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Sanguinarine suppresses IgE induced inflammatory responses through inhibition of type II PtdIns 4-kinase(s)

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ABSTRACT

The effects of sanguinarine on IgE mediated early signaling mechanisms leading to inflammatory mediators release were investigated. Pretreatment of RBL 2H3 cells with sanguinarine inhibited IgE induced activation of type II PtdIns 4-kinase activity. Concomitant with type II PtdIns 4-kinase inhibition, sanguinarine also inhibited IgE induced degranulation and β hexosaminidase release in RBL 2H3 cells. *In vitro* assays showed sanguinarine inhibited type II PtdIns 4-kinase activity in a dose dependent fashion with no effect on PtdIns 3-kinase activity. Fluorescence spectroscopic studies suggested that sanguinarine binds to type II PtdIns 4-kinases α and β isoforms with a K_d of 2.4 and 1.8 μM, respectively. Kinetic studies showed that sanguinarine competes with PtdIns binding site of type II PtdIns 4-kinase β. These results suggest that the anti-inflammatory effects of sanguinarine on PtdIns 3-kinase signaling pathway are more likely an indirect effect and emphasize the importance of the cross talk between type II PtdIns 4-kinases and PtdIns 3-kinases.

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Introduction

Sanguinarine, a plant derived quaternary benzo[c]phenanthridine alkaloid (Fig. 1) has several biological properties such as anti-inflammatory, anti-cancer, anti-oxidant, anti-bacterial, anti-fungal, anti-viral and anti-protozoan activities [1–5]. The physiological target molecules for sanguinarine have not been clearly identified. Several key molecules and pathways involved in inflammatory responses, cell cycle and apoptosis are affected by sanguinarine [5–9]. Pretreatment of neutrophils with sanguinarine has shown to inhibit degranulation, oxidative burst and phagocytosis [10–12]. In human prostate cancer cell lines, sanguinarine induces cell cycle arrest at G0/G1 and trigger apoptosis [13]. Sanguinarine also inhibits VEGF induced AKT phosphorylation and angiogenesis suggesting that PtdIns 3-kinase signaling pathway may be preferentially affected [14].

However, the effects of sanguinarine on classical phosphatidylinositol[2] 4,5P_2 (PtdIns 4,5P_2) cycle are largely unknown. This classical pathway contributes to two important signaling pathways (i) PLC mediated hydrolysis of PtdIns 4,5P_2 and generation of second messengers, inositol 1,4,5 trisphosphate and 1,2 diacyl glycerol (ii) PtdIns 3-kinase mediated signaling pathway in which PtdIns 3,4,5P_3 are generated and activation of AKT [15]. Following hydrolysis, hormone/receptor sensitive PtdIns 4,5P_2 pool is rapidly resynthesized by phosphatidylinositol 4-kinases (PtdIns 4-kinases) and phosphatidylinositol phosphate kinases. Inhibition of these enzymes may lead to defective signaling in PLC mediate signaling and/or PtdIns 3-kinase signaling cascades [16].

Type II PtdIns 4-kinase(s) are shown to be part of many immune cell receptors including FcεRI, TCR-CD3, CD4 and CD7 receptor signaling [17–21]. Inhibition of type II PtdIns 4-kinase activity has been shown to abrogate degranulation and oxidative burst in neutrophils and IgE induced mediator release in RBL 2H3 cells [19, 22]. These results mimic inhibition of PtdIns 3-kinase signaling pathways and suggest that some of the sanguinarine effects may be due to inhibition of type II PtdIns 4-kinases and their cross signaling pathways with PtdIns 3-kinases. This idea has been investigated in the present manuscript. The results show that sanguinarine indeed inhibits type II PtdIns 4-kinase activities with no effect on PtdIns 3-kinase activity and underlines the importance of cross signaling pathways mediated by type II PtdIns 4-kinases and PtdIns 3-kinases.

Materials and methods

Chemical reagents and antibodies

All the chemicals unless otherwise mentioned were obtained from Sigma–Aldrich chemicals, Bangalore India. DMEM

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[2] *Abbreviations used:* PtdIns, phosphatidylinositol; DMEM, Dulbecco's modified Eagle's medium.

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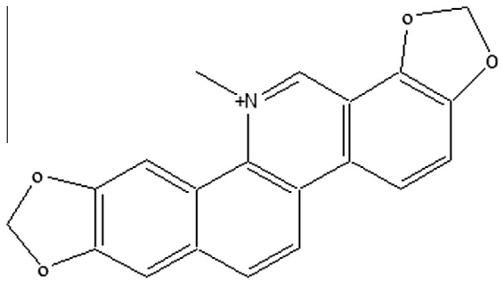

Fig. 1. Structure of Sanguinarine.

(Dulbecco's Modified Eagle's Medium) was from Himedia laboratories, India. Phenylmethylsulfonylfluoride was from SRL India, [γP^{32}] ATP was from Board of Radiation and Isotope Technology, Hyderabad, India. Ni–NTA beads were from Qiagen. Glutathione beads were from GE health care life sciences. P6 resin was from Bio-Rad.

Cell culture

RBL 2H3 cells were maintained as monolayer cultures in DMEM supplemented with 10% heat inactivated fetal bovine serum, 2 mM glutamine, penicillin (100 U/mL), streptomycin (25 μg/mL), amphotericin B (0.25 μg/mL), in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were harvested with trypsin–EDTA solution (0.05% trypsin-0.2% EDTA) at 37 °C for 3–5 min. Cell suspension was washed twice with complete DMEM prior to the experiments.

Cell stimulation and lysis

RBL 2H3 cells were stimulated with IgE DNP-HSA as described earlier [19]. Briefly, RBL 2H3 cells (1×10^6 cells in 0.2 mL of complete DMEM) were incubated with DNP-specific-IgE (1 μg) at 37 °C for 3 h. IgE sensitized RBL 2H3 cells were washed twice with DMEM and the bound IgE were cross-linked with DNP-HSA (1 μg/mL) in a final volume of 200 μL for 3 min. The reaction was terminated by adding 1 mL of ice cold DMEM and immediately transferred into an ice bucket. Cells were washed once with ice cold phosphate buffered saline prior to lysis in 0.2 mL of lysis buffer [Triton X-100 (1%), Tris–HCl (25 mM pH 7.4), NaCl (150 mM), EGTA (2 mM), sodium orthovanadate (0.25 mM) and PMSF (1 mM)]. The lysates were centrifuged at 30,000*g* at 4 °C for 30 min. The supernatants were assayed for PtdIns 4-kinase activity or used for immunoprecipitation.

PtdIns 4-kinase assay

PtdIns 4-kinase activity was assayed as described earlier [23]. Briefly the enzymatic reaction was carried out in final volume of 50 μL at room temperature (~25 °C) in 50 mM Tris (pH 7.6), 10 mM $MgCl_2$, 0.25 mM EGTA, 0.1 mM sodium orthovanadate, 20 μg/mL phosphatidylinositol, 100 μM [γ-^{32}P] ATP and 0.4% Triton X-100 with 10 μL of cell lysate. The reaction was initiated by adding [γ-^{32}P] ATP and incubated for 6 min. The reaction was terminated by adding 50 μL of 12 N HCl. Phospholipids were extracted with 500 μL of chloroform: methanol: water (15:15:5, v/v/v), washed once with methanol: 1 N HCl (1:1 v/v) and analyzed on thin layer chromatography. Thin layer chromatography plates were pre-treated with 2% sodium potassium tartarate and 60 mM EDTA in 50% ethanol and dried in oven before loading samples. The chromatograms were developed with chloroform: methanol: water: ammonium hydroxide (90:90:20:7, v/v/v). Labeled phospholipids were visualized by autoradiography and quantified using scintillation counting.

Immunoprecipitation and PtdIns 3-kinase activity

RBL 2H3 cell lysates were prepared as described above [19]. Cell lysates were incubated with p85 or p110 antibodies at 4 °C for 90 min. At the end of incubation, protein A agarose beads were added and further incubated for 60 min. The protein A agarose beads were washed thrice with ice cold phosphate buffer saline (pH 7.4) and assayed for PtdIns 3-kinase activity in 100 μL reaction volume containing 50 mM Tris (pH 7.6), 10 mM $MgCl_2$, 0.25 mM EGTA, 0.1 mM sodium orthovanadate, 20 μg/mL PtdIns, 100 μM [γ-^{32}P] ATP. The reaction was initiated with the addition of [γ-^{32}P] ATP and incubated at room temperature (~25 °C) for 6 min. The reaction was terminated with 50 μL of 12 N HCl. The labeled phospholipids were extracted and separated as described in PtdIns 4-kinase assay. The phospholipids were visualized by autoradiography and quantified by scintillation counting.

Purification of His-tagged proteins

His-tagged human type II PtdIns 4-kinase α and β were cloned in pET 28 vectors and were expressed in*Escherichia coli* BL 21 cells [21]. Mid log culture of bacterial cells were induced with 0.5 mM IPTG at 37 °C for 3 h. At the end of incubation, cells were centrifuged at 7000 rpm for 15 min and lysed on ice with lysis buffer [50 mM Na_2HPO_4 (pH8.0), 300 mM NaCl, 10 mM imidazole, and-lysozyme 100 μg/mL]. After 15 min incubation, cells were sonicated for 1 min in presence of 1 mM PMSF. The lysate was centrifuged at 23,469*g* for 20 min. The supernatant was loaded on Ni–NTA column. The Ni–NTA column was washed with 6 column volumes of wash buffer [50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 70 mM imidazole 1%]. Bound PtdIns 4-kinases were eluted with elution buffer [50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 150 mM imidazole, 1%]. Imidazole is removed from purified protein solution using P6 column.

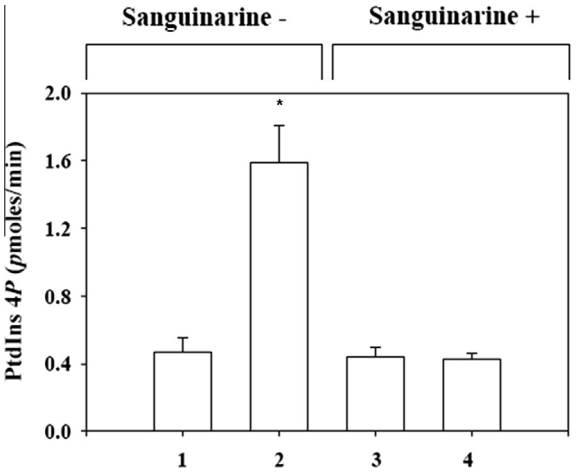

Fig. 2. Sanguinarine inhibits PtdIns 4-kinase activation in FcεRI cross linked RBL 2H3 cells. RBL 2H3 cells were pre-incubated with sanguinarine and FcεRI receptors were cross linked with IgE and DNP-HSA. Control and stimulated cells were lysed and cell lysates were assayed for PtdIns 4-kinase activity. Lane 1 and 2, PtdIns 4-kinase activity in unstimulated and antigen stimulated cell lysates in the absence of sanguinarine. Lane 3 and 4, PtdIns 4-kinase activity in unstimulated and antigen stimulated cell lysates, which were preincubated with sanguinarine. Data points represent the mean ± SEM ($N \geqslant 3$). Statistical analysis was done to compare PtdIns 4-kinase activity upon stimulation and decrease in PtdIns 4-kinase activity upon sanguinarine treatment with graph pad prism and P value is lesser than 0.05 (one way ANOVA).

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