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Sinomenine potentiates degranulation of RBL-2H3 basophils *via* up-regulation of phospholipase A_2 phosphorylation by Annexin A1 cleavage and ERK phosphorylation without influencing on calcium mobilization

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ABSTRACT

Sinomenine (SIN), an alkaloid derived from the Chinese medicinal plant *Sinomenium acutum*, is the major component of Zhengqing Fongtong Ning (ZQFTN), a pharmaceutical drug produced by Hunan Zhengqing Pharmaceutical Co. Ltd. in China for the treatment of rheumatoid arthritis and other autoimmune diseases. Some clinic reports indicate that ZQFTN may induce an anaphylactic reaction *via* potentiating the degranulation of immune cells. In the current study, we aimed to examine whether SIN is capable of inducing the degranulation of basophilic leukemia 2H3 (RBL-2H3) cells to elucidate how the anaphylactic reaction occurs. The results revealed that SIN could up-regulate β-hexosaminidase levels in RBL-2H3 cells without significant cytotoxicity, suggesting that SIN could induce the degranulation of RBL-2H3 cells. Furthermore, SIN increased the release of prostaglandin D_2 (PGD_2) and prostaglandin E_2 (PGE_2) in RBL-2H3 cells *via* promoting the expression of phosphorylated-extracellular signal-regulated kinase (P-ERK), the cleavage of Annexin A1 (ANXA1), and phosphorylated-cytosolic phospholipase A_2 (P-cPLA$_2$), as well as cyclooxygenase-2 (COX-2). The ERK inhibitor, PD98059, significantly attenuated the up-regulatory effect of SIN on cPLA$_2$ phosphorylation. Interestingly, SIN did not significantly increase Ca^{2+} influx in the cells. These findings not only explored the anaphylactic reaction and underlying mechanism of ZQFTN in RBL-2H3 cells, but may promote the development of relevant strategies for overcoming the adverse effects of the drug.

1. Introduction

Sinomenine (C19H23NO4, SIN, Fig. 1A) is a purified alkaloid derived from the roots of *Sinomenium acutum*, a medicinal plant broadly used in China for treating rheumatism and arthritic diseases [1]. SIN reportedly possesses significant anti-inflammatory and immunosuppressive activities *via* modulating the activation and function of immune cells, including T cells and B cells [2]. Currently, SIN has been developed as a marketed drug in China, called Zhengqing Fengtong Ning (ZQFTN), for the treatment of rheumatoid arthritis and other autoimmune conditions [3]. However, immune-mediated drug hypersensitivity reactions (IDHRs) occur in some cases, resulting in pruritus, flushing and macula in patients administered ZQFTN injection within 10 min [4]. The information sheet of ZQFTN states that the drug may induce intensive release of histamine associated with the development of IDHRs [5]. However, the mechanism of these anaphylactic reactions of the drug remains unknown.

According to literature reports, the development of IDHR is likely associated with the release of inflammatory mediators from mast cells and basophils [6], two types of key effector cells in IDHRs that mediate other inflammatory processes, especially allergenic reactions. Upon stimulation, mast cells and basophils undergo degranulation to release various mediators, including histamine and β-hexosaminidase, which are stored inside the vesicles, or eicosanoids, including PGD_2 and PGE_2, which are immediately synthesized upon stimulation [7].

Annexin A1 (ANXA1) is the first characterized member of the phospholipid-binding proteins and plays an important role in the pathogeneses of various diseases, including cancer and inflammation [8]. ANXA1 consists of two domains: an N-terminal trail and a C-terminal core domain. The N terminus is unique within the superfamily with varying amino acid sequences and lengths and is thought to be responsible for different biological activities and the specific function of ANXA1 [9]. ANXA1 is cleaved in some activated cells and inflammatory animal models, such as mast cells, neutrophils, basophils and OVA-induced allergenic conditions that are characterized by IDHRs [10]. The

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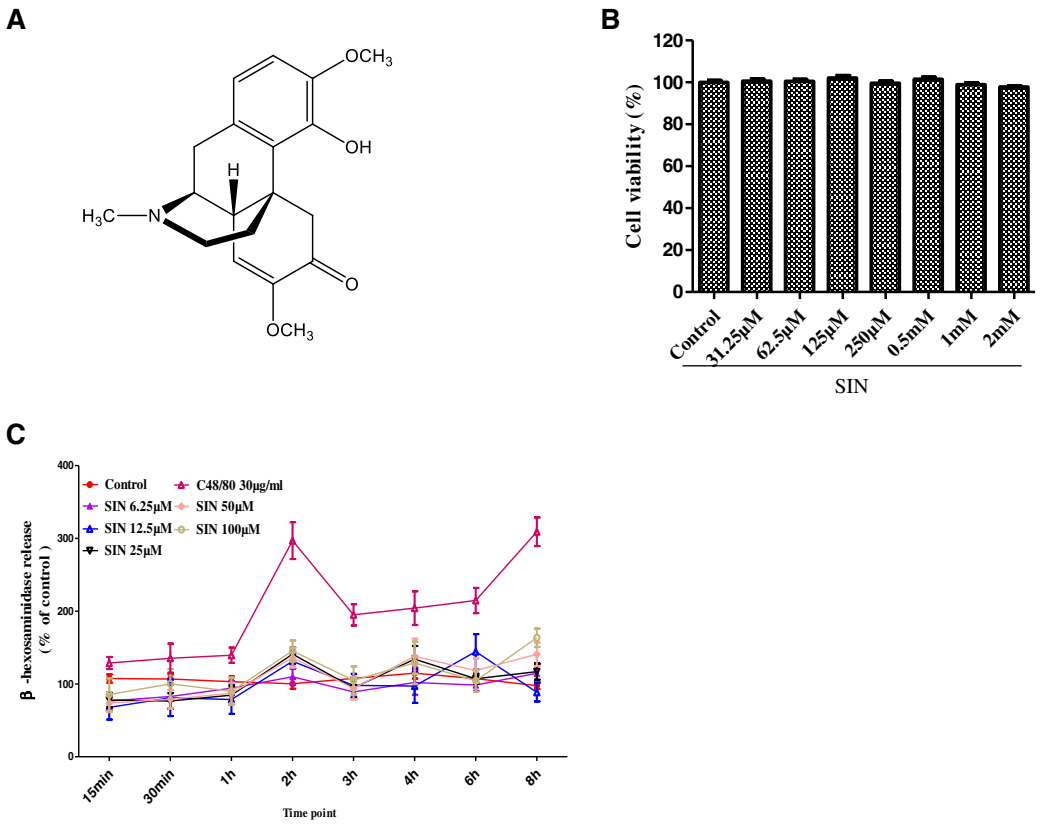

Fig. 1. Effect of SIN on the degranulation of RBL-2H3 basophils. (A) The chemical structure of SIN. (B) The cytotoxicity of SIN on RBL-2H3 cells. RBL-2H3 cells (1×10^5 cells/ml) were treated with the indicated concentrations of SIN for 48 h. Cytotoxicity was determined by the MTT assay. The percent cell viability was calculated relative to untreated cells. (C) SIN increases the degranulation of RBL-2H3 basophils. RBL-2H3 cells (2×10^5 cells/ml) were treated with different doses of SIN or C48/80 for the indicated time points. The β-hexosaminidase level of the supernatants was measured. Each value represents the mean ± SEM of three independent experiments.

cleavage of ANXA1 can increase the levels of inflammatory mediators and induce inflammatory reactions by enhancing the phosphorylation of another phospholipid-associated protein, cytosolic phospholipase A_2 ($cPLA_2$).

$cPLA_2$ plays a key role in allergic diseases, arthritis and IDHRs [11] and is a member of a superfamily of enzymes that hydrolyze the ester bond of phospholipids at the *sn*-2 position. $cPLA_2$ preferentially hydrolyzes arachidonoyl phospholipids and generates arachidonic acid (AA). In basophils and mast cells, phosphorylation of ERK and $cPLA_2$ mediates the release of AA, which is further converted to PGs by COX [12]. Additionally, AA is thought to be dependent on the phosphorylation of $cPLA_2$ by ERK in RBL-2H3 cells [13]. ERK belongs to the MAPK family and it is believed that MAPKs and intracellular Ca^{2+} concentrations are the primary synergistic signals required for the secretion of TNF-α in mast cells [14]. In the current study, we first investigated the sensitize effect of SIN on RBL-2H3 cells, and elucidate the underlying mechanism so as to explore the anaphylactic reactions of ZQFTN.

2. Materials and methods

2.1. Cell line and reagents

Rat basophilic leukemia-2H3 basophils (RBL-2H3) were purchased from the American Type Culture Collection (ATCC), ATCC cat #CRL-2256 (RBL-2H3). RBL-2H3 cells were cultured in DMEM (GIBCO, Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS, Clontech, Mountain View, CA, USA) and 100 U/ml penicillin and 100 U/ml streptomycin (Invitrogen, Scotland, UK) at 5% CO_2 and 37 °C in a humidified incubator. The primary antibodies of p-$cPLA_2$, p-ERK, COX-2, GAPDH, ANXA1 and β-actin were obtained from Cell Signaling (Cell Signaling Technology, USA). Ionomycin was obtained from Calbiochem (Merck Millipore, Germany). Flo-3AM was purchased from BD Bioscience (BD Bioscience, USA). The MAP kinase kinase inhibitor, PD98059, was obtained from Sigma-Aldrich (St. Louis, MO, USA). SIN (>98% purity verified by HPLC) was provided by Hunan Zhengqing Pharmaceutical Co. Ltd (Hunan Province, China) and was dissolved in DMSO and stored at −40 °C as the stock solution.

2.2. MTT assay for cell viability

The cell viability was examined using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. In brief, RBL-2H3 cells were seeded at a density of 1×10^5 cells/ml in 96-well plates. Cells without any drug treatment served as controls. Different concentrations of SIN (31.25 μM to 2 mM) were added into each well and incubated with the cells for 48 h. MTT dissolved in PBS (5 mg/mL, Sigma) was added to each well for 4 h, following by the addition of 150 μl of dimethyl sulfoxide (DMSO) to dissolve the formazan formed during the reaction. The absorbance was measured using a microplate reader at 490/630 nm (Infinite M200PRO microplate spectrophotometer, TECAN, USA). The percentage of cell viability was calculated using the following formula: cell viability (%) $= A_{treated}/A_{control} \times 100$. The reported data represent three independent experiments.

2.3. Measurement of the release of β-hexosaminidase

The degranulation response of RBL-2H3 basophils was quantified by measuring the level of released β-hexosaminidase in the supernatant according to the method previously described [15]. In brief, RBL-2H3 cells were seeded at a density of 2×10^5 cells/ml in 96-well plates.