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Haloperidol, a sigma receptor 1 antagonist, promotes ferroptosis in hepatocellular carcinoma cells

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ABSTRACT

Ferroptosis is a novel form of cell death, which is characterized by accumulation of reactive oxygen species (ROS). Sigma 1 receptor (S1R) has been suggested to function in oxidative stress metabolism. Both erastin and sorafenib significantly induced S1R protein expression. Haloperidol strongly promoted erastin- and sorafenib-induced cell death, which was blocked by ferrostatin-1 but not ZVAD-FMK or necrosulfonamide. During ferroptosis, haloperidol substantially increased the cellular levels of Fe^{2+}, GSH and lipid peroxidation. Furthermore, several ferroptosis-related protein targets were up-regulated in the absence of haloperidol. Thus, Our study identified an association between haloperidol and ferroptosis for the first time. Our analyses of a combination of drugs may provide a novel strategy of hepatocellular carcinoma (HCC) therapy.

1. Introduction

Ferroptosis was recently identified as a new form of regulated cell death (RCD) by Brent R. Stockwell's laboratory in 2012. Ferroptosis differs from apoptosis and other major forms of RCD (e.g., necroptosis and autophagic cell death) in many aspects. For example, it is characterized by cell volume shrinkage and increased mitochondrial membranes and is mediated by iron-dependent lipid peroxide accumulation [1]. Ferroptosis may occur in both physiological and pathological processes and was specifically found in several types of tumor cells [2–4]. The iron-dependent accumulation of ROS finally induces ferroptosis through inactivation of glutathione peroxidase 4 (GPX4) or glutathione (GSH) deficiency [2]. To date, several ferroptosis-related targets have been identified, including nuclear factor erythroid 2-related factor 2 (NRF2) [5], haeme oxygenase 1 (HO-1) [6], GPX4 and the well-known p53 [7]. However, detailed signal transduction pathways and key transcriptional regulators of ferroptosis remain unknown.

S1R is a protein modulator that is associated with a variety of neurological diseases and ischaemia-reperfusion injury [8]. S1R is generally localized at the endoplasmic reticulum (ER), mitochondrial membrane [9] and the plasma membrane [10]. Recently, it was observed at the nuclear envelope [11]. However, its localization was altered following intracellular oxidative stress [12]. S1R agonists have been shown to contribute to cellular protection by suppressing ischaemia-induced ROS production. In contrast, S1R antagonists decreased tumor cell survival [9,13,14]. Furthermore, a recent metabolic study showed an increase in oxidative stress markers (including oxidized GSH and GSH) in the liver of S1R-knockout (S1RKO) mice compared to those of wild-type (WT) mice [15]. Meanwhile, another study reported elevated ROS levels and decreased GSH levels in Müller cells harvested from S1RKO mice compared with those of cells from WT mice [16]. Although the exact role of S1R has not been confirmed, these findings strongly suggest that S1R plays an important role in oxidative stress metabolism.

S1Rs are found in many tissues in humans, rats and mice, but their expression is generally concentrated in the central nervous system. S1R is highly abundant in the liver as well. Various classes of psychotropic drugs bind to S1R, including haloperidol, which has a relatively high affinity for S1R [17]. In this study, we identified haloperidol as a promoter of ferroptosis in hepatocellular carcinoma (HCC) cells, in both erastin- and sorafenib-induced conditions. Furthermore, we demonstrated that haloperidol increases

Abbreviations: ROS, reactive oxygen species; S1R, sigma 1 receptor; S1RKO, S1R-knockout; RCD, regulated cell death; CCK-8, Cell Counting Kit-8; GPX4, glutathione peroxidase 4; GSH, glutathione; NRF2, nuclear factor erythroid 2-related factor 2; HO-1, haeme oxygenase 1; MDA, malondialdehyde; ER, endoplasmic reticulum; ARE, antioxidant response element.

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iron accumulation and lipid peroxidation in ferroptosis and contributes to this process by inducing the expression of S1R and other regulators involved in ferroptosis. Collectively, our results indicate the novel pharmacological action of haloperidol in promoting cellular ferroptosis.

2. Methods

2.1. Regents

Antibodies to NRF2 (ab62352), S1R (ab151288), HO-1 (ab68477) and GPX4 (ab125066) were all obtained from Abcam (Shanghai, China). Erastin (No. S7242), sorafenib (No. S7397), ZVAD-FMK (No. S7023), and ferrostatin-1 (No. S7243) were obtained from Selleck (Shanghai, China). Haloperidol (H1512) and necrosulfonamide (ab143839) were obtained from Sigma Aldrich (Shanghai, China) and Abcam (Shanghai, China), respectively.

2.2. Cell culture

The human HCC cell lines Hep G2 and Huh-7, maintained at our institute, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (HyClone) in a humidified incubator with 5% CO_2 and 95% air.

2.3. Cell viability assay

Cell viability was evaluated with a Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Shanghai, China) according to the manufacturer's instructions. WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5(2,4-disulfophenyl)-2H-tetrazolium monosodium salt], which is a highly water-soluble tetrazolium salt, was used to evaluate cell viability by CCK-8 assays. WST-8 is a next-generation, sensitive reagent that serves as a chromogenic indicator for NADH. Following the reaction, NADH can reduce WST-8 at neutral pH values in the presence of 1-methoxy PMS to produce a water-soluble formazan dye, which is used as a cell viability indicator in cell proliferation and death assays by measuring absorption at 450 nM.

2.4. Western blot

Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor complexes to harvest proteins. Then, the proteins in the supernatants of each sample were separated by 8%–12% SDS poly-acrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (pore size 0.45 μm). After the membrane was blocked (5% milk) for approximately 1 h, it was incubated overnight at 4 °C with each primary antibody, followed by incubation with secondary antibody for 2 h at room temperature. Finally, we visualized the blots using an enhanced chemiluminescence system.

2.5. Iron assay

The intracellular iron concentration in cell lysates was assessed using an iron colorimetric assay kit purchased from Biovision (Milpitas, California, USA) according to the manufacturer's instructions. In this assay, ferric carrier proteins dissociate ferric iron into solution in the presence of acid buffer. After reduction to the ferrous form (Fe^{2+}), iron reacts with Ferene S to produce a stable colored complex. This kit can thus measure iron level in samples.

2.6. Lipid peroxidation assay

The intracellular malondialdehyde (MDA) concentration in cell lysates was assessed using a lipid peroxidation colorimetric assay kit purchased from Biovision (Milpitas, California, USA) according to the manufacturer's instructions. Lipid peroxidation, which produces MDA as an end product, occurs as a result of oxidative damage and is a marker for oxidative stress.

2.7. GSH assay

The intracellular GSH level in cell lysates was assessed using a GSH colorimetric assay kit purchased from Biovision (Milpitas, California, USA) according to the manufacturer's instructions.

2.8. Statistical analysis

All data are expressed as the mean ± SD. Data were analyzed using two-tailed Student's t tests for comparison of two groups or ANOVA LSD tests for comparisons among multiple groups. Significance was defined as $\boldsymbol{p} < 0.05$.

3. Results

3.1. Identification of haloperidol as a promoter of erastin- and sorafenib-induced cell death

As previously described [15,16], S1R is involved in oxidative stress metabolism. To determine whether inducing ferroptosis by erastin and sorafenib affects S1R, we analyzed the protein levels of S1R in two HCC cell lines. The results indicated that both erastin and sorafenib significantly induced S1R protein expression (Fig. 1A and B). Next, we examined the effect of haloperidol (an S1R antagonist) on ferroptosis. Cell viability was measured in Hep G2 and Huh-7 cell lines following treatment with 10 μM erastin or 5 μM sorafenib in the absence or presence of haloperidol. Indeed, haloperidol promoted erastin- and sorafenib-induced cell death in a dose- (Fig. 1C and D) and time- (Fig. 1E and F) dependent manner in both HCC cell lines. These findings suggest that haloperidol promotes both erastin- and sorafenib-induced cell death.

3.2. Haloperidol promotes cellular ferroptosis but not necroptosis or apoptosis

Next, we added ferrostatin-1 (a strong ferroptosis inhibitor), ZVAD-FMK (a strong apoptosis inhibitor) and necrosulfonamide (a strong necroptosis inhibitor) to address the mechanism of haloperidol in ferroptosis. Ferrostatin-1 inhibits ferroptosis by depressing cellular lipid peroxidation. Similar to results from previous studies [1,2,18–20], erastin- and sorafenib-induced cell death in both HCC cell lines was blocked by ferrostatin-1 but not ZVAD-FMK or necrosulfonamide. As expected, in the presence of 10 μM haloperidol, the aggravated erastin- and sorafenib-induced cell death was also blocked by ferrostatin-1 in both HCC cell lines, but the other inhibitors had no significant effects (Fig. 2A and B). These findings indicate that haloperidol at 10 μM promotes cellular ferroptosis but not necroptosis or apoptosis.

3.3. Haloperidol accelerates iron accumulation and lipid peroxidation, while facilitating GSH depletion, in ferroptosis

Accumulation of ferrous iron and lipid peroxidation products were reported to participate in erastin- and sorafenib-induced ferroptosis [5]. Thus, we analyzed MDA and Fe^{2+} levels to determine whether haloperidol-promoted ferroptosis is associated with

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