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● *Original Contribution*

SONODYNAMIC THERAPY MEDIATED BY EMODIN INDUCES THE OXIDATION OF MICROTUBULES TO FACILITATE THE SONODYNAMIC EFFECT

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Abstract—In previous studies, sonodynamic therapy mediated by emodin (emodin-SDT) induced cytoskeletal filament disruption and apoptosis of THP-1-derived macrophages. In this research, we investigated the underlying mechanism. THP-1-derived macrophages were incubated with emodin and exposed to ultrasound irradiation. After emodin-SDT, we measured the production of reactive oxygen species (ROS) and analyzed the level of amino acid oxidation in microtubules, the cleavage of microtubules and the mitochondrial membrane potential (MMP). We found that intracellular emodin accumulated mainly on microtubules. After emodin-SDT, generation of ROS was evident. Analysis of the carbonyl content of proteins suggested oxidation of microtubules. Microtubules were disrupted after emodin-SDT, and the antioxidant *N*-acetyl-L-cysteine prevented this disruption. MMP decreased after emodin-SDT, and this effect could be prevented by *N*-acetyl-L-cysteine. We conclude that emodin-SDT induces the generation of ROS. The oxidation of microtubules leads to its cleavage and the subsequent decline in MMP.
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Key Words: **Emodin, Sonodynamic therapy, Macrophages, Oxidation.**

INTRODUCTION

Sonodynamic therapy (SDT) is a promising new treatment for cancer with impressive effects in both *in vitro* and *in vivo* studies (Hu et al. 2016; Lv et al. 2017; Trendowski 2015). It also offers new therapeutic possibilities for the treatment of atherosclerosis. Our research group has been involved in this field for 9 y. We discovered that SDT can stabilize the vulnerable atherosclerotic plaque by inducing the apoptosis of macrophages in the plaque and attenuating the inflammatory activity (Jiang et al. 2017; Li et al. 2015a, 2015b; Tian et al. 2016).

In previous *in vitro* studies, macrophages exposed to ultrasound irradiation exhibited cytoskeletal filament disruption and subsequent apoptosis. Emodin, a compound found in a traditional Chinese herb, reinforced this effect of ultrasound irradiation, so it was suggested that emodin could act as a new sonosensitizer (Gao et al. 2011). However, the mechanisms involved in emodin-mediated SDT (emodin-SDT) remain unclear. Among cytoskeletal filaments, microtubules are known to possess several hydrophobic domains, and emodin is a hydrophobic molecule. We hypothesized that emodin could bind to the hydrophobic domains of microtubules and induce the events that follow exposure to ultrasound irradiation. The goal of this study was to explore the mechanisms that underlie cytoskeletal filament disruption and apoptosis of macrophages after emodin-SDT.

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Conflicts of interest: The authors have no conflicts of interest.

METHODS

Reagents

Emodin was provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The reagent was a commercial product of analytical grade with a purity >98% that was supplied at 20 mg per package. The product was dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C in the dark. Stock solutions were diluted 10- to 1000-fold for final use under experimental conditions. The final concentration of DMSO in the cells was 0.1%. RMPI medium (1640) and fetal bovine serum were obtained from HyClone Chemical Company (ThermoFisher Biotechnology, Beijing, China). Benzylpenicillin–streptomycin was obtained from Beyotime Biotechnology (Jiangsu, China). Phorbol-12-myristate-13-acetate (PMA) was obtained from Calbiochem, an affiliate of Merck KGaA, Darmstadt, Germany. Hoechst 33342 and DAPI (4',6-diamidino-2-

phenylindole) were purchased from Sigma Chemical Company (Santa Clara, CA, USA). Tubulin-Tracker Red, JC-1 and DAFH-DA were obtained from Beyotime Biotechnology (Shanghai, China). Alexa Fluor Hydrazide 488 was purchased from Life Technologies (New York, NY, USA).

Cell culture

THP-1 (human acute monocytic leukemia cell line) cells, obtained from American Type Culture Collection (Manassas, VA, USA) were used in the experiments. THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 50×10^{-3} g/L benzylpenicillin–streptomycin. Cells were maintained at 37 °C and 5% CO_2/95% air in a humidified incubator and were harvested for passage when they reached confluence. For the experiments, cells were seeded onto microculture plates (Costar, Corning, USA) containing RPMI-1640 medium plus PMA at a concentration of 10×10^{-3} g/L and incubated for 72 h. Then, the medium was removed and replaced with fresh medium without PMA.

Drug intracellular distribution

Cells were seeded onto 35-mm Petri dishes at a density of 1×10^4 cells/mL. Emodin was added at a final concentration of 15×10^{-3} g/L. After 2 h, the cells were washed twice with phosphate-buffered saline (PBS), and the emodin taken up by the cells was examined by means of a fluorescence microscope (IX71, Olympus, Japan) using an excitation filter with a wavelength of 420–480 nm and an emission filter with a wavelength of 480–550 nm. Adherent cells were then dissociated with trypsin to obtain a cell suspension, centrifuged, washed twice with PBS and analyzed with a fluorescence spectrophotometer (Ocean Optics, Dunedin, FL, USA). Next, cells were disrupted with cell lysis buffer, and their microtubules extracted at 0 °C. After centrifugation and two more washes with PBS, the microtubule fluorescence was also analyzed.

Ultrasound exposure system

The system is the same one used in previous work (Gao et al. 2011). The ultrasound exposure system is illustrated in Figure 1 (838 A-H-O-S ultrasonic device, Sheng Xiang Technology, Shenzhen, China). The ultrasound transducer, with a diameter of 38 mm, was submerged in a container filled with degassed water. The overall resonant frequency of the transducer was 0.86 MHz. The reading output power intensity from the amplifier was 2 W/cm^2. The target depth of the ultrasound was 10 cm below the transducer surface. The output acoustic pressure was measured in degassed water 10 cm from the transducer surface. The output intensity was 0.44 W/cm^2.

For all experiments, degassed water was used as the ultrasonic medium. The solution inside the Petri dishes was buffered from overheating by the water around the Petri dishes, and the temperature was set to room temperature (2 °C–25 °C). During the sonication procedure, the temperature of the solution inside the Petri dishes did not rise more than 0.1 °C, as measured with a thermometer.

Sonodynamic therapy

Cell viability. As mentioned earlier, cells (1×10^5 cells/mL) were seeded in 35-mm Petri dishes. Cells were incubated with emodin at a concentration of 15×10^{-3} g/L for 2 h in the dark. They were then exposed to pulse ultrasound for 5–15 min. Control Petri dishes were sham-exposed to ultrasound. After SDT, each Petri dish was incubated for 6 h before the MTT assay was performed. Mitochondrial function was expressed as a percentage of viable treated cells relative to untreated control cells (without ultrasound and drug). All experiments were repeated three times independently (Gao et al. 2011).

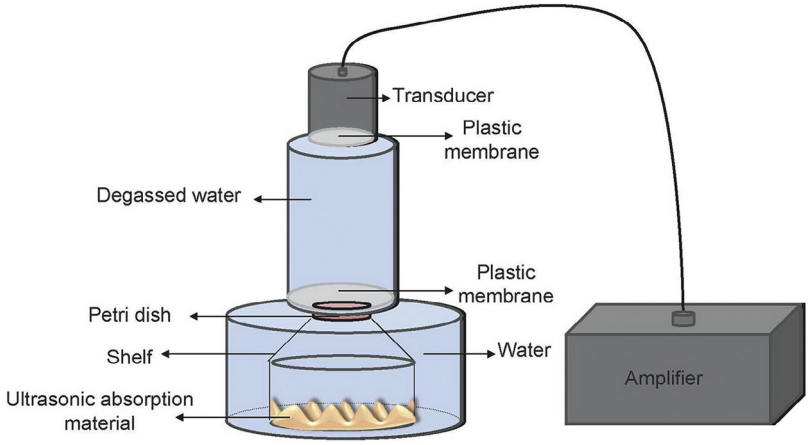

Fig. 1. Ultrasound exposure system.