Selenious- β -lactoglobulin induces the apoptosis of human lung cancer A549 cells via an intrinsic mitochondrial pathway

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Abstract In this study, the cytotoxic activity of selenious- β -lactoglobulin (Se- β -Lg) and the anti-cancer mechanism were investigated in human lung cancer A549 cells in vitro. MTT assay showed that Se- β -Lg at 200 μ g/mL exhibited a significant suppression effect on A549 cells and the maximum inhibition rate reached 90% after 72 h treatment. Flow cytometry analysis revealed that 200 μ g/mL of Se- β -Lg induced cell cycle arrest at G₀/G₁ phase. Cell apoptosis was induced via the generation of reactive oxygen species (ROS) and the decrease of mitochondrial membrane potential ($\Delta\Psi_m$) in a time-dependent manner. Furthermore, Se- β -Lg suppressed the expression of Bcl-2 and improved the level of Bax, leading to the release of cytochrome c and a higher expression of caspase-3 in A549 cells. In summary, Se- β -Lg could induce

apoptosis in A549 cells via an intrinsic mitochondrial pathway and it might serve as a potential therapeutic agent for human lung cancer.

Keywords Selenious- β -lactoglobulin (se- β -lg) · Antitumor activity · Mitochondrial pathway

Introduction

Lung cancer refers to the rampant and uncontrolled growth of tissues in the lung, A549 cells were categorized into non-small-cell lung cancer (NSCLC), which accounts for more than 80% of lung cancer (Tirino et al. 2013). At present, chemotherapy is the main therapeutical approach toward to NSCLC, however, it would generally cause drug resistance of cancer cells and toxic side effects on host (Yu et al. 2018b). Selenium is an essential mineral trace and naturally present in many foods. Selenium compounds, as potential therapeutic agents of cancer, have been researched in epidemiological, preclinical and clinical applications (Chen et al. 2017; Chen and Wong 2009). Selenium compounds could induce cancer cells apoptosis consisting of both organic and inorganic forms. In addition, selenoproteins were considered as effective compounds for tumor prevention with low toxicity, which could exhibit better effects compared with inorganic selenium (Abdulah et al. 2005). The antitumor activity of

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selenomethionine (Se-Met) has been proven to inhibit growth of human colon cancer cells which was associated with S-G2/M cell cycle arrest (Goulet et al. 2005). Moreover, Chen et al. demonstrated that selenocysteine (Sec) could induce human melanoma A375 cells apoptosis through activating ROS mediated mitochondrial pathway and P53 phosphorylation (Chen and Wong 2008).

Apoptosis, a process of programmed cell death, is vital in maintaining cellular homeostasis which can be triggered via death receptor-dependent (extrinsic) or mitochondrial (intrinsic) pathway (Xie et al. 2016). In cancer cells, the over expression of intracellular reactive oxygen species (ROS) can damage DNA and protein, leading to cell cycle arrest or apoptosis. Studies have demonstrated that the generation of ROS can dissipate the mitochondrial membrane potential ($\Delta\Psi_m$) and release cytochrome c into cytosol from mitochondria (Tao et al. 2018; Yu et al. 2018a). Finally, the caspase cascades are activated and induce cancer cells apoptosis. Selenium compounds could induce apoptotic cells death with the involvement of mitochondrial signaling pathway (Fernandes and Gandin 2015).

Recently, milk proteins have been widely used in the food industry due to their unique structures and high nutritional value. β -Lactoglobulin (β -Lg) is comprised of 162 amino acid residues, with one free cysteine residue and two disulphide bridges, accounting for about 58% of the whey protein in the milk (Cheng et al. 2017; Jones et al. 2010). Since β -Lg has the unique tridimensional structure, consisting of eight antiparallel β -strands that are folded into a hydrophobic β -barrel and an α -helix structure, it has received considerable attention in nutraceutical delivery strategies. Furthermore, β -Lg nanocapsules containing oxali-palladium and low methoxyl pectin can be utilized in antitumor drug delivery (Ghalandari et al. 2014). Jiang and Liu have proven that β -lactoglobulin-conjugated linoleic acid complex could also be used for colon cancer therapy (Jiang and Liu 2010). Hence, β -Lg was selected to combine with selenium dioxide as a potential antitumor drug against human lung cancer A549 cells.

β -Lg functional properties can be easily modified by heating and high pressure, the synthesis of selenium methionine resulted in harsh and unsafe operational conditions along with expensive production costs. To overcome these obstacles, L-arginine-selenic acid

(selenoarginine) was successfully produced in the vacuum and low temperature (40 °C) conditions by arginine and selenium dioxide and it was found that selenic acid could combine with arginine guanidine's primary amine (Liu et al. 2012). A novel organic selenium-enriched compound (Se- β -Lg) was chemically synthesized by β -lactoglobulin and SeO_2 under the safe and ambient conditions, and its structure characteristics and inhibition effect on K562 were investigated in our previous research (Zheng et al. 2016). However, the anti-tumor mechanisms of Se- β -Lg on A549 cells are unclear. In this study, the antitumor activity of Se- β -Lg on A549 cells and the underlying mechanisms were investigated, which will provide a theoretical basis for its clinical application.

Materials and methods

Materials

SeO_2 was purchased from Sigma (St. Louis, MO, USA) and β -Lg was separated in our lab. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma. Annexin V apoptosis detection kit was purchased from Keygen Biotech (Nanjing, Jiangsu Province, China). Reactive oxygen species (ROS) assay kit, Rhodamin 123 assay kit and Enhanced chemiluminescence (ECL) kit were purchased from Solarbio Science and Technology Co, Ltd (Beijing, China). RNA Extraction kit, Reverse Transcript kit and SYBR[®]Premix Ex Taq[™] II kit were purchased from Takara Bio Inc (Shiga, Japan). The antibodies against caspase-3, Bax, Bcl-2, cytochrome c and β -actin were obtained from Sungene Biotech (Tianjin, China).

Cell culture

Human lung cancer A549 cells and mouse embryonic fibroblasts NIH-3T3 cells were purchased from Chinese Academy of Medical Sciences (Beijing, China) and cultured in RPMI 1640 medium (Thermo Scientific Hyclone, Shanghai, China) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Thermo Scientific Hyclone), 100 U/mL streptomycin and penicillin (Gibco, Burlington, Canada) in a humidified 5% CO_2 at 37 °C.

Preparation of selenious- β -lactoglobulin

Selenious- β -lactoglobulin (Se- β -Lg) was synthesized by selenium dioxide and β -Lg, based on the method described previously (Liu et al. 2012; Zheng et al. 2016). Briefly, β -Lg was slowly added to the seleninic acid solution buffer and the mixture was reacted in a vacuum reactor with 6% H_2O_2 at 40 °C for 5 h. Finally, Se- β -Lg was performed after precipitation by ethanol and drying under the vacuum condition. After analyzed by Fourier Transform Infrared (FTIR), the results showed that Se- β -Lg had a characteristic peak at 878 cm^{-1} attributed to $\text{Se}=\text{O}$ compared with β -Lg. Hydride generation atomic fluorescence spectrometry showed that the content of selenium in Se- β -Lg was 75.85 mg/g (Zheng et al. 2016).

Cell proliferation assay

Inhibition effect of Se- β -Lg on A549 cells growth was analyzed by MTT assay as described previously (Jia et al. 2016; Song et al. 2013). Briefly, human lung cancer A549 cells were seeded in 96-well plates with 5×10^3 cells per well overnight. Then cells were exposed to various concentrations of Se- β -Lg for 24 h, 48 h and 72 h. After incubation with 20 μL 5 mg/mL MTT for another 4 h, DMSO was added to dissolve the purple formazan crystals. The absorbance was read at 570 nm using a microplate reader (Model 680; Bio-Rad, Hercules, CA, USA). The inhibitory effect of Se- β -Lg on NIH-3T3 cells proliferation was also measured in the same way. In addition, we evaluated the growth inhibition of SeO_2 and β -Lg on A549 cells by MTT assay under the same conditions. Each assay was conducted in triplicate. The inhibition ratio was calculated by the following formula.

$$\begin{aligned} &\text{The inhibition ratio (\%)} \\ &= (\text{Absorbance of untreated cells} \\ &\quad - \text{Absorbance of treated cells}) \\ &\quad / \text{Absorbance of untreated cells} \times 100. \end{aligned}$$

Annexin V-FITC/PI staining

The apoptosis ratio of A549 cells was investigated based on translocation of phosphatidylserine to the exterior of the membrane and detected by Annexin V-FITC/PI staining (Ma et al. 2014). Briefly, after

treatment with 200 $\mu\text{g/mL}$ Se- β -Lg for 24 h, 48 h and 72 h, the cells were harvested and stained by Annexin V-FITC and PI. Subsequently, the levels of apoptosis rate were analyzed by flow cytometry with Cell Quest software (BD Biosciences, San Jose, CA, USA).

Cell cycle detection

Cell cycle phase distribution was assessed by flow cytometry (Hua et al. 2015). In brief, after incubation with 200 $\mu\text{g/mL}$ Se- β -Lg for 24 h, 48 h and 72 h, the cells were harvested and fixed with ice-cold 70% ethanol and stored at 4 °C for overnight. Then the cells were stained with propidium iodide (excitation wavelength of 535 nm and emission wavelength of 615 nm) and RNase A for 30 min at 37 °C in the dark. Eventually, the DNA content and the percentages of cells in each phase of the cell cycle were evaluated by ModFit LT software.

Mitochondrial membrane potential disruption assay

Mitochondrial membrane potential ($\Delta\Psi_m$) disruption was estimated with the stain of Rhodamine 123 (Ma et al. 2014). Briefly, the cells were incubated with 200 $\mu\text{g/mL}$ Se- β -Lg for 24 h, 48 h and 72 h. Then, cells were harvested and incubated with 1 μM of Rhodamine 123 (excitation wavelength of 488 nm and emission wavelength of 515 nm) for 30 min at 37 °C in the dark. After centrifugation, the mitochondrial membrane potential was detected by flow cytometry with Cell Quest software.

Intracellular reactive oxygen species (ROS) assay

In order to determine the intracellular changes of ROS caused by Se- β -Lg, A549 cells were stained with fluorogenic probe 2',7'-dihydrofluorescein diacetate (DCFH-DA) (Ma et al. 2014). In brief, the cells were treated with 200 $\mu\text{g/mL}$ Se- β -Lg for 24 h, 48 h and 72 h. Subsequently, the cells were harvested and re-suspended in PBS containing 10 μM DCFH-DA for 20 min at 37 °C in the dark, and DCFH-DA (non-fluorescent with excitation wavelength of 485 nm) would be hydrolyzed and oxidized to form fluorescent DCF (dichlorofluorescein, excitation wavelength of 485 nm and emission wavelength of 525 nm). After washing twice with PBS, the ROS

levels were analyzed by flow cytometry with Cell Quest software (BD Biosciences).

Quantitative real-time PCR analysis

The expressions of apoptosis related genes were measured by Real-time PCR according to the method described by Zhang et al. (2013). After incubation with 200 µg/mL Se-β-Lg for different time (24 h, 48 h, 72 h), total RNA was isolated and transcribed into cDNA by the Reverse Transcript kit according to the manufacturer's protocol. Then quantitative Real-time PCR was implemented by the SYBR® Premix Ex Taq™ II strictly according to the manufacturer's instructions. The expression levels of different mRNA in Se-β-Lg-treated cells were compared with GAPDH, which was used as an endogenous control, according to the comparative cycle threshold (Ct). The results were calculated by the $2^{-\Delta\Delta C_t}$ method. The primers used for PCR were synthesized by Takara Bio (Beijing, China) and summarized in Table 1.

Western blotting analysis

To evaluate the expression changes of the apoptosis-related proteins in A549 cells induced by Se-β-Lg, western blotting assay was performed as previously described (Song et al. 2013). Briefly, after exposure to 200 µg/mL of Se-β-Lg for 24 h, 48 h and 72 h, cells were harvested and re-suspended in cell lysis buffer. After centrifugation, protein concentrations of lysates were determined by BCA protein assay kit. Then, the protein samples were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The membranes were blocked

with 5% BSA and incubated with primary antibodies (β-actin, Caspase-3, Bax, Bcl-2, cytochrome c) (Tianjin Sungene Biotech Co., Tianjin, China) at 4 °C for overnight. After incubation with horseradish peroxidase-conjugated secondary antibody (Tianjin Sungene Biotech Co., Tianjin, China), signals were visualized using enhanced chemiluminescence (ECL) detection kit and images were acquired with a ChemiDoc™ XRS + imaging System (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All experiments were repeated at least three times. Significant differences between groups were analyzed using SPSS version 17 software. The $p < 0.05$ was considered statistically significant and $p < 0.01$ was considered to be highly statistically significant. Data were expressed as mean ± SD.

Results

Inhibition effect of Se-β-Lg on A549 cells

The anti-proliferative effects of Se-β-Lg on A549 cells and NIH-3T3 cells were measured by MTT assay. The data (Fig. 1a) showed that Se-β-Lg (25 µg/mL or more) could significantly inhibit the proliferation of A549 cells in a dose-dependent manner from 24 to 72 h. After treatment with 200 µg/mL Se-β-Lg for 48 h, the inhibition rate was $81.76 \pm 2.56\%$, while the inhibitory ratio on A549 cells would not remarkably increase when treated with higher concentration or longer co-culture duration. Therefore, 200 µg/

Table 1 List of primers for Real time PCR

Gene name	Primer sequences (5'–3')	Orientation
GADPH	GGAGCGAGATCCCTCCAAAAT	Forward
	GGCTGTTGTCATACTTCTCATGG	Reverse
Caspase-3	TGGAGCGAATCAATGGACT	Forward
	AGGACTCAATTCTTGCCAC	Reverse
Bax	GCGAGTGTCTCAAGCGCATC	Forward
	CCAGTTGAAGTTGCCGTCAGAA	Reverse
Bcl-2	TCGCCCTGTGGATGACTGAG	Forward
	CAGAGTCTTCAGAGACAGCCAGGA	Reverse
Cytochrome c	GACTCTGGAATATCCCTGGACAACA	Forward
	CTGAGGTTTGCTGCATCGACA	Reverse

Fig. 1 **a** Se-β-Lg inhibited the growth of A549 cells by MTT assay. The cells were treated with 25–400 μg/mL of Se-β-Lg for 24 h, 48 h and 72 h. **b** Se-β-Lg induced growth inhibition of NIH-3T3 cells. **c** Effect of 20 μg/mL SeO₂ on A549 cells was

determined by MTT assay. **d** Inhibitory effect of β-Lg on the proliferation of A549 cells was studied. Results are expressed as mean ± SD of three independence experiments

mL was selected as the most suitable concentration for further experiments. In addition, Fig. 1b shows that Se-β-Lg had no growth inhibitory effect on NIH-3T3 cells, used as a normal cell model, which indicated that Se-β-Lg showed specificity inhibition effects on A549 cells with few side effects on normal cells.

Meanwhile, in order to compare the inhibition effect of selenium dioxide and β-Lg with Se-β-Lg, MTT was performed under the same conditions. Figure 1c showed that 20 μg/mL SeO₂ (selenium content equal to 200 μg/mL Se-β-Lg) also could decrease A549 cells viability in a time-dependent manner and the inhibition rate was $78.15 \pm 3.98\%$ after 48 h treatment, these results were relatively lower than those after treatment with 200 μg/mL of Se-β-Lg. Besides, it was observed that β-Lg could

barely affect the viability of A549 cells at any of the test concentrations (Fig. 1d).

Annexin V-FITC/PI staining results

To further evaluate the relationship between the inhibition effect and apoptosis of Se-β-Lg on A549 cells, apoptotic rate was measured by flow cytometric analysis after staining with Annexin V-FITC/PI. The proportion of normal cells in the control group was significantly higher than that of Se-β-Lg-treated cells across the entire treatment period. As depicted in Fig. 2, 200 μg/mL of Se-β-Lg could induce cell apoptosis in a time-dependent manner especially after 48 h treatment. The percentages of early apoptotic cells were distinctively increased from $3.88 \pm 0.17\%$ at 24 h,

Fig. 2 The apoptosis rate of Se-β-Lg on A549 cells. * $p < 0.05$ versus control, ** $p < 0.01$ versus control (mean $\pm$ SD, $n = 3$)

36.97 $\pm$ 1.89% at 48 h to 43.12 $\pm$ 2.10% at 72 h in the experimental groups than in the control group (0.55 $\pm$ 0.01%). The apoptotic rate was in accordance with the cell growth inhibition of Se-β-Lg at 24 h. The results demonstrated that Se-β-Lg would induce A549 cell apoptosis and externalization of phosphatidylserine was obvious in Se-β-Lg-treated cells.

Results of cell cycle detection

It was generally believed that apoptosis and cell cycle arrest are two main mechanisms in the inhibition of cell growth. Cell cycle arrest is a critical pathway impacting on the process of cell growth and proliferation (Xia et al. 2014). In order to elucidate apoptosis effects of Se-β-Lg on cell cycle arrest at a specific phase, DNA contents in A549 cells were analyzed by flow cytometry. Numerous anticancer agents could induce cells arrested at G0/G1, S or G2/M phase by abrogation the cell cycle checkpoints (Hua et al. 2015). As illustrated in Fig. 3a, b, cells treated with 200 μ g/mL of Se-β-Lg presented an accumulation in the G0/G1 phase with the increase from 60.76 $\pm$ 4.57% in control group to 64.12 $\pm$ 3.50%, 70.39 $\pm$ 3.49% and 71.58 $\pm$ 4.42% in the experimental groups at 24 h, 48 h and 72 h, respectively. Meanwhile, the percentages of S and G2/M phase were gradually decreased in Se-β-Lg-treated cells in a time-dependent manner, which indicated that the growth suppression effect of Se-β-Lg on A549 cells was associated with the cell cycle arrest at G0/G1 phase.

Effects of Se-β-Lg on mitochondrial membrane potential

The reduction of mitochondrial membrane potential ($\Delta\Psi_m$) acts as an important role in intrinsic mitochondrial pathway by regulating the opening of mitochondrial permeability transition pores (PTP). Therefore, in order to explore whether the apoptotic effects of Se-β-Lg were involved in the depletion of $\Delta\Psi_m$, the Rhodamine 123 staining was employed. As one of the early apoptogenic factors, the dissipation of $\Delta\Psi_m$ was considered as an irreversible pathway in the death cascade. The results (Fig. 4a, b) indicated that the difference of mitochondrial membrane potential between the control group (96.13 $\pm$ 2.49%) and Se-β-Lg-treated group at 24 h (92.09 $\pm$ 2.59%) was insignificant, but a remarkable disruption of $\Delta\Psi_m$ was determined at 48 h (21.33 $\pm$ 1.49%) and 72 h (11.03 $\pm$ 0.91%) in the experimental groups which was correlated with the previous results. These findings revealed that the disruption of $\Delta\Psi_m$ and the apoptosis effects of Se-β-Lg on A549 cells might be associated with the mitochondrial dysfunction. A similar apoptotic effect with selenocystine on A375 cells was also reported previously (Chen and Wong 2008).

Generation of intracellular ROS by Se-β-Lg

The excessive cellular ROS could oxidize membrane proteins of mitochondria and disrupt the mitochondria membrane potential, finally resulting in cells apoptosis (Cao et al. 2017; Ya 2017). In this study, the generation of intracellular ROS was determined by flow cytometry using staining with fluorescent probe DCFH-DA. The results demonstrated a time-dependent elevation of ROS generation in the right shift of the histogram (Fig. 5a). A significant accumulation of intracellular ROS in A549 cells after treatment with 200 μ g/mL Se-β-Lg was detected especially at 48 h (31.30 $\pm$ 2.31%) and 72 h (58.08 $\pm$ 3.91%) compared with the control group (3.01 $\pm$ 0.25%). The data showed that the increase of intracellular ROS was correlated with the apoptotic effects (Fig. 2) and the depletion of $\Delta\Psi_m$ (Fig. 4a) induced by Se-β-Lg in A549 cells. Our results were consistent with the evidences which suggested that the increased level of ROS can induce oxidative stress, damage DNA and protein in cancer cells (Pelicano et al. 2004).

Fig. 3 The effect of Se- β -Lg on cell cycle. **a** A549 cells (Control, 200 μ g/mL Se- β -Lg treated for 24 h, 48 h and 72 h) were analyzed by flow cytometry. **b** The distribution of cell cycle is shown by histograms. * $p < 0.05$ versus control, ** $p < 0.01$ versus control (mean $\pm$ SD, $n = 3$)

Effects of Se- β -Lg on the expression of the apoptosis-related genes

Intrinsic mitochondrial pathway plays a pivotal role in selenium compounds-induced apoptosis in numerous cancer cell lines (Xiang et al. 2009). In the present

study, the expression of apoptosis related genes was investigated by Real-time PCR and the results (Fig. 6a) showed a double expression level of Bax at 72 h treatment in comparison to the control group, whereas a moderately suppressed Bcl-2 was detected in Se- β -Lg-treated A549 cells compared with the

Fig. 4 Disruption of $\Delta\Psi_m$ in A549 cells caused by Se- β -Lg was detected by staining with Rhodamine 123. **a** The $\Delta\Psi_m$ of A549 cells caused by 200 $\mu\text{g}/\text{mL}$ Se- β -Lg was analyzed by flow cytometry. **b** The $\Delta\Psi_m$ level is shown by histograms. M1 represents cells with disruption of $\Delta\Psi_m$ and M2 represents normal cells. * $p < 0.05$ versus control, ** $p < 0.01$ versus control (mean $\pm$ SD, $n = 3$)

control group. The unbalanced expression of Bax and Bcl-2 in Se- β -Lg-treated cells was highly relevant with the disruption of $\Delta\Psi_m$, which was consistent with the selenocystine-induced apoptosis in MCF-7

human breast carcinoma cells (Chen and Wong 2009). As known, the released proapoptotic molecules from mitochondria into cytosol and activation of caspase-3 are the main steps in the mitochondria regulated

Fig. 5 Effect of Se- β -Lg on the generation of intracellular ROS in A549 cells. **a** The ROS of A549 cells caused by 200 μ g/mL Se- β -Lg was analyzed by flow cytometry. **b** The ROS level is shown by histograms. M1 represents normal cells and M2 represents cells with accumulation of intracellular ROS. * $p < 0.05$ versus control, ** $p < 0.01$ versus control (mean $\pm$ SD, $n = 3$)

apoptotic pathway. Thus, the expression of cytochrome c (cyt-c) and the activation of caspase-3 were also determined. As we expected, gradually increased expression levels of cytochrome c in cytosolic can be

determined by Real-time PCR, especially at 72 h treatment (Fig. 6). The results suggested that the damaged mitochondrial outer membrane induced by Se- β -Lg (Fig. 4) could release apoptogenic proteins

Fig. 6 Effect of Se-β-Lg on the expression of caspase-3, Bax, Bcl-2 and cytochrome c involved in the mitochondria pathway was determined by Real-time PCR (a) and western blotting (b). * $p < 0.05$ versus control, ** $p < 0.01$ versus control (mean $\pm$ SD, $n = 3$)

from mitochondria into the cytosol (Hua et al. 2015), and a notable up-regulated expression level of activated caspase-3 was also observed in a time-dependent manner after the 24 h treatment (Fig. 6a) which demonstrated that apoptotic cell death in A549 cells was caused by Se-β-Lg. Our data showed a similar mechanism in Se-methylselenocysteine-induced apoptosis in SKOV-3 ovarian cancer cells (Yeo et al. 2002). These results further suggested that the mitochondria play a central role in apoptotic cells death induced by Se-β-Lg on A549 cells.

Effects of Se-β-Lg on the expression of the apoptosis-related proteins

To further verify the intrinsic mitochondria pathway in the apoptotic pathway induced by Se-β-Lg, the expression of mitochondria-associated proteins was

determined by Western blotting. As shown in Fig. 6b, an up-regulation of Bax was markedly observed in Se-β-Lg-treated groups at 24 h treatment compared to the control group. On the contrary, a down-regulation of anti-apoptotic protein Bcl-2 was determined in a time-dependent manner in Se-β-Lg-treated A549 cells compared with control cells. Besides a significant accumulation in cytoplasm matrix cytochrome c can be detected after treatment with 200 $\mu\text{g/mL}$ Se-β-Lg for 24 h, 48 h and 72 h, which was consistent with the disruption of $\Delta\Psi_m$ (Fig. 4a) and accumulation of intracellular ROS (Fig. 5a). Subsequently, the activation of caspase-3 was observed gradually in a time-dependent manner in the Se-β-Lg-treated groups in accordance with the translocation of cytochrome c in cytoplasm. These results were in agreement with the study of exopolysaccharide, possessing antitumor effects on MCF-7 cells through an mitochondrial pathway (Wang et al. 2016). Our results demonstrated that the mechanism of cell apoptosis in A549 cells induced by Se-β-Lg was mainly correlated with the intrinsic mitochondrial pathway.

Discussion

Selenium is an essential element in human and low selenium concentration in human body can increase risk of cancer, poor immune function, cognitive and immune system dysfunction (Rayman 2012; Sun et al. 2017). β-Lg is the most abundant protein in whey protein containing 162 amino acid residues in each monomer. Moreover, β-Lg can be used as a transporter for hydrophobic and amphiphilic ligands such as retinol, vitamin D and fatty acids due to its unique tridimensional structure (Ghalandari et al. 2014). Thus, it has received considerable attention in nutraceutical delivery strategies. A new selenium-rich compound, Se-β-Lg, was synthesized based on the reaction of selenium and primary amine of arginine guanidine and lysine under vacuum and low temperature conditions. A high selenium content ($75.85 \times 10^3 \text{ mg/kg}$) and relevant molecular structure of Se-β-Lg has reported previously.

In the present study, we investigated the apoptotic death effects of Se-β-Lg on human non-small-cell lung cancer A549 cells in vitro. MTT assay showed that 200 $\mu\text{g/mL}$ Se-β-Lg could dramatically reduce the proliferation of A549 cells in a time-dependent

manner (Fig. 1a) with few cytotoxic effects on normal NIH-3T3 cells (Fig. 1b). During the early stage of apoptosis, translocation of phosphatidylserine (PS) from the interior of the plasma membrane to the outer leaflet has been recognized as an important characteristic of cell apoptosis (Mesner and Kaufmann 1997; Perumalsamy et al. 2017). Hence, the staining of Annexin-FITC/PI was employed and the results illustrated a significant increase in apoptotic rates of Se- β -Lg treated A549 cells (Fig. 2). Furthermore, cell cycle represents a series of tightly integrated events which consist of four typical phases: S phase and M phase are separated by gaps of G1 and G2 and many studies have shown that many antitumor agents could induce apoptosis by cell cycle arrest at a specific phase (Pan et al. 2005). At present, an accumulation at G0/G1 phase was determined in Se- β -Lg-treated groups (Fig. 3a), combined with the apoptotic rate (Fig. 2), these results confirmed that Se- β -Lg possessed significant inhibitory effects on A549 cells due to the apoptotic pathway.

ROS, a mediator of intracellular signal, is a kind of byproduct in the process of aerobic metabolism. Selenium compounds can result in cell cycle arrest or apoptosis via accumulation of intracellular ROS (Jiang et al. 2001; Sanmartín et al. 2012). In our study, $\Delta\Psi_m$ of A549 cells was disrupted and excessive cellular ROS was generated (Figs. 4, 5) after 48 h treatment with Se- β -Lg. Our results were consistent with the studies of other selenium compounds which could also induce apoptosis in many different cancer cell lines due to the excessive generation of ROS from aberrant mitochondria (Chen and Wong 2009; Zhu et al. 2017).

In the therapies of carcinoma, apoptosis may occur through cell death receptor triggered extrinsic pathway or mitochondria regulated intrinsic pathway. Mitochondrial integrity was mediated by Bcl-2 proteins family, including a group of Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic) proteins (Birkinshaw and Czabotar 2017; Kim et al. 2015). When cancer cells were stimulated by antitumor agents, the up-regulated expression of Bax and down-regulated expression of Bcl-2 could control mitochondrial outer membrane permeabilization. Subsequently, mitochondrial permeability transition pores (PTP) would open and cause cytochrome c and other proapoptotic molecules to be released from mitochondria into cytosol (Chan et al. 2015). In this study, we observed that Se- β -Lg could

deplete the $\Delta\Psi_m$ dramatically after 48 h treatment, which was highly associated with the up-regulated expression level of Bax and down-regulated expression level of Bcl-2 (Fig. 6). Further study indicated that a time-dependent increased expression of cytochrome c in the cytosol on Se- β -Lg-treated A549 cells was observed and consistent with the regulation of Bcl-2 proteins family. It has been reported that the released cytochrome c along with Apaf-1 and caspase-9 can activate downstream executioner caspase-3, which plays a pivotal role in the progress of cell apoptosis, finally resulting in cell apoptosis (McArthur and Kile 2018; Zhang et al. 2013). As described in Fig. 6, Real-time PCR and Western blotting assay both demonstrated a gradually increased expression of caspase-3, especially after 48 h treatment, corresponding to the increase in expression of Bax, decrease in expression of Bcl-2, disruption of $\Delta\Psi_m$ and the release of cytochrome c in Se- β -Lg-treated A549 cells in a time-dependent manner. Thus, our results suggested that the apoptotic effects of Se- β -Lg in A549 cells were mainly modulated by the mitochondrial pathway.

Conclusions

In this study, the cytotoxic activity of Se- β -Lg on human lung cancer A549 cells in vitro was investigated and the anticancer mechanism was illustrated. Our results indicated that the anti-proliferative effects of Se- β -Lg were dose-dependent in A549 cells. In addition, the results revealed that Se- β -Lg-induced apoptosis was associated with the cell cycle arrest at G0/G1 phase, the generation of ROS and disruption of $\Delta\Psi_m$. Further study showed that Se- β -Lg could up-regulate the expression level of Bax, down-regulate the expression level of Bcl-2 then leading to the release of cytochrome c and higher expression of caspase-3 in A549 cells which were associated with intrinsic mitochondrial pathway. These findings indicate that Se- β -Lg might be a potent anticancer agent in human lung cancer.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

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