Enhancement of the release of inflammatory mediators by substance P in rat basophilic leukemia RBL-2H3 cells

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Summary

Substance P (SP), a neurotransmitter, may play an important role in neurogenic inflammation. Ginseng has been used extensively in traditional medicine; however, few studies were focused on their anti-allergic effect. Therefore, the effect and mechanism of ginsenoside Rb1 on the SP enhancement of allergic mediators were explored. In this study, SP and dinitrophenyl-bovine serum albumin (DNP-BSA) were used to activate rat basophilic leukemia (RBL)-2H3 cells. The cultured supernatants were assayed for histamine, leukotriene C$_4$(LTC$_4$) and interleulin-4 (IL-4) production. The mitogen-activated protein kinases (MAPKs) signaling pathway was determined by Western blotting analysis. We found that IgE/DNP-BSA, SP, ginsenoside Rb1, or MAPK specific inhibitors had no effect on cell viability and cytotoxicity. SP (30 μM) alone, did not induce histamine and LTC$_4$ release, but it enhanced allergen-induced histamine and LTC$_4$ release. In addition, SP significantly induced and enhanced allergen-activated IL-4. Ginsenoside Rb1 dose-dependently inhibited these effects. SP enhanced the allergen-activated ERK pathway in RBL-2H3 cells, and Rb1 effectively inhibited the ERK pathway activation. Although MAPK specific inhibitors suppressed LTC$_4$ and IL-4, only U0126 inhibited the SP enhanced histamine release. These results demonstrate that Rb1 dose-dependently inhibited SP enhanced allergen-induced mediator release and its mechanism was through the inhibition of the ERK pathway.

Introduction

Neurogenic inflammation is orchestrated by a large number of neuropeptides including tachykinins such as substance P (SP) and neurokinin A, or calcitonin gene-related peptide [1, 2]. SP and neurokinin A, and its receptors are present in human and animal airways [1, 3]. Local release of tachykinins affects blood vessels and broncho-constrition and hyperresponsiveness. Tachykinins are important to mast cells, and they are chemotactic for neutrophils and eosinophils [1]. SP can stimulate histamine release from human mast cells of bronchoalveolar lavage and enhance anti-IgE-induced histamine release from rat peritoneal mast cells [4, 5].

SP and neurokinin A, act through neurokinin (NK)-1 and NK-2 receptors. NK-1 and NK-2 receptors are expressed in the lungs, and SP is released in the airways of rat sensitized to ovalbumin (OVA). The selective NK-1 or NK-2

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antagonists decreased OVA-induced late airway responses in challenged animals [6]. On the other hand, interleukin-4 (IL-4) and IgE-dependent mast cell activation play important roles in allergic airway inflammation [7, 8] and leukotriene (LT) induce contraction of the human airway smooth muscle, chemotaxis and increased vascular permeability [9–12]. These mediators play important roles in asthma and allergic rhinitis. SP induces LT-B2 and prostaglandin E2 in mice [13]. LT-receptor antagonist, montelukast is effective and safe in the treatment of patients with asthma and allergic rhinitis [9].

Ginsenoside Rb1 from *Panax ginseng* (Araliaceae) has a partial neurotrophic and neuroprotective role in dopaminergic cell cultures under glutamate stress [14]. Ginsenoside Rh2 and the metabolite of Rb1 have anti-inflammatory activities to LPS-induced macrophages [15, 16]. Pretreatment with Rb1 significantly decreased histamine and LT in a dose-dependent manner during mast cell activation [17]. Rh2 had the most potent inhibitory activity on *beta*-hexosaminidase release from rat basophilic leukemia (RBL) cells and in the passive cutaneous anaphylaxis reaction [15]. However, the effects of SP and Rb1 on signaling pathway of mast cells have not been studied. Therefore, we were interested in studying the signaling pathway of SP enhancement on allergen-induced RBL-2H3 cells and the effect of Rb1 on this neurogenic inflammation model.

Materials and methods

Ginsenoside Rb1, anti-dinitrophenyl (DNP) IgE monoclonal antibody, substance P and *o*-phthalaldehyde were obtained from Sigma (St. Louis, MO, USA). Dinitrophenyl-bovine serum albumin (DNP-BSA) was obtained from Calbiochem (San Diego, CA, USA) and MAP kinase inhibitors, SB203580, SP600125, and U0126 were obtained from Biomol (Plymouth Meeting, PA, USA). Fetal calf serum (FCS) was obtained from BIOLOGICAL INDUSTRIES (Kibbutz Beit Haeme, Israel). Penicillin, streptomycin, and Dulbecco's Modified Eagle's medium (DMEM) were purchased from GIBCO (Grand Island, NY, USA). Mammalian protein extraction reagent (M-PER) was obtained from PIERCE (Rockford, IL, USA). Anti-phospho-p38, ERK, JNK, or *β*-actin antibodies were purchased from Abcam (Cambridge, UK).

Cell culture and mediator assay

The RBL-2H3 cell line was maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified incubator under 5% CO_2. Confluent cultures were passed by trypsinization. The RBL cells were seeded at 2×10^5cells/well. and sensitized with 10 ng/ml of anti-DNP IgE for 1 h at 37 °C. The cells were then washed with Hank's balanced salt solution (HBSS) buffer, incubated with ginsenoside Rb1 or medium for 1 h at 37 °C and then stimulated with 10 ng/ml DNP-BSA plus 30 μM substance P for 30 min at 37 °C. Histamine content in the supernatant and in the cells was measured by the *o*-phthalaldehyde fluorometric according to the procedure described by Yoshimura et al. [18]. Histamine release (%) was expressed as the percentage of total histamine content.

IL-4 and LTC$_4$ content in culture supernatants were determined by ELISA kits (R&D, Minneapolis, MN and Cayman chemical, Ann Arbor, MI, USA; respectively). The absorbance at 410 nm was determined using a microplate reader (spectraMAX 340, Molecular Devices, Sunnyvale, CA, USA).

Western blot analysis

Cells were washed and protein extracted by using M-PER reagent (Rockford, IL, USA). Lysates were clarified by centrifugation at $12,000 \times g$ for 15 min at 4 °C. The protein content was determined with the aid of Bio-Rad DC Protein Assay Kit. Equal amounts of samples were subjected to SDS-PAGE using 10% running-gels. Proteins were transferred into PVDF membrane and blocking with 5% non-fat milk 2 h at room temperature. To study the mitogen-activated protein kinases (MAPKs) signaling pathway, the membranes were then incubated overnight at 4 °C with anti-phospho-p38, ERK, JNK, or *β*-actin antibodies. Membranes were then incubated for 2 h with the appropriate horseradish peroxidase-conjugated anti-IgG. Proteins were detected by the ECL system (Amersham, Berkshire, UK) and quantified by densitometric analysis (Digital Image Analysis System, PDI, Huntington Station, NY, USA).

Statistical analysis

Results are expressed as mean $\pm$ standard error of the means from at least three experiments. Comparisons among several groups were performed with non-parametric ANOVA. Comparisons between two groups were performed with the student's t-test. A value of $p < 0.05$ was considered statistically significant.

Results

Effect of SP on allergen-induced mediators

RBL-2H3 cells have been used for the study of IgE-mediated degranulation, secretion of inflammatory mediators, such as histamine, LTC_4 release and IL-4 [19]. Our results were consistent with the previous reports on DNP-induced mediator release. The DNP-BSA dose-dependently affected histamine, LTC_4 and IL-4 release from RBL cells with a sub-optimal concentration of 10 ng/ml IgE. (data not shown). SP (0.001–30 μM) alone did not induce histamine and LTC_4 release, but it enhanced allergen-induced histamine and LTC_4 release from RBL-2H3 cells. However, SP significantly induced IL-4 alone and with allergen (Figure 1).

Effect of Rb1 on SP-enhanced mediator production

To investigate whether ginsenoside Rb1 has anti-allergic activity, we used SP-enhancement of IgE/DNP-activated RBL-2H3 cells for the study. We found that Rb1 dose-dependently (50–100 μM) inhibited histamine and LTC_4 release far below the levels induced by IgE/DNP-BSA. In addition, Rb1 dose-dependently (20–100 μM) inhibited SP-enhanced IL-4 secretion (Figure 2).

MAPK activation in RBL-2H3 cells

SP alone or IgE/DNP induced MAPK activation in RBL-2H3 cells. SP dose-dependently enhanced the IgE/DNP-activated ERK pathway more prominently than p38 or JNK MAPKs (Figure 3). U0126 specifically inhibited SP enhancement of allergen-activated ERK and Rb1 dose-dependently (20–100 μM) inhibited the activated ERK1/2 pathway (Figure 4). Among MAPK inhibitors (from 0.01 to 1 μM of concentrations),
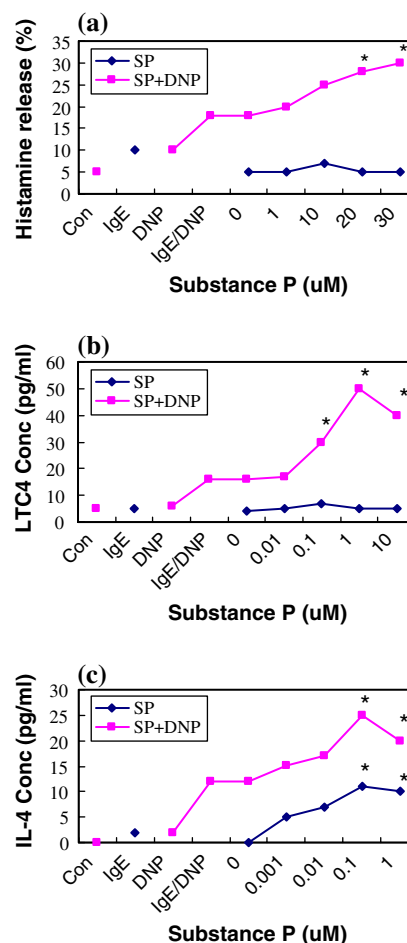

Figure 1. Effects of SP on the allergen-induced mediators from RBL-2H3 cells. SP enhanced DNP-BSA (10 ng/ml)-induced histamine (a), LTC_4(b), and IL-4 (c) release from RBL-2H3 cells (2×10^5) with a sub-optimal concentration of 10 ng/ml IgE. Histamine release was assayed by a fluorometric method, IL-4 and LTC_4 by ELISA kits. Values represent means from five separate experiments. *$p < 0.01$ as compared with the IgE/DNP group (mean $\pm$ SEM): histamine release (20 $\pm$ 2%); LTC_4(15 $\pm$ 5 μg/ml), and IL-4 (12 $\pm$ 2 μg/ml). SP (μM): concentration in μM.

U0126 inhibited SP enhanced IgE/DNP-activated histamine release whereas SB203580 or SP600125 did not (Figure 5a). In contrast, all three inhibitors inhibited SP-enhanced allergen-activated LTC_4 and IL-4 in RBL-2H3 cells (Figure 5b, c).

Discussion

Symptoms of asthma/allergic rhinitis are induced by inflammatory mediators, which are released upon activation of mast cells by allergen-IgE
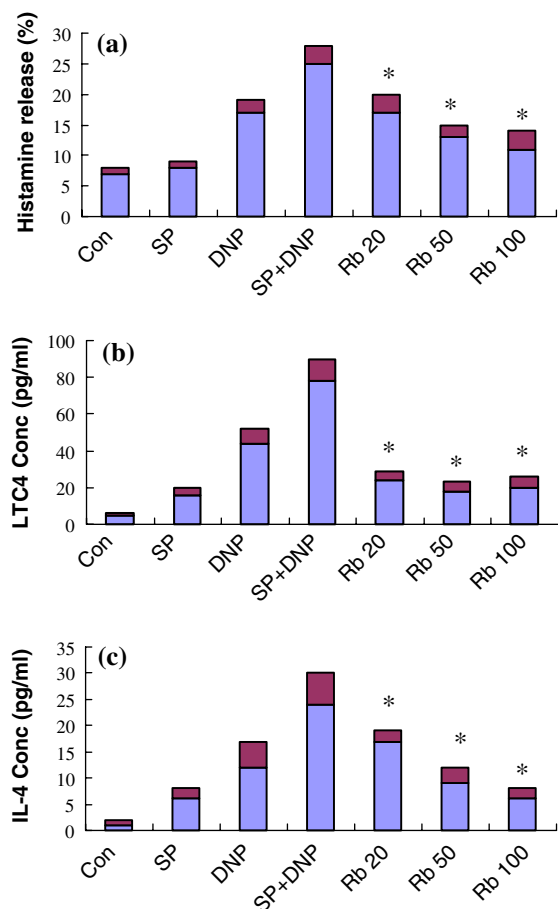
Figure 2. Effects of ginsenoside Rb1 on the SP enhancement of allergen-induced mediators. Pretreatment (1 h) with Rb1 inhibited histamine (a), LTC_4 (b), and IL-4 (c) release from the SP enhancement of allergen-induced RBL-2H3 cells. Data represent means ± SEM from five separate experiments. *$p < 0.01$ as compared with the SP/allergen group. Rb1 concentrations in μM: Rb 20: 20 μM; Rb 50: 50 μM; Rb100: 100 μM.

interaction. These mediators target the end organs directly or indirectly. For instance, stimulation of sensory nerves by histamine may lead to sneezing, pruritus, rhinorrhea, nasal congestion, and asthma. These symptoms are characterized by the phenomenon of hyperresponsiveness to non-allergic stimuli, such as cold air and various irritants and these symptoms may also cause by allergic inflammation on the sensory nerves that supply the upper airway [20, 21]. Therefore, neurogenic inflammation involved airway and other organs, is orchestrated by a large number of neuropeptides including SP and neurokinin A [22]. Allergic inflammation in turns, can induce SP synthesis in large-diameter (A-fiber) ganglion
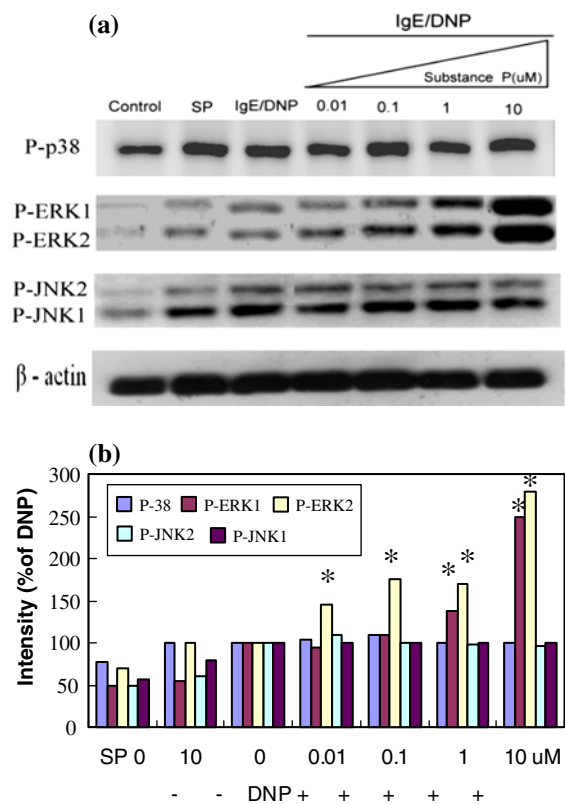

Figure 3. Effect of SP on enhancement of allergen-induced MAPK pathway. RBL-2H3 cells were stimulated with 10 ng/ml IgE/DNP-BSA and substance P for 20 min at 37 °C and then were extracted and subjected to Western blotting as described in the Materials and methods. The blotted membranes were examined with anti-phospho-p38, ERK1/2, JNK, or β-actin antibodies. Upper panel (a): representive blot results. Lower panel (b): densitometry data represented the means from three experiments. *$p < 0.05$ as compared with the IgE/DNP group.

neurons innervating guinea pig lungs [23]. Although a recent study showed that SP induced histamine release in human skin mast cells [24], present result agreed with other studies that SP alone did not induce histamine release but enhanced allergen-effect in RBL cells [5, 25]. The difference may be related to the allergic state of the cells sources.

SP induces LTC_4 release from murine mast cells [26], but RBL-2H3 cells in our study did not. The discrepancy may be due to the different sources of the mast cells, similar to the controversy of histamine release [24, 25]. However, we found that SP significantly enhanced allergen-induced LTC_4 release. LTC_4 and its metabolites are extremely potent bronchoconstriction agents that
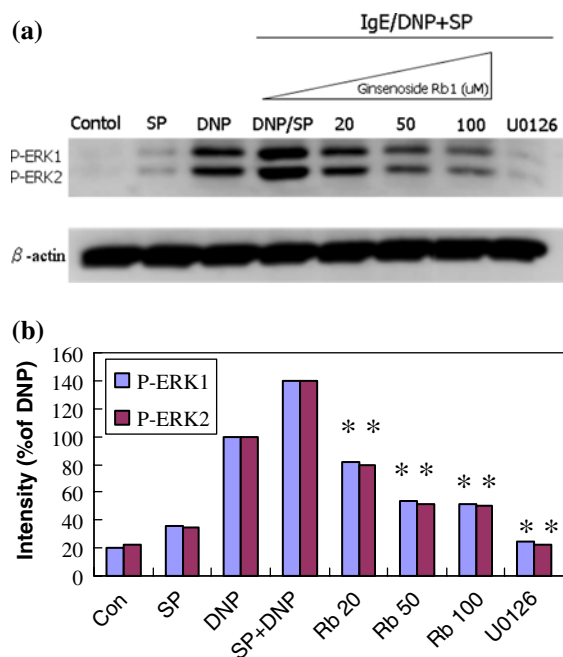
Figure 4. Effect of Rb1 on SP enhancement of IgE/DNP-induced MAPK activation. RBL-2H3 cells were pretreated with Rb1 for 1 h and subjected to Western blotting analysis for MAPK phosphorylation. Upper panel (a): representative blot results showed that Rb1 (20–100 μM) and U0126 (1 μM) specifically inhibited the SP enhancement of allergen-activated ERK1/2. Lower panel (b): densitometry data represented the means from three experiments. *$p < 0.05$ as compared with the SP/allergen group.
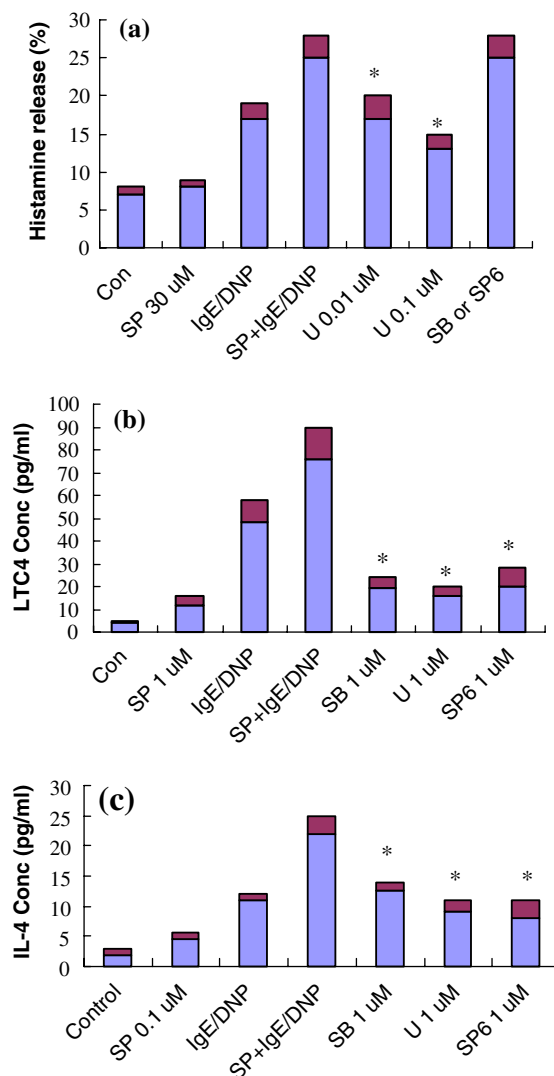

Figure 5. Effects of MAPK inhibitors on SP enhancement of allergen-induced mediators from RBL-2H3 cells. Cells were pretreated with U0126 (U), SB203580 (SB) and SP600125 (SP6) for 1 h and then assayed the mediators from SP enhanced IgE/DNP-activated cells. Suppression of histamine (a), LTC$_4$ (b) and IL-4 (c) release by inhibitors of MAPKs are compared with each SP/allergen group. Data represent means $\pm$ SEM from three experiments. *$p < 0.05$ as compared with the SP/allergen group.

are pathogenic in both asthma and allergy [10–12]. In addition, SP alone induced IL-4 similar to the other report [27] and it enhanced allergen-induced IL-4 from RBL-2H3 cells. Mast cell activation provides a rapid and local pulse of IL-4 into the local environment essential for the triggering of T lymphocytes into sustained IL-4 production [28, 29]. Human mast cells storing IL-4 within the granules are likely to have an important influence during the initiation and maintenance of the allergic response [30]. In corroborate with this, a recent study with combined analyses of genetic alterations in the IL-4/IL-13 pathway reveals its actual significance to the development of atopy and childhood asthma [31].

Ginseng has long been used for enhancing brain function, immunity and anti-inflammation. Ginsenoside Rb1 potentiates the effects of nerve growth factor on nerve fiber outgrowth [32] and increases choline uptake by the brain [33]. Previously, pretreatment of Rb1 (91 μM) had been

shown to decrease histamine and LT in allergen-activated lung mast cells [17]. Just as expected, we observed that Rb1 (20–100 μM) significantly inhibited histamine, IL-4, and LTC$_4$ release from SP-enhanced allergen-activated RBL-2H3 cells. Thus, the suppression of Rb1 on SP-enhanced inflammatory mediators may provide a new approach for treating neurogenic inflammation.

SP stimulated TNF- α production in rat peritoneal mast cells is involved with MAPKs signaling pathways [33]. We found that SP or allergen induced ERK, JNK and p38 MAP kinase pathways in RBL-2H3 cells and SP enhanced allergen-induced ERK1/2 more prominently than other MAP kinases. Our result shows that Rb1 dose-dependently inhibited the activated ERK1/2 pathway. Furthermore, all three inhibitors of MAP kinases suppressed LTC$_4$ and IL-4 from SP-enhanced RBL-2H3 cells. However, U0126 selectively suppressed histamine release from the SP enhanced cells. This suggests that the ERK signaling pathway is more important to RBL-2H3 cells under SP and/or allergen activation. A previous study showed that U0126 slightly reduced OVA-induced release of histamine but significantly inhibited the release of LTs from guinea pig lung fragments [34]. In contrast, a study with rat peritoneal mast cells showed that p38 MAPK and JNK, but not ERK, were induced 5 min after exposure to SP and the specific p38 MAPK inhibitor SB203580 abolished SP-induced increase in TNF-α and reduced histamine secretion [35]. Exposure of human basophils to allergens results in a rapid secretion of mediators such as histamine, LTC$_4$, IL-4 and IL-13 which involves PI 3-kinase and the subsequent activation of p38 MAPK and ERK1/2 but not JNK [36]. These differences in the activation of MAPK pathway are possibly due to different responses in cell types. Nevertheless, these MAPK-activated mediators are targets of current asthmatic therapy consisting of anti-histamines, LT antagonists, anti-inflammatory glucocorticoid, and humanized monoclonal antibodies against IgE and IL-4 [37].

In summary, neurogenic factor such as SP plays a role in enhancing the allergen-activation of mast cells. Rb1 may exert their effects by stabilizing nerves from secreting neuropeptides and reducing neurogenic inflammation. Therefore, the inhibitory effects of Rb1 on neurogenic inflammation may provide an additional therapeutic strategy for asthma.

Acknowledgements

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