Original Article
Zinc wave during the treatment of hypoxia is required for initial reactive oxygen species activation in mitochondria

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Abstract: Mitochondrial reactive oxygen species (ROS) are known to accumulate during chemical hypoxia, causing adverse effects on cell function and survival. Recent studies show important role zinc accumulation plays in dysfunction associated with hypoxia. It is well known that ROS accumulation also plays a major role in cellular damage by hypoxia. In this study, fluorescent imaging and pharmacological methods were used in live HeLa cells to determine role of zinc in initial ROS accumulation in mitochondria during chemical hypoxia (oxygen glucose depravation with 4 mM sodium dithionite). Accumulation of both was observed as a very rapid phenomenon with initial rapid zinc increase (zinc wave) within 60 seconds of hypoxia onset and ROS increase within 4.5 minutes. Zinc chelation with TPEN removed the initial zinc wave which in turn abolished ROS accumulation. Influx of exogenous zinc induced rapid ROS accumulation. Inhibition of NADPH oxidase with apocynin, a NADPH oxidase inhibitor, showed significant and prolonged reduction in zinc induced ROS accumulation. We proposed a novel mechanism of intracellular zinc increase that activates NADPH oxidase which in turn triggers mitochondrial ROS production.

Keywords: Hypoxia, free zinc, reactive oxygen species, mitochondria

Introduction

Reactive oxygen species (ROS) produced by mitochondria have been of interest for many years due to their involvement in pathological mechanisms of cell death [1-4]. Many studies have focused on ROS and hypoxia because increasing evidence indicates that hypoxic conditions induce ROS accumulation, which subsequently damages intracellular functions. For example, studies show that ROS plays a major role in the pathogenesis of hypoxia in the brain [5, 6]. ROS increase is not the only outcome of hypoxia, as intracellular zinc accumulation has been observed shortly after onset of hypoxia [7-11]. Zinc accumulation precedes neuronal cell death [7, 9, 11] and calcium increases [12, 13]. Recent evidence indicates that zinc increases play an important role in the pathology of hypoxia in neurons [11, 14, 15] and removal of zinc with chelators like calcium-EDTA [7, 8, 16, 17] and TPEN [18] have been shown to reduce cell death, suggesting that zinc plays an important role in pathology of hypoxia.

There have been numerous reports that zinc is sequestered in mitochondria, which contributes to mitochondrial dysfunction during hypoxia [13, 19-22]. Without this sequestration, exogenous zinc is known to cause cell death regardless of hypoxic conditions [23]. Recently, there is emerging evidence that a membrane enzyme, NADPH oxidase, contributes significantly to ROS generation [24-26]. While the molecular mechanism for 'burst' or ROS generation remains largely uncertain, recent evidence shows that NADPH oxidase and mitochondria are in direct communication and that NADPH produced ROS can trigger mitochondrial ROS production [27]. NADPH oxidase activity and ROS formation increase progressively with increasing the duration of hypoxia [28, 29]. We don't know the cross-talk between the zinc- and ROS-mediated signaling systems during the course

of hypoxic stress. Both zinc and NADPH oxidase are thought to be involved with mitochondrial ROS production. Therefore, there appears to be a relationship between them. Zinc has been shown to increase the activity of NADPH oxidase that may be an enzyme mediating zinc-induced oxidative injury [30-32].

This study was undertaken to further understand the temporal relationship between zinc accumulation and mitochondrial ROS production and how zinc is involved in ROS generation within mitochondria. We show that zinc increase (zinc wave) is a very rapid response to chemical hypoxia and it precedes mitochondrial ROS induction and accumulation. The zinc wave was required for mitochondrial ROS induction because when zinc was removed during chemical hypoxia, the initial mitochondrial ROS accumulation is abolished. Importance of zinc in induction of mitochondrial ROS was shown with a second layer of experiments which demonstrated that exogenous zinc in normoxic conditions induced mitochondrial ROS accumulation. Pharmacological inhibition of NADPH oxidase showed marked decrease in zinc induced mitochondrial ROS production, suggesting a key role this enzyme may play in zinc induced mitochondrial ROS.

Experimantal procedures

Materials

Most chemicals were purchased from Sigma-Aldrich (St. Louis, MO), with exception of apocynin which was purchased from Santa Cruz Biotechnologies (Dallas, TX). Fluorescent dyes were purchased from Molecular Probes (Eugene, OR) or Life Technologies (Grand Island, NY). HeLa cells were purchased from ATCC (Manass, VA).

Cell culture

HeLa cells were used between passages 4-14. They were split every other day using the standard trypsanization method and maintained in EMEM medium supplemented with 5% fetal bovine serum (ATCC, Manass, VA) in 5% CO_2 -95% humidity air at 37°C (as suggested by ATCC).

Fluorescent experiments

HeLa cells were trypsinized and seeded at medium density onto glass bottom Petri dishes

(P35G-4.5-14-C, MatTek Corp, Ashland, MA). Cells were incubated in 5% CO_2 -95% air at 37°C for at least 24 hours before experimentation. On the day of the experiment, the cells were washed three times with 1 mL of HEPES buffer (in mM) 25 HEPES, 125 NaCl, 3 KCl, 1.28 $CaCl_2$, 1.1 $MgCl_2$, 5 glucose. For mitochondrial superoxide detection, MitoSOX Red dye was used at final concentration of 5 μM and cells were loaded for 10 min at 37°C. For zinc detection, FluoZin-3 AM was used at a concentration of 1 μM and loaded onto cells for 60 minutes at room temperature. After incubation with respective dyes for each treatment, cells were washed three times with HEPES buffer and left to "rest" at room temperature for 10 minutes before experimentation. Images were collected with a Motic AE31 microscope using Olympus U Plan FL 40X, 075 NA, with QImaging Retiga 1300i camera. Image-Pro Plus 6.2 (Media Cybernetics, Rockville, MD) was used to collect and analyze the data. Images were collected every 10-60 seconds (specified for each experiment).

Chemical hypoxia

Chemical hypoxia was induced using 4 mM final concentration of sodium dithionite (DT) in oxygen and glucose deprived (OGD) HEPES buffer. The composition of the buffer was the same as in the fluorescent experiments but without 5 mM glucose. To achieve OGD, nitrogen gas was bubbled through the HEPES buffer for at least 10 minutes. This OGD and 4 mM sodium dithionite buffer was added by pipetting as 2x concentrated solution into the petri dish holding the cells to induce rapid hypoxia, after at least one minute of the baseline fluorescence was observed. The recordings lasted 10-15 minutes.

Exogenous zinc

Cells were loaded with MitoSOX Red dye (5 μM final concentration) using the same method as the fluorescent experiments. Zinc influx was induced by pipetting 2x concentrated solution of HEPES buffer with final concentrations of 50 μM $ZnCl_2$ and 10 μM Na-pyrithione, a zinc ionophore, which allows zinc ions to enter the cells.

NADPH oxidase inhibition

Cells were prepared using the same protocols as above. Cells were loaded with MitoSOX Red (5 μM final concentration). The NADPH oxidase
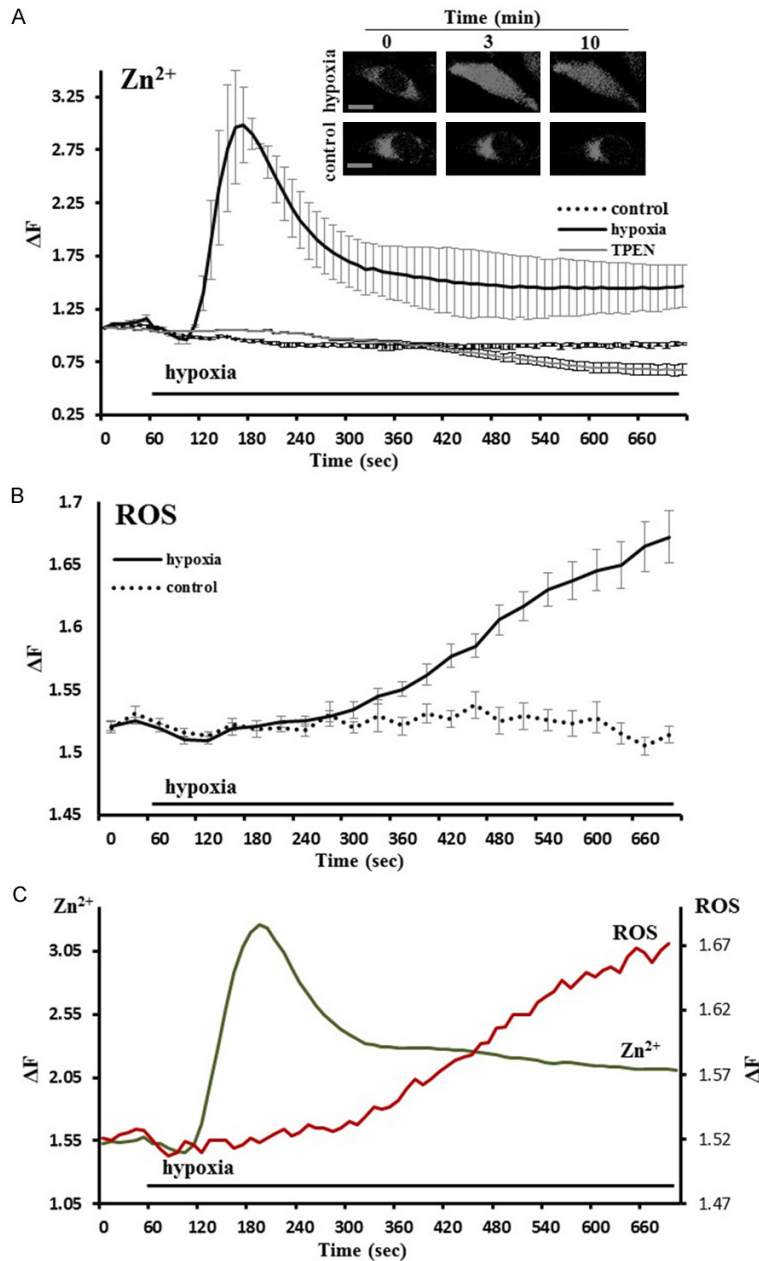
Figure 1. The initial and rapid zinc wave in HeLa cells induced by hypoxia precedes initial mitochondrial ROS accumulation. A. Zinc transients (1 µM FluoZin-3, AM) showing the zinc wave began 60 seconds after the induction of hypoxia. Zinc chelation using TPEN (35 µM) abolishes the response, indicating that the zinc wave is zinc dependent. Control shows zinc transient during normoxic conditions with the addition of a physiological buffer in place of hypoxia. Hypoxic treatment is indicated with a black line below the transients. Data represent mean ± SD of 14-20 cells in three separate experiments. Inserts are representative images of HeLa cells showing the changes of zinc fluorescence at various time points in hypoxia treatment or in control condition. Scale bar = 10 µm. B. Mitochondrial ROS transients (MitoSOX Red 5 µM) during hypoxia compared to ROS accumulation during normoxic control. Data represent mean ± SD of 15-17 cells in three separate experiments. Hypoxia is indicated with a black line below the transients. C. Two representative traces of ROS and zinc accumulation showing the relationship between zinc and the ROS transients during hypoxia. Zinc concentration is on the left y-axis, and ROS concentration is on the right y-axis. Hypoxia is indicated by a black line below the traces.

inhibitor apocynin was dissolved in DMSO and used at 60 µM final concentration with endogenous zinc (50 µM) and sodium pyrithione (10 µM). Apocynin was added at the same time as other solutions. All the solution had 0.1% final concentration of DMSO. Fluorescent intensity was measured at 10 minutes after treatments were added.

Data analyses

For the zinc and mitochondrial ROS transients the cytosol excluding the nucleus was analyzed for each cell. The changes were normalized to baseline and average was plotted on the graph with standard deviation bars. For the NADPH oxidase inhibition the percent change in fluorescence was calculated following formula: $((F-F_0/F_0) \times 100)$, where F_0 is averaged baseline fluorescence intensity before addition of zinc and apocynin, and F is fluorescence intensity value at 20 minutes of incubation with zinc and/or apocynin. The significance was measured by simple two-group comparison and analyzed by Student's paired t test (or single-factor ANOVA), with $p < 0.05$ considered significant.

Results

Zinc wave and ROS accumulation during hypoxia

To detect zinc transients in cells during the treatment of hypoxia we used FluoZin-3 AM, a cell permeant zinc fluorescent indicator. This fluorescent probe has high affinity to zinc with K_D about 15 nM and exhibits a 50-fold increase in fluorescence in response to saturating zinc
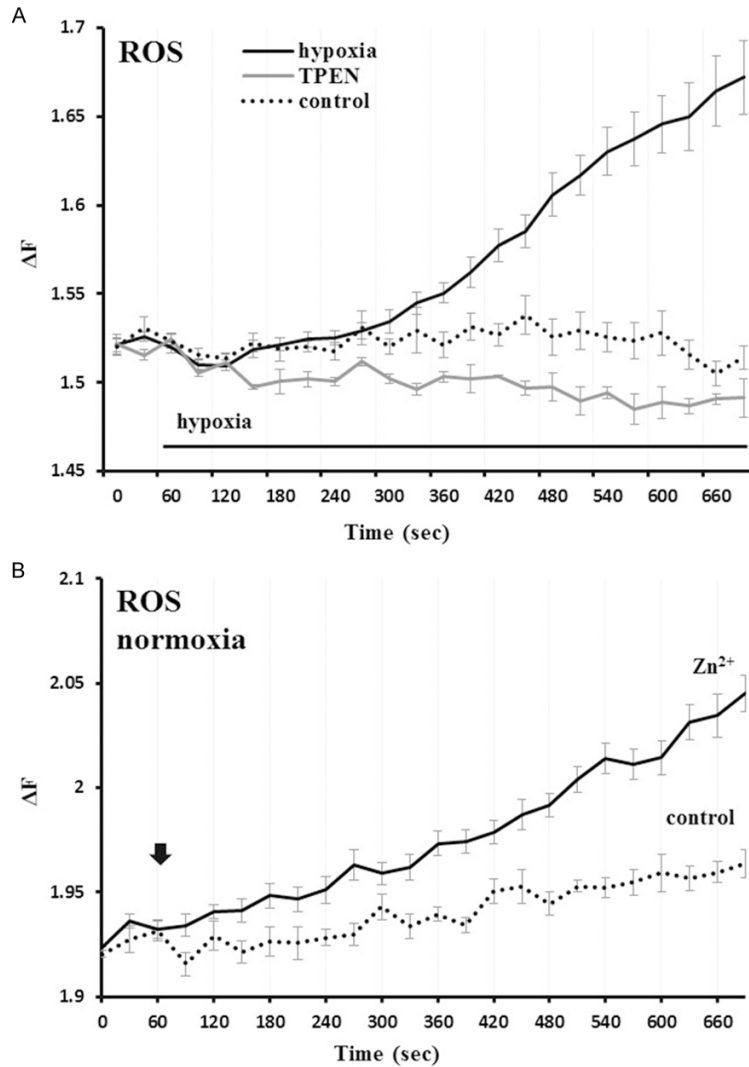
Figure 2. Zinc is required for mitochondrial ROS accumulation. A. ROS accumulation during hypoxia showing increase of MitoSOX Red (5 μM) fluorescence within 4.5-5 minutes of hypoxia and a trace showing that zinc chelation with TPEN (35 μM) abolishes the initial ROS accumulation. Hypoxia is indicated with a black line below the transients. Data represent mean ± SD of 15-17 cells in three separate experiments. B. Zinc-induced ROS increase, measured with MitoSOX Red (5 μM) in HeLa cells with addition of exogenous zinc. The additions of 50 μM zinc/10 μM sodium pyrithione and a control (10 μM sodium pyrithione alone) are indicated by an arrow. Data represent mean ± SD of 7-15 cells in three different experiments.

cent zinc indicator. When cells were treated with chemically induced hypoxia, there was a rapid increase in zinc concentration initiated usually within 60 seconds of hypoxic treatment, causing a brief upsurge of zinc fluorescence or a zinc wave (**Figure 1A**). Zinc wave reached the peak in about 1 minute and then declined, with the wave lasting about 3 or 4 min. However, as shown in **Figure 1A**, the overall zinc concentration in HeLa cells didn't return to the basal level but maintained elevated throughout the observation period.

To ensure that the observed increase was zinc dependent, a chelator specific to zinc N,N,N',N'-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN), was added at final concentration of 35 μM five minutes prior to induction of hypoxia. When zinc was chelated, zinc wave was not observed; instead the zinc transient was similar to control. This suggests that the observed zinc wave with fluorescent zinc indicator was zinc dependent.

To investigate ROS changes during hypoxia, we used Mito-SOX Red, a fluorescent indicator specific to superoxide production in mitochondria. This probe is live-cell permeable and is selectively targeted to mitochondria. The distribution of the dye was similar to mitochondrial distribution in cytosol of HeLa cells loaded with mitochondria specific indicators, like MitoTracker Red [35-37]. When HeLa cells were exposed to hypoxic treatment, there was accumulation of ROS production initiated within 4-5 min of hypoxia and continued to increase steadily (**Figure 1B**). The increase of mitochondria ROS followed closely behind the zinc wave (**Figure 1C**).

levels [33, 34]. Zinc fluorescence or the labile zinc distribution had an uneven appearance in HeLa cells labeled with the fluorescent zinc indicator. Some brighter fluorescence was found in the cytosol around the nucleus. The nucleus appeared dark and had nearly no detectable zinc fluorescence, due to zinc in the nucleus being tightly bound to nuclear proteins and being unavailable for chelation by fluores-
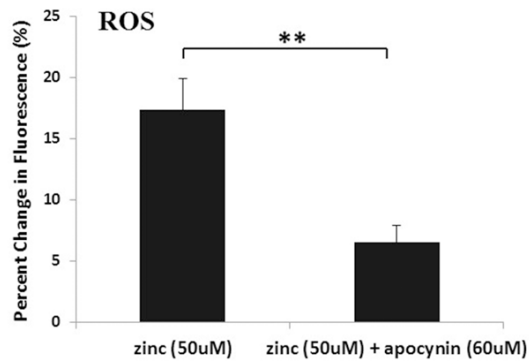
Figure 3. NADPH oxidase is an important contributor to zinc wave induced mitochondrial ROS accumulation. HeLa cells loaded with 5 µM MitoSOX Red showing percent change in baseline fluorescence compared to zinc-induced mitochondrial ROS with or without 60 µM Apocynin, a NADPH oxidase inhibitor, under normoxic conditions. Cells were incubated 10 minutes with 50 µM zinc/10 µM sodium pyrithione prior to the measurements. Both groups had 0.1% DMSO added. Data represent mean ± SD of 12-15 cells in three separate experiments (**$p < 0.01$).

Effect of zinc on mitochondrial ROS accumulation

To determine if the zinc wave played a role in initiating ROS production, zinc was removed with 35 µM TPEN five minutes prior to induction of hypoxia. The mitochondrial ROS production was compared between HeLa cells that were under hypoxia treatment and HeLa cells that were treated with zinc chelation in addition to hypoxia (**Figure 2A**). When the zinc wave was removed by TPEN, as shown in **Figure 2A**, there was no mitochondrial ROS accumulation detected in hypoxic treatment. Next, we investigated how mitochondrial ROS changes in response to the addition of exogenous zinc (50 µM) in normoxic conditions. Zinc was added together with the zinc ionophore sodium pyrithione (10 µM), a lipid soluble molecule that transports zinc through the cell membrane. We observed an increase in mitochondrial ROS measured by MitoSOX Red (5 µM) (**Figure 2B**). The increase of mitochondrial ROS production became significant, comparing to the control, after 3 minutes of zinc application, which was generally consistent with hypoxia induced ROS production (**Figure 1B**). The application of sodium pyrithione alone (10 µM) caused an increase in mitochondrial ROS, as expected, due to the cytotoxic effect of sodium pyrithione. Taken together, zinc, either endog-

enously released during hypoxia or exogenously applied to cells, plays a critical role in the increase of mitochondrial ROS production.

Effect of NADHP oxidase inhibitor on zinc wave induced mitochondrial ROS response

NADPH oxidase is one of enzymes that seem to predominate in producing ROS in mammalian cells [29]. Studies suggest that NADPH oxidase mediated ROS production depends on the function of mitochondria [2, 27, 38]. In the present study, we investigated the effect of apocynin, a NADPH oxidase inhibitor, on exogenous zinc induced ROS production. As shown in **Figure 3**, the application of apocynin (60 µM) decreased zinc induced mitochondrial ROS production in HeLa cells, after 10 min of treatment.

Discussion

The major findings of this study show an early increase or zinc wave in the intracellular free zinc concentration and a late increase of ROS in the mitochondria of HeLa cells during the treatment with hypoxia. Our study demonstrates clearly that the zinc wave precedes the increase of mitochondrial ROS production and is required for mitochondrial ROS increase during hypoxia. The application of exogenous zinc also induced the increase of mitochondrial ROS production. NADPH oxidase contributes to the zinc wave induced increase of mitochondrial ROS.

Labile or free zinc is tightly regulated in a healthy cell and is maintained in the picomolar range, because free zinc is toxic to the cells [10, 14, 15, 39, 40]. Studies have shown that there are zinc increases in early stages of hypoxia [12, 41], which collaborates with hypoxic stress induced neuronal cells death [11]. We show here that a zinc wave can be observed within 60 seconds of hypoxia which is consistent with previous studies and adds evidence to support the hypothesis that zinc signal is important in the early stages of cellular responses to hypoxia. Such a rapid and significant source of free intracellular zinc increase is still elusive. The available evidence suggests that it can be metallothioneins that free zinc during redox changes [42-44]. Studies also show that zinc released from organelle storages such as endoplasmic reticulum [45].

We show that the zinc wave is followed by a significant mitochondrial ROS increase which starts almost right after zinc wave reaches the peak. Without zinc (by removing zinc with zinc chelation), the ROS response couldn't be initiated and ROS accumulation was not detected. In the light of these findings, we suggest that an initial and rapid zinc wave triggers cellular responses that quickly lead to ROS accumulation. Other studies have shown that removal of zinc with zinc chelator would cause the reduction of hypoxia induced cell death [11, 18, 46]. If mitochondrial ROS response is zinc dependent, then in the absence of zinc the mechanism of cell damage is not turned on and mitochondrial ROS elevation is delayed, resulting in positive outcomes on cell health and survival. This indicates that zinc is an important player in mitochondrial ROS increase during hypoxia. This may explain a 3 minute lag in ROS response after intracellular zinc wave. The results in present study further clarify the role of zinc in cell injury and offer a mechanism as to why this happens. To further investigate the involvement of zinc in mitochondrial ROS increase, we investigated mitochondrial responses to exogenous zinc in normoxic conditions. Mitochondrial ROS started to accumulate shortly after zinc addition (**Figure 2B**), with significant change within 3 minutes of incubating with zinc. These data suggest that an increase of cytosolic labile zinc by itself will trigger mitochondrial ROS accumulation further supporting that zinc plays a role in induction of ROS in mitochondria.

In the light of several lines of research, NADPH oxidase becomes a key player in the explanation of the zinc effect on mitochondrial ROS increase. NADPH oxidase is one of the major contributors to ROS production during hypoxia as well as during normoxic conditions [29]. Studies suggest that NADPH oxidase can be induced within minutes [47] and that zinc can induce NADPH oxidase [30, 31], indicating an early and fast reaction between zinc and NAPDH oxidase. Phosphorylation is required for p47phox that activates NADPH oxidase [48-51]. Zinc may be a phosphorylation modulator of NADPH oxidase assembly [31, 52]. We investigated the hypotheses that ROS increase would be altered by inhibition of this enzyme during exogenous zinc induced ROS accumulation. In these experiments, zinc was present however the effect of zinc on ROS increase was significantly reduced by NADPH oxidase inhibitor (**Figure 3**), supporting that NADPH oxidase is likely a major player in zinc induced initial ROS accumulation. The interplay between specific ROS sources is still elusive. The recent studies on cross talk between two major ROS sources, mitochondria and NADPH oxidases suggest that one can cause the other [27]. The potential role of zinc in the cross talk, as shown in the present study, is of particular interest.

In summary, the present study shows that a zinc wave precedes mitochondrial ROS increase during hypoxia. Zinc is required for the initial increase in mitochondrial ROS during both hypoxia and normoxia. We suggest a novel mechanism of zinc action on mitochondrial ROS induction involving NADPH oxidase, with zinc as a possible phosphorylation modulator. The initial zinc wave facilitates phosphorylation and subsequent activation of NADPH oxidase, which will induce initial ROS accumulation. We propose that these results may not be limited to HeLa cells and can occur in many cells types (e.g, neurons during stroke, cardiac muscle cells during infarction, and lung cells during hypoxia induced by pulmonary embolism). Additional studies are needed to further understand the action and ramifications of the initial zinc wave and its role in phosphorylation of NADPH oxidase.

Disclosure of conflict of interest

None.

Abbreviations

ROS, reactive oxygen species; TPEN, N,N,N',N'-tetrakis-(2-pyridylmethyl) ethylenediamine; DT, sodium dithionite; OGD, oxygen glucose depravation; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMSO, dimethyl sulfoxide.

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References

[1] Dan Dunn J, Alvarez LA, Zhang X, Soldati T. Reactive oxygen species and mitochondria: A nexus of cellular homeostasis. Redox Biol 2015; 6: 472-85.

[2] Starkov AA. The role of mitochondria in reactive oxygen species metabolism and signaling. Ann N Y Acad Sci 2008; 1147: 37-52.

[3] Wang CC, Fang KM, Yang CS, Tzeng SF. Reactive oxygen species-induced cell death of rat primary astrocytes through mitochondria-mediated mechanism. J Cell Biochem 2009; 107: 933-43.

[4] Orrenius S. Reactive oxygen species in mitochondria-mediated cell death. Drug Metab Rev 2007; 39: 443-55.

[5] Kalogeris T, Bao Y, Korthuis RJ. Mitochondrial reactive oxygen species: a double edged sword in ischemia/reperfusion vs preconditioning. Redox Biol 2014; 2: 702-14.

[6] Chan PH. Reactive oxygen radicals in signaling and damage in the ischemic brain. J Cereb Blood Flow Metab 2001; 21: 2-14.

[7] Koh JY, Suh SW, Gwag BJ, He YY, Hsu CY, Choi DW. The role of zinc in selective neuronal death after transient global cerebral ischemia. Science 1996; 272: 1013-6.

[8] Suh SW, Listiack K, Bell B, Chen J, Motamedi M, Silva D, Danscher G, Whetsell W, Thompson R, Frederickson C. Detection of pathological zinc accumulation in neurons: methods for autopsy, biopsy, and cultured tissue. J Histochem Cytochem 1999; 47: 969-72.

[9] Stork CJ, Li YV. Intracellular zinc elevation measured with a "calcium-specific" indicator during ischemia and reperfusion in rat hippocampus: a question on calcium overload. J Neurosci 2006; 26: 10430-7.

[10] Frederickson CJ, Maret W, Cuajungco MP. Zinc and excitotoxic brain injury: a new model. Neuroscientist 2004; 10: 18-25.

[11] Stork CJ, Li YV. Rising zinc: a significant cause of ischemic neuronal death in the CA1 region of rat hippocampus. J Cereb Blood Flow Metab 2009; 29: 1399-408.

[12] Medvedeva YV, Lin B, Shuttleworth CW, Weiss JH. Intracellular Zn2+ accumulation contributes to synaptic failure, mitochondrial depolarization, and cell death in an acute slice oxygen-glucose deprivation model of ischemia. J Neurosci 2009; 29: 1105-14.

[13] Medvedeva YV, Weiss JH. Intramitochondrial Zn2+ accumulation via the Ca2+ uniporter contributes to acute ischemic neurodegeneration. Neurobiol Dis 2014; 68: 137-44.

[14] Sensi SL, Paoletti P, Koh JY, Aizenman E, Bush AI, Hershfinkel M. The neurophysiology and pathology of brain zinc. J Neurosci 2011; 31: 16076-85.

[15] Li YV. Zinc Overload in Stroke. In: Li YV and Zhang JH, editors. Metal Ion in Stroke. New York: Springer; 2012. pp. 167-189.

[16] Lee JY, Kim YJ, Kim TY, Koh JY, Kim YH. Essential role for zinc-triggered p75NTR activation in preconditioning neuroprotection. J Neurosci 2008; 28: 10919-27.

[17] Calderone A, Jover T, Mashiko T, Noh KM, Tanaka H, Bennett MV, Zukin RS. Late calcium EDTA rescues hippocampal CA1 neurons from global ischemia-induced death. J Neurosci 2004; 24: 9903-13.

[18] Bossy-Wetzel E, Talantova MV, Lee WD, Schölzke MN, Harrop A, Mathews E, Götz T, Han J, Ellisman MH, Perkins GA, Lipton SA. Crosstalk between nitric oxide and zinc pathways to neuronal cell death involving mitochondrial dysfunction and p38-activated K+ channels. Neuron 2004; 41: 351-65.

[19] Bonanni L, Chachar M, Jover-Mengual T, Li H, Jones A, Yokota H, Ofengeim D, Flannery RJ, Miyawaki T, Cho CH, Polster BM, Pypaert M, Hardwick JM, Sensi SL, Zukin RS, Jonas EA. Zinc-dependent multi-conductance channel activity in mitochondria isolated from ischemic brain. J Neurosci 2006; 26: 6851-62.

[20] Stork CJ, Li YV. Elevated Cytoplasmic Free Zinc and Increased Reactive Oxygen Species Generation in the Context of Brain Injury. Acta Neurochir Suppl 2016; 121: 347-53.

[21] Granzotto A, Sensi SL. Intracellular zinc is a critical intermediate in the excitotoxic cascade. Neurobiol Dis 2015; 81: 25-37.

[22] Dineley KE, Richards LL, Votyakova TV, Reynolds IJ. Zinc causes loss of membrane potential and elevates reactive oxygen species in rat brain mitochondria. Mitochondrion 2005; 5: 55-65.

[23] Sensi SL, Paoletti P, Bush AI, Sekler I. Zinc in the physiology and pathology of the CNS. Nat Rev Neurosci 2009; 10: 780-91.

[24] Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev 2007; 87: 245-313.

[25] Zhang QG, Laird MD, Han D, Nguyen K, Scott E, Dong Y, Dhandapani KM, Brann DW. Critical role of NADPH oxidase in neuronal oxidative damage and microglia activation following traumatic brain injury. PLoS One 2012; 7: e34504.

[26] Abramov AY, Scorziello A, Duchen MR. Three distinct mechanisms generate oxygen free radicals in neurons and contribute to cell death during anoxia and reoxygenation. J Neurosci 2007; 27: 1129-38.

[27] Dikalov S. Cross talk between mitochondria and NADPH oxidases. Free Radic Biol Med 2011; 51: 1289-301.

[28] Brennan AM, Suh SW, Won SJ, Narasimhan P, Kauppinen TM, Lee H, Edling Y, Chan PH, Swanson RA. NADPH oxidase is the primary source of superoxide induced by NMDA receptor activation. Nat Neurosci 2009; 12: 857-63.

[29] Rathore R, Zheng YM, Niu CF, Liu QH, Korde A, Ho YS, Wang YX. Hypoxia activates NADPH oxidase to increase [ROS]i and [Ca2+]i through the mitochondrial ROS-PKCepsilon signaling axis in pulmonary artery smooth muscle cells. Free Radic Biol Med 2008; 45: 1223-31.

[30] Kim YH, Koh JY. The role of NADPH oxidase and neuronal nitric oxide synthase in zinc-induced poly (ADP-ribose) polymerase activation and cell death in cortical culture. Exp Neurol 2002; 177: 407-18.

[31] Noh KM, Koh JY. Induction and activation by zinc of NADPH oxidase in cultured cortical neurons and astrocytes. J Neurosci 2000; 20: RC111.

[32] Matsunaga Y, Kawai Y, Kohda Y, Gemba M. Involvement of activation of NADPH oxidase and extracellular signal-regulated kinase (ERK) in renal cell injury induced by zinc. J Toxicol Sci 2005; 30: 135-44.

[33] Martin JL, Stork CJ, Li YV. Determining zinc with commonly used calcium and zinc fluorescent indicators, a question on calcium signals. Cell Calcium 2006; 40: 393-402.

[34] Haugland RP. The Handbook - A Guide to Fluorescent Probes and Labeling Technologies. Tenth Edition ed, ed. M.T.Z. Spence. 2005, Eugene, Oregon: Molecular Probes, Invitrogen.

[35] Zhang J, Liu W, Liu J, Xiao W, Liu L, Jiang C, Sun X, Liu P, Zhu Y, Zhang C, Chen Q. G-protein β2 subunit interacts with mitofusin 1 to regulate mitochondrial fusion. Nat Commun 2010; 1: 101.

[36] Nagaraj R, Gururaja-Rao S, Jones KT, Slattery M, Negre N, Braas D, Christofk H, White KP, Mann R, Banerjee U. Control of mitochondrial structure and function by the Yorkie/YAP oncogenic pathway. Genes Dev 2012; 26: 2027-37.

[37] Zhao T, Huang X, Han L, Wang X, Cheng H, Zhao Y, Chen Q, Chen J, Cheng H, Xiao R, Zheng M. Central role of mitofusin 2 in autophagosome-lysosome fusion in cardiomyocytes. J Biol Chem 2012; 287: 23615-25.

[38] Wenzel P, Mollnau H, Oelze M, Schulz E, Wickramanayake JM, Müller J, Schuhmacher S, Hortmann M, Baldus S, Gori T, Brandes RP, Münzel T, Daiber A. First evidence for a crosstalk between mitochondrial and NADPH oxidase-derived reactive oxygen species in nitroglycerin-triggered vascular dysfunction. Antioxid Redox Signal 2008; 10: 1435-47.

[39] Frederickson CJ, Koh JY, Bush AI. The neurobiology of zinc in health and disease. Nat Rev Neurosci 2005; 6: 449-62.

[40] Weiss JH, Sensi SL, Koh JY. Zn(2+): a novel ionic mediator of neural injury in brain disease. Trends Pharmacol Sci 2000; 21: 395-401.

[41] Aras MA, Hara H, Hartnett KA, Kandler K, Aizenman E. Protein kinase C regulation of neuronal zinc signaling mediates survival during preconditioning. J Neurochem 2009; 110: 106-17.

[42] Jacob C, Maret W, Vallee BL. Control of zinc transfer between thionein, metallothionein, and zinc proteins. Proc Natl Acad Sci U S A 1998; 95: 3489-94.

[43] Maret W. Zinc coordination environments in proteins as redox sensors and signal transducers. Antioxid Redox Signal 2006; 8: 1419-41.

[44] Maret W, Vallee BL. Thiolate ligands in metallothionein confer redox activity on zinc clusters. Proc Natl Acad Sci U S A 1998; 95: 3478-82.

[45] Stork CJ, Li YV. Zinc release from thapsigargin/IP3-sensitive stores in cultured cortical neurons. J Mol Signal 2010; 5: 5.

[46] Choi DW. Zinc neurotoxicity may contribute to selective neuronal death following transient global cerebral ischemia. Cold Spring Harb Symp Quant Biol 1996; 61: 385-7.

[47] Tolando R, Jovanovic A, Brigelius-Flohé R, Ursini F, Maiorino M. Reactive oxygen species and proinflammatory cytokine signaling in endothelial cells: effect of selenium supplementation. Free Radic Biol Med 2000; 28: 979-86.

[48] Ago T, Kuribayashi F, Hiroaki H, Takeya R, Ito T, Kohda D, Sumimoto H. Phosphorylation of p47phox directs phox homology domain from SH3 domain toward phosphoinositides, leading to phagocyte NADPH oxidase activation. Proc Natl Acad Sci U S A 2003; 100: 4474-9.

[49] Dang PM, Fontayne A, Hakim J, El Benna J, Périanin A. Protein kinase C ζ phosphorylates a subset of selective sites of the NADPH oxidase component p47phox and participates in formyl peptide-mediated neutrophil respiratory burst. J Immunol 2001; 166: 1206-13.

[50] El-Benna J, Dang PM, Gougerot-Pocidalo MA, Marie JC, Braut-Boucher F. p47phox, the phagocyte NADPH oxidase/NOX2 organizer: structure, phosphorylation and implication in diseases. Exp Mol Med 2009; 41: 217-25.

[51] Hoyal CR, Gutierrez A, Young BM, Catz SD, Lin JH, Tsichlis PN, Babior BM. Modulation of p47PHOX activity by site-specific phosphorylation: Akt-dependent activation of the NADPH oxidase. Proc Natl Acad Sci U S A 2003; 100: 5130-5.

[52] Aras MA, Aizenman E. Aizenman, Redox regulation of intracellular zinc: molecular signaling in the life and death of neurons. Antioxid Redox Signal 2011; 15: 2249-63.