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♦Copper (II) ions modulate Angiogenin activity in human endothelial cells

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ABSTRACT

Angiogenin (ANG), a member of the secreted ribonuclease family, is a potent angiogenesis stimulator that interacts with endothelial cells inducing a wide range of responses. Metal ions dyshomeostasis play a fundamental role in the onset of neurodegenerative diseases, in particular copper that is also involved in angiogenesis processes. It is known that vascular pathologies are present in neurodegenerative diseases and Angiogenin is down-regulated in Alzheimer and Parkinson diseases, as well as it has been found as one of the mutated genes in amyotrophic lateral sclerosis (ALS). Copper (II) induces an increase of Angiogenin binding to endothelial cells but, so far, the relationship between copper-ANG and angiogenesis induction remain unclear. Herein, the effects of copper (II) ions on Angiogenin activity and expression were evaluated. The binding of copper was demonstrated to affect the intracellular localization of the protein decreasing its nuclear translocation. Moreover, the ANG-copper (II) system negatively affects the protein-induced angiogenesis, as well as endothelial cells migration. Surprisingly, copper also reveals the ability to modulate the Angiogenin transcription. These results highlight the tight relationship between copper and Angiogenin, pointing out the biological relevance of ANG-copper system in the regulation of endothelial cell function, and revealing a possible new mechanism at the basis of vascular pathologies.

1. Introduction

Angiogenin (ANG) is a 14.2 kDa angiogenic protein overexpressed in many types of cancers (Tello-Montoliu, 2006) as well as down-regulated in several neurodegenerative diseases including amyotrophic lateral sclerosis (ALS) (Aparicio-Erriu and Prehn, 2012), Parkinson's disease (PD) (Steidinger, 2011) and Alzheimer's disease (AD)(Kim and Kim, 2012). Furthermore, loss-of-function mutation of ANG has been related to the onset of PD and ALS (Crabtree, 2007; Greenway, 2006; Padhi, 2012; Steidinger, 2011; Wu, 2007). The onset and progression of neurodegenerative disease have been related for long time to the accumulation of insoluble misfolded protein such as β-amyloid (Cavallucci, 2012; Hardy and Higgins, 1992) and, more recently, to alteration of metallostasis (metal dys-homeostasis) (Ayton, 2013; Milardi and Rizzarelli, 2011; Tiiman, 2013) as well as to the neurovascular abnormalities (Chen, 2013; Drachman, 2014; Lyros, 2014). Thus, the understanding of the link between metal ions, angiogenic proteins and angiogenesis may be helpful to develop new therapeutic strategies concerning of cancer and neurodegenerative disease such as AD and ALS.

ANG plays a crucial role as stress-regulator balancing cell growth and survival depending on cellular status (Del Giudice, 2014; Li and Hu, 2012). Under growth conditions, ANG is rapidly translocated into the nucleus of cancer cells (Yoshioka, 2006), motoneurons (Kieran, 2008; Subramanian, 2007) and endothelial cells (Hu, 2000; Li, 1997) and promotes the ribosomal RNA (rRNA) transcription (Sheng, 2014). Under stress conditions, ANG relocates into the cytoplasm where it is accumulated in stress granules (SGs) and mediates the production of stress-induced tRNA-derived ~40 nucleotide non coding RNAs (designated as 3′-tiRNAs) (Pizzo, 2013; Yamasaki, 2009).

ANG interacts with endothelial and smooth muscle cells inducing a wide range of effects including cell migration (Hu, 1994; Wei,

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2011), invasion (Hu, 1994), proliferation (Hu, 1997, Hu, 1998a; Sadagopan, 2009), and tube formation (Miyake, 2014). The nuclear translocation, ribonucleolytic activity and signalling transduction of ANG are needed to maintain its angiogenic activity (Gao and Xu, 2008).

Interestingly, divalent copper increases the apparent number of ANG molecules bound to endothelial cells even if the apparent dissociation constant remains unchanged (Badet, 1989; Soncin, 1997a). However, other data suggested that ANG and Copper act through different mechanisms in regulating angiogenesis (Hu, 1998a). Copper is one of the most abundant transition metal in the body and it is essential for multiple biological pathways (Peña, 1999). Copper is a well-known angiogenic factor in vivo even though the molecular and cellular mechanism by which this action takes place is still unclear (D'Andrea, 2010). Copper dyshomeostasis has been also related to the onset and progression of neurodegenerative diseases (Iakovidis, 2011; Noda, 2013; Park, 2014). Thus, the metal ion interaction with angiogenic factor such as ANG could be part of the mechanism and/or angiogenic signalling of copper. In this context, a more detailed characterization of ANG-copper interaction could represent a valuable aid in a better understanding of potential mutual biological influences.

Soncin et al. (1997a) have reported that the recombinant ANG tightly binds about 2.4 copper ions per molecule at physiological pH. It has been shown that ANG binds copper ion, involving preferentially the ribonucleolytic site and/or the putative receptor binding site of the protein (La Mendola, 2010, 2012). Herein, we investigated the influence of copper binding to ANG on the properties and functions of the protein in endothelial cells. Copper reduces the ribonucleolytic and angiogenic activity of ANG. Furthermore, in endothelial cells, copper favours the cytosolic localization of ANG without effects on the proliferative and survival activity of the protein. Although the exact mechanism has to be discovered, these results pointed out a tight relation of ANG and copper.

2. Materials and methods

2.1. Material

Human umbilical vein endothelial cells (HUVECs) and cell culture medium (EGM®-2 BulletKit®) were purchased from Lonza srl (Milan, Italy). All other reagents were obtained from standard commercial sources and were of the highest commercially available grade.

2.2. Protein expression of ANG and its mutants

Expression of recombinant human ANG was carried out following a previously reported method (Holloway, 2001). The protein was refolded from inclusion bodies according to the procedure developed by Jang et al. (2004). Conversion of Met-(-1) ANG to the authentic <Glu-1 protein was achieved as described in supplementary data. The H114Y mutant was generated using the Quikchange Site-Directed mutagenesis kit according to manufacturer's instructions (Supplementary data).

2.3. Ribonucleolytic activity

The ribonucleolytic activity toward tRNA was determined by measuring the formation of perchloric acid-soluble fragments as reported (La Mendola, 2012). Briefly, ANG (0.5 μM) and tRNA (2 mg/mL) were incubated at pH 7.4 (33 mM MOPS, 33 mM NaCl) in the presence or absence of $CuSO_4$ (0-15 μM). After 2 h at 37 °C, the solution (300 μL) was diluted with ice-cold 3.4% $HClO_4$ (700 μL) and kept on ice for 10 min. Finally, the sample was centrifuged (10,000 × *g*) for 10 min and the absorbance of perchloric acid soluble fragments was measured at 260 nm.

2.4. Cell lines and drug treatments

HUVECs were maintained in EGM®-2 BulletKit® medium. Cells were grown at 37 °C in a humid atmosphere in the presence of 5% CO_2. HUVECs were grown to 80% confluence and used in successive experiments until the sixth detaching passage. When ANG was used in combination with copper, in order to allow the formation of the complex, ANG and copper was combined 2 h prior the cells treatment.

2.5. ERK 1/2 phosphorylation assays

HUVECs were seeded in 96-well plates at a density of 5×10^3 cells/well for 24 h in EGM®-2 medium. Cells were starved for 12 h before the experiment using EBM®-2 medium and were treated with various concentration of ANG in the presence or absence of $CuSO_4$ for different times (0–60 min). Then, the ERK 1/2 phosphorylation and the total ERK 1/2 were evaluated as previously described (Wong, 2004) with few modification (for detailed see Supplementary data). In parallel, a western blot analysis of total ERK1/2 and phosphor-ERK1/2 was performed as previously described (Schafe, 2008) with few modifications (Supplementary data). The primary antibodies used were anti-GAPDH (G9545 Sigma–Aldrich), anti-phospho ERK1/2 (sc-7383 SantaCruz) and anti-ERK1/2 (#4695 Cell Signaling Technology). The secondary antibodies were goat anti-rabbit IgG-HRP (12-348 Millipore) and goat anti-mouse IgG-HRP (12-349 Millipore).

2.6. ANG and 45S rRNA expression levels

In order to evaluate the 45S rRNA and ANG mRNA expression a real-time RT-PCR was performed as previously described (Nolan, 2006). Briefly, HUVECs were treated with ANG or $CuSO_4$ alone or in combination in growth medium. Total RNA was extracted using the RNeasy® Mini Kit (Quiagen) according to manufacturer's instructions, and the real-time RT-PCR reactions was performed (Supplementary data). mRNA levels for each sample were normalised against β-actin mRNA levels, and relative expression was calculated using the Ct value.

2.7. Immunofluorescence and confocal microscopy

HUVEC cells were cultured on coverslips at 2×10^4 cell/cm^2 in EGM®-2 and treated with ANG (100 nM) in the presence or absence of $CuSO_4$ (100 nM–500 nM) for different times (5-60 min). The antibodies used were anti-ANG (0.4 μg/ml, dil. 1:500, sc-9044 Santa-Cruz) and Alexa-Fluor-488-conjugated goat anti-rabbit (2 μg/ml, dil. 1:1000, A11008 Life Technologies). Non-specific binding was controlled by omitting the primary antibody or by substituting the same concentration of non specific isotypic immunoglobulin (Data not shown). Nuclei were stained with DAPI (1:5000 diluition; Sigma–Aldrich). Confocal microscopy was performed with an Olympus FV1000 confocal laser scanning microscope (LSM) equipped UV/visible lasers: 405 nm (50 mW), 20 mW Multiline Argon laser (457 nm, 488 nm, 515 nm, total 30 mW), HeNe(G) laser (543 nm, 1 mW), HeNe(R) laser (633 nm, 1 mW); oil immersion objective (60xO PLAPO) and spectral filtering system. Emitted light was detected in sequential mode. Acquisition parameters were: 405 nm excitation at 12% laser power, emission filter SDM490 (band pass) 425–475 nm, PMT voltage at 530 V (channel 1, blue); 488 nm excitation at 16% laser power, emission filter SDM560, 500–600 nm, PMT voltage at 695 V (channel 2, green). The detector gain was fixed at a constant value and images were taken