AMPK/ULK1 and JNK/p38/ERK Regulate Serotonin (5-hydroxytryptamine) -induced Autophagy in Hepatoma Cells

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Abstract

It has been reported that serotonin (5-hydroxytryptamine, 5-HT), autophagy and ROS are involved in the pathogenesis of cancer, here, we used HepG2 and Hep3B cells as model cells to investigate whether 5-HT could induce hepatoma cells autophagy and its possible mechanisms involved in ROS. First, the immunofluorescence results showed that 5-HT could induce the production of LC3 in hepatoma cells, and western blot results showed that 5-HT induced a complete autophagy process in hepatoma cells. Secondly, our results showed that 5-HT induced autophagy through the AMPK/ULK1 instead of the mTOR-dependent pathway in hepatoma cells. Thirdly, 5-HT also could stimulate autophagy through JNK/p38/ERK pathway activation in hepatoma cells. Finally, the DHR 123 assay showed that 5-HT could induce ROS production in hepatoma cells and the ROS scavenger NAC significantly inhibited the 5-HT-induced LC3 in hepatoma cells, this showed that ROS was necessary for 5-HT-induced autophagy in hepatoma cells. Therefore, we demonstrated that 5-HT induced autophagy in hepatoma cells, which was mediated by respective AMPK/ULK1 and JNK/p38/ERK pathways not by inhibiting phosphorylation of the mTOR pathway, and ROS participated in the occurrence of 5-HT-induced autophagy. This provided a theoretical basis for the important effects of 5-HT in the treatment or prevention of cancer, and provided new ideas for the development of new anti-cancer drugs targeting 5-HT receptors.

Introduction

Autophagy is a highly conservative process, which ensure high intracellular quality through which cytoplasmic components including long-lived proteins, protein aggregates, organelles, and invading pathogens could be degraded and recycled [1]. When the intracellular or extracellular environment changes (such as starvation), the level of autophagy will increase. Since the discovery of autophagy in the 1950s, significant progress has been made in this area. Three types of autophagy including macroautophagy, microautophagy and chaperone-mediated autophagy have been clearly revealed according to their physiological function and the way the substrate transported [2]. In most cases, autophagy is a pathway that promotes survival, particularly in response to growth factors, hormones or cellular stress such as nutrient deprivation [3]. Autophagy involves in many physiological processes including embryonic development, cell remodeling and differentiation, and it protects against aging, tumors and invasive pathogens [4]. However, in some cases, autophagy may cause cell death [5]. Defective autophagy involves the pathogenesis of many diseases such as cardiovascular, autoimmune diseases, neurodegenerative diseases, muscular dystrophies, cancer and aging [6–9]. Therefore, autophagy is of particular importance for maintaining cellular homeostasis, nutrient metabolism and cellular stress [10, 11].

In the case of cancer, extensive attention has been devoted to understanding the paradoxical roles of autophagy in tumor suppression and tumor promotion. There are two signaling pathways associated with autophagy play important roles in cancer cells, one is PI3P kinase/Akt/mTOR/P70S6K signaling pathway and the other is Ras/Raf-1/MEk1/2/ERK1/2 pathway. The Akt/mTOR pathway negatively regulated autophagy whereas the ERK1/2 pathway positively regulates autophagy [12, 13].

Serotonin (5-hydroxytryptamine, 5-HT), an anthraquinone derivative, could be found in fungi, animals and plants and most commonly in mammalian tissues [14]. 5-HT appears frequently in cerebral cortex and synapses platelets which mainly secreted by human peripheral blood. Under certain physiological and pathological conditions, 5-HT and 5-HT receptor (5-HTR) specifically bind and exert their biological effects. There are seven subtypes of 5-HTRs, divided into active receptors (HTR2A and HTR2B) and inhibitory receptors (HTR1) [15]. It been has well known that the relationship between 5-HT and human emotions, sleep, depression, appetite and sex. Previous studies have shown that 5-HT has been used as a tumor marker for gastrointestinal carcinoids and to some extent as a bronchial, liver cancer and ovarian carcinoid [16]. In addition, 5-HT could also be used as a specific tumor marker for islet cells and intestinal neuroendocrine tumors [17]. Serum 5-HT levels have been found to be useful for the evaluation of prognosis in urothelial, prostate and renal cell carcinoma [17]. The effect of 5-HT on prostate cancer is mediated through several receptor subtypes that exist at different stages of the tumor. 5-HT1A, 5-HT1B, 5-HT2B and 5-HT4 receptors have been identified in human prostate cancer cell lines such as PC3, DU145 and LNCaP. In addition, 5-HT is involved in its growth [18]. Invasive prostate cancer with high Gleason grade shows strong expression of 5-HT1A and 5-HT1B receptors compared to benign prostatic hyperplasia stroma tissue (BPH). Immunostaining of the 5-HT1A receptor was also confirmed in prostate cancer cells that metastasized to human lymph nodes and bones and in PC3 cells transplanted subcutaneously in athymic nude mice [19]. Moreover, 5-HT showed a stronger pro-invasive effect on glioma cell lines [20]. 5-HTR antagonists have been successfully used to inhibit cancer cell growth and the concept of 5-HTR-oriented drug therapy has emerged. One of the focuses of future research is to develop 5-HTR antagonists or selective 5-HT uptake inhibitors that could be used for tissue-targeted therapy. Especially, a recent report showed that attenuation of peripheral serotonin inhibits tumor growth and enhances immune checkpoint blockade therapy in murine tumor models [21]. However, the mechanism of the effect of 5-HT on cancer still needs further study.

The imbalance of redox reactions and the increase of oxidative stress are one of the important features of cancer cell metabolism [22]. It is an important factor in the development of cancer. An important source of oxidative stress is ROS, which promotes cancer cell proliferation, angiogenesis and metastasis, and helps cancer progression and invasion [23]. ROS-induced cancer cell proliferation has been found in breast cancer, lung cancer, liver cancer and many other cancer cells [24].

Based on the close relationship between autophagy, ROS, 5-HT and cancer, we used hepatoma cell HepG2 and Hep3B as model cells to investigate whether 5-HT could induce autophagy and related mechanisms. This will provide a scientific theoretical basis for the important effects of 5-HT in the treatment or prevention of cancer, and provide new ideas for the development of new anti-cancer drugs targeting 5-HT receptors.

Materials And Methods

Cell culture, antibodies and the plasmid

The HepG2 and Hep3B cells (ATCC, Manassas, VA, USA) were cultured in DMEM complete medium and MEM complete medium containing 10% fetal bovine serum and placed in a cell culture flask. Place in a cell incubator with a 5% CO_2 concentration. All cell culture reagents were purchased from Gibco Laboratories (Gibco, NY, USA). The LC3, GAPDH, p62, p-mTOR, p-P70S6K, p-4E-BP1, p-AMPKα, p-AMPKβ, p-ULK1, p-ERK, p-p38 and p-JNK antibodies used in the experiments were purchased from Cell Signaling Technology (Maryland, USA). The pEGFP-LC3 is mammalian expression of rat LC3 fused to EGFP. The ptfLC3 is mammalian expression of rat LC3 fused to mRFP and EGFP.

Treatment with 5-HT

5-HT was diluted with cell culture medium to concentrations from 0.25 μM to 32 mM and 0.5 h to 24 h. The cells were treated with DMSO served as blank control and positive control was used with the autophagy inducer rapamycin (5 μM) in advance for 12 h. Then the cells were treated with 5-HT with or without wortmannin (100 nM, 1 h), CQ (25 μM, 1 h) and NAC (1 μM, 1 h). The cells were seeded into 6-well flat-bottom plates (1×10^6 cells/well) or 24-well glass-bottom plates (2×10^5 cells/well) in serum-free and antibiotic-free DMEM or MEM medium.

Cell transfection

For the fluorescence microscope image collection, the cells needed to be transiently transfected with pEGFP-LC3 or ptfLC3plasmids. The HepG2 and Hep3B Cells were incubated in 6-well flat-bottom plates. The instructions to complete cell transfection were followed. Then, we replaced the medium with new serum-free DMEM or MEM. The cells transfected with plasmids were used in other experiments.

Immunofluorescence

Cells or transfected cells were seeded in 24-well glass-bottom plates. The images were obtained by fluorescencemicroscope (Olympus BX53). Image analyses and exports were performed using a Fluoviewver. 1.7.3.0 (Olympus, Tokyo, Japan).

Western blot analysis

The HepG2 and Hep3B cells were treated with 5-HT as described above and the total protein was collected by centrifugation and was quantified using the BCA reagent (Beyotim, P0012). The images were obtained by a CanoScan LiDE 100 scanner (Canon). Protein blots were measured using Image-J software.

ROS detection

Refer to Bi Yuntian's ROS Test Kit instructions to detect intracellular ROS. The specific steps are as follows: HepG2 and Hep3B cells were cultured in the logarithmic growth phase, counted in trypsin, and plated in 24-well cell culture plates at 2×10^4 cells/500 μL/well. After 12 h of culture in serum-free medium, they were replaced with phenol red-free medium containing 5 μM DHR 123 for 30 min. Change

to a phenol red-free medium containing 1 μM 5-HT for 2 h, aspirate the medium, wash each well three times with PBS, and then add 500 μL of PBS per well into a fluorescence microplate reader.

Statistical analysis

The group means were compared using a one-way ANOVA, and Student's *t*-test was used to determine the significance of differences. For *p* values,* $p< 0.05$, ** $p< 0.01$, or *** $p< 0.001$ compared with the control were considered statistically significant. The data are representative of triplicate experiments and are presented as the mean value ± the SD.

Results

5-HT induced autophagic response in hepatoma cells.

Expression of LC3-II in autophagy is considered to be an important sign of autophagy [25]. To determine whether 5-HT could induce autophagy, the HepG2 and Hep3B cells were transfected with GFP-LC3 plasmid. Following successful transfection, the HepG2 and Hep3B cells were treated with 5-HT at a concentration of 1 μM for 2 h. The rapamycin (an autophagy inducer) and wortmannin (an autophagy inhibitor) were also used to treat the transfected cells. The results showed that the expression of LC3 puncta were significantly increased in HepG2 and Hep3B cells when treatment with rapamycin or 5-HT, whereas, wortmannin (100 nM) could significantly inhibit rapamycin- or 5-HT-induced LC3 puncta (Fig. 1A and Fig. S1A). These date indicated that 5-HT might induce autophagy in hepatoma cells.

To further confirm the autophagy induced by 5-HT. The LC3 levels of 5-HT-treated hepatoma cells (HepG2 and Hep3B) were determined by western blotting. The HepG2 and Hep3B cells were treated with 1 μM 5-HT for 8 time points (0.5 h to 24 h) respectively. Rapamycin was used as a positive control. The results showed that the ratio of LC3-II/GAPDH was significantly increased in 5-TH-treated HepG2 and Hep3B cells in a time-dependent manner compared with that of untreated cells, and the expression of LC3 reached its peak in 2 h and 4 h in HepG2 and Hep3B cells, respectively (Fig. 1B). Then, the HepG2 and Hep3B cells were treated with 5-HT at a concentration gradient of 0.25 to 32 μM for 2 h or 4 h, respectively. We found that the ratio of LC3-II/GAPDH were increased in a dose-dependent manner and the expressions of LC3-II reached their peak in 1 μM and 8 μM in HepG2 and Hep3B cells, respectively (Fig. 1C). In summary, these data confirmed that 5-HT could induce autophagy in hepatoma cells.

5-HT induced a complete autophagic process in hepatoma cells.

The polyubiquitin-binding protein p62/SQSTM1 is a marker of complete autophagy, it was selectively incorporated into autophagosomes through indirect binding to LC3 and is efficiently degraded by autophagy [26]. So, we explored if 5-HT could induce degradation of p62 in hepatoma cells. After treatment of HepG2 and Hep3B cells with 5-HT for 0.5 to 24 h, the expression of p62 protein was detected by western blot. The results showed that the p62 protein was significantly degraded in 2 h and 4 h after treatment with 5-HT in HepG2 cells and in Hep3B cells, respectively (Fig. 2A and Fig. S1B). Then western

blot were used to detect the effect of 5-HT concentration (0.25 to 32 μM) on the p62 protein in HepG2 and Hep3B cells for 2 h and 4 h, respectively. The results showed that the degradation of p62 reached its peak when cells were treated with 5-HT at 32 μM in HepG2 cells and 8 μM in Hep3B cells (Fig. 2B and Fig. S1B).

To future examine the occur of complete autophagic, HepG2 and Hep3B cells were transfected with RFP-GFP-LC3 plasmid, and then the HepG2 and Hep3B cells were treated with 5-HT alone or pretreated with CQ and then treated with 5-HT, the number of RFP-GFP-LC3 puncta in HepG2 and Hep3B cells was observed under fluorescence microscope. Chloroquine (CQ), an agent that impairs lysosomal acidification, which could block autophagy by block LC-II turnover, thus CQ not only increases the number of GFP-LC3 puncta, but also allows LC3-II to accumulate in cells. Our results showed that the 5-HT-treated groups contained more green and red LC3 puncta than the control groups. Interestingly, the number of red puncta was significantly increased than the green puncta (Fig. 2C and Fig. S1E), however, the number of GFP-LC3 puncta in CQ pretreated groups was significantly increased than 5-HT alone group (Fig. 2C and Fig. S1E), this data indicating that some green puncta had vanished during the autophagic process and suggesting that 5-HT induces a complete process.

We also measured the accumulation of the LC3-II used western blot. Our results showed that LC3-II accumulated upon CQ treatment (Fig. 2D and Fig. S1C). Most importantly, the level of LC3-II was obviously higher after treatment with the combination of 5-HT and CQ compared with that of 5-HT treatment alone (Fig. 2D and Fig. S1C), demonstrating that autophagy induced by 5-HT included the process of the fusion of autophagosomes with lysosomes. Taken together, these data suggested that 5-HT induced a complete autophagic process in HepG2 and Hep3B cells.

5-HT activated autophagy through the AMPK/ULK1 pathway instead of the mTOR-dependent pathway in hepatoma cells

Previous studies reported that many autophagy occurs by inhibiting the phosphorylation of mTOR, such as rapamycin [27]. To verify whether the autophagy induced by 5-HT in HepG2 and Hep3B cells was involved in mTOR pathway, the HepG2 and Hep3B cells were treated with 5-HT for 0.5 to 24 h, respectively, and the phosphorylation of mTOR pathway proteins were detected by western blot. The results showed that 5-HT did not inhibit the phosphorylation of mTOR and its related proteins (p70S6K and 4E-BP1) during the treatment period (Fig. 3A). Then HepG2 and Hep3B cells were treated with 5-HT in a concentration gradient from 0.25 μM to 32 μM for 2 h and 4 h, respectively. Western blot assays showed that the concentrations of 5-HT did not inhibit the expressions of mTOR and its associated proteins (Fig. 3B). Therefore, 5-HT activated autophagy through the mTOR-independent pathway in hepatoma cells.

ULK1 is an important protein in autophagy initiation process regulated by mTOR and AMPK [28,29,8] then we investigate whether 5-HT could induced the expression of ULK1 and AMPK. 5-HT were used to treat the HepG2 and Hep3B cells for 0.5 h to 24 h. Western blot results showed that the expressions of p-AMPK and ULK1 proteins first were increased and then decreased with elongated treated time in HepG2 and

Hep3B cells (Fig. 3A). Furthermore, the western blot showed that 5-HT unregulated the phosphorylation of AMPK and ULK1 in HepG2 and Hep3B cells treated with 5-HT at concentrations from 0.25 to 32 mM for 2 h or 4 h, respectively (Fig. 3B).

In order to further explore the relationship between ULK1 and AMPK pathway related proteins and autophagy, ULK1 inhibitor (SBI-0206965) and AMPK inhibitor (Compound C) were used to detect the change of autophagy marker protein LC3. Our results showed that compared with 5-HT treatment alone, pretreated with SBI-0206965 or Compound C significantly reduced LC3-II expression in hepatoma cells (Fig. 3C). Therefore, 5-HT induced autophagy through AMPK/ULK1 pathway activation.

5-HT stimulated autophagy through JNK/p38/ERK pathway activation

It has been confirmed that MAPK-related signal pathways including ERK, JNK and p38 play important roles in autophagy [30], and then we speculated whether 5-HT induced autophagy through ERK/JNK/p38 pathway. The HepG2 and Hep3B cells were treated with 5-HT at 8 time points (0.5 h to 24 h). The western blot results showed that 5-HT could induce the phosphorylation of JNK, ERK and p38 and presented a trend that increased at first and then fell with elongated treated time, and the proteins expression reached its peak in 2 h and 4 h (Fig. 3A). After treatment with 5-HT at different concentrations range from 0.25 to 32 μM, it was found that the levels of phosphorylation of proteins were increased in a dose-dependent manner, then decreased (Fig. 3B).

In order to explore the relationship among ERK, JNK and p38 in 5-HT-induced autophagy. We used PD98059 (ERK Inhibitors, 20 μM), SP600125 (JNK Inhibitors, 10 μM) and SB203580 (p38 inhibitor, 10 μM) to pretreat HepG2 and Hep3B cells for 1 h, and then added 5-HT to treat HepG2 and Hep3B cells for 2 h and 4 h, respectively. The results showed that PD98059 could inhibit the phosphorylation of ERK but not the phosphorylation of JNK and p38. Moreover, SB203580 could inhibit the phosphorylation of p38 and ERK but not the phosphorylation of JNK. Nevertheless, SP600125 could inhibit the phosphorylation of ERK, JNK and p38. Furthermore, the treatment of all of inhibitors significantly inhibited the expression levels of LC3-II (Fig. 3D). Thus, we concluded that 5-HT-induced autophagy in HepG2 and Hep3B cells is dependent on the JNK/p38/ERK pathway, ERK is downstream of JNK and p38, and the ERK phosphorylation was regulated by JNK and p38, the phosphorylation of p38 was regulated by JNK.

ROS participated in 5-HT-induced autophagy in hepatoma cells.

The HepG2 and Hep3B cells were treated with 5-HT for 0.5 h to 24 h, respectively. And the hepatoma cells treated by 16 μM PMA for 2 h was used as a positive control for ROS induction. Fluorescence microplate reader was used to detect fluorescence intensity. The results showed that the ROS were significantly increased in HepG2 and in Hep3B in 2 h and in 4 h after treated with 5-HT, respectively (Fig. 4A). Then, HepG2 and Hep3B cells were treated with 5-HT or rapamycin at concentrations of 0.25 to 32 μM for 2 h and 4 h, respectively. And ROS production were significantly increased in 5-HT-treated HepG2 and Hep3B cells compared with that of untreated cells in a dose-dependent manner, and the production of ROS reached its peak when HepG2 and Hep3B cells were treated with 5-HT for 1 μM and 8 μM, respectively

(Fig. 4B). In addition, The ROS scavenger NAC effectively decreased the production of 5-HT-induced ROS (Fig. 4C).

To further verify the relationship between ROS and autophagy, HepG2 and Hep3B cells were transfected with GFP-LC3 plasmid and treated with 5-HT or 5-HT pretreated with NAC. The GFP-LC3 puncta was observed under a fluorescencemicroscope. Compared with the 5-HT group, the GFP-LC3 puncta formation was significantly reduced in the NAC pretreated group (Fig. 4D and Fig. S1D). Further, western blot was used to assay the LC3 levels of 5-HT-treated hepatoma cells (HepG2 and Hep3B). The results showed that the level of protein LC3 was significantly increased in the 5-HT-treated group compared with that of the NAC-pretreated group and control group (Fig. 4E and Fig. S1D). All the data suggested that ROS was involved in 5-HT-induced autophagy of HepG2 and Hep3B cells.

Discussion

5-HT is a neurotransmitter in the central nervous system that affects human emotions, sleep, and appetite. In fact, the content of 5-HT in the human central nervous system accounts for only a small part of the body's total 5-HT content, and most of them are secreted by the intestinal metachorocytes. In the past few decades, apart from the extensive research on 5-HT as a neurotransmitter, there has been no breakthrough in the study of other effects of 5-HT *in vivo*. Studies have shown that 5-HT plays an important role in the occurrence and development of cancer, including bladder cancer, lung cancer, glioma, liver cancer and other malignancies [20]. However, the underlying mechanism by which 5-HT affects cancer remains unclear. Therefore, we conducted a preliminary study on the molecular mechanism of 5-HT treatment of hepatoma cells. Our results show that 5-HT could induce autophagy in hepatoma cells through the non-suppressive mTOR pathway.

Autophagy is a very complex physiological process. Only under the proper control of autophagy-related genes, autophagy signaling pathways and autophagy complexes could ensure the orderly progress of this process. Microtubule-associated protein 1 light chain 3, namely LC3/Atg8, is a very important autophagosome marker protein in autophagy. Therefore, the number of autophagosomes could be determined by observing LC3. Autophagy in the process of fusion of autophagosomes with lysosomes is called complete autophagy. Autophagy in the absence of this process is called incomplete autophagy. To determine the cause of upregulation of LC3-II protein and the type of 5-HT-induced autophagy, we added lysosomal inhibitor chloroquine (CQ) to 5-HT-induced HepG2 and Hep3B cells to inhibit autophagosome degradation. In this experiment, we first constructed a GFP-LC3 and RFP-GFP-LC3 fluorescent plasmid transient transfection HepG2 and Hep3B cells. The fluorescence microscope results showed that after the cells were treated with 1 μM 5-HT for 30 min, GFP-LC3 puncta were observed. After HepG2 and Hep3B cells were treated with 5-HT at 1 μM and 8 μM for 2 h and 4 h, respectively, significant GFP-LC3 puncta were observed. The results showed that 5-HT could induce an increase of autophagosome in hepatoma cells. Moreover, 5-HT-treated groups contained more green and red LC3 puncta than the control groups, and the number of red puncta was significantly increased than the number of green puncta. However, the number of GFP-LC3 puncta in CQ pretreated groups was significantly increased than 5-HT alone group,

this data indicating that some green puncta had vanished during the autophagic process and suggesting that 5-HT induced a complete process.

P62/SQSTMI is an ubiquitinated receptor in the cytoplasm that is capable of being degraded by autophagy and therefore could be used as a marker protein to reflect the intensity of autophagy [31]. The p62 protein has a structural region that directly interacts with LC3, and p62 could participate in autophagy and gradually degrade during autophagy. The process of autophagy is inhibited and the expression level of p62 protein is increased. Therefore, p62 is also a marker for judging the intensity of autophagy [26]. In this experiment, we treated HepG2 and Hep3B cells with 5-HT at a concentration of 1 μM and 8 μM for 2 h and 4 h, respectively, and the expression of p62 protein was significantly reduced. The results showed that 5-HT promoted the degradation of p62 protein in HepG2 and Hep3B cells.

The mTOR signaling pathway plays an important role in the regulation of autophagy and is the most thoroughly studied autophagy-signaling pathway. Many factors such as cellular hypoxia, Triazine drugs, and certain metals induce autophagy through the mTOR signaling pathway. ULK1 is a very important protein in the process of autophagy initiation, and it is also an autophagy initiator molecule. Under normal conditions, ULK1 is relatively stable in the cytoplasm. ULK1 is not only regulated by mTOR but also regulated by AMPK. Because the activity of mTOR is inhibited, the inhibitory effect on ULK1 is attenuated, and then the phosphorylation of ULK1 is activated to initiate autophagy. The regulation of ULK1 by AMPK is positive. When the cell nutrient is deficient, it will increase the activity of AMPK, and then activate the phosphorylation of ULK1 to initiate the occurrence of autophagy [32]. In this experiment, we used western blot technique to detect the phosphorylation levels of mTOR, 4E-BP1 and p70S6K, to demonstrate the important role in autophagy. The experimental results showed that different concentrations of 5-HT could not inhibit the phosphorylation of mTOR, 4E-BP1 and p70S6K at different time, indicating that 5-HT could not induce autophagy through the mTOR signaling pathway. Moreover, our results showed that the phosphorylation of ULK1 and AMPK in HepG2 and Hep3B cells could be induced with the increase of induction time showing a trend of first increase and then decrease. The experimental results showed that although 5-HT could not inhibit the activity of mTOR, it could activate the phosphorylation of AMPK/ULK1 and induce the occurrence of autophagy.

MAPK kinases are a class of serine/threonine protein kinases involved in autophagy, including ERK, JNK and p38. The role played by these three kinases varies with inducing factors and cell types. For example, autophagy in human colon cancer cells induced by amino acid starvation was achieved by inducing phosphorylation of ERK [33]. However, bromelain induces autophagy in cells by inhibiting phosphorylation of ERK [34]. In rat hepatocytes, the phosphorylation of p38 could be activated by amino acid transport to inhibit the occurrence of autophagy. However, the phosphorylation of p38 could also be activated by the aggregation of glial fibrillary acidic protein to induce autophagy [35]. Activation of JNK induces autophagy in sarcoma cells [36]. In mouse fibroblasts, autophagy was activated by phosphorylation of JNK [37]. In this experiment, the western blot results showed that 5-HT treatment with different concentrations could upregulate the phosphorylation of ERK, JNK, and p38 at different times, and showed a trend of increasing first and then decreasing. The results indicate that 5-HT could induced

autophagy by activating the phosphorylation of ERK, JNK, and p38 of the MAPK kinase family. The specific inhibitors corresponding to ERK, JNK and p38 were PD98059, SP600125 and SB203580, respectively. The inhibitor experimental results showed that PD98059 (ERK inhibitor) inhibited the phosphorylation of ERK and could not inhibit the phosphorylation of JNK and p38. SB203580 (p38 inhibitor) inhibits the phosphorylation of p38 and ERK and does not inhibit the phosphorylation of JNK.SP600125 (JNK inhibitor) inhibited phosphorylation of ERK, JNK, and p38.Therefore, in the autophagy process of HepG2 and Hep3B cells induced by 5-HT, JNK is the upstream regulator of p38 and ERK, and p38 is the upstream regulator of ERK.

ROS is an important source of oxidative stress, mainly including O2-, H2O2, 1O2, OH- and O3. Normal cellular metabolism produces ROS, and physiological processes such as cell proliferation, differentiation, and apoptosis involve ROS. ROS could activate cancer-related signaling pathways, promote cancer cell proliferation, angiogenesis, and metastasis [38]. Moreover, recent studies have shown that ROS could participate in the regulation of autophagy [39]. Studies have shown that the production of ROS in cancer cells was significantly increased compared with normal cells [39]. Some chemical drugs could induce apoptosis and autophagy by stimulating cells to produce intracellular ROS [40]. In this experiment, the results showed that the HepG2 and Hep3B cells intracellular ROS level was significantly increased, and the peaked at 2 h and 4 h, respectively. NAC is an antioxidant commonly used in experiments and is used to remove reactive oxygen species from cells. In this experiment, the results showed that compared with the 5-HT group, the intracellular ROS levels in HepG2 and Hep3B cells were significantly decreased after NAC treatment. We observed in fluorescence microscopy that NAC pretreatment significantly reduced GFP-LC3 spotting in HepG2 and Hep3B cells. The detection of NAC pretreatment significantly reduced the expression of autophagy marker protein LC3-II by western blot. These results indicate that 5-HT-induced autophagy in HepG2 and Hep3B cells could be prevented by NAC. These results indicate that ROS participates in 5-HT-induced autophagy in HepG2 and Hep3B cells.

Declarations

Competing interests The authorsdeclare that they have no conflict of interest.

Ethical statement The animal and cell experiments in this study were performed in accordance with the recommendations,and protocols approved by the Ethics Committee of Jilin University(No.SY202111019).

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Authors' contributions Chao Wang: Conceptualization, Methodology, Software;Chao Wang: Data curation, Writing- Original draft preparation;Yang Wang, Shulin Li、Xudong Tang: Visualization, Investigation;Shulin Li、Xudong Tang、Hong yue Xu , Jin yang Shi , Yunxiao Ma , Hong Zhou , Yanan An, Keshu Shen and Lu Yu: Supervision;Chao Wang, Yang Wang: Software, Validation;Xinhua Cui: Writing-Reviewing and Editing.

Consent for publication All authors and corresponding authors agree to publish the paper.

Availability of data and materials The data is reliable in this article.

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Figures

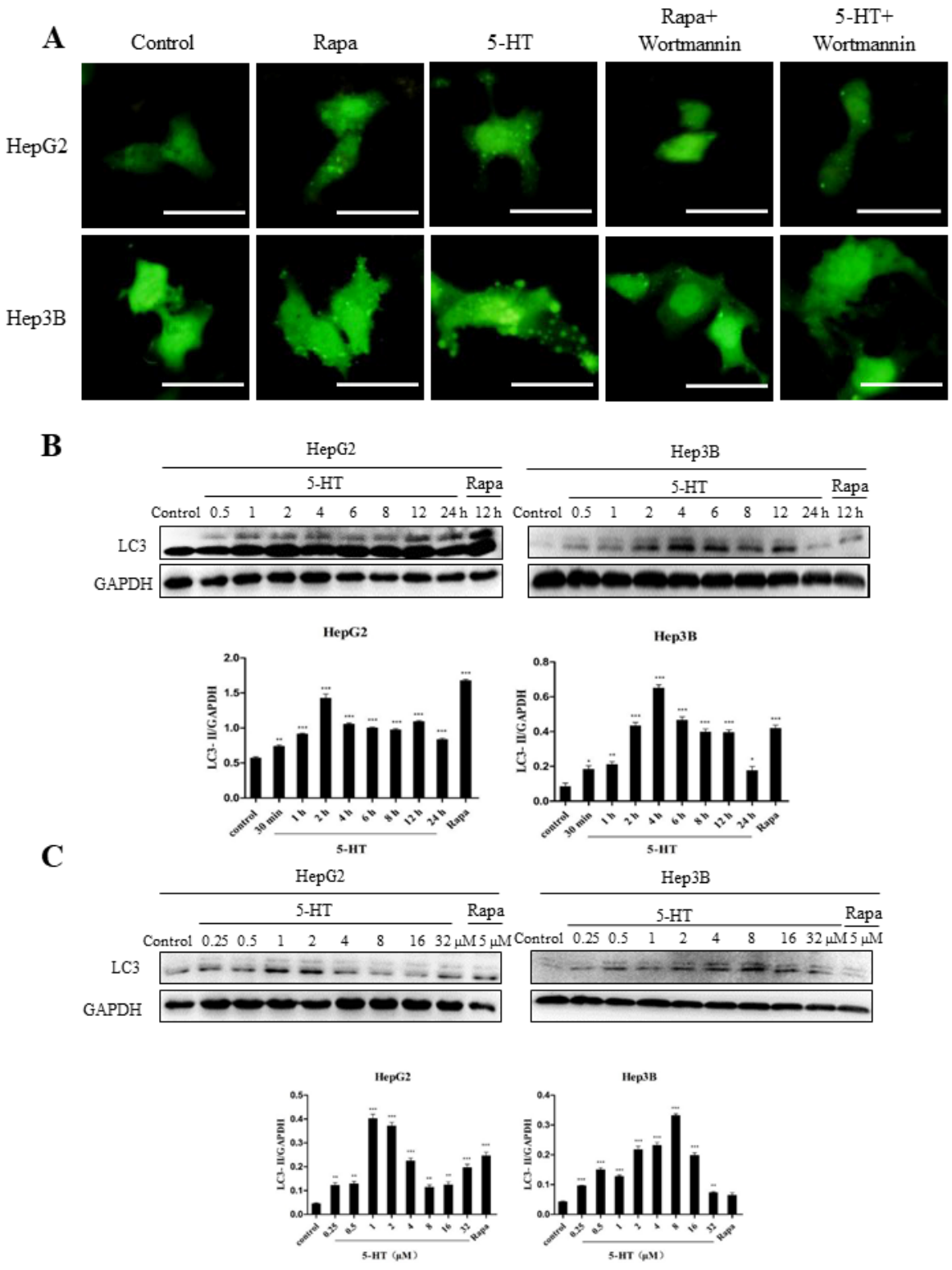

Figure 1

5-HT induced autophagic response in hepatoma cell. (A) The GFP-LC3 puncta detected by fluorescence microscopy of in Hep3B and HepG2 cells treated with 5-HT, rapamycin (rapa), 5-HT + wortmannin and rapa + wortmannin. (B) Western blot was used to detect the expression of LC3-I and LC3-II protein in HepG2 and Hep3B cells after 5-HT treatment for different times. (C) Western blot analysis of LC3-I and

LC3-II protein expression in HepG2 and Hep3B cells treated with different concentrations of 5-HT. Compared to respective controls, * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$.

Figure 2

5-HT induced a complete autophagic process in hepatoma cell. (A) Western blot was used to detect the expression of p62 protein in HepG2 and Hep3B cells after 5-HT treatment for different times. (B) Western blot was used to detect the expression of p62 protein in HepG2 and Hep3B cells treated with different concentrations of 5-HT. (C) The GFP-LC3 and RFP-LC3 detected by fluorescence microscopy of in HepG2 cells treated with 5-HT or 5-HT + CQ. (D) Western blot analysis of LC3-I and LC3-II protein expression in HepG2 and Hep3B cells treated with CQ, 5-HT and 5-HT + CQ.

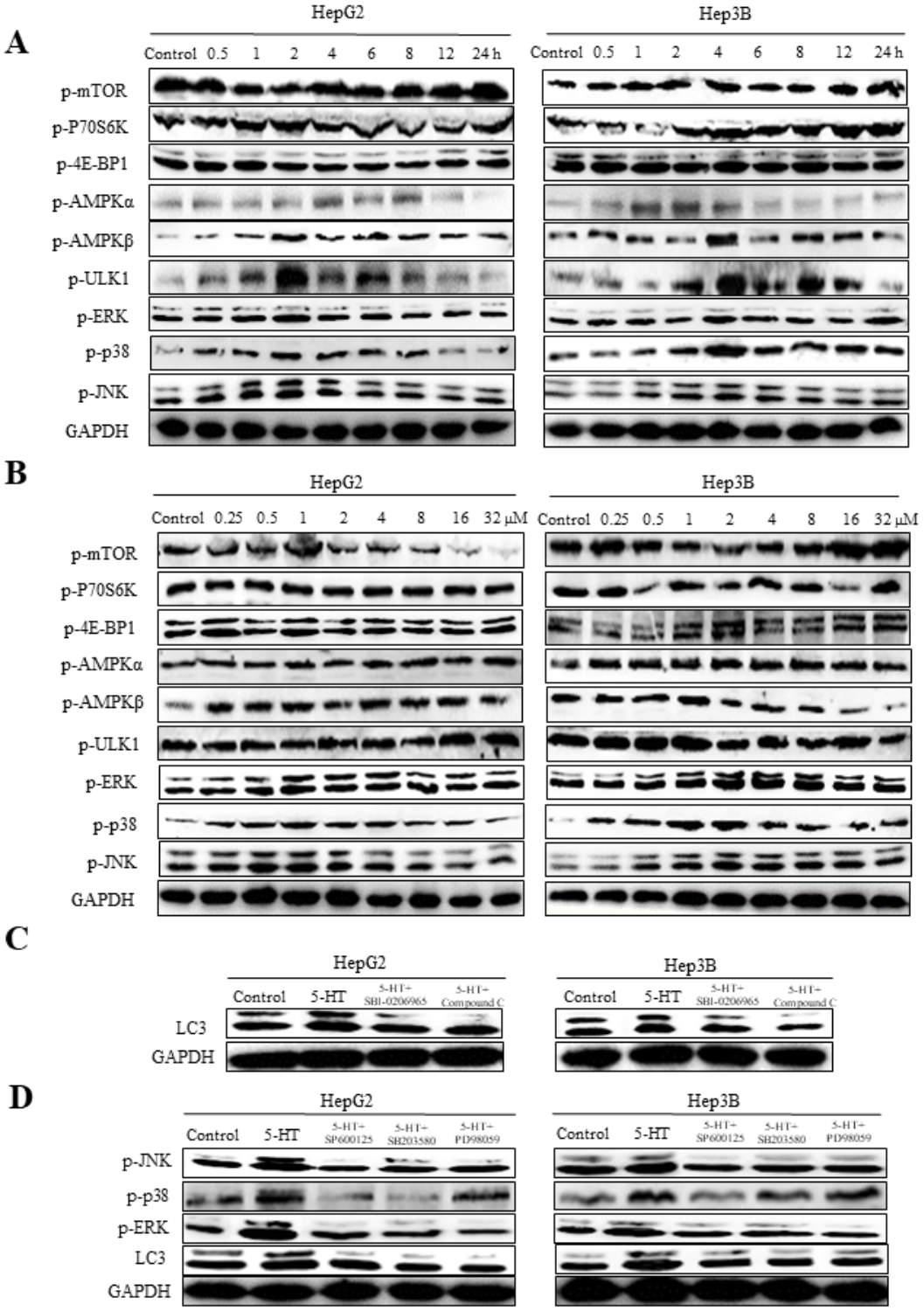

Figure 3

AMPK/ULK1 and JNK/p38/ERK play an important role in 5-HT-induced hepatoma cell autophagy. (A) Western blot was used to detect the phosphorylation levels of p-mTOR, p-P70S6K, p-4E-BP1, p-AMPKα, p-AMPKβ, p-ULK1, p-ERK, p-p38 and p-JNK in 5-HT-treated HepG2 and Hep3B cells with different times. (B) Western blot was used to detect the phosphorylation levels of p-mTOR, p-P70S6K, p-4E-BP1, p-AMPKα, p-AMPKβ, p-ULK1, p-ERK, p-p38 and p-JNK in 5-HT-treated HepG2 and Hep3B cells with different does. (C)

Western blot was used to detect the levels of LC3-II in 5-HT-treated HepG2 and Hep3B cells with ULK1 inhibitor SBI-0206965 (10 mM) or AMPK inhibitor Compound C (5 mM). (D) Western blot was used to detect the phosphorylation levels of ERK, JNK and p38 in HepG2 and Hep3B cells treated with ERK inhibitor PD98059 (20 μM), JNK inhibitor SP600125 (10 μM) and p38 inhibitor SB203580 (10 μM).

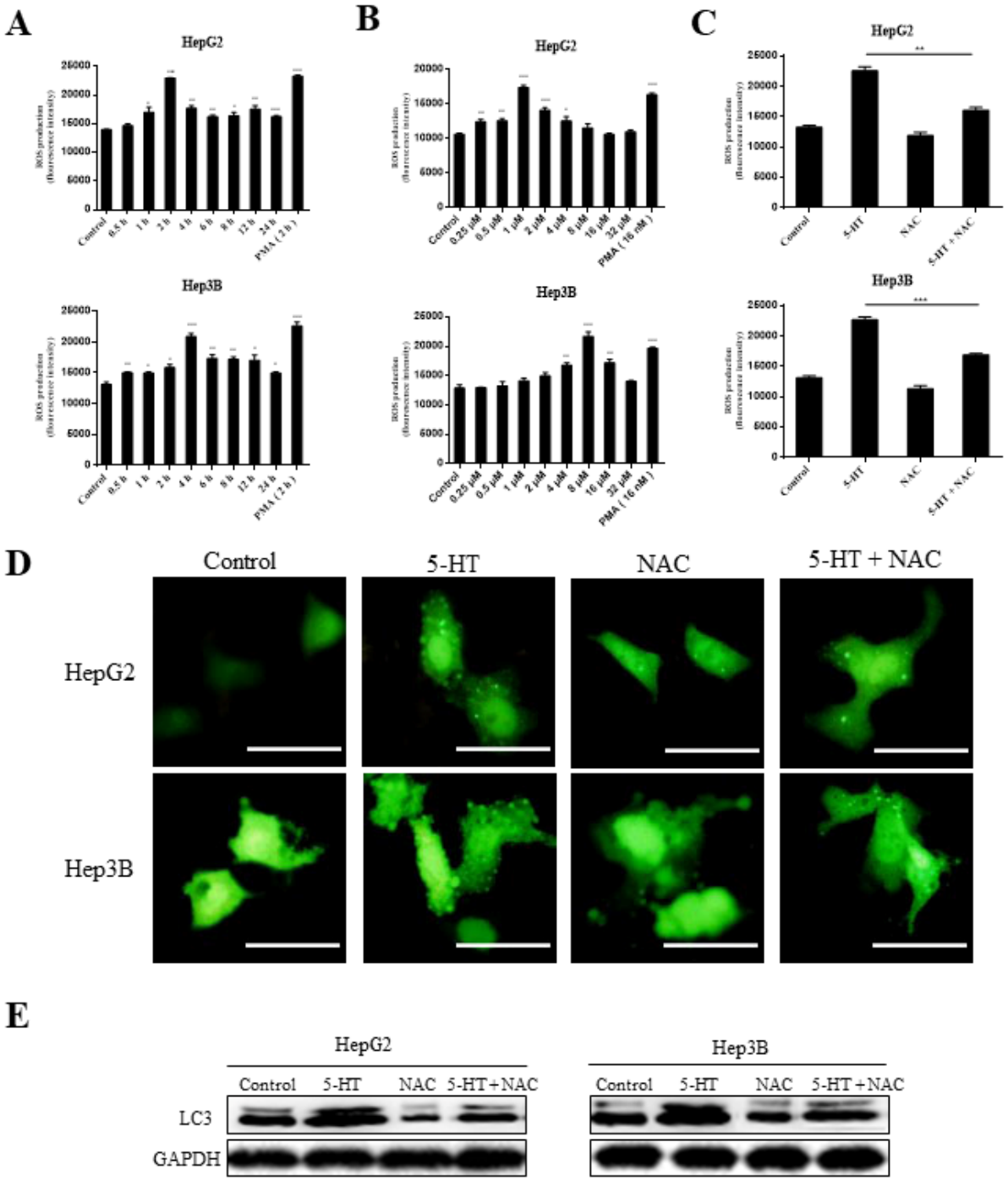

Figure 4

ROS participated in 5-HT-induced autophagy in hepatoma cell. (A) Intracellular of ROS levels in HepG2 and Hep3B cells after treated by 5-HT for different time. (B) Intracellular of ROS levels in 5-HT-treated HepG2 and Hep3B cells with different doses. (C) Intracellular ROS levels in HepG2 and Hep3B cells treated with 5-HT alone or in combination with NAC. (D) The GFP-LC3 puncta detected by fluorescence microscopy of in Hep3B and HepG2 cells treated with 5-HT alone or in combination with NAC. (E) LC3-II protein level detected by western blot in HepG2 and Hep3B cells treated with 5-HT alone or in combination with NAC. Compared to respective controls, * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$.

Supplementary Files

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- Supplementaryfigure1.png