SWCNH (Single walled carbon nanohorn) supervises ER (Endoplasmic reticulum) stress through triggering autophagy process of hepatocytes, especially in hepatoma cell line HepG2

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Abstract

Backgrounds. The cellular homeostasis is major maintained by the catabolic pathway of autophagy. Our previous work indicated that SWCNH were associated with endoplasmic reticulum (ER) stress mediated by calcium flow and autophagic response. But, its mechanism was unclear. Methods. The regulation of SWCNH on the calcium flow then autophagy of liver cells were investigated through inducing ER stress with tunicamycin and SWCNH. The calcium flow was determined using Fluo-3, then autophagy was examined with immunofluorescence or western blot for LC3, Beclin-1, ATG-5, and p62. Moreover, the apoptotic protein of Bax and Bcl-2 was detected, too. Results. Tunicamycin-induced ER stress in hepatocytes was related to calcium flow, especially for hepatoma cell line HepG2. Moreover, SWCNH participated in the regulation of endoplasmic reticulum stress-related calcium flow. Besides, SWCNH induced hepatocyte autophagy and inhibited cell apoptosis, then mediated the process of hepatocyte autophagy. Conclusions. Tunicamycin-induced ER stress in hepatocytes was related to calcium flow. Moreover, SWCNH induced hepatocyte autophagy, inhibited cell apoptosis, and participated in the autophagy regulation of hepatocyte, especially for hepatoma cell line.

Introduction

The cellular homeostasis is major maintained by the catabolic pathway of autophagy [1], and plays an important role in many pathologies, such as carcinoma, neurodegeneration, aging, etc [2, 3]. The formation of phagophore is the first step of autophagy [4], the membrane edges of phagophore prolong and then devour cytoplasm portions [5]. Consequently, the completed structure of autophagosome generates after membrane fusion [6]. Furthermore, the formation of autolysosome combined autophagosome with lysosome, then the content of this single membrane vesicle is degraded [7]. But, the recent biogenesis mechanism of autophagosome is unknown.

Whereas, the current studies have confirmed that the plasma membrane of different cellular compartments, including mitochondria, endoplasmic reticulum (ER), and Golgi may be associated with the precursors of autophagosome membrane [8]. Moreover, the autophagic response can be induced by ER stress [9], which is caused by activation of unfolded protein response come from misfolded proteins accumulation in ER [10]. Furthermore, the proteins expressions of recovery process and their chaperones occur [11], meanwhile, the structure of pre-antophagosome assembles after stimulation of ER stress [12].

Some investigators have explored the bio-effect role of single-walled carbon nanohorn (SWCNH) [13] in promising carrier for drug delivery systems based on its specific surface structure and large surface area, especially its affinity for biomolecules [14–16]. Moreover, we found the toxicity of SWCNH itself in hepatocytes,
but the mechanisms remained unclear. Furthermore, The calcium signals in liver carcinoma cells, both calcium entry and calcium liberation from internal pools could be regulated, and the mobilization of calcium appeared to critically contribute to the crucial intracellular pathway for hepatocellular carcinoma progression.

However, the death of cells could be regulated by two processes, included autophagy and apoptosis [17]. The complex interactive regulation of the two process between autophagy and apoptosis can be activated by a variety of regulatory molecules, stimuli of stress, and even calcium signals [18]. Therefore, the roles of SWCNH in ER stress and autophagy associated with intracellular calcium flow was investigated in hepatocytes.

Materials and methods

SWCNH characteristics

SWCNH were synthesized in air by arc discharge method and dried in air at 100 °C for 3 h. C, H, N analysis was performed on the Vario EL III elemental analyzer (The elementar Analysysysteme GmbH). The content of other elements was measured on an S4-Explorer x-ray fluorescence spectrometer (Bruck Company) with 1 kW power and wavelength dispersion mode. The surface area and mesopore size of SWCNH were measured on the ASAP2010 V3.02E surface area analyzer (Micromeritics Instrument Corporation, USA) by the B.E.T method. The particle density of SWCNH was measured on AccuPyc 1330 pycnometer (Micromeritics Instrument Company, USA) using high-pressure He buoyancy effect at 291.3K. The particle size of SWCNH in a 10 μg ml⁻¹ SWCNH aqueous suspension was determined by dynamic light scattering at 298.3K on Zetasizer Nano ZS (Malvern Instrument Ltd).

Preparation and characterization of SWCNH-coated dishes

To prepare the homogeneous SWCNH coating, a dilute solution of SWCNH in ultrapure water (produced with Milli-Q system, Millipro, USA) was dispersed. An aliquot (10 μg ml⁻¹) of the dispersed SWCNH was immediately spotted onto a 60 mm non-treated polystyrene dish (Normal PS), which has a low adhesive surface for suspension culture in order to reduce the influence of the base material layer. The dishes were dried at 60 °C in air for 3 h and sterilized by UV irradiation (DM-5; Daishin Co., Ltd, Osaka, Japan) for 16 h. The SWCNH-coated PS dishes (0.85 μg cm⁻²) with a bottom area of about 1 cm² were prepared for SEM measurements and contact angle determinations. Uncoated PS dishes were used as control. After pretreated by spraying gold on films of samples, SEM measurements were carried out on SIRION field emission scanning electronic microscope (FEI Corporation Ltd) with accelerating voltage of 10.0 kV. Contact angles of water droplets (volumes 2–5 μl) on SWCNH/PS and uncoated PS surfaces were determined on Dataphysics OCA20 Contact Angle Measuring System at 293K.

Cell culture

The immortalized human normal liver cell line L02 was obtained form Peking Union Medical College, and Homo sapiens hepatoma cell line HepG2 (ATCC® HB-8065TM), was purchased from ATCC (American Type Culture Collection). All cell lines were cultured using DMEM medium contained with FBS. The abbreviations (SW10, SW30, and SW50) correspond to the concentrations of SWCNH in each dish: 0.21, 0.64, and 0.96 μg cm⁻², respectively.

Western blot analysis

The proteins electrophoresis were conducted with SDS-PAGE gel. Moreover, blocked membrane transferred with proteins was dealt with skim milk and probed primary antibody. The primary antibodies were utilized in our work: β-actin (13E5) (Cell Signaling, Danvers, MA, USA; CST 4970S, dilution 1: 2000), anti-GRP78/BiP antibody (ab21685, 1:1000; Abcam, Cambridge, MA, USA); anti-CHOP antibody (L63F7, CST2895, 1:800; Cell Signaling, Danvers, MA, USA); anti-LC3 (M152-3; dilution 1:2,000; MBL, USA), anti-Beclin-1 (CST 3495; dilution 1:1,500; Cell Signaling, Danvers, MA, USA); anti-ATG-5 (CST 8540; dilution 1:1,200; Cell Signaling, Danvers, MA, USA); anti-p62 (D5E2, CST 8025: dilution 1:1,500; Cell Signaling, Danvers, MA, USA); anti-Bax (2D2, CST 89477: dilution 1:1,000; Cell Signaling, Danvers, MA, USA); anti-Bcl-2 (124, CST 15071: dilution 1:1,500; Cell Signaling, Danvers, MA, USA). Finally, the chemiluminescence examination was performed based on the secondary HRP antibody using exposed film.

Co-localization

The fixed cells with paraformaldehyde were immunostained using appropriated antibody after staining with 4′,6-diamidino-2-phenylindole. Furthermore, confocal images were obtained with Zeiss 510 META microscope.
Calcium flow

Cells were washed two times and suspended in buffer A (IMDM, which was containing 10 mM Hepes, pH 7.0) at a concentration of $1 \times 10^6 / \text{ml}$. Frua-2 AM and SNARF-1 AM (both from Molecular probes, Eugene, OR) were dissolved in DMSO at a concentration of 1 mM and were added directly to cells suspended in buffer A. Cells were incubated at 37°C for 30 min. Next, an equal volume of buffer B (IMDM, which was containing 10 mM Hepes, pH 7.4, and 5% FBS) was added and the cells were incubated for an additional 10 min at 37°C. Cells were washed two times and resuspended in buffer C (IMDM containing 10 mM Hepes, pH 7.2, 5% FBS, and 10 μg ml⁻¹ DNAse [Sigma]) at a final concentration of $1 \times 10^6 / \text{ml}$.

Cells were dealt with Frua-2 AM for record of Ca²⁺ ions based on the controller of temperature at 37°C utilizing the platform for life imaging service (Efringerstrasse 79, 4057 Basel, Switzerland). After washing with HBSS solution, the intensity and location of intracellular Fluo-3 fluorescence were recorded and monitored at 340–380 nm [19]. The concentration variation of free calcium in cells was represented with the ratio of F340/F380 fluorescence intensity, which was the key factor for the cytosolic changes of Ca²⁺ concentrations [20]. The image and fluorescence were obtained with an fluorescence microscope coupled to camera, then analyzed using Fluorescence Ratio Imaging Software (version 7.0). Total of 50–100 cells were measured individually in each experiment group, and which were repeated three times.

Besides, Fluo-3 AM was used to determine intracellular calcium flux with confocal microscopy.

Statistics

The data were showed as mean ± SD (standard deviation). The differences between the two groups were analyzed with the Student’s t-test. Kruskal-Wallis ANOVA was used to analyzed the abnormally distributed data among groups. SPSS software (version 18.0) was utilized to perform these statistical analyses. $P < 0.05$ was significant statistically.

Results

Characterization of SWCNH

Considering that cellular biological behavior could be affected by the physicochemical characteristics of the material, SWCNH. Its elemental composition, surface adsorptive isotherm, particle density, and particle size distribution were measured. The results showed that the material contained 95.3% C, and the content of each of the transition metals was less than 0.1%, e.g., Fe was 0.0863%, Cr 0.004%, Cu 0.0396% etc, total metal content was about 0.25%. Due to catalyst free preparation method of the material, its metal impurities should be from the graphite raw material. The result for adsorptive isotherm plot of SWCNH showed that B.E.T surface area was 631.55 m² g⁻¹. Single point total pore volume of pores (diameter less than 308.7 nm at P/Po 0.994) was 1.57 cm³ g⁻¹.

Figure 1. Particle sizes (diameter, nm) distribution of SWCNH in aqueous suspension (10 μg ml⁻¹). The result for adsorptive isotherm plot of SWCNH showed that B.E.T surface area was 631.55 m² g⁻¹. Single point total pore volume of pores (diameter less than 308.7 nm at P/Po 0.994) was 1.57 cm³ g⁻¹.
Characterization of SWCNH

The results of SEM images (figure 3) show that on the PS surface, SWCNH were individual spherical particles with diameters of 60–100 nm. After evaporation of water in aqueous SWCNH suspension, the structure of SWCNH aggregates were disintegrated into individual spherical aggregates of SWCNH on PS surface. It was probable that this effect could be explained by stronger π–π stacking interactions between the benzene ring on surface of PS and SWCNH than that between SWCNH aggregates.

Tunicamycin-induced endoplasmic reticulum (ER) stress in hepatocytes was related to calcium flow

We first used tunicamycin to induce endoplasmic reticulum (ER) stress in liver cells [20]. After tunicamycin treatment, L02 (figure 4(A)) and HepG2 (figure 4(B)) cell lines both evoked endoplasmic reticulum (ER) stress. The protein expression of ER stress marker GRP78 showed a dose-dependent increase with the the enhanced tunicamycin concentration, especially in HepG2.

Our experimental data indicated that CHOP [21–23] was a key protein of the ER stress signaling pathway, also increased in a dose-dependent manner with the enhanced tunicamycin concentration, and the expression was more significant in HepG2 (figures 4(A) and (B)).

In addition, the detection experiment of intracellular calcium flow found that with the increase of the tunicamycin concentration, the intracellular calcium flow of hepatocytes showed a dose-dependent increase, and it was more significant in HepG2 (figures 4(C) and (D)). The laser confocal microscope was further used to
detect the intracellular calcium current, and the ER localization protein calreticulin was used as an internal reference [24]. The results indicated that the calcium current in HepG2 was significantly stronger than that of L02 cells, and the calcium current was induced by tunicamycin in the two cell lines were both significantly increased, but the calcium flow of HepG2 was enhanced more significantly (figure 5).

Figure 4. Tunicamycin-induced ER stress in hepatocytes was related to calcium flow. After treatment with tunicamycin, L02 (A) and HepG2 (B) both evoked ER stress. The protein expression of ER stress marker GRP78 showed a dose-dependent increase with the enhanced tunicamycin concentration, especially in HepG2. Moreover, the expression of CHOP also increased in a dose-dependent manner follow with the enhanced tunicamycin concentration, and it was more significant in HepG2 (A) and (B). In addition, with the enhanced tunicamycin concentration, the intracellular calcium flow showed a dose-dependent increase, and it was more significant in HepG2 (C) and (D).
SWCNH participated in the regulation of ER stress-related calcium flow

Furthermore, it was aimed to clear whether SWCNH regulated the process of ER stress in L02 (figure 6(A)) and HepG2 (figure 6(B)). Through examining the expression levels of marker protein of ER stress GRP78, and its key signal transduction pathway protein CHOP [25], we found that the inhibitor of ER stress 4-PBA resulted in a decreased expression levels of GRP78 and CHOP in both the two cell lines, and it was more obviously in HepG2.
cell line. On the contrary, SWCNH (SW50) caused a significant increased expression levels of GRP78 and CHOP in both the two cell lines, and it was more significant in hepatoma cell line HepG2 (figures 6A and B).

Furthermore, through intracellular calcium flow detection experiments, it was found that the inhibitor of ER stress 4-PBA significantly suppressed intracellular calcium flow ($P < 0.01$), especially for the liver cancer cell line HepG2 (C) and (D). On the contrary, SWCNH resulted in a significant promotion of intracellular calcium flow ($P < 0.01$), especially in HepG2 (C) and (D).

**SWCNH induced autophagy and inhibited apoptosis of hepatocytes**

After treatment with SWCNH, L02 (figure 8(A)) and HepG2 (figure 8(B)) cell lines both evoked autophagy. However, the autophagy marker, LC3-II protein expression [26] increased in a dose-dependent manner with the increase of the SWCNH concentration, and it was more significant in HepG2 (figures 5C and D). At the same time, the expression of autophagy key regulatory proteins Beclin-1 [27], and ATG-5 [28] or autophagy protein p62 [29] all showed a dose-dependent decline with the increase of SWCNH concentration, and the expression of these marker proteins in hepatoma cell line HepG2 were more obviously decreased. On the contrary, the apoptosis-promoting gene Bax of the Bcl-2 family [30], its expression also decreased in a dose-dependent manner follow as the increased SWCNH concentration. In the mean time, the expression of the apoptosis-inhibiting protein Bcl-2 increased significantly, and it was more prominent in the hepatoma cell line HepG2 (figures 8(A) and B).

Further application of laser confocal microscopy to detect the autophagy status of cells, the results suggested that after treatment with SWCNH, autophagy appeared both in L02 and HepG2, and the autophagy status of...
HepG2 was more significant (figure 9). Moreover, SWCNH induced down-regulation expression of calreticulin in both the cell lines (figure 9).

**SWCNH participated in the regulation of hepatocyte autophagy**

Besides, with the purpose of confirming whether SWCNH regulated the autophagy process of L02 (figure 10(A)) and HepG2 (figure 10(B)), we examined the autophagy marker protein LC-3 and its subtypes LC-3I/LC-3II expression levels, and then found that the inhibitor of ER stress 4-PBA resulted in a decreased expression ratio of the autophagy marker protein LC3-II in both the two cell lines. Moreover, it was more prominent in hepatoma cell line HepG2. On the contrary, SWCNH made for a visible increased expression ratio of the
autophagy marker protein LC3-II in the two cell lines, but it was more significant in normal cell line L02 at this time (figures 10(A)–(D)).

Meanwhile, the inhibitor of ER stress 4-PBA resulted in decreased expression of the key regulatory protein complexes of autophagy Beclin-1 and ATG-5, or autophagy protein p62 in the two cell lines, and this was also more pronounced in HepG2. In addition, SWCNH inhibited the expression level of apoptosis-promoting protein Bax, and promoted the expression of apoptosis-inhibiting protein Bcl-2, besides this was more marked in HepG2 (figures 10(A) and (B)).

Further application of laser confocal microscopy was utilized to detect the autophagy status of cells, and the results suggested that the inhibitor of ER stress 4-PBA restrained the autophagy process of L02 and HepG2, and the autophagy status of HepG2 was more distinct (figure 11). However, SWCNH promoted the autophagy process of both the two cell lines (figure 11).

**Discussion**

The intracellular calcium signaling pathways are referred to the variety of biological processes in tumor cells, such as growth, apoptosis, differentiation, autophagy and metastasis [31]. Moreover, the in-depth and comprehensive researches involved in the interaction mechanism between apoptosis and autophagy must take breakthroughs on the treatment and cognition of cancers and other diseases [32].
Therefore, we firstly induced endoplasmic reticulum (ER) stress with tunicamycin in hepatocytes, then its relationship between calcium flow regulated by SWCNH was further studied. Our results identified that L02 and HepG2 both evoked endoplasmic reticulum (ER) stress after treatment with tunicamycin. Moreover, the marker of endoplasmic reticulum stress, GRP78 showed a dose-dependent increased expression with the enhanced concentration of tunicamycin, especially in the hepatoma cell line HepG2. Besides, the key protein of the ER stress signaling pathway, CHOP also increased in a dose-dependent manner with the enhanced tunicamycin concentration, and its expression was more significant in HepG2.

CHOP is a homologous protein of the transcription factor C/EBP, and can inhibit the transcription factor C/EBP and LAP [21]. Some cell stress, such as starvation, can induce the expression of CHOP, and CHOP can inhibit cell cycle transition from G1 to S phase [22]. Recent studies have found that the expression level of CHOP

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**Figure 9.** The confocal images indicated that SWCNH induced hepatocyte autophagy. The fluorescence intensity of LC-3 indicated that the degree of autophagy, then the higher brightness of LC-3 corresponded to the stronger autophagy, but the opposite images meant the reverse results. Moreover, the expression of calreticulin demonstrated its cellular location in ER. Further application of laser confocal microscopy was used to detect the autophagy status of cells, the results suggested that after treatment with SWCNH, autophagy appeared both in L02 and HepG2, and the autophagy status of HepG2 was more significant. (Scale bars is 0.25 μm).
is up-regulated under ER stress, CHOP can mediate programmed cell death or apoptosis and the activation of GADD34 during the process of ER stress [23]. The above studies are consistent with the results of our study.

Furthermore, the detection experiments of intracellular calcium flow were conducted, and it confirmed that with the increased tunicamycin concentration, the intrahepatic calcium flow showed a dose-dependent enhance, and it was more significant in hepatoma cell line HepG2. Moreover, our results indicated that the calcium current in HepG2 was significantly stronger than that in L02, and the calcium current was induced by tunicamycin in the two cell lines were both significantly increased, and the calcium flow of HepG2 was enhanced more significantly. In a word, we confirmed that tunicamycin-induced endoplasmic reticulum (ER) stress in hepatocytes was related to calcium flow. Some researchers confirmed that calcium signals was essential for maintaining homeostasis of liver cells. It sustained our results in this paper.

In recent years, we have explored the toxicity of nanomaterials in hepatocytes, and then found that SWCNH regulated the process of ER stress in human liver cell lines. Our results indicated that the inhibitor of ER stress 4-PBA resulted in a decreased expression levels of GRP78 and CHOP in the two cell lines, and the more obvious expression exhibited in HepG2. On the contrary, SWCNH caused a significant enhancement of GRP78 and CHOP expression levels in both the two cell lines, and it was more significant in HepG2. Additionally, through intracellular calcium flow detection experiments, we found the inhibitor of ER stress 4-PBA significantly suppressed intracellular calcium flow, but SWCNH resulted in a significant increase of intracellular calcium flow, especially for the liver cancer cell line HepG2. It validated that SWCNH participated in the regulation of ER stress-related calcium flow.

According to the different regulation methods of apoptosis and autophagy, the interaction can be roughly summarized into three types: cooperative relationship, confrontation relationship and promotion relationship [33–37]. The multiple interaction modes between autophagy and apoptosis must have common signaling pathways and regulatory proteins, which researchers call interaction regulators [38].

Our further work sustained that L02 and HepG2 both evoked autophagy after treatment with SWCNH, and it was more significant in HepG2. At the mean time, SWCNH inhibited apoptosis of hepatocyte, especially in HepG2. These process were all mediated by ER stress. So, we thought that SWCNH participated in the regulation of hepatocyte autophagy. The multiple interaction modes of apoptosis and autophagy in liver cells, especially
for hepatoma cells must have the common signaling pathway and regulatory protein involved in SWCNH, which maybe the key interaction regulator. However, Zhang et al had demonstrated that SWNH particles could penetrate into human liver cells with the different interacted mechanisms on human normal cell lines compared to hepatoma cell lines. They thought that single-walled carbon nanohorn (SWNH) aggregates inhibited proliferation of human liver cell lines and promoted apoptosis, especially for hepatoma cell lines [39]. But, their conclusions were not consistant with our work. Therefore, further research on the mechanisms and application in treatment of hepatocellular carcinoma with SWNHs is needed.

Conclusions

Tunicamycin-induced endoplasmic reticulum (ER) stress in hepatocytes was related to calcium flow. Moreover, SWCNH induced ER stress and inhibited cell apoptosis, then participated in the regulation of hepatocyte autophagy. SWCNH maybe the key interaction regulator of apoptosis and autophagy in liver cells, especially for hepatoma cells.
Acknowledgments

Not applicable.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).
Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Authors’ contributions
JLD, and TTW contributed to the conception of the study; JLD, YZ, and ZHX contributed significantly to performing these experiments; JLD, YZ, ZHX, JH, and TTW performed the data analyses and wrote the manuscript; JLD and TTW helped perform the analysis with constructive discussions.

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