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Effects of intracellular iron overload on cell death and identification of potent cell death inhibitors

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ABSTRACT

Iron overload causes many diseases, while the underlying etiologies of these diseases are unclear. Cell death processes including apoptosis, necroptosis, cyclophilin D-(CypD)-dependent necrosis and a recently described additional form of regulated cell death called ferroptosis, are dependent on iron or iron-dependent reactive oxygen species (ROS). However, whether the accumulation of intracellular iron itself induces ferroptosis or other forms of cell death is largely elusive. In present study, we study the role of intracellular iron overload itself-induced cell death mechanisms by using ferric ammonium citrate (FAC) and a membrane-permeable Ferric 8-hydroxyquinoline complex (Fe-8HQ) respectively. We show that FAC-induced intracellular iron overload causes ferroptosis. We also identify 3-phosphoinositide-dependent kinase 1 (PDK1) inhibitor GSK2334470 as a potent ferroptosis inhibitor. Whereas, Fe-8HQ-induced intracellular iron overload causes unregulated necrosis, but partially activates PARP-1 dependent parthanatos. Interestingly, we identify many phenolic compounds as potent inhibitors of Fe-8HQ-induced cell death. In conclusion, intracellular iron overload-induced cell death form might be dependent on the intracellular iron accumulation rate, newly identified cell death inhibitors in our study that target ferroptosis and unregulated oxidative cell death represent potential therapeutic strategies against iron overload related diseases.

1. Introduction

Certain amounts of iron are essential for cell metabolic processes and organismal function [1], while abnormal iron accumulation and ROS production are both indicated in a numerous number of diseases [2]. Intracellular iron deposition in the parenchymal cells of the liver and other body organs caused by inherited diseases can lead to cellular injury [3,4]. In addition, aberrant accumulation of iron within neurons has been implicated in the etiology of neurodegenerative diseases [5]. However, how intracellular iron accumulation actually promotes cell death and cellular injury in these pathological conditions is elusive.

Both iron and iron-dependent ROS-producing enzymes are thought to participate in different kinds of regulated forms of cell death. Aberrant accumulation of mitochondrial ROS is essential to trigger AMP-activated protein kinase (AMPK) dependent apoptosis [6]. Tumor necrosis factor (TNF)-induced labile iron levels and subsequent mitochondrial ROS could promote RIP1 autophosphorylation, which is essential for the effective induction of necroptosis [7,8]. In addition, translocation of iron from lysosomes to mitochondria is required for CypD-dependent necrosis during ischemia reperfusion injury [9]. More specifically, ferroptosis is a form of regulated cell death whose execution requires the iron-catalyzed accumulation of lipid ROS [10]. Under both circumstances of glutathione depletion with erastin and inactivation of the phospholipid peroxidase glutathione peroxidase 4 with the direct inhibitor (1 S, 3 R)-RSL3 (RSL3), iron-containing enzymatic effectors, including lipoxygenases (LOXs), mediated lipid hydroperoxides will accumulate to lethal levels [11]. Iron and iron-derived lipid ROS are required for ferroptosis under different conditions. However, whether the accumulation of intracellular iron itself induces ferroptosis or other forms of cell death mentioned above remains unclear.

Here, we applied ferric ammonium citrate (FAC) and a membrane-permeable ferric 8-hydroxyquinoline complex (Fe-8HQ) to establish slow and rapid intracellular iron overload-induced cell death models respectively, and aimed to identify the cell death mechanisms.

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2. Materials and methods

2.1. Chemicals

(1S, 3R)-RSL3, Nordihydroguaiaretic acid, Liproxstatin-1, Cyclosporin A, VX-765, Staurosporine, Camptothecin, Z-VAD-FMK, Baicalein, Zileuton, Molidustat, custom compounds library, ALLN and E64D were from MedChemExpress. Trolox was obtained from Abcam. Adaptaquin was from R&D Systems. Unless otherwise indicated, all other chemicals were from Sigma-Aldrich.

2.2. Cell lines and cell culture

HeLa cells (PARP1$^{-/-}$ and wild type) were obtained from Professor Jun Huang (Life Sciences Institute, Zhejiang University, Hangzhou). All other cell lines were from American Type Culture Collection (ATCC, Manassas, VA, USA). Mouse hepatocytes AML12 were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), 10 μg/ml insulin, 5 μg/ml transferrin, 7 ng/ml selenium (1:100 dilution of ITS, GIBCO), and 100 nM dexamethasone. HeLa and HT-1080 fibrosarcoma cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). All cell lines were grown in humidified tissue culture incubator (SANYO) at 37 °C with 5% CO_2.

2.3. Light microscopy

To examine the morphology of cell death, phase contrast images of static bright field cells were captured using the LEICA DCF295 microscope equipped with 20× phase-contrast objective. All image data shown are representative samples from three random fields.

2.4. Cellular iron staining

Cells were seeded in 6-well plates. After treating with test compounds for the indicated time, cells were washed with PBS twice and stained with 100 nM of Calcein-AM (Ab14140, Abcam) in PBS for 15 min in culture incubator. Cells were released with Accutase™ Cell Detachment Solution (561527, BD Biosciences), harvested in 2 ml PBS, and centrifuged at 2500 rpm for 5 min. The cell pellet was resuspended in 500 μL of PBS and analyzed using flow cytometer from FL1 channel (FACSCalibur, BD Biosciences). A minimum of 10,000 cells were analyzed per condition, and data were processed in the software FlowJo for all other flow cytometry experiments.

2.5. Cell death and viability

Cell viability was assessed by Cell Counting Kit-8 (MedChemExpress). CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega Corporation) was used to measure the released lactate dehydrogenase (LDH). Additionally, Dead Cell Apoptosis Kit with Annexin V Alexa Fluo 488/PI (V13241, Invitrogen) was used to detect cell death by following the manufacturer's instruction.

2.6. Assessment of reactive oxygen species production

After one day of cell seeding on 6-well plates, cells were treated as indicated and harvested by accutase, washed and incubated in 500 ml warm Hanks Balanced Salt Solution (HBSS, Gibco) containing 5 μM CM-H_2DCFDA (C6827, Invitrogen) for 10min, or 5 μM BODIPY 581/591 C11 (D3861, Invitrogen) for 1 h at 37 °C in culture incubator. Cells were then centrifuged and resuspended in 500 ml of fresh HBSS. Data were collected by flow cytometer from the FL1 channel.

2.7. Caspase-3 activity measurement

Caspase-3 activity assay kit (Jiancheng Bioengineering, Nanjing, China) was used to determine caspase-3 activities. Briefly, 200,000 cells were seeded on 6-well plates. After 24 h, cells were treated with DMSO, apoptosis inducers and iron for indicated times. Cells were harvested and lysed 30 min in the lysis buffer. The lysate was centrifuged at 12,000 rpm at 4 °C for 15 min, and cleared lysate was used to determine the amount of protein and caspase-3 activity in the sample. Caspase-3 activity is normalized to same protein levels and reported as a percentage relative to the negative control.

2.8. Protein analysis and western blot

HeLa cells (PARP1−/− and wild type) were lysed with Mammalian Protein Extraction Reagent, supplemented with Protease Inhibitor Cocktails (all from Fudebio-tech, Hangzhou, China). The total protein was determined by Pierce BCA Protein Assay Kit (Beyotime, Haimen, China). Equivalent amounts of proteins were loaded on a 12.5% SDS gel and transferred to a PVDF membrane. The membranes were incubated with primary antibody including PARP-1 (A0942, ABclonal) and β-actin (AC004, ABclonal) overnight at 4 °C. After incubation with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, blots were then washed three times as above. Blots were incubated for 5 min with the Clarity Western ECL Substrate (BioRad), and the LAS-4000 CCD camera system (Fujifilm) was used to detect chemiluminescence signals.

2.9. Statistical analyses

All data are represented as mean + or ± SD. Statistical analyses were performed by using one-way ANOVA with post hoc Bonferroni test on GraphPad Prism (version 5.01) software. P values < 0.05 were considered significantly different.

3. Results and discussion

3.1. Intracellular iron overload-induced cell death models establishment

Periportal hepatocytes are the primary iron loads cells of the liver in hereditary hemochromatosis [4]. Mouse hepatocytes AML12 cells display many properties of differentiated hepatocytes [12]. Thus, we selected AML12 cells for studies of iron-induced cell death mechanisms that might contribute to the diseases of liver iron overload. We hypothesized that ferroptosis is likely to be involved in iron overload-induced cell death. Accordingly, we selected NRAS mutant HT-1080 fibrosarcoma cells, a ferroptosis sensitive cell line [10], to further study the possible role of ferroptosis. Depending on the severity of iron overload, iron accumulation might vary from slow to rapid forms [4]. Thus, we selected FAC, which is a physiological form of non−transferrin-bound iron, to investigate the slow intracellular iron overload-induced cell death mechanism. On the contrary, we investigated rapid intracellular iron overload-induced cell death by using a highly lipophilic Fe-8HQ that permeates the cell membrane rapidly.

To detect intracellular iron accumulation, we used calcein-AM, a fluorescent probe that is quenched upon binding to intracellular iron [13]. Before treating with iron sources, we defined a 90% basal level of fluorescence. FAC treatment gradually shifted the fluorescence level to 40% about 2 h in AML 1 cells (Fig. 1a), while cells

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