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Effects of oleic acid on SP-B expression and release in A549 cells

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Abstract. – OBJECTIVE: Pulmonary surfactant-associated protein B (SP-B), which is synthesized and secreted by alveolar epithelial type II cells, is crucial for normal functioning of pulmonary surfactant. Degeneration of pulmonary surfactant is the essential cause of acute lung injury (ALI). ALI is often studied in animal models using oleic acid, and the effects of oleic acid on pulmonary surfactant and SP-B are not clear. In this study, we examined the effects of oleic acid on the A549 cell line which resembles the alveolar epithelial type II cells.

MATERIALS AND METHODS: A549 cells were exposed for 24 hours to 300, 400, 500 or 600 μM of oleic acid. Cell morphological changes were observed using an inverted microscope, and cell proliferation was quantified with the Cell Counting Kit-8. Extracellular SP-B levels were assessed by ELISA, whereas intracellular SP-B expression by Western blot.

RESULTS: Oleic acid caused dose-dependent changes in cell morphology of A549 cells and decreased their proliferation. This was accompanied by release of SP-B into extracellular supernatants and corresponding decrease of intracellular levels of this protein.

CONCLUSIONS: Oleic acid causes a dose-dependent injury to A549 cells, release of SP-B into extracellular compartment, and decrease of intracellular SP-B expression. Our findings provide mechanistic insights into animal modeling of ALI with oleic acid.

Key Words:
Acute lung injury, Surfactant protein B, Oleic acid, Intervention.

Introduction

Degeneration of pulmonary surfactant is the essential cause of acute lung injury (ALI)[1-3]. The function of pulmonary surfactant mainly depends on the pulmonary surfactant-associated protein B (SP-B), which is synthesized, packaged and secreted by alveolar epithelial type II cells[4-6]. This protein is crucial in decreasing the surface tension of alveoli, preventing the end-expiratory pulmonary alveoli collapse, promoting the recirculation of pulmonary surfactant, as well as for promoting maturity and secretion of SP-C[7-10].

ALI is often studied in animal models. The optimal animal model should be able to demonstrate the mechanism, occurrence and development of ALI[11,12]. The current animal models of ALI include the endotoxin induced injury model, hyperoxia induced injury model, mechanical ventilation induced injury model, and oleic acid induced injury model[13,14]. The latter model has been established in 1968[15] and has since then become the most commonly used animal model of ALI[16-19].

In mammals, oleic acid is the most abundant free fatty acid, comprising about 60% of the total free fatty acid content[20,21]. Oleic acid in ALI model directly affects capillary endothelial cells[22]. In fact there is hyperemia in lung tissue and extensive bleeding in pulmonary alveoli. Formation of microthrombus in pulmonary alveoli and epithelial cell necrosis cause direct injury to blood capillary endothelium by an unknown mechanism[23,24]. To explain the mechanisms of the oleic acid induced ALI, the oxidative stress, increased procoagulant activity, and endothelin mechanisms have been proposed[25,26]. There have been no studies on the effects of oleic acid on SP-B. In this study, we examined the effects of oleic acid using the A549 cell line exposed to different concentrations of this compound. This cell line has the same phenotype and characteristics as alveolar epithelial type II cells. A549 cells

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synthesize, transport and secrete SP-B *in vitro* and are, thus, the most commonly used cell type to study ALI in cell culture[27-29].

Materials and Methods

Materials

Foetal bovine serum, Dulbecco's Modified Eagle Medium (DMEM), dimethyl sulfoxide (DMSO), and oleic acid were from Sigma-Aldrich (St. Louis, MO, USA). Streptomycin and penicillin were provided by the Pharmacy of Daping Hospital, Third Military Medical University (Chongqing, China). Pancreatin was from Thermo Fisher Scientific (Waltham, MA, USA), SP-B ELISA kit and monoclonal antibody were, respectively, from Wuhan HuaMei Biological Engineering Co. (Wuhan, China) and (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The CCK-8 Cell Counting Kit was purchased from Dojindo Laboratories (Kyushu, Japan).

Cell Culture and Preparation of Oleic Acid Solution

The human pulmonary adenocarcinoma A549 cells represented the alveolar type II epithelial cells. The cells were provided by the Cancer Center of Daping Hospital, Third Military Medical University. A549 cells were cultured in DMEM containing 100 μg/ml streptomycin, 100 μg/ml penicillin, and 10% foetal bovine serum (FBS).

Oleic acid was prepared as follows. Oleic acid (0.49 ml) was mixed with 3.5 ml of absolute ethyl alcohol; the mixture was filtered 2 times in the dark and kept aliquoted at -20° C. For experiments, oleic acid stock was mixed with 0.15 M NaOH to prepare 0.2 M working.

In the experiments described below, A549 cells were exposed for 24 hours to serum-free DMEM (control cells) or 300, 400, 500 or 600 μM of oleic acid.

Cell Morphology and Proliferative Activity

For experiments, 200 μl of cell suspension (concentration of 4×10^4 cells/ml) were plated onto a 96-well culture plate. After 24 hours of cell growth, 200 μl of different concentrations of oleic acid were added to corresponding wells (all experimental conditions done in quintuplicate), and cells were cultured for further 24 hours. Cells were then observed under the microscope (X200 magnification). After that, 10 μl of CCK-8 solution was added, and cells were incubated for 2 hours. Then, optical densities were measured at 450 nm.

SP-B Detection by ELISA and Western Blot

For ELISA, cells (4×10^4/ml) were plated onto a 96-well culture plate and treated with oleic acid as above. ELISA was conducted per manufacturer's instructions to detect SP-B levels in cell supernatants.

For Western blot analysis, the cells were counted to prepare culture flasks with 2×10^6 cells per flask. Five flasks were prepared, and 4 ml of serum-free medium or different concentrations of oleic acid were added to corresponding flasks. After 24 hours, supernatants and cells were collected. Western Blot was used to detect the SP-B levels.

Statistical Analysis

The SPSS16.0 statistical package (SPSS Inc., Chicago, IL, USA) was used to analyze the data. Data were presented as mean ± SD and compared using a *t*-test for independent samples. A p value of < 0.05 was considered as statistically significant. The Origin software was used to prepare graphs.

Results

Cell Morphological Changes

Control A549 cells showed polygonal shape with abundant cytoplasm and evident protuberance (Figure 1A). The cells adhered well to a culture plate and fused together. There was no karyopyknosis. When the cells were treated with 300 μM of oleic acid (Figure 1B), A549 cells rounded up, their volume was slightly decreased, and there were granules of different sizes. These changes became more pronounced with higher concentrations of oleic acid. Thus, cells treated with 400 μM of oleic acid became substantially rounder than control cells; this was accompanied by a decrease in cell volumes, shortening of cell protuberance, and increase in number of different size granules (Figure 1C). When exposed to 500 μM of oleic acid, A549 cells started to break, and the volume was significantly decreased. The organelles were disintegrated and fragmented (Figure 1D). At 600 μM of oleic acid, cells

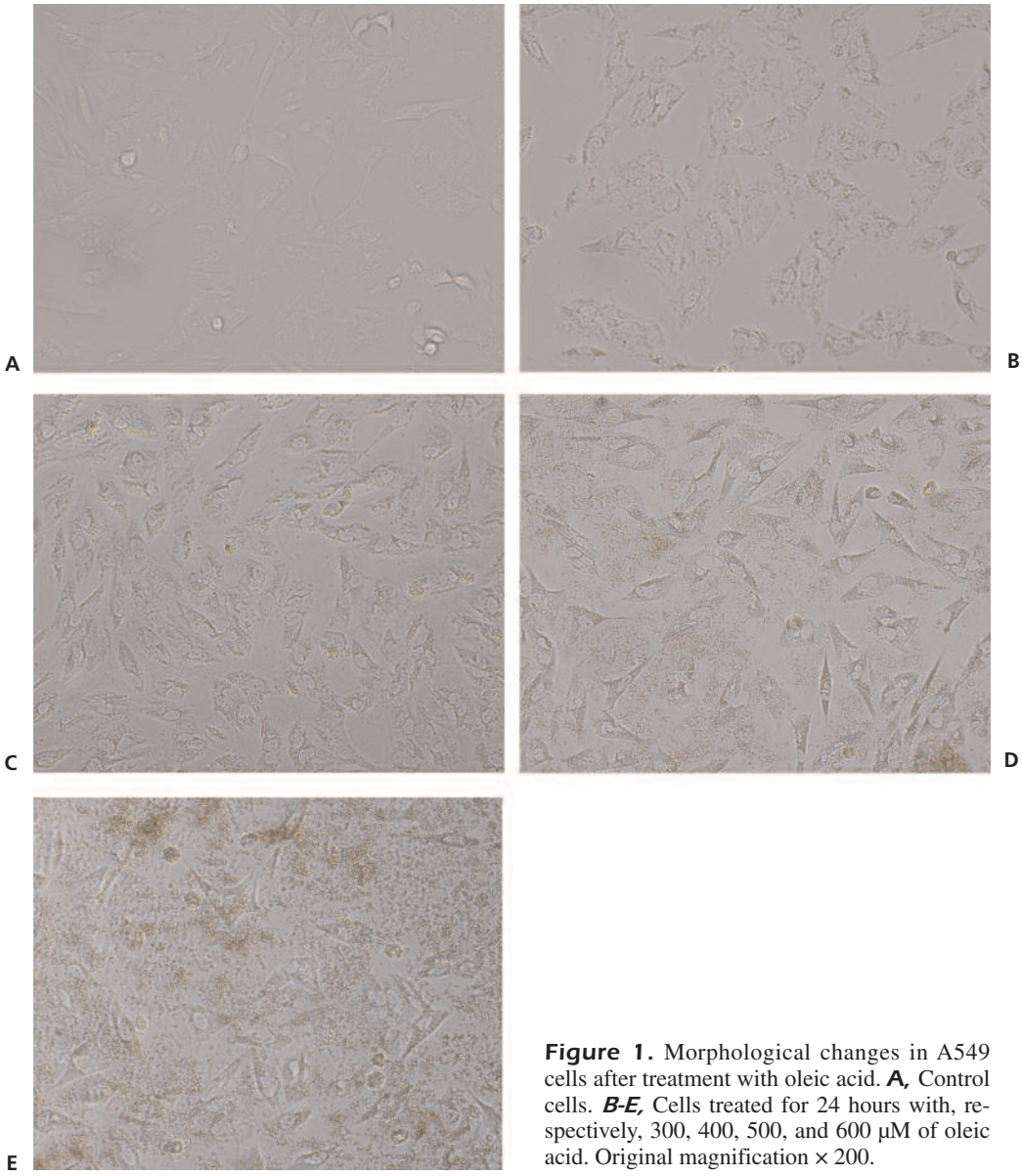

Figure 1. Morphological changes in A549 cells after treatment with oleic acid. ***A,*** Control cells. ***B-E,*** Cells treated for 24 hours with, respectively, 300, 400, 500, and 600 μM of oleic acid. Original magnification × 200.

were mostly broken, there was a shirking of the nucleus, and the organelles were visibly disintegrated and fragmented (Figure 1E).

Cell Proliferation

We next tested cell proliferation. Judging by the optical density, A549 cells showed a dose-dependent decrease in cell proliferation (Table I).

Expression of SP- in Supernatants Detected by ELISA

Subsequently, SP-B expression in cell supernatants was detected by ELISA. The extracellular levels of SP-B increased along with increas-

Table I. Optical density (OD) of cell proliferation after treatment with oleic acid.

Experimental conditions	OD
Control cells	2.92 ± 0.14
300 μM of oleic acid	$2.54 \pm 0.16^{*}$
400 μM of oleic acid	$2.21 \pm 0.14^{*\#}$
500 μM of oleic acid	$1.88 \pm 0.11^{*\#+}$
600 μM of oleic acid	$1.66 \pm 0.07^{*\#+\$}$

Data are mean ± SD of five experiments. $^{*}p < 0.05$ vs. control cells; $^{\#}p < 0.05$ vs. cells treated with 300 μM of oleic acid; $^{+}p < 0.05$ vs. cells treated with 400 μM of oleic acid; $^{\$}p < 0.05$ vs. cells treated with 500 μM of oleic acid.

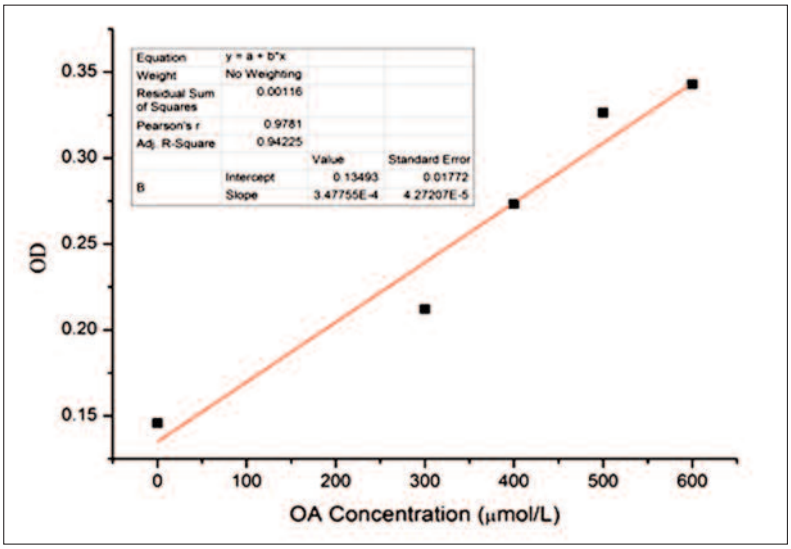

Figure 2. Change of optical density (OD) of SP-B after treatment with oleic acid.

ing concentrations of oleic acid (Table II and Figure 2).

Intracellular SP-B Expression Detected by Western Blot

Finally, we quantified intracellular expression of SP-B expressions. This concentration decreased dose-dependently after treatment with oleic acid (Figure 3). We also observed the presence of fragments of SP-B of lower molecular weight (around 30 kDa).

Table II. Change of SP-B optical density (OD) after treatment with oleic acid.

Experimental conditions	OD
Control cells	0.15 ± 0.02
300 μM oleic acid	0.21 ± 0.01 *
400 μM oleic acid	0.27 ± 0.01 * #
500 μM oleic acid	0.32 ± 0.01 * # &
600 μM oleic acid	0.34 ± 0.01 * # & ¶

Data are mean ± SD of five experiments. $^{*}p < 0.05$ vs. control cells; $^{\#}p < 0.05$ vs. cells treated with 300 μM of oleic acid; $^{+}p < 0.05$ vs. cells treated with 400 μM of oleic acid; $^{\S}p < 0.05$ vs. cells treated with 500 μM of oleic acid.

Discussion

In this study, we tested the effects of oleic acid on A549 cells. Oleic acid caused dose-dependent changes in cell morphology and decreased proliferation of A549 cells. This was accompanied by increased release of SP-B into extracellular supernatants and corresponding decrease of intracellular levels of this protein. It suggests that in ALI models, cell injury caused by oleic acid causes gradual release of intracellular SP-B into extracellular compartment.

SP-B is first expressed as a 42 kDa monomer pro-form which further undergoes post-translational modifications. After pyrolysis, mature SP-B is formed and preserved in the lamellar bodies. It can interact with phospholipid membrane to

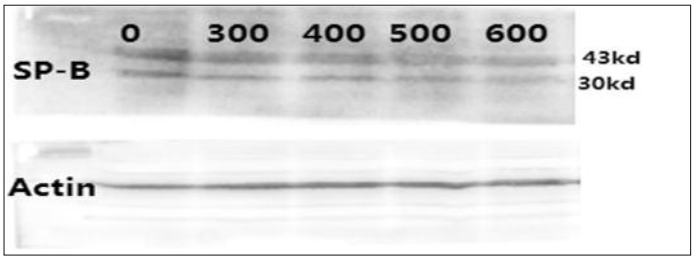

Figure 3. Changes in SP-B expression after treatment with different concentrations of oleic acid. SP-B expression was detected by Western blot. At the top, concentrations of oleic acid (μM) are shown. The 43 kDa bands represent pro-form of SP-B, and 30 kDa bands represent proteolytically cleave fragments of SP-B.

initiate the secretion, which requires proteolytic cleavage and formation of active SP-B comprising 79 amino acid residues with a molecular weight of about 18 kDa[30-32]. Using Western blot, we demonstrate that A549 cells treated with oleic acid exhibit decreased expression of the 43 kDa proform and presence of incomplete SP-B fragments with a molecular weight of 28-32 kDa.

Conclusions

Oleic acid causes a dose-dependent injury to A549 cells which is accompanied by release of SP-B into extracellular compartment and decrease of intracellular SP-B expression. Our findings provide mechanistic insights into animal modeling of ALI with oleic acid.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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