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Autophagy mediates astrocyte death during zinc potentiated ischemia reperfusion injury

Rong Pan^{*}, Graham S. Timmins^{*}, Wenlan Liu^{*}, and Ke Jian Liu^{*,†}

^{*}Department of Pharmaceutical Sciences, University of New Mexico Health Sciences Center, Albuquerque, NM 87131.

[†]Department of Neurology, University of New Mexico Health Sciences Center, Albuquerque, NM 87131.

Abstract

Pathological release of excess zinc ions and the resultant increase in intracellular zinc has been implicated in ischemic brain cell death, although the underlying mechanisms are not fully understood. Since zinc promotes the formation of the autophagic signal, reactive oxygen species (ROS), and increases autophagy, a known mechanism of cell death, we hypothesized that autophagy is involved in zinc-induced hypoxic cell death. To study this hypothesis, we determined the effect of zinc on autophagy and ROS generation in C8-D1A astrocytes subjected to hypoxia and reoxygenation (H/R), simulating ischemic stroke. C8-D1A astrocytes subjected to 3-hr hypoxia and 18-hr reoxygenation exhibited dramatically increased autophagy and astrocyte cell death in the presence of 100 μ M zinc. Pharmacological inhibition of autophagy decreased zinc-potentiated H/R induced cell death, while scavenging ROS reduced both autophagy and cell death caused by zinc-potentiated H/R. These data indicate that zinc-potentiated increases in ROS lead to over-exuberant autophagy and increased cell death in H/R treated astrocytes. Furthermore, our elucidation of this novel mechanism indicates that modulation of autophagy, ROS and zinc levels may be useful targets in decreasing brain damage during stroke.

Keywords

Hypoxia; Zinc; cell death; autophagy; reactive oxygen species

INTRODUCTION

As a leading cause of death and adult disability for Americans, stroke has a great effect on public health. Many efforts have been directed toward understanding the molecular events involved in brain injury from cerebral ischemia. Following ischemic stroke, zinc is released from a subset of glutamatergic terminals in the brain [1]. A previous study shows that extracellular zinc could be brought to more 100 μ M by synaptically released zinc [2]. This

Address correspondence to: Dr. Ke Jian Liu, College of Pharmacy, University of New Mexico Health Sciences Center, Albuquerque, NM 87131, USA. Fax: (505) 272-0704; Tel: (505) 272-9546; kliu@salud.unm.edu..

COMPETING INTERESTS

The authors declare that they have no competing interests.

pathological release of excess zinc ions following ischemia is considered important in ischemic brain injury [3-8]. Nowadays, ischemic brain injury research is focused on not only neuronal but also astrocytic injury and death. Studies show that astrocytic death can even occur prior to neuronal death [9-12]. Our previous work shows that zinc dramatically potentiates hypoxia-reoxygenation-induced astrocytic death [13], although the molecular mechanism of this zinc potentiation is still not well understood.

Zinc regulates hundreds of biological processes including autophagy [14]. Autophagy, through its many roles such as degrading long-lived proteins and dysfunctional organelles, is a major mechanism of cell homeostasis, and is essential for cellular health and survival. It can enable cell survival in nutrient-limiting conditions by generating free amino acids and fatty acids to maintain cellular function, and keep cells healthy by removing damaged organelles and proteins. However, an excess of autophagy can also cause cell death through excessive self-digestion or triggering apoptosis [15-17]. Autophagy is one of the three possible mechanisms of cell death, which include: Type I, apoptosis; Type II, autophagic cell death; and Type III, necrosis [18]. Until recently, most researchers thought that neurons died from necrosis or apoptosis after ischemic injury. However, new studies have demonstrated that ischemia-reperfusion can activate autophagic cell death that can be modulated through compounds such as propofol, a known ROS scavenger [19-21]. The importance of reactive oxygen species (ROS) in autophagic signaling has been found in a wide range of cell types [22-25]. Furthermore, reports show that zinc can induce oxidative stress and promote reactive oxygen generation [26,27].

Based on the possible nexus of zinc, ROS and autophagy in ischemic injury during stroke, we hypothesized that autophagy drives zinc-potentiated astrocyte death in hypoxia/reoxygenation (H/R) injury.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagles's medium (DMEM), Fetal bovine serum (FBS), antibiotic-antimycotic solution, Mito-tracker and Lyso-tracker were purchased from Invitrogen. Zinc chloride and 3-Methyladenine (3-MA) were purchased from Sigma.

Cell culture

C8-D1A astrocyte cell line was from ATCC. The cells were cultured in DMEM containing 10% FBS and 1% (v/v) antibiotic-antimycotic solution at 37°C in 95% air/5% CO₂ incubator.

Hypoxic cellular model

Immediately upon placing cells in a hypoxia chamber (1% O₂, 5% CO₂ at 37°C), the medium was replaced with oxygen free experimental media (DMEM containing 100 µM zinc chloride), which has been deoxygenated with nitrogen gas for 15 min. Cells were then incubated in the chamber for 3 hrs. After taking cells out from the chamber, FBS was added

to media (10 % v/v) and then incubated the cells at 37° C in 95% air/5% CO₂ incubator for 18 hrs.

Immunofluorescence for LC-3B

Astrocyte cells were plated onto glass coverslips. After 3-hr hypoxia and 18-hr reoxygenation, cells were fixed in 4% paraformaldehyde (PFA) for 15 min. Following 3 washes with PBS, cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min. The samples were blocked with 10% normal goat serum (Invitrogen, USA). After aspiration of blocking solution, anti-LC-3B antibody (1:400, overnight at 4 °C) was applied (Cell signaling, USA). After washing cells in PBS 3 times, the specimens were incubated with Alexa Fluor 488 goat anti-rabbit antibody (1:500, room temperature for 2hrs, Invitrogen, USA). Fluorescence signal of LC-3B was captured by Olympus IX71 fluorescence microscopy using a GFP dichroic mirror.

Measuring of intracellular protein radicals by immuno-spin trapping

Immuno-spin trapping is a potent, sensitive and specific method to detect macromolecule-derived radicals produced by their reaction with ROS [28-30]. Anti-5,5-Dimethyl-1-pyrroline N-oxide (DMPO) antibody (Alexis Biochemicals, USA) was used to measure intracellular protein radical by immunofluorescence. DMPO (100mM) was added to the cell culture media 3 hrs before the end of reoxygenation. At the end of reoxygenation treatment, samples were collected, and then fixed in 4% PFA. Cells were subsequently processed for immunostaining using rabbit anti-DMPO (1:200, overnight at 4°C), in order to determine ROS exposure. Alexa Fluor 488 Goat Anti-Rabbit IgG (Invitrogen, USA) was used as previously and the fluorescence signal was captured by Olympus IX71 fluorescence microscopy with a GFP dichroic mirror. Identical exposure time (500 ms) was used to ensure microscopy fluorescence level was correlated with actual DMPO bound radicals.

Western-blot analysis

At the end of the experiment, cells were quickly scraped, collected and lysed in RIPA buffer (Santa Cruz, USA). Cell extracts were centrifuged at 18,000 g for 15 min at 4°C. Proteins were separated by Any kD™ Mini-PROTEAN® TGX™ (Bio-Rad, USA) gel electrophoresis and transferred to FL PVDF Membranes (Millipore, USA). Membranes were blocked with Odyssey Blocking Buffer (37°C for 1 hr, Li-Cor, USA) and then incubated with antibodies against LC-3B (diluted 1:1,000, overnight at 4°C, Sigma, USA). RDye 800CW Goat Anti-Rabbit Secondary Antibody (diluted 1:10,000, Li-cor, USA) was used. Blots were imaged using the Odyssey® Infrared Imaging System (Li-cor, USA) with Molecular Imaging Software V4.0.

Cell viability assay

Cell viability was measured by using Cytotox 96 non-radioactive cytotoxicity assay kit (Promega, USA), which quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. C8-D1A astrocytes (5 × 10³ cells/well) were seeded into 96-well plates. Following treatment, 50 µl supernatant from each well was transferred to a flat-bottom 96-well enzymatic assay plate. 50 µl of the reconstituted

Substrate Mix was added to each well of the plate, and the plate was incubated for 30 min at room temperature. 50 μ l of the Stop Solution was then added to each well of the plate, and absorbance was measured at 490 nm using a microplate reader.

Data analysis

Each experiment was independently repeated at least 3 times. Data were presented as means SE. *P* values were determined using one-way analysis of variance (ANOVA). A value of * *P* < 0.05 was considered statistically significant.

RESULTS

Zinc chloride increased autophagy level in C8-D1A astrocytes

Excess zinc release following ischemic stroke significantly contributes to ischemic brain injury and we have shown that zinc significantly increases cell death under hypoxic condition [13]. However the molecular mechanism is still not clear. It is known that autophagy takes an important role in cell fate determination. Therefore, we would like to know if and how autophagy contributes to zinc-induced hypoxic cell death.

To answer the question, we first tested whether autophagy increases in zinc-treated hypoxic cells. The cell culture medium was replaced with oxygen free experimental media (DMEM containing 100 μ M zinc chloride and/or 2.5 mM 3-Methyladenine (3-MA, autophagy inhibitor)), which had previously been bubbled with nitrogen for 15 min before hypoxic treatment. Cells were then incubated in a polymer hypoxic glove chamber (Coy Laboratory Products Inc. Grass Lake, MI) with 1% O₂ at 37°C for 3 hrs. After 3-hr hypoxic treatment, cells were placed at 37°C with 95% air/5% CO₂ in an incubator for 18 hrs. Then, the autophagy level of the cell was assessed by the ratio of LC3B-II/LC3B-I signal intensity by Western Blot. As shown in Fig. 1A and 1B, after zinc chloride addition, the ratio of LC3B-II/LC3B-I signal intensity was increased significantly even in normoxia, and this was further increased by the hypoxic/reoxygenation condition. The autophagy inhibitor 3MA was used, and 3MA reversed the zinc and hypoxia-reoxygenation-induced increase of LC3B-II/LC3B-I signal intensity. To confirm the role of zinc, the specific zinc chelator N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) was added to cell culture medium 30 min before zinc addition. In accord with a role for zinc, its chelation by TPEN decreased zinc-induced increase of LC3B-II/LC3B-I signal intensity. To further confirm the function of zinc on inducing autophagy in hypoxic astrocytes, autophagy level in astrocytes was detected by immunofluorescence with anti-LC-3B antibody. As shown in Fig 1 C, at normoxic condition, little LC3B puncta were detectable, while LC3B puncta were visible at 3-hr hypoxia/18-hr reoxygenation with zinc condition. Based on these data, our results indicate that zinc indeed increased autophagy in the hypoxic/reoxygenic astrocytes.

Autophagy was involved in zinc-induced hypoxic/reoxygenic cell death

Since zinc potentiated autophagy in hypoxic/reoxygenation-treated astrocytes its potential role in zinc-induced hypoxic/reoxygenation cell death was studied. The extent of cell death between zinc treated cells and non-treated cells is shown in Fig 2. 100 μ M zinc or hypoxia/reoxygenation treatment alone resulted in relatively minor cell death. However, the

combination of these two treatments significantly increased cell death to over 30%. TPEN pre-treatment decreased cell death rate, confirming the cell death increase was caused by zinc. The reduction of cell death rate by 3MA pre-treatment indicated that autophagy was involved in zinc induced hypoxic cell death.

ROS involved in zinc-induced hypoxia/reoxygenation autophagy

A major mode of cell death in ischemic stroke is oxidative-stress [31]. Oxidative stress causes mitochondrial dysfunction, leading to ROS generation [32]. ROS is a known signaling molecule in autophagy in different cell types [22-25]. However, a role for ROS involved in zinc-induced autophagy has not been established. Immuno-spin trapping is widely used to detect macromolecule radicals, produced by reaction of low molecular weight ROS, and so increases in DMPO immunotrapping indicate increased ROS flux. As shown in Figure 3, in normoxia, the ROS flux level was low, and was barely increased by zinc treatment alone. However, hypoxia/reoxygenation increased ROS flux, which was further potentiated by zinc addition. Notably, 50 μ M N-acetyl-L-cysteine (NAC), a scavenger of ROS, removed the majority of immunofluorescence signal, indicating that the DMPO bound radicals were produced by ROS. To resolve whether the zinc-induced ROS in hypoxia-reoxygenation is involved in autophagy, the ratio of LC3B-II/LC3B-I in astrocytes was measured after ROS scavenging by NAC (Fig. 4A, B and C). The ratio of LC3B-II/LC3B-I signal intensity in zinc-treated hypoxic/reoxygenation astrocytes was significantly decreased by NAC treatment, indicating a role of zinc-induced ROS in control of autophagy. Furthermore NAC significantly reduced the zinc-induced cell death in hypoxia-reoxygenation treated astrocytes (Fig. 4D).

DISCUSSION

The present study demonstrates that the combination of 100 μ M zinc and 3-hr hypoxia/18-hr reoxygenation significantly increased autophagy levels and cell death in astrocytes (Figure 1 and 2) while 100 μ M zinc or 3-hr hypoxia/18-hr reoxygenation alone only has a relatively small effect. The role of the excess zinc in this process includes zinc-potentialization of ROS levels, and the scavenging of ROS decreased both autophagy and cell death (Figure 4). Overall this data indicates that excess zinc promotes autophagy and hypoxia-reoxygenation induced astrocytic death through ROS generation in hypoxic astrocytes.

Although it is known that zinc is involved in brain damage after ischemia and zinc has serious toxicity in hypoxic cells [13], the mechanism is still not clear. Increased autophagy by zinc and its role involved in cell fate determination has been found in ethanol exposed human hepatoma cells [33], cultured retinal pigment epithelial and photoreceptor cell [34], cancer cells [35,36], H₂O₂ [37] treated astrocytes and Cliquinol treated astrocytes and neurons [38]. However, the role of zinc on autophagy in hypoxia-reoxygenation damaged astrocytes has remained unappreciated. We found that excess zinc dramatically increased autophagy, leading to cell death, in hypoxic/reoxygenation treated astrocytes (Fig 1 and 2). Use of TPEN, a specific zinc chelator reversed the zinc-induction of autophagy. Even TPEN alone reduced hypoxia-induced autophagy level. Our previous results show that hypoxia remarkably increases intracellular free zinc [13]. The hypoxia-induced intracellular free zinc

increase may raise the autophagy level, and this may be the reason why TPEN treatment decreased autophagy level in hypoxic astrocytes without exogenous zinc addition. Astrocyte death was reduced by both TPEN and the autophagy inhibitor 3MA, demonstrating that excess zinc-induced autophagy was involved in hypoxic astrocytic death. Since autophagy has dual role in cell fate determination [15-17], the effect of autophagy on cerebral ischemia is controversial in current literature. Most studies show that autophagy is harmful after cerebral ischemia, mainly through the overactivation of autophagy, autophagic neuron death and caspase dependent apoptosis [19-21]. However, others have shown that autophagy can be neuroprotective by removing dysfunctional mitochondria [39]. Autophagic neuroprotection may also involve immune responses and other neuroprotective pathways [40]. Overall, the effect of autophagy in cerebral ischemia is likely complex with the extent and context of autophagy being key factor. We noticed that 3MA alone caused significant cell death. It was known that autophagy is essential for cell by removing damaged organelles and proteins [41]. Thus we speculated that the 3MA induced cell death was due to the toxicity from the damaged organelles and proteins that should be removed by autophagy.

Since 3MA only partially reversed zinc-induced cell death, autophagy may not be the only pathway for zinc-induced hypoxic/reoxygenation cell death. Zinc can cause hypoxic cell death through both caspase-dependent and -independent pathways [13,42,43], and it has also been reported as a factor in neuron necrosis pathway in ischemic rats [8]. Therefore, the mechanism of zinc-induced hypoxic cell death needs further investigation.

Mitochondria produce large quantities of ATP for maintaining neuronal homeostasis. In addition, mitochondria are the major site of ROS production, and are thought to be key activators of programmed cell death [44]. Excessive zinc causes loss of membrane potential in mitochondria, and mitochondrial function can be blocked by zinc in all physiologically relevant substrate conditions [26]. These dysfunctional mitochondria enhance ROS generation, leading to cell death [45]. In addition, many reports demonstrate that ROS serves as a signaling molecule in autophagy [22-25]. Our results showed that excess zinc increased ROS generation in astrocytes exposed to hypoxia/reoxygenation. Moreover, reduction of ROS successfully decreased zinc-induced autophagy and cell death.

In conclusion, our results demonstrate that excess zinc can promote autophagy in hypoxic/reoxygenation treated astrocytes through ROS signaling, leading to cell death. Our work suggests that modulation of zinc, ROS and autophagy, either separately or in concert, may provide opportunities to reduce tissue damage in stroke.

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Fig. 1. Zinc increased autophagy at hypoxia/reoxygenation

C8-D1A astrocytes were treated with 100 μ M zinc chloride under normoxia or 3-hr hypoxia/18-hr reoxygenation. The autophagy biomarker LC3B protein level was measured by western blotting. Zinc chelator TPEN (1 μ M) was used to confirm the zinc's function on autophagy. The autophagy inhibitor 3-methyladenine (3MA, 2.5 mM) was used as negative control for autophagy. The concentration of β -actin in the astrocyte was measured as loading control. (A) Representative Western blots. (B) The fold change of LC3B-II/LC3B-I signal intensity. (C) C8-D1A Cells were antibody labeled with fluorescent probes to LC3B. LC3B

puncta were indicative of LC3B-II in autophagosomes. Examples are marked by white arrows.

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Fig. 2. Inhibition of autophagy decreased zinc-induced cell death

C8-D1A astrocytes were treated with zinc chloride with or without 3MA. After 3-hr hypoxic/18-hr reoxygenation, cell viability was assayed by Cytotox 96 non-radioactive cytotoxicity assay kit.

Fig. 3. DMPO bound protein radical level increased after zinc and hypoxia/reoxygenation treatment

Astrocytes were exposed to 3-hr hypoxia/18-hr reoxygenation in the presence of 100 μ M zinc. DMPO bound macromolecular radical levels in astrocytes were measured by immuno-spin trapping (A). (B) Fold change of the mean densitometry of immuno-fluorescence signal.

Fig. 4. Inhibition of ROS decreased zinc-induced hypoxic autophagy

50 μ M N-acetyl-L-cysteine was added to astrocytes culture media 30 min before the cells exposed to 3-hr hypoxia/18-hr reoxygenation in the presence of 100 μ M zinc. Autophagy in astrocytes was measured through Western Blotting with representative blot shown in (A). (B) The fold change of LC3B-II/LC3B-I signal intensity. (C) C8-D1A Cells were antibody labeled with fluorescent probes to LC3B. LC3B puncta were indicative of LC3B-II in the outer wall of autophagosomes. Examples are marked by white arrows. (D) C8-D1A cells viability was assayed by Cytotox 96 non-radioactive cytotoxicity assay kit.