JOURNAL OF PROTEOMICS XX (2013) XXX–XXX

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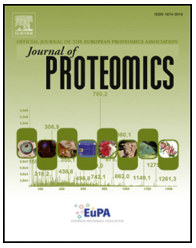

Angiogenin induces modifications in the astrocyte secretome: Relevance to amyotrophic lateral sclerosis

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ARTICLE INFO

Article history:
Received 24 April 2013
Accepted 26 July 2013

Keywords:
Astrocyte
Secretome
SILAC
Angiogenin
Amyotrophic lateral sclerosis

ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease affecting lower and upper motoneurons. Recent studies have shown that both motor neurons and non-neuronal neighbouring cells such as astrocytes and microglia contribute to disease pathology. Loss-of-function mutations in the angiogenin (*ANG*) gene have been identified in ALS patients. Angiogenin is enriched in motor neurons and exerts neuroprotective effects *in vitro* and *in vivo*. We have recently shown that motoneurons secrete angiogenin, and that secreted angiogenin is exclusively taken up by astrocytes, suggesting a paracrine mechanism of neuroprotection. To gain insights into astrocyte effectors of angiogenin-induced neuroprotection, we examined alterations in the astrocyte secretome induced by angiogenin treatment using quantitative proteomics based on Stable Isotope Labelling by Amino Acids in Cell Culture (SILAC). We identified 2128 proteins in conditioned media from primary cultured mouse astrocytes, including 1247 putative secreted proteins. Of these, 60 proteins showed significant regulation of secretion in response to angiogenin stimulation. Regulated proteins include chemokines and cytokines, proteases and protease inhibitors as well as proteins involved in reorganising the extracellular matrix. In conclusion, this proteomic analysis increases our knowledge of the astrocyte secretome and reveals potential molecular substrates underlying the paracrine, neuroprotective effects of angiogenin.

Biological significance

This study provides the most extensive list of astrocyte-secreted proteins available and reveals novel potential molecular substrates of astrocyte–neuron communication. It also identifies a set of astrocyte-derived proteins that might slow down ALS disease progression. It should be relevant to a large readership of neuroscientists and clinicians, in particular those with an interest in the physiological and pathological roles of astrocytes and in the molecular and cellular mechanisms underlying neurodegenerative disorders.

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1874-3919/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jprot.2013.07.028

1. Introduction

Astrocytes represent the most numerous cell population in the mammalian brain. They have attracted a lot of attention lately, as they have been involved in many processes beyond their traditional role as physical support for neurons. Astrocytes optimize the environment of neurons by controlling local ion and pH homeostasis, delivering glucose and providing metabolic substrates [1–3]. They control neuro-developmental processes such as neuronal migration, axon and dendrite growth, and formation of synapses, thereby contributing to the establishment of the correct architecture of the brain [1,2]. Astrocytes have also been identified as key regulators of synaptic transmission and plasticity [4]. Finally, recent evidence has revealed previously unsuspected roles of astrocytes in higher brain functions, such as sleep homeostasis [5] and memory consolidation [6]. Many of these functions require a close interplay of astrocytes with neighbouring neurons and are mediated by the release of diffusible factors. These include peptides and proteins that influence neuronal development and survival or control the immune response, such as growth factors, cell adhesion molecules, extracellular matrix components, proteases and protease regulators [7]. Accordingly, identification of proteins secreted by astrocytes by proteomics approaches has emerged as a valuable strategy to identify the molecular mechanisms underlying astrocyte–neuron communication [7–14].

Besides their role in brain homeostasis and neuronal function, astrocytes have been implicated in the development and progression of several neurodegenerative diseases [15–18]. These include amyotrophic lateral sclerosis (ALS), a fatal, progressive disorder characterized by the relatively selective loss of both upper and lower motor neurons [19]. Around 10% of ALS cases show a confirmed hereditary component, of which expanded non-coding GGGGCC repeats in *C9ORF72*, and mutations at the superoxide dismutase 1 (SOD1) locus represent the most common genetic determinants [20,21]. The search for other gene mutations segregating within ALS pedigrees has led to the identification of mutations in the angiogenin (*ANG*) gene [22]. Angiogenin is a member of the RNase A family of pancreatic ribonucleases recently renamed as vertebrate secreted RNases [23], which exhibits low RNAse activity and potently induces angiogenesis [24,25]. We have previously shown that angiogenin is enriched in motor neurons [22], and protects them against various ALS-associated insults such as excitotoxicity, hypoxia and endoplasmic reticulum stress [26,27]. We also showed that angiogenin produced by motor neurons is selectively taken up by astrocytes and that uptake of angiogenin into astrocytes is necessary for its protective effects against stress-induced motor neuron injury [28].

In the present study, we hypothesized that angiogenin taken up by astrocytes might promote the release of protective proteins, an issue we addressed by a proteomics strategy based on the Stable Isotope Labelling by Amino Acids in Cell Culture (SILAC) technology in combination with liquid chromatography coupled to Fourier transform tandem mass spectrometry (LC–FT-MS/MS). To directly characterize the quantitative variations in the astrocyte secretome induced by angiogenin treatment, we used a procedure based on two different sets of heavy amino acids (L-[$^{13}C_6$]arginine and L-[$^{2}H_4$] lysine, or L-[$^{13}C_6$-$^{15}N_4$]arginine and L-[$^{13}C_6$-$^{15}N_2$]lysine). We provide the most comprehensive astrocyte secretome map available and a list of 60 angiogenin-regulated, astrocyte-secreted proteins that are potential molecular substrates of the paracrine neuroprotective effects of angiogenin.

2. Materials and methods

2.1. Primary cultures of cortical astrocytes

Primary astrocyte cultures were prepared from two day-old C57 Black 6 mouse pups. Cortices were dissected and the meninges removed prior to incubation of tissue pieces in Minimum Essential Medium (Gibco) containing 0.025% Trypsin (Sigma) and 0.1 mg/mL DNase I (Sigma) for 15 min at 37 °C. The tissue was triturated three times in Dulbecco's modified eagle medium supplemented with glucose (DMEM; 4.5 g/L glucose; Lonza) containing 0.1 mg/mL DNase I to dissociate cells. The resulting cell suspension was centrifuged at 200 ×*g* for 5 min and the cell pellet resuspended in either DMEM (Pierce) containing 2 mM L-glutamine, 100 U/mL Penicillin/Streptomycin and 10% foetal bovine serum (all from Sigma) or DMEM depleted of L-arginine and L-lysine (SILAC DMEM, Pierce) supplemented with 2 mM L-glutamine, 100 U/mL, Penicillin/Streptomycin, 10% dialysed foetal bovine serum (Pierce), additionally substituted with isotope-labelled L-arginine and L-lysine (L-[$^{13}C_6$]arginine (Arg6) and L-[$^{2}H_4$]lysine (Lys4), or L-[$^{13}C_6$-$^{15}N_4$]arginine (Arg10) and L-[$^{13}C_6$-$^{15}N_2$]lysine (Lys8), Euriso-top) for SILAC experiments. Cells were grown for 14 days in T75 flasks (3 cortices per flask) in presence of 5% CO_2 and 95% air and the medium was replaced after 7 and 13 days in culture. At this stage, cultures were found to be highly enriched in astrocytes, as assessed by immunostaining using a glial fibrillary acidic protein antibody [28].

2.2. Cell treatment and media conditioning

Cultures were washed 6 times in PBS to completely remove serum proteins and treated with 1 μg/mL angiogenin (R&D Systems) in 50 μg/mL bovine serum albumin (BSA; Sigma) or with BSA alone (Vehicle) in Neurobasal medium (containing 100 U/mL Penicillin/Streptomycin and 2 mM L-glutamine) for 6 h to ensure uptake of angiogenin by astrocytes. Following two washes in PBS, astrocytes were covered with 6 mL fresh SILAC DMEM (containing 100 U/mL Penicillin/Streptomycin and 2 mM L-glutamine) for 18 h. This incubation period allowed the accumulation of protein amounts sufficient for mass spectrometry analysis in the extracellular medium [11] and enabled the supernatant of angiogenin-treated cultures to protect motor neurons against ALS-associated insults [28]. After the 18-h secretion period, conditioned media from vehicle- and angiogenin-treated cultures were collected, mixed and centrifuged at 200 ×*g* for 5 min and then at 20,000 ×*g* for 25 min to remove non-adherent cells and cell debris, respectively. Proteins

Please cite this article as: Skorupa A, et al, Angiogenin induces modifications in the astrocyte secretome: Relevance to amyotrophic lateral sclerosis, J Prot (2013), http://dx.doi.org/10.1016/j.jprot.2013.07.028