Original Article
Reduced metallothionein expression induced by Zinc deficiency results in apoptosis in hepatic stellate cell line LX-2

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Abstract: The present study is to investigate the molecular mechanism by which Zinc (Zn) deficiency induces apoptosis in hepatic stellate cells. LX-2 cells were incubated with N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamine (TPEN; 5 µM, 10 µM and 25 µM) for 24 h. MTT assay was used to test the proliferation ability of LX-2 cells. Flow cytometry was performed to detect cell apoptosis. Western blotting assay was employed to determine the expression of metallothionein (MT). Atomic absorption spectroscopy was performed to measure intracellular reactive oxygen species content. To test the activity of mitochondria, respiratory control rate was tested. To investigate the activation of apoptotic signaling pathway, cytochrome C oxidase activity was determined. TPEN effectively decreased the content of Zn in LX-2 cells. Zn deficiency led to the inhibition of proliferation and enhancement of apoptosis of LX-2 cells. Zn deficiency induced the inhibition of MT expression in LX-2 cells. Inhibition of MT expression induced by Zn deficiency resulted in enhanced reactive oxygen species content, impaired mitochondrial function and inhibition of cytochrome C oxidase activity. Intracellular MT content in LX-2 cells is reduced by Zn deficiency. Reduction in MT expression further increases intracellular ROS content, enhances oxidative stress, inhibits cytochrome C oxidase activity, impairs mitochondrial function, and finally leads to cell apoptosis.

Keywords: Zinc, metallothionein, apoptosis, hepatic stellate cells, LX-2 cells

Introduction

Before the formation of liver cirrhosis from liver fibrosis, hepatocytes undergo apoptosis and necrosis under the stimulation by inflammation, induce excessive deposition of extracellular matrix proteins, and lead to hyperplasia of fibrous connective tissue in the liver [1, 2]. Zinc (Zn) is an essential trace element in human body. It has multiple biological functions such as anti-oxidization, anti-inflammation, and anti-apoptosis [3]. Zn content in normal ranges inhibits cell apoptosis, while that in abnormal ranges induces cell apoptosis [4]. A study shows that abnormal metabolism of Zn can induce the apoptosis of several types of cells such as hepatocytes, renal cells, and fibroblasts [5]. T lymphocyte apoptosis induced by Zn deficiency is the main reason for thymic

atrophy [6]. Apoptosis induced by Zn deficiency is closely related to body growth, development, immune function regulation and various diseases [7]. Truong-Tran et al. report that apoptosis induced by Zn deficiency may be related to the apoptotic signal transduction pathway that is activated by increased oxidative stress in the body. These authors suggest that Zn may be the key to the connection between oxidation-reduction and apoptotic pathway in the body [8].

Metallothionein (MT) is a kind of low molecular weight metal-binding protein that has high metal and cysteine contents. MT has varying degrees of relation to liver diseases, and is found to have inhibitory effect on hepatic fibrosis progression [9]. MT exerts its protective effect on livers with lesions by its antioxidant

effect, cytoprotection, and promotional effect on the regeneration of liver cells [10, 11]. In the acute phase of lesions, MT expression is significantly enhanced, reducing oxidative stress in the liver and lowering the degree of liver lesions. After the disease progresses into chronic stage, the expression of MT is reduced [12]. During liver fibrosis and cirrhosis, the content of MT is only 25-30% of that in normal liver tissues [13-15].

MT is closely related to Zn metabolism. MT provides Zn ions that are needed to sustain the activities of proteases and transcription factors via Zn ion-binding protein [16-18]. MT that binds Zn acts as the regulator of Zn metabolism by eradicating free radicals. When MT synthesis is increased, intracellular binding of Zn is promoted, leading to controlled free Zn ions. Reversely, Zn regulates the expression of MT [19-23]. Zn binds metal-responsive transcription factor (MTF) of metal response element (MRE), and enters cell nucleus, where MTF recognizes the specific sequence (MRE) of MT gene promoter and binds DNA, followed by the initiation of gene transcription [24].

The activity of mitochondria is closely related to cell apoptosis. When the electric potential difference across mitochondrion membrane is reduced, a series of biochemical changes may be induced inside and outside mitochondrion membrane, including the release of cytochrome C (a caspase activator that regulates both energy metabolism and cell apoptosis), changes in mitochondrion membrane permeability, and activation of Bcl-2 family and caspase. These processes may induce the cascade reactions of cell apoptosis and finally lead to cell apoptosis [25]. During the increase of oxidative stress, factors like H_2O_2 may induce the opening of mitochondrial permeability transition pore (MPTP), as well as membrane potential collapse, leading to proteolytic cleavage of Opal and mitochondrial fission [26]. In the mean time, Drp1 accumulates in the membrane of mitochondria, leading to mitochondrial fission and cell apoptosis stimulated by small ubiquitin-related modifier [26]. In the present study, we investigate whether Zn inhibits MT expression, leads to intracellular oxidative stress, impairs mitochondrial activities, and finally causes cell apoptosis.

Materials and methods

Cells

Hepatic stellate LX-2 cells were cultured in high-dulbecco's modified eagle medium supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. The cells were divided into groups according to N,N,N',N'-tetrakis (2-pyridylmethyl) ethane-1,2-diamine (TPEN) concentrations. In control group, the cells were cultured for 24 h without any treatment before determination. In 5 μM group, the cells were incubated with 5 μM TPEN for 24 h before determination. In 10 μM group, the cells were incubated with 10 μM TPEN for 24 h before determination. In 25 μM group, the cells were incubated with 25 μM TPEN for 24 h before determination.

Atomic absorption spectroscopy

LX-2 cells were digested using 1% TNE before collection by centrifugation (200 × g; model 5415D, Eppendorf, Hamburg, Germany). After addition of 1% sodium dodecyl sulfate (100 μl), the cells were vortexed and lysed by sonication (Sonifier 400 W Digital Cell Disruptor, Branson Ultrasonics Corporation, Emerson Electric, Ferguson, MO, USA). Then, the cells were centrifuged at 12000 × g under 4°C for 10 min. The supernatant that contained target proteins was saved. Five times of volume of concentrated nitric acid was added to the protein, and left standing for 2 days before detection. The content of Zn was determined using atomic absorption spectroscopy (iCE 3300, Thermo Fisher Scientific, Waltham, MA, USA) and normalized to the total protein content in LX-2 cells.

MTT assay

MTT assay was performed at 48 h after changing medium. After removing old medium, 200 μL MCDB131 medium was added into each well, followed by addition of 20 μL MTT with shaking. After incubation at 37°C for 3.5 h, the supernatants were discarded, followed by the addition of 100 μL dimethyl sulfoxide. After incubation for 5-10 min, the supernatants were transferred to another clean 96-well plate for the measurement of optical density values at 490 nm in a microplate reader (Model 680, Bio-Rad, Hercules, CA, USA). All tests were performed in quintuplicate.
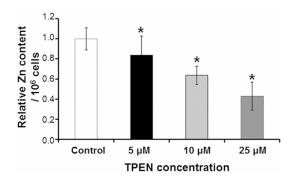
Figure 1. Effect of TPEN (5 µM, 10 µM and 25 µM) on the content of Zn in LX-2 cells after incubation for 24 h. LX-2 cells were digested using 1% TNE before collection by centrifugation. After addition of 1% sodium dodecyl sulfate (100 µL), the cells were vortexed and lysed by sonication. Then, the cells were centrifuged at 12000 × g under 4°C for 10 min. The supernatant that contained target proteins was saved. Five times of volume of concentrated nitric acid was added to the protein, and left standing for 2 days before detection. The content of Zn was determined using atomic absorption spectroscopy and normalized to the total protein content in LX-2 cells. The data were expressed as means ± standard deviation. *P < 0.05 compared with control.

Flow cytometry

The cells were trypsinized before centrifugation at 200 × g for 5 minutes, followed by washing with phosphate-buffered saline. The cells were incubated with 100 µl Annexin-V-FLUOS staining reagent containing 2% propidium iodide (Roche, Basel, Switzerland) under room temperature for 10-15 minutes. Cell apoptosis was detected using flow cytometry (MoFlo Astrios EQ, Beckman Coulter, Brea, CA, USA). Apoptotic index was obtained from the percentage of cells double-stained by Annexin-V/propidium iodide. Each test was performed in triplicate.

Western blotting

A total of 30 µg of protein per well was resolved using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes. After blocking and washing, the membrane was incubated with monoclonal goat anti-mouse MT (1:1,000, Santa Cruz, CA, USA), or with mouse anti-β-actin (1:1,000, Santa Cruz, CA, USA) at 4°C for 12 h, followed by the incubation with horseradish peroxidase-labeled rabbit anti-goat secondary antibody (1:2,000, Santa Cruz, CA, USA) for 1 h at room temperature. The
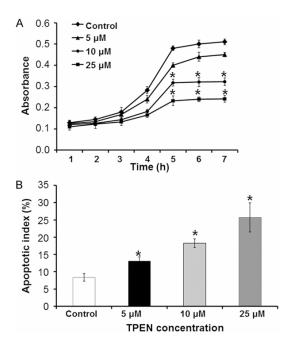

Figure 2. Effect of different concentrations of TPEN on LX-2 cell (A) proliferation and (B) apoptosis. Cell proliferation was measured using MTT assay. Apoptosis was detected using flow cytometry. The data were expressed as means ± standard deviation. *P < 0.05 compared with control.

immunoreactive bands were visualized by enhanced chemiluminescence (Santa Cruz, CA, USA). The level of protein expression in each sample was determined by normalizing target band intensity to β-actin band intensity.

Determination of reactive oxygen species (ROS) content

The cells were digested using trypsin, followed by centrifugation at 300 g for 5 min. Then, the cells were processed using fluorescent assay kit (GENMED, Shanghai, China) following the manufacturer's protocol. Fluorescence intensity was measured using light-sheet microscopy (LaVision BioTec GmbH, Bielefeld, Germany) with an excitation wave length of 540 nm and an emission wave length of 590 nm. Intracellular ROS content was calculated using the obtained relative fluorescence units.

Determination of mitochondrial respiratory control rate (RCR)

In the reaction chamber of respirometry (Oxygraph-2k, Oroboros Instruments Corp., Innsbruck, Austria), a mixture of 225 mM mannitol,
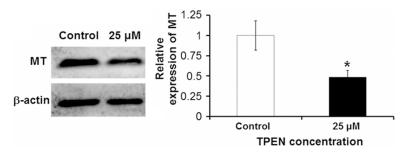
Figure 3. Effect of Zn deficiency on metallothionein protein expression in LX-2 cells. Western blotting was performed in control group and 25 μM TPEN group. The data were expressed as means ± standard deviation. *P < 0.05 compared with control.

70 mM sucrose, 1 mM EDTA, 0.1% bovine serum albumin, and 10 mM potassium phosphate (pH 7.4) was added and maintained for 8-10 min. The maximal relative oxygen concentration of the instrument was set to 20. Then, the suspension of extracted mitochondria was added. After recording of baseline for a while, 10 μL exogenous substrate (0.2 M disodium succinate) was added and IV stage respiration appeared. After recording for 2 min, 8 μL adenosine diphosphate (50 mM) was added and III stage respiration appeared. After all adenosine diphosphate was consumed, IV stage respiration appeared again. RCR = III stage respiratory rate/IV stage respiratory rate. RCR reflects the integrity of mitochondrial membrane and coupling degree with oxidative phosphorylation.

Cytochrome C oxidase (COX) activity test

LX-2 cells were trypsinized and collected by centrifugation (200 × g; model 5415D, Eppendorf, Hamburg, Germany) in Eppendorf tubes. The cells were then resuspended in 1 ml MSTE buffer, and transferred to Dounce homogenizer (Thomas Scientific, Swedesboro, NJ, USA) for homogenization on ice for 200 times. Cell homogenates were then transferred to a new 2 ml Eppendorf tube for centrifugation at 600 × g under 4°C for 5 min. The supernatant was then centrifuged at 4°C and 1000 × g for 5 min. Subsequently, the supernatant was collected for another centrifugation at 4°C and 7000 × g for 10 min. After discarding supernatant, the sediments were then resuspended in 1 mL MSTE buffer, followed by centrifugation at 4°C and 7000 × g for 10 min. Then the supernatant was discarded and the sediments were resuspended in 1 mL MSTE buffer, followed by centrifugation at 4°C and 10000 × g for 10 min to collect mitochondria.

The collected mitochondria samples were mixed with 40 μL 1% sodium dodecyl sulfate and place on ice. The mixture was then vortexed for 10 s every 5 min, for a total of 6 times and 30 min. Then, the mixture was centrifuged at 4°C and 12000 × g for 10 min to collect supernatant, which was mitochondrial protein lysates. The concentration of mitochondrial protein was determined using DAC protein detection kit (Bradford Protein Assay Kit, Beyotime, Shanghai, China). COX activity was tested using COX activity detection kit (Genmed, Shanghai, China).

Statistical analysis

The results were analyzed using SPSS 13.0 software (IBM, Armonk, NY, USA). The data were given in means ± SD. Two groups of mean values were compared using Student's t-test. P value less than 0.05 was considered statistically significant.

Results

TPEN effectively decreases the content of Zn in LX-2 cells

To determine the content of Zn in LX-2 cells, atomic absorption spectroscopy was performed. The data showed that TPEN (5 μM, 10 μM and 25 μM) significantly reduced the content of Zn in LX-2 cells compared with control, with higher concentration of TPEN resulting in more significant reduction in Zn content (P < 0.05) (**Figure 1**). The result suggests that TPEN effectively decreases the content of Zn in LX-2 cells.

Zn deficiency leads to the inhibition of proliferation and enhancement of apoptosis of LX-2 cells

To test the effect of TPEN on LX-2 cell proliferation and apoptosis, MTT assay and flow cytometry were performed, respectively. MTT data showed that TPEN (10 μM and 25 μM) significantly inhibited the proliferation of LX-2 cells compared with control (P < 0.05). However, TPEN (5 μM) only slightly inhibited the proliferation of LX-2 cells without statistical significance
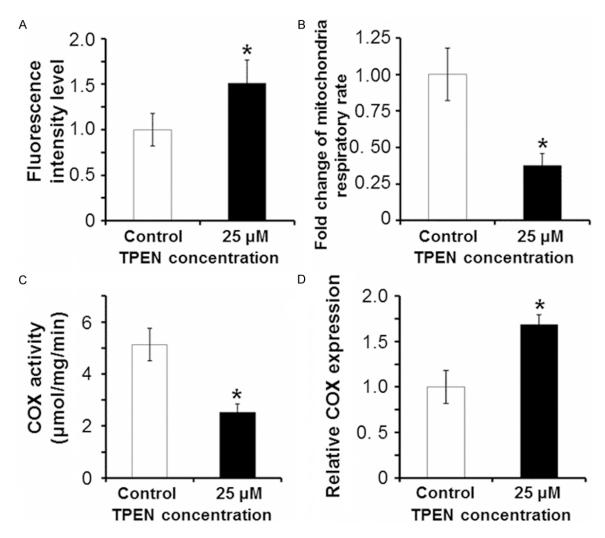
Figure 4. Effect of Zn deficiency on oxidative stress, mitochondrial function and apoptosis-related signaling pathways in LX-2 cells. (A) Reactive oxygen species content, (B) mitochondrial respiratory control rate, (C) cytochrome C oxidase activity, and (D) cytochrome C oxidase expression in control group and 25 μM TPEN group were determined. The data were expressed as means ± standard deviation. *P < 0.05 compared with control.

(P > 0.05) (**Figure 2A**). Consistent with these data, flow cytometry showed that the apoptotic index of LX-2 cells was significantly enhanced by TPEN (10 μM and 25 μM) (P < 0.05), but only slightly increased by TPEN (5 μM) (**Figure 2B**). These results indicate that Zn deficiency leads to the inhibition of proliferation and enhancement of apoptosis of LX-2 cells.

Zn deficiency induces the inhibition of MT expression in LX-2 cells

To determine the effect of Zn deficiency on MT protein expression in LX-2 cells, Western blotting was performed in control group and 25 μM TPEN group. The data showed that TPEN (25 μM) induced significantly decreased MT protein

expression in LX-2 cells (**Figure 3**). The result suggests that Zn deficiency results in the inhibition of MT expression in LX-2 cells.

Inhibition of MT expression induced by Zn deficiency results in enhanced oxidative stress, impaired mitochondrial function and activation of apoptotic signaling pathways

To study the effect of Zn deficiency on oxidative stress, mitochondrial function and apoptosis-related signaling pathways in LX-2 cells, ROS content, mitochondrial RCR, and COX activity in control group and 25 μM TPEN group were determined. The data showed that TPEN (25 μM) significantly enhanced the ROS fluorescence intensity in LX-2 cells (P < 0.05) (**Figure**

4A). In addition, mitochondrial respiratory rate was reduced in 25 μM TPEN group compared with control group ($P < 0.05$) (**Figure 4B**). Furthermore, TPEN (25 μM) significantly inhibited COX activity in LX-2 cells compared with control ($P < 0.05$) (**Figure 4C**). Western blotting analysis of cytochrome C oxidase content showed that its expression was significantly enhanced in 25 μM TPEN group compared with control ($P < 0.05$) (**Figure 4D**). These results indicate that inhibition of MT expression induced by Zn deficiency results in enhanced oxidative stress, impaired mitochondrial function and activation of apoptotic signaling pathways, finally leading to the promotion of LX-2 cell apoptosis.

Discussion

The present study shows that addition of TPEN induces Zn deficiency in LX-2 cells, which subsequently leads to cell apoptosis. In addition, the degree of apoptosis is enhanced with the increase in the degree of Zn deficiency. After treatment with TPEN, intracellular MT expression was significantly reduced, suggesting that MT might play important roles in cell apoptosis induced by Zn deficiency. MT is an important enzyme that regulates intracellular oxidative stress. In the acute phase of lesions, MT expression is significantly enhanced to reduce the oxidative stress and damages in the liver. In the present study, intracellular oxidative stress in LX-2 cells was significantly enhanced after MT expression was reduced by Zn deficiency for 24 h. Further investigations showed that elevated oxidative stress impaired mitochondrial function in the cells, such as significantly reduced mitochondrial respiratory rate. The activity of mitochondria is closely related to cell apoptosis. The present study also showed that the activity of apoptosis-related signaling pathway was significantly enhanced after mitochondrial activity impairment by reduced MT expression. These results demonstrate that reduced MT expression induced by Zn deficiency is the key to elevated intracellular oxidative stress, enhanced apoptosis-related signaling pathway, and promoted cell apoptosis. However, the present study fails to elucidate the molecular mechanism by which Zn deficiency induces the decrease in MT expression. This will be investigated in future studies. In conclusion, the present study demonstrates that intracellular MT content in LX-2 cells is reduced by Zn deficiency that is induced by incubation with TPEN (25 μM) for 24 h. Reduction in MT expression further increases intracellular ROS content, enhances oxidative stress, inhibits cytochrome C oxidase activity, impairs mitochondrial function, and finally leads to cell apoptosis.

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Disclosure of conflict of interest

None.

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