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Human alpha 1-antitrypsin protects neurons and glial cells against oxygen and glucose deprivation through inhibition of interleukins expression

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ABSTRACT

Background: Death due to cerebral stroke afflicts a large number of neuronal populations, including glial cells depending on the brain region affected. Drugs with a wide cellular range of protection are needed to develop effective therapies for stroke. Human alpha 1-antitrypsin (hAAT) is a serine proteinase inhibitor with potent anti-inflammatory, anti-apoptotic and immunoregulatory activities. This study aimed to test whether hAAT can protect different kind of neurons and glial cells after the oxygen and glucose deprivation (OGD).
Methods: Addition of hAAT to mouse neuronal cortical, hippocampal and striatal cultures, as well as glial cultures, was performed 30 min after OGD induction and cell viability was assessed 24 h later. The expression of different apoptotic markers and several inflammatory parameters were assessed by immunoblotting and RT-PCR.
Results: hAAT had a concentration-dependent survival effect in all neuronal cultures exposed to OGD, with a maximal effect at 1–2 mg/mL. The addition of hAAT at 1 mg/mL reduced the OGD-mediated necrotic and apoptotic death in all neuronal cultures. This neuroprotective activity of hAAT was associated with a decrease of cleaved caspase-3 and an increase of MAP2 levels. It was also associated with a reduction of pro-inflammatory cytokines protein levels and expression, increase of IL-10 protein levels and decrease of nuclear localization of nuclear factor-kappaB. Similar to neurons, addition of hAAT protected astrocytes and oligodendrocytes against OGD-induced cell death.
Conclusions: Human AAT protects neuronal and glial cells against OGD through interaction with cytokines.
General significance: Human AAT could be a good therapeutic neuroprotective candidate to treat ischemic stroke.

1. Introduction

Ischemic stroke is a cerebrovascular disease associated with high risk of mortality and is a leading cause of disability in adults [1]. The infarcted area resulting from the cerebral ischemia is characterized by the presence of a primary necrotic core and a secondary apoptotic area. This neuronal damage is promoted by the temporal activation of different extrinsic and intrinsic events [2]. First, the presence of oxidative stress, excitotoxicity and blood brain barrier (BBB) disruption contribute to the development of the infarcted area [3–5]. Then, the presence of pro-inflammatory cytokines activates infiltrated neutrophils that release neutrophil elastase contributing to exacerbate the neuronal damage [6]. Altogether, these events activate pro-inflammatory cytokines and proteases conducing to neuronal death [7–10]. A large number of neuronal populations could be afflicted depending on the brain region affected. Moreover, glial cells are also damaged in cerebral ischemia, contributing to neurological deficits [11, 12]. Therefore, molecules with capacity to avoid the activity of neutrophils and to inhibit the activation of pro-inflammatory cytokines and proteases in different neuronal types and glial cells could help to prevent cerebral ischemia mediated-cell death.

Human alpha 1-antitrypsin (hAAT) is the most abundant serum serine protease inhibitor, primarily synthesized in the liver [13]. The inhibitory action of hAAT on serine proteases confers protection through the reduction of the serine proteases-mediated inflammation [13–15]. There is an increase of plasmatic hAAT levels in response to ischemic injury, such as in patients with acute coronary syndrome [16] and hypoxia in human cell lines [17], suggesting the activation of a

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protective response against myocardial infarction or hypoxia. In addition, administration of hAAT to mouse models subjected to myocardial infarction or brain ischemic injury protects the cells, thereby reducing the infarct size [18, 19]. This beneficial action has been associated with the capacity of AAT to restrict the activity of cells from innate and adaptive immune system [20]. However, a protective effect of hAAT independent of serine protease inhibition and associated with its capacity to enter the cells has been reported [21]. Moreover, AAT has been described to have a protective effect in pancreatic or liver cells through inhibition of caspases mediated-apoptosis, reduction of pro-inflammatory cytokines, and increasing anti-inflammatory cytokines [22, 23].

In this study we wanted to explore the therapeutic capacity of hAAT to protect different neuronal populations and glial cells after ischemic injury. To this aim, we induced oxygen and glucose deprivation (OGD) in different neuronal and glial cultures, followed by the addition of different concentrations of hAAT. Then, we studied the protective capacity of hAAT against the OGD-mediated cell death, and we analyzed the ability to inhibit the activation of caspase-3 and the induction of pro-inflammatory cytokines.

2. Materials and methods

2.1. Cell cultures

Primary neuronal cultures were obtained from 18-days old C57BL/6 mouse embryos. Hippocampi and cerebral cortex were dissected and cells were seeded at 100,000 cells/cm^2. Dissociated striatal cells were plated at a density of 50,000 cells/cm^2. All neuronal cultures were seeded on plates pre-coated with 0.1 mg/mL poly-D-lysine (Sigma Chemical Co., St. Louis, MO, USA). Neurons were cultured in Neurobasal medium (Gibco-BRL, Renfrewshire, Scotland, UK) supplemented with B27 (Gibco-BRL) and glutamax (Gibco-BRL). Neuronal cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Half of the culture medium was replaced at 7 days *in vitro* (div) and cultures were maintained until 12 div before the induction of OGD.

Primary cortical astrocyte cultures were obtained from P1 C57BL/6 mouse pups. Cortical tissue was dissociated and placed in 25 cm^2 flasks with MEM medium (Gibco-BRL) supplemented with 20% fetal bovine serum (FBS; Biological Industries, Beit Haemek, Israel), 90 mM D-Glucose (Buchs, Switzerland), 200 mM L-glutamine and Earle's salts (Gibco-BRL) and maintaned in an incubator at 37 °C with 5% CO_2. After two passages, cultures were purified by rocking for 10 min at 400 r.p.m. Medium with undesired floating cells was replaced, and flasks were placed in an incubator for 2 h at 37 °C. Next, flasks were rocked again for 16–18 h at 250 r.p.m. Finally, medium with floating cells was replaced with new medium. Confirmation of an astrocyte phenotype was based in > 95% of cells exhibiting positive staining for the astrocytic marker glial fibrillary acid protein (GFAP).

Primary cortical oligodendrocyte precursor cells (OPCs) were obtained from P1 C57BL/6 mouse pups. After dissection, the tissue was dissociated and the cells were placed on poly-D-lysine (Sigma Chemical Co) precoated 25 cm^2 flasks in Dulbecco's-modified Eagle's medium (DMEM with high glucose; Gibco-BRL) supplemented with 15% FBS, 200 mM L-glutamine at 37 °C with 5% CO_2 humidified atmosphere. Cells were incubated during 10 days and then flasks were agitated for 4 h at 150 r.p.m. to remove microglia from the culture, the media were changed and flasks were placed in the incubator for 2 h at 37 °C with 5% CO_2 humidified atmosphere. Next, flasks were shacked again overnight at 230 r.p.m. to dislodge OPCs. OPCs were further purified from astrocytes and microglia by plating on uncoated culture dishes for 1 h. The nonadherent cells were collected and replated in the medium into poly-D-lysine coated culture dishes. Six hours after plating, the culture medium was replaced with a low serum DMEM medium (1% FBS) supplemented with 10 ng/ml human recombinant PDGF-AA and 10 ng/ml human recombinant basic fibroblast growth factor (bFGF; both from PeproTech, London, UK) to stimulate proliferation. Cultures contained less than 1% GFAP-positive cells or CD11-positive cells.

All animals were treated in compliance with the Animal Experimentation Ethics Committee of the University of Barcelona, in compliance with Spanish (RD 53/2013) and European (2010/63/UE) regulations for the care and use of laboratory animals.

2.2. Induction of OGD

OGD experiments with cultured neurons and cultured glia were conducted as previously described [24, 25]. Briefly, primary neurons on div 12 were washed with deoxygenated glucose-free Earle's balanced salt solution (BSS) and transferred to an anaerobic chamber (CBS Scientific, CA, USA) containing a gas mixture of 5% CO_2 and 95% N_2 for 60 min. Glial cells to 90% of confluence were washed with deoxygenated glucose-free Earle's BSS and transferred to an anaerobic chamber (CBS Scientific) containing the same gas mixture for 120 min. After OGD, neurons and glia were kept in pre-OGD culture medium and returned to normoxic conditions. Control sister cultures were exposed to oxygenated Earle's BSS containing 5.5 mmol/L glucose in normoxic conditions.

2.3. Human alpha 1-antitrypsin treatment

Administration of human alpha 1-antitrypsin (hAAT; Prolastin®-C, Grifols Therapeutics Inc., Clayton, NC, USA) was performed 30 min after induction of OGD. To measure cell viability using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay, hAAT was added to the culture medium at 0.1, 0.5, 1, 2, 4, 6 mg/mL. For lactate dehydrogenase (LDH) assay, DAPI staining, analysis of protein levels and mRNA expression, a hAAT concentration of 1 mg/mL was used. In all cases, analysis was performed 24 h after OGD induction.

2.4. MTT assay

Cell viability was assessed by the MTT (Sigma Chemical Co.) assay. Cells were incubated with MTT (0.25 mg/mL) for 30 min at 37 °C. MTT was converted in the mitochondria of viable cells to formazan, a water-insoluble precipitate. The formazan derivative was solubilized in 500 μL of dimethyl sulfoxide (DMSO; Sigma Chemical Co.) and dual wavelength was measured at 560 nm and 620 nm in a microplate reader (Bio-Rad, Munich, Germany). Data shown are the mean ± standard error of the mean (SEM) of values obtained in 5 independent experiments performed in triplicate.

2.5. LDH assay

Necrosis was determined evaluating the plasma membrane integrity, by measuring the LDH (Sigma Chemical Co.) released from damaged cells. LDH release was measured according to the manufacturer's instructions. Levels of LDH in the media were detected using a microplate reader (492 nm, Bio-Rad). Data shown are mean ± SEM of values obtained in 5 independent experiments performed in triplicate.

2.6. Hoechst 33258 staining

Apoptosis was studied by nuclear DNA staining with Hoechst 33258 (Invitrogen, Eugene, OR, USA) as previously described [26]. Neuronal cells were fixed with 4% para-formaldehyde for 10 min, and washed twice in phosphate-buffered saline (PBS). Then, cells were stained with Hoechst 33258 (2 μg/mL) for 5 min, washed twice with PBS, and the coverslips were mounted with Mowiol. Nuclear DNA staining was observed with a fluorescence microscope (Olympus, Tokio, Japan). Apoptotic cells were detected by condensation or fragmentation of the nucleus. More than 250 cells were counted per coverslip in a blinded