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

Kira G Slepchenko, Qiping Lu, Yang V Li

Department of Biomedical Science and Biological Science, Ohio Heritage College of Osteopathic Medicine, Ohio University, Athens Ohio, USA

Received January 28, 2016; Accepted February 9, 2016; Epub April 25, 2016; Published April 30, 2016

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
Zinc wave is required for mitochondrial ROS activation

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.

**Experimental 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 trypsinization method and maintained in EMEM medium supplemented with 5% fetal bovine serum (ATCC, Manass, VA) in 5% CO₂ -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% CO₂ -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 CaCl₂, 1.1 MgCl₂, 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 ZnCl₂ and 10 µM Na-pyritione, 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
Zinc wave is required for mitochondrial ROS activation

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: \((\frac{F - F_0}{F_0}) \times 100\), 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...
Zinc wave is required for mitochondrial ROS activation


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 fluorescent 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 MitoSOX 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 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).
Zinc wave is required for mitochondrial ROS activation

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

Effect of NADPH 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].
Zinc wave is required for mitochondrial ROS activation

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.

Acknowledgements

This work was supported in part by NIH grant NS081629 to YVL.

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 deprivation; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMSO, dimethyl sulfoxide.

Address correspondence to: Dr. Yang V Li, Department of Biomedical, Ohio Heritage College of Osteopathic Medicine, 342 Irvine Hall, Ohio University, Athens Ohio, OH 45701 Science, USA. Tel: 740-593-2384; Fax: 740-593-2778; E-mail: lyl1@ohio.edu
Zinc wave is required for mitochondrial ROS activation

References


Zinc wave is required for mitochondrial ROS activation


