP breakdown followed by nucleoside efflux. *N*-ethylcarboxamidoadenosine (NECA), an unselective adenosine receptor agonist, protected from cell death following hypoxia. Cytotoxicity was more pronounced in A_1R KO astrocytes and tended to be higher in WT cells in the presence of the A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX). Genetic deletion of A_{2A} receptor resulted in less prominent effects. A_3R KO glial cells were more affected by hypoxia than WT cells. Accordingly, the A_3 receptor agonist 2-chloro-N^6-(3-iodobenzyl)-*N*-methyl-5′-carbamoyladenosine (CL-IB-MECA) reduced ATP depletion caused by hypoxic conditions. It also reduced apoptosis in human astroglioma D384 cells after oxygen deprivation. In conclusion, the data point to a cytoprotective role of adenosine mediated by both A_1 and A_3 receptors in primary mouse astrocytes.

1. Introduction

Astrocytes, the predominant glial cell type in the brain, play an important role in physiological and pathological neuronal activities as well as in the control of central nervous system (CNS) homeostasis (Panickar and Norenberg, 2005). Because of their close proximity to capillaries, changes in astrocytes are among the earliest events following ischemia (Panickar and Norenberg, 2005). Astrocytes activate several mechanisms that tend to decrease neuronal injury, e.g. they produce trophic factors, regulate transmitter and ion concentrations and remove excessive glutamate from the extracellular milieu (Aschner et al., 2002). Thus, astrocytes have a direct impact on neuronal survival and synaptic function, as well as neurogenesis and neural repair (Aschner et al., 2002; Swanson et al., 2004). Although astrocytes are more resistant to oxidative stress than neurons, strong energetic demands during ischemia can compromise astrocytic function and viability. Both necrotic and apoptotic cell death in astrocytes have been reported after exposure to ischemia in vitro (Yu et al., 2001). ATP levels in rat cortical astrocytes have been shown to decline to different extent during hypoxia, resulting in apoptosis and necrosis (Gregory et al., 1990; Yu et al., 1989, 2001).

Adenosine is important as a modulator of glial function in emergency situations (Boison, 2006; Daré et al., 2007). The concentration of adenosine is known to rise quickly during stressful events (i.e. lack of oxygen) to reset the energy balance in the cells. Extracellular adenosine is derived from the breakdown of intra- and/or extracellular ATP (Fredholm, 2007). Its effects are mediated by G protein coupled adenosine receptors of four distinct subtypes (A_1, A_{2A}, A_{2B}, A_3) with tissue-specific distribution (Fredholm et al., 2000). A_1 and A_3 receptor activation stimulates G_i proteins, which inter alia cause inhibition of adenylyl cyclase and thus decrease levels of cAMP, whereas A_{2A} and A_{2B} receptors enhance cAMP formation through the interaction with stimulatory G_s/G_{olf} proteins (Fredholm et al., 2000). All four adenosine receptor subtypes are present in astrocytes (Daré et al., 2007). Activation of adenosine receptors in glial cells seems to mediate many protective effects on the surrounding neurons after hypoxic insult. During transient hypoxia astrocytes contribute to regulation of the excitatory synaptic transmission by discharging adenosine, which reduces presynaptic transmitter release by acting on A_1 receptors (Martin et al., 2007; Pascual et al., 2005) and potentiates glutamatergic transmission by acting on glial A_{2A} receptors (Li et al., 2001). A_1 receptors were also found to inhibit reactive astrogliosis, one

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of the main features of glial response to ischemic injury (Ciccarelli et al., 1994). Intriguingly, adenosine acting on A_{2A} and A_3 receptors was reported to increase the number of reactive astrocytes (Brambilla et al., 2003). The A_3 receptors has been found to mediate both protection and cell death, depending on the degree of receptor activation and type of toxic insult (Abbracchio et al., 1997a,b; Di Iorio et al., 2002). In addition, stimulation of A_3 receptors in astrocytes was shown to induce release of neuroprotective cytokines (Wittendorp et al., 2004).

The majority of the studies addressing the role of adenosine receptors in modulating astrocyte functions during hypoxia have employed pharmacological tools, i.e. agonists and antagonists. However, these tools are sometimes insufficiently specific, especially in the case of the rodent A_3 receptors. Here we have used an alternative approach to eliminate the function of specific adenosine receptors, i.e. their genetic deletion. We have investigated the cytotoxic effects of hypoxia-like conditions in primary astrocytes prepared from specific adenosine receptor knock-out (KO) mice. Besides using oxygen deprivation, we have treated the cells with cobalt chloride ($CoCl_2$), which activates the hypoxia inducible factor-1α (HIF-1α) pathway. Release of purines and regulation of hypoxia effects by adenosine receptors were also studied in the human astrocytoma D384 cell line. Our results confirm that hypoxia raises adenosine levels in astrocyte cultures. They also indicate that the pro-survival action of adenosine during conditions of oxygen deprivation involves the activation of adenosine A_1 and A_3 receptors in mouse astrocytes.

2. Materials and methodology

2.1. Chemicals

All cell culture reagents, including media, antibiotics, fetal bovine serum (FBS) and phosphate buffered saline (PBS) were purchased from Gibco-Life Technologies (Täby, Sweden) and cell culture plastics from Corning Incorporated (Schipol-Rijk, The Netherlands). $CoCl_2$ (cell culture tested), the lactate dehydrogenase (LDH) detection kit, adenosine deaminase, *N*-ethylcarboxamidoadenosine (NECA) and the rest of the chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2-Chloro-N^6-(3-iodobenzyl)-*N*-methyl-5′-carbamoyladenosine (CL-IB-MECA) was obtained from Tocris Cookson (Ellisville, MO) and dipyridamole from Boehringer-Ingelheim (Ingelheim, Germany). Antibodies against β-III tubulin and glial fibrillary acidic protein (GFAP), as well as FITC-conjugated isolectin B4 were purchased from Sigma-Aldrich Chemie (Schnelldorf, Germany).

2.2. Animals

Four types of mice were used: *adenosine A_1 receptor knock-out* (A_1R KO) (Johansson et al., 2001), and *adenosine A_{2A} receptor knock-out* (A_{2A}R KO) *mice* (Chen et al., 1999), *adenosine A_3 receptor deficient mice* (A_3R KO) generated by Merck Research Laboratories (Salvatore et al., 2000) and *C57BL/6 mice* (wild type, WT). To generate the A_1R KO mice, the second coding exon of the mouse adenosine A_1 receptor gene was inactivated in mouse E14.1 embryonic stem cells, and 129/OlaHsd/C57BL/6 hybrid mice were generated. A_{2A}R KO mice were made on mixed 129-Steel×C57BL/6 and the A_3R KO mice on 129×C57BL/6 genetic background. All adenosine receptor KO mice were backcrossed for more than 10 generations with C57BL/6 to achieve essentially pure congenic lines. Animals were bred at the Department of Physiology and Pharmacology, Karolinska Institutet. Mice were housed at a constant room temperature (22 °C; 12 h light/dark cycle, lights on at 6 am) with *ad libitum* access to food and water and were routinely genotyped by PCR. The study was performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and all procedures were approved by the Animal Ethics Committee of Northern Stockholm.

2.3. Primary astrocyte cultures

Primary cultures of mouse astrocytes were prepared from newborn mice sacrificed by decapitation. The brains were rapidly dissected out, cerebellum and olfactory bulb were removed and the meninges and blood vessels were carefully stripped off at the stereomicroscope. The tissue was chopped and transferred to complete Dulbecco's modified Eagle medium (DMEM) (i.e. supplemented with 10% FBS, 50 units/ml penicillin and 50 mg/ml streptomycin-sulphate) and dissociated by pipetting in order to generate a cell suspension, which was filtered through a 70-μm pore size mesh cell strainer. Cells were seeded at a density of 28,000 cells/cm^2 in poly-L-lysine-coated bottles and maintained in complete DMEM at 37 °C in a humidified atmosphere with 5% CO_2. The culture medium was changed on day in vitro (DIV)1, DIV5 and DIV7. On DIV9 the microglia were shaken loose from the astrocyte cultures and discarded. The astrocytes were harvested from the flask with trypsin and reseeded at a density of 28,000 cells/cm^2. On DIV12 the cells were exposed to the various chemicals in F-12 medium supplemented with 10% FBS. Purity of the cultures was monitored by immunocytochemical detection of the astrocyte marker GFAP and the neuronal marker β-III tubulin. Absence of microglial cells was confirmed using FITC-conjugated isolectin B4.

2.4. D384 cell line

The human astrocytoma D384 cells were routinely seeded at a density of 10,000 cells/cm^2 in DMEM (Invitrogen AB, Gibco BRL, Lidingö, Sweden) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. The cell culture flasks were kept in an incubator at 37 °C in a humidified atmosphere with 5% CO_2.

2.5. Exposure to neurotoxic conditions

D384 cells were seeded 24 h prior to exposure to the hypoxic conditions. For studies on primary astrocytes, the culture medium was changed on DIV12 to F-12 supplemented with 10% FBS before neurotoxic challenge. Cells were either treated with $CoCl_2$ or 2-deoxy-glucose (2-DG), or were transferred to a CO_2 incubator (Queue, LABEQUIPTM, Ontario, Canada) connected to a nitrogen source to obtain hypoxia (1% O_2, 5% CO_2, 94% N_2). The measurements of O_2 concentration inside the incubator were performed with the Draeger Pac III analyzer (Draeger Safety mc., Pittsburg, PA, USA). In some experiments cells were pre-treated for 1 h with various substances (adenosine receptor agonists and antagonists) prior to treatment with cobalt or hypoxia.

2.6. Luminescence ATP detection assay system

Cells were plated in 96-well plates and exposed to $CoCl_2$, 2-DG or hypoxia. In some experiments WT astrocytes were pre-treated with various substances (adenosine receptor agonists and antagonist) for 1 h prior to neurotoxic challenge. At the end of the treatments, the total ATP level in each well was measured with the ATP LiteTM kit, which is based on the production of light caused by the reaction of ATP with added firefly luciferase and D-luciferin. The luminescence was measured with a Trilux Micro Beta luminescence counter (Wallac, Upplands Väsby, Sweden).

2.7. Trypan blue exclusion test

Cells were harvested with trypsin and pooled together with cells floating in the culture medium. An aliquot of this cell suspension was mixed with an equal volume of 0.4% trypan blue in phosphate buffered saline (PBS). Cells were scored at the phase contrast microscope using