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ROS-Ca^{2+} is associated with mitochondria permeability transition pore involved in surfactin-induced MCF-7 cells apoptosis

Xiao-hong Cao, Si-si Zhao, Dong-yue Liu, Zhuo Wang, Li-li Niu, Li-hua Hou, Chun-ling Wang*

Key Laboratory of Food Nutrition and Safety (Tianjin University of Science & Technology), Ministry of Education, China

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ABSTRACT

The surfactin can inhibit proliferation and induce apoptosis in cancer cells. Moreover, surfactin can induce cell death in human breast cancer MCF-7 cells through mitochondrial pathway. However, the molecular mechanism involved in this pathway remains to be elucidated. Here, the reactive oxygen species (ROS) and Ca^{2+} on mitochondria permeability transition pore (MPTP) activity, and MCF-7 cell apoptosis which induced by surfactin were investigated. It is found that surfactin evoked mitochondrial ROS generation, and the surfactin-induced cell death was prevented by N-acetylcysteine (NAC, an inhibitor of ROS). An increasing cytoplasmic Ca^{2+} concentration was detected in surfactin-induced MCF-7 apoptosis, which was inhibited by 1,2-bis (2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid (BAPTA-AM, a chelator of calcium). In addition, the relationship between ROS generation and the increase of cytoplasm Ca^{2+} was determined. The results showed that surfactin initially induced the ROS formation, leading to the MPTP opening accompanied with the collapse of mitochondrial membrane potential ($\Delta\Psi_m$). Then the cytoplasmic Ca^{2+} concentration increased in virtue of the changes of mitochondrial permeability, which was prevented by BAPTA-AM. Besides, cytochrome c (cyt c) was released from mitochondria to cytoplasm through the MPTP and activated caspase-9, eventually induced apoptosis. In summary, surfactin has notable anti-tumor effect on MCF-7 cells, however, there was no obvious cytotoxicity on normal cells.

1. Introduction

Breast cancer, a deadly disease, may be caused by many risk factors such as family history of breast cancer at a young age, early menarche, and late menopause [1]. Until now, surgery, radiotherapy [2], cytotoxic chemotherapy, endocrine therapy [3], biologic therapy, or combinations of these are used for the clinical treatment of breast cancer. New anti-cancer strategies are underway and play important roles in clinical trials for combating cancer in future [4]. Besides, C-phycocyanin [5], surfactin [6] and other biomolecules were reported to have significant anti-cancer effects.

Surfactin exhibits antibacterial, antiviral, antitumor and hemolytic action [6–10]. This anionic cyclic lipopeptide is constituted by a heptapeptide interlinked with a β-hydroxy fatty acid [11]. In our previous studies surfactin purified from Bacillus subtilis natto TK-1, exhibits an anti-breast cancer function, which can induce cell death in human breast cancer MCF-7 cells through mitochondrial pathway [12–14]. However, the mechanisms involved in this pathway remain to be elucidated.

In recent years, the role of MPTP in cell apoptosis has received considerable attention. The mitochondrial membrane permeability transition, a critical process in cell apoptosis [15,16], occurs after the opening of a channel, so called the MPTP. MPTP consists of VDAC, ANT, CyP-D and other molecules. VDAC, the core components of MPTP, is modulated by many factors, such as NADH (nicotinamide adenine dinucleotide), Ca^{2+}, ATP level, glutamate and hexokinase [17–20]. ANT locates in the inner mitochondrial membrane, which is a specific ATP/ADP transporter [21,22]. CyP-D, a mitochondrial peptidyl-prolyl cis–trans isomerase, is directly associated with ANT in the matrix of mitochondria, which modulated the MPTP affinity for Ca^{2+}, and it is the target of the desensitizing effects of CsA on the MPTP [16].

Oxidative stress, overloading of mitochondrial Ca^{2+} ($[Ca^{2+}]_m$), and low ATP concentration have been considered to induce the

Abbreviations: MPTP, mitochondria permeability transition pore; VDAC, voltage-dependent anion channel; ANT, adenine nucleotide transporter; CyP-D, cyclophilin D; ATP, adenosine triphosphate; ADP, adenosine diphosphate; CsA, cyclosporin A; $\Delta\Psi_m$, mitochondrial membrane potential; cyt c, cytochrome c; ROS, reactive oxygen species; NAC, N-acetylcysteine; BAPTA-AM, 1,2-bis (2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid; DCFH-DA, 2′ 7′-dichlorofluorescein diacetate; calcein-AM, calcein acetoxymethyl ester; PBS, phosphate-buffered saline; $[Ca^{2+}]_c$, cytoplasmic calcium concentration; LSCM, laser scanning confocal microscope; FCM, flow cytometry.

* Corresponding author at: No. 29, 13th Avenue, Tianjin Economy Technological Development Area, Tianjin 300457, China. Tel.: +86 22 60601428; fax: +86 22 60601332.
E-mail address: wangchunling@tust.edu.cn (C.-l. Wang).

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opening of MPTP [23–25]. CsA prevents opening of the MPTP, which is well known as a typically inhibitor of calcineurin and P-glycoprotein, but it is also a strong inhibitor of the MPTP [26,27]. Due to the opening of MPTP, $\Delta\Psi_m$ collapses which is required to drive oxidative phosphorylation, ATP depletion and mitochondrial matrix swelling [28–30]. Subsequently, the outer membrane ruptures, and intermembrane proteins are released into the cytoplasm, such as cyt c, which triggers downstream caspase cascades to initiate cellular fragmentation and eventually causes cell apoptosis [31].

However, given the complexity of the functional and molecular interplay of the MPTP, few studies have detected the activity of the MPTP in intact cell and its mechanism is still the matter of intense investigation [32,33]. In this study, investigation was been performed to measure the role of ROS and Ca^{2+} on MPTP activity, which resulted in MCF-7 cell apoptosis.

2. Materials and methods

2.1. Materials

Human breast cancer MCF-7 cell lines and human liver L-02 cells lines were obtained from the Institute of Biochemistry and cell Biology, SIBS, CAS (Shanghai, China). The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100-U/mL penicillin, 100-U/mL streptomycin, in 25-cm^2 culture flasks at 37 °C in a humidified atmosphere with 5% CO_2.

Surfactin from Bacillus natto TK-1 was purified via acidic precipitation, methanol extract, thin-layer chromatography (TLC), and analytical C18-reversed-phase highperformance liquid chromatography (HPLC) system.

The NAC (inhibitor of ROS), BAPTA-AM (1,2-bis (2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid, the intracellular Ca^{2+} chelator), CsA (inhibitor of MPTP) and the molecular probe of DCFH-DA, Flou-3/AM, calcein-AM, Rhodamine-123 were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Rabbit polyclonal antibodies against cytochrome c, caspase-9, β-actin and horseradish peroxidase conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals were of the highest commercial grade available.

2.2. MTT assay of cellular toxicity

MCF-7 cells and L-02 cells were transferred to 96-well plates containing RPMI-1640 medium supplemented with 10% fetal bovine serum. The optimal cell number was found to be 2×10^4 cells per well by bacteria count. The cells were allowed to settle overnight and were treated with varying concentrations of surfactin (0, 20, 40, 60, 80, 100 μg/mL) for 24 or 48 h at 37 °C in a humidified 5% CO_2 incubator. After incubation, MTT solution (0.5 mg/mL in PBS) was added for 4 h at 37 °C. The precipitated formazan was dissolved in 150 μL DMSO and the plates were read at 570 nm and reference absorbance at 630 nm with a microplate reader (Model 680, Bio-Rad, Hercules, CA, USA). All experiments were carried out in triplicate. Cell inhibition rate (%) = [(absorbance of control cells − absorbance of cells treated with surfactin or/and inhibitors)/absorbance of control cells] × 100%

2.3. WST-1 cell proliferation assay

Cells were transferred to flat bottomed 96-well plates with 200 μL per well culture medium (included 10% fetal bovine serum) and cultured at 37 °C in a humidified 5% CO_2 incubator. The optimal cell number was about 1×10^4 cells per well. After overnight incubation, the cells were treated with varying concentrations of NAC (0, 1.5, 1, 2, 5 mM), BAPTA-AM (0, 10, 20, 30, 40 μM), CsA (0, 25, 50, 100, 200 μM) combined with/without 30 μg/mL surfactin for 24 h, respectively. Then added 20 μL premixed WST-1 cell proliferation reagent to each well (1:10 final dilution) and incubated for 2 h at 37 °C. The absorbance of each well was measured at 450 nm and referenced absorbance at 650 nm with a microplate reader (Model 680; Bio-Rad, Hercules, CA, USA). Each test was performed in triplicate experiments.

2.4. Measurement of ROS production

The intracellular generation of ROS was inspected by DCFH-DA. The nonfluorescent ester penetrated into the cells and was hydrolyzed to DCFH by the cellular esterases. The probe is rapidly oxidized to the highly fluorescent compound 2′7′-dichlorofluorescein (DCFH) in the presence of cellular peroxidase and ROS such as hydrogen peroxide or fatty acid peroxides. Briefly, cells (1×10^6) were cultured with 30 μg/mL surfactin for 1.5, 3, 6 and 9 h and loaded with 10 μM DCFH-DA for 30 min at 37 °C in the dark, then washed with PBS (pH 7.2) three times. The fluorescence was measured at an excitation of 488 nm and an emission of 525 nm.

2.5. Measurement of cytoplasmic calcium concentration ($[Ca^{2+}]_c$)

Changes of cytoplasmic Ca^{2+} were analyzed with the fluorescent Ca^{2+} indicator Fluo-3/AM by LSCM. MCF-7 cells were cultured and treated with 30 μg/mL surfactin for 3, 6 and 9 h, respectively, then incubated with Fluo-3/AM for 30 min at 37 °C, washed with PBS (pH 7.2) thrice. The change of $[Ca^{2+}]_c$ were evaluated by means of fluorescence intensity measurements with excitation at 488 nm and emission at 525 nm.

2.6. Measurement of mitochondrial permeability transition pore (MPTP) activity

calcein-AM is a cell-permeant and non-fluorescent compound, which is widely used for determining cell viability. In live cells the non-fluorescent calcein-AM is hydrolyzed by intracellular esterases into the strongly green fluorescent anion calcein. The fluorescent calcein is well-retained in the cytoplasm in live cells [34]. Thus, this reagent was used to monitor the activity of MPTP. Considering the positive correlation between the surfactin applied and the ROS generation, Ca^{2+} accumulation, MPTP opening, the activity of MPTP was mediated in succession. MCF-7 cells were cultured and treated with 30 μg/mL surfactin for 1.5–12 h, then incubated with calcein-Am and $CoCl_2$ for 1 h at 37 °C in the dark, and washed with PBS (pH 7.2) twice. The change of fluorescence intensity was measured with excitation at 488 nm and emission at 525 nm.

2.7. Analysis of $\Delta\Psi_m$

Mitochondrial membrane potential was analyzed by staining with Rhodamine-123, a cationic membrane-permeant fluorescent probe, which was accumulated rapidly and selectively within mitochondria. The cells were treated with 30 μg/mL surfactin for 6, 9 and 12 h, respectively, then incubated with 5 μg/mL Rhodamine-123 for 30 min. After washing twice with PBS (pH 7.2), the fluorescence intensity of Rhodamine-123 was measured with excitation at 488 nm and emission at 525 nm.

2.8. Western blotting analysis

MCF-7 cells were treated with surfactin for various time, and washed with PBS buffer, then suspended in cell lysis buffer (1% Triton X-100, 0.015-M NaCl, 10-mM Tris–HCl, 1-mM EDTA, 1-mM PMSF, 10 μg/mL of each leupeptin and pepstatin A), followed by