Redox Biology 2 (2014) 8–14

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research Paper

# Over-expressed copper/zinc superoxide dismutase localizes to mitochondria in neurons inhibiting the angiotensin II-mediated increase in mitochondrial superoxide ☆

Shumin Li [a,1], Adam J. Case [a,1], Rui-Fang Yang [a], Harold D. Schultz [a,b], Matthew C. Zimmerman [a,b,*]

[a] Cellular and Integrative Physiology, University of Nebraska Medical Center, Omaha, NE, USA
[b] Redox Biology Center, University of Nebraska – Lincoln, Lincoln, NE, USA

ARTICLE INFO

*Article history:*
Received 6 November 2013
Accepted 7 November 2013
Available online 18 November 2013

*Keywords:*
Mitochondria
Angiotensin II
CuZnSOD
Superoxide
Neurons
Potassium current

ABSTRACT

Angiotensin II (AngII) is the main effector peptide of the renin–angiotensin system (RAS), and contributes to the pathogenesis of cardiovascular disease by exerting its effects on an array of different cell types, including central neurons. AngII intra-neuronal signaling is mediated, at least in part, by reactive oxygen species, particularly superoxide ($O_2^{\bullet -}$). Recently, it has been discovered that mitochondria are a major subcellular source of AngII-induced $O_2^{\bullet -}$. We have previously reported that over-expression of manganese superoxide dismutase (MnSOD), a mitochondrial matrix-localized $O_2^{\bullet -}$ scavenging enzyme, inhibits AngII intra-neuronal signaling. Interestingly, over-expression of copper/zinc superoxide dismutase (CuZnSOD), which is believed to be primarily localized to the cytoplasm, similarly inhibits AngII intra-neuronal signaling and provides protection against AngII-mediated neurogenic hypertension. Herein, we tested the hypothesis that CuZnSOD over-expression in central neurons localizes to mitochondria and inhibits AngII intra-neuronal signaling by scavenging mitochondrial $O_2^{\bullet -}$. Using a neuronal cell culture model (CATH.a neurons), we demonstrate that both endogenous and adenovirus-mediated over-expressed CuZnSOD (AdCuZnSOD) are present in mitochondria. Furthermore, we show that over-expression of CuZnSOD attenuates the AngII-mediated increase in mitochondrial $O_2^{\bullet -}$ levels and the AngII-induced inhibition of neuronal potassium current. Taken together, these data clearly show that over-expressed CuZnSOD in neurons localizes in mitochondria, scavenges AngII-induced mitochondrial $O_2^{\bullet -}$, and inhibits AngII intra-neuronal signaling.



## Introduction

The brain renin–angiotensin system (RAS) plays an essential role in the pathogenesis of hypertension [1]. Angiotensin II (AngII) is the main effector peptide of the RAS, and elicits its pressor response primarily through angiotensin type 1 receptors ($AT_1R$) [2]. $AT_1R$ stimulation initiates a signaling cascade that activates NADPH oxidases (NOX) and increases intra-cellular levels of superoxide ($O_2^{\bullet -}$) [3–5]. While the importance of $O_2^{\bullet -}$ in AngII intra-neuronal signaling has been repeatedly and convincingly shown, the exact mechanism of how $O_2^{\bullet -}$ acts as an effector molecule and the subcellular localization of its production in AngII signaling is not fully understood.

Superoxide is formed in numerous subcellular compartments, but recent evidence indicates a major location of AngII-induced $O_2^{\bullet -}$ production is the mitochondria. For example, mitochondrial $O_2^{\bullet -}$ has been shown to mediate both the pressor response and sympathoexcitation induced by AngII in the rostral ventrolateral medulla of the brain [6,7]. Additionally, we have demonstrated that AngII-mediated mitochondrial $O_2^{\bullet -}$ leads to the inhibition of neuronal potassium ($K^+$) current [8]. Recently, we reported that one of the NOX isoforms (i.e. NOX4) is located in neuron mitochondria, and contributes to the increase in mitochondrial $O_2^{\bullet -}$ flux after AngII stimulation [9]. Due to the significant role

*Abbreviations:* AngII, angiotensin II; RAS, renin–angiotensin system; MnSOD, manganese superoxide dismutase; CuZnSOD, copper/zinc superoxide dismutase; $AT_1R$, angiotensin type 1 receptor; NOX, NADPH oxidase; MIMS, mitochondrial inter-membrane space; $I_{kv}$, neuronal potassium current; ROS, reactive oxygen species



* Corresponding author at: Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, 985850 Nebraska Medical Center, Omaha, NE 68198-5850, USA. Tel.: +1 402 559 7842; fax: +1 402 559 4438.
*E-mail address:* mczimmerman@unmc.edu (M.C. Zimmerman).
[1] These authors contributed equally to this work.

2213-2317/$ - see front matter © 2013 The Authors. Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.redox.2013.11.002

mitochondrial $O_2^{\bullet -}$ plays in AngII-mediated signaling, increased scavenging of this reactive oxygen species (ROS) could prove beneficial in the treatment of AngII-mediated hypertension.

The superoxide dismutase (SOD) family of enzymes possesses the unique ability to convert $O_2^{\bullet -}$ to hydrogen peroxide ($H_2O_2$) and oxygen [10]. In mammalian systems, two intra-cellular isoforms of SOD exist: copper/zinc (CuZnSOD) and manganese (MnSOD). MnSOD is exclusively located within the mitochondrial matrix, while CuZnSOD is thought to be primarily located in the cytoplasm of cells [10]. Previous studies have demonstrated that adenovirus-mediated over-expression of either CuZnSOD or MnSOD in the brain decreases blood pressure in hypertensive animal models [5,8,11,12], but how these differentially-located enzymes elicit the same effect is unknown.

Previous studies have shown CuZnSOD to be expressed not only in the cytoplasm, but also in the mitochondrial intermembrane space (MIMS) of certain cell types including neurons [13–17]. However, the function of mitochondrial-localized CuZnSOD as it relates to AngII intra-neuronal signaling has not been fully elucidated. In addition, it remains unclear if adenoviral-mediated over-expression of CuZnSOD results in increased expression of active CuZnSOD in mitochondria. Here, we present data demonstrating that both endogenously and adenovirus-mediated over-expressed CuZnSOD are associated with the mitochondria of central neurons. Furthermore, we reveal that over-expression of CuZnSOD in neurons attenuates mitochondrial-derived $O_2^{\bullet -}$ and inhibition of neuronal potassium current ($I_{kv}$) in response to AngII. Overall, this study shows the presence of active CuZnSOD in neuron mitochondria following adenovirus-mediated gene transfer, and furthers the importance of mitochondrial $O_2^{\bullet -}$ in AngII intra-neuronal signaling.

## Methods and materials

### *Cell culture*

Mouse catecholaminergic neurons (CATH.a cell line, ATCC #11179), were cultured in the RPMI 1640 medium (supplemented with 8% normal horse serum, 4% fetal bovine serum, and 1% penicillin–streptomycin) and maintained in 5% $CO_2$ at 37 °C. Prior to experimentation, neurons were differentiated utilizing $N^6$,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (1 mM, Sigma, St. Louis, MO, USA) for 6–8 days [8,18].

### *Adenovirus transduction*

For over-expression studies, replication-deficient recombinant adenovirus (Ad5-CMV) encoding human CuZnSOD (AdCuZnSOD) or control vector (AdEmpty) was obtained from ViraQuest Inc. (North Liberty, IA). On day 3 of differentiation, CATH.a neurons were transduced with 50 multiplicity of infection (MOI) of respective virus for 24 h in serum-free media. Complete media was replaced for an additional 4 days post-infection prior to experimentation [19].

### *Immunofluorescence and confocal microscopy*

Differentiated and transduced CATH.a neurons were incubated with 250 nM MitoTracker Red (Invitrogen, Molecular Probes, Carlsbad, CA) for 20 min, as previously described [8], to localize mitochondria. Following this, cells were fixed in 4% paraformaldehyde and subjected to CuZnSOD immunofluorescence staining. Neurons were incubated with blocking buffer (phosphate buffer supplemented with 10% normal horse serum and 0.3% Triton X-100) for 1 h at room temperature, then incubated with primary CuZnSOD antibody (1:500 dilution, The Binding Site Limited, Birmingham, UK) overnight at 4 °C. Following washout of the primary antibody, neurons were incubated with FITC-conjugated secondary antibody (1:500 dilution, The Binding Site Limited, Birmingham, UK) for 2 h at room temperature. Fluorescent images were acquired with a Zeiss 510 Meta Confocal Laser Scanning Microscope.

### *Mitochondrial isolation*

Mitochondria were isolated as previously described [8,20]. Briefly, CATH.a neurons were homogenized in ice-cold buffer A (225 mM mannitol, 65 mM sucrose, 10 mM HEPES, and 1 mM EGTA) using a glass Dounce homogenizer. The homogenates were centrifuged at 500*g* for 6 min at 4 °C to eliminate cellular debris. The supernatant was collected and centrifuged at 10,000*g* for 10 min at 4 °C to obtain mitochondria-enriched pellet. This mitochondria-enriched pellet was resuspended with ice-cold buffer B (225 mM mannitol, 65 mM sucrose, and 10 mM HEPES), and washed twice by centrifugation. The final mitochondrial fraction was subjected to standard Western blot analysis.

### *Western blot analysis*

Immunoblotting was performed on whole cell lysates and isolated mitochondrial fractions. Briefly, samples were separated on 4–20% gradient pre-casted denaturing gels, followed by a transfer to nitrocellulose membranes. After blocking, membranes were incubated with primary antibody (CuZnSOD – 1:1000 dilution, Santa Cruz Biotechnology, CA; MnSOD – 1:2000 dilution, Upstate Biotech/Millipore, Billerica, MA; cytochrome c oxidase subunit IV, COXIV – 1:1000 dilution, Abcam, Cambridge, MA; lactate dehydrogenase, LDH – 1:1000 dilution, Abcam, Cambridge, MA; calnexin – 1:1000 dilution, Abcam, Cambridge, MA) overnight at 4 °C. Following washout of primary antibody, membranes were incubated with secondary antibody (1:10,000, Thermo Scientific, Rockford, IL) for 1 h at room temperature. After addition of chemiluminescence substrate (Pierce Enhanced Detection System, Thermo Scientific, Rockford, IL), images were acquired by a UVP Bioimaging System.

### *SOD activity assay*

CuZnSOD and MnSOD activity in whole cell lysates and mitochondrial fractions from CATH.a neurons was determined by a semi-quantitative in-gel activity assay as previously reported [21]. Briefly, 60 μg of protein was separated by electrophoresis on a 12.5% native gel, which was then stained with 2.4 mM nitroblue tetrazolium, 28 μM riboflavin, and 28 mM N,N,N,N-tetramethylethylenediamine for 20 min in dark. Following washout of the staining solution with distilled water, the gel was illuminated under a fluorescent light until achromatic bands appeared. SOD enzymatic activity is indicated by the intensity of achromatic bands.

### *Mitochondrial superoxide analysis*

CATH.a neurons were incubated with MitoSOX Red (Invitrogen, Molecular Probes, Carlsbad, CA), a $O_2^{\bullet -}$ sensitive fluorogenic probe, and MitoTracker Green (Invitrogen, Molecular Probes, Carlsbad, CA), a mitochondrial marker, as previously described [8,22]. Briefly, non-transduced control, AdEmpty, and AdCuZnSOD-transduced CATH.a neurons were loaded with 1 μM of MitoSOX Red (excitation: 405 nm and emission: 505–550 nm) for 20 min and 50 nM of MitoTracker Green (excitation: 488 nm and emission: 575–615 nm) for 30 min. Fluorescence images were acquired with a Zeiss 510 Meta Confocal Laser Scanning Microscope before and after addition of 100 nM AngII for 30 min. Individual neurons within an image were identified as a region of interest (ROI) and