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2-aminoethoxydiphenyl borate reduces degranulation and release of cytokines in a rat mast cell line

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Abstract. – BACKGROUND AND OBJECTIVE, 2-aminoethoxydiphenyl borate (2-APB) has been reported to be a useful pharmacological tool in the study of calcium signaling. It is used either as a membrane-permeable inhibitor for inositol 1,4,5-trisphosphate (IP3) receptors or a store-operated calcium (Ca^{2+}) entry (SOCE) inhibitor. The present study aimed to evaluate the effects of 2-APB on degranulation and cytokine production after antigen activation in a rat mast cell line, RBL-2H3 cells.

MATERIALS AND METHODS, Degranulation levels were analyzed by beta-hexosaminidase assay. Intracellular calcium concentrations were measured by Fluo-3 assay. The mRNA expression of interleukin-4 (IL-4) and tumor necrosis factor (TNF)-alfa were analyzed by Real-time PCR.

RESULTS, The intracellular Ca^{2+} levels were greatly suppressed in the absence or presence of 2 mmol/L Ca^{2+} in the extracellular medium when RBL-2H3 cells were pretreated with 100 μmol/L 2-APB for 15 min. The beta-hexosaminidase activity as well as the mRNA expression of IL-4 and TNF-alfa levels were significantly decreased after application of 2-APB.

CONCLUSION, This study indicates that the application of 2-APB may be a useful method to inhibit mast cell activation.

Key Words:
RBL-2H3 cells, Degranulation, Calcium signaling, Calcium channel blockers.

Introduction

Mast cells play a key role in allergic disorders such as bronchial asthma, allergic rhinitis, urticaria and pollinosis. They are located in skin, gut, respiratory tract and urinary tract where pathogens and allergens are frequently encountered[1]. When they are activated by cross-linking of the high affinity IgE receptor (FcεRI), preformed mediators that are stored in cytoplasmic granules such as histamine, tryptase, chymase and leukotriene C_4 are released. In addition, mast cell activation also leads to the *de novo* synthesis of proinflammatory lipid mediators, cytokines and chemokines[2,3].

Calcium signals are essential for mast cell activation and degranulation. 2-aminoethoxydiphenyl borate (2-APB) is a commonly used pharmacological blocker for calcium signals and its effects are comprehensive. It is first reported to be a membrane-permeable blocker of the inositol 1,4,5-trisphosphate (IP3)-induced calcium release[4], and further research revealed that it is also an inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) Ca^{2+} pumps[5]. In addition, it is found to have inhibitory effects on store-operated calcium (Ca^{2+}) (SOC) entry[6,7]. Although significant progress has been made in the molecular and functional characterization of Ca^{2+} influx pathways on mast cells during the past few years, the effect of 2-APB on mast cell activation is still unclear.

Rat basophilic leukemia cell line (RBL-2H3) is a tumor analog of mast cells that possesses phenotypic characteristics of mucosal mast cells. It is widely used in mast cell-associated studies such as IgE-FcεRI interactions, signalling pathways for degranulation and to test novel mast cell stabilizers[8-11]. The aim of the present study is to investigate the effects of 2-APB on Ca^{2+} signals, degranulation levels as well as interleukin-4 (IL-4) and tumor necrosis factor (TNF)-α production in antigen-activated RBL-2H3 cells.

Methods

Cell Culture

RBL-2H3 cells were obtained from the American Type Culture Collection (ATCC). Cells were

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routinely maintained in Dulbecco's Modified Eagle Medium (DMEM) (HyClone, Logan, UT, USA) containing 10% of (v/v) fetal bovine serum (FBS) (HyClone, Logan, UT, USA). Cultures were maintained at 37°C in a 5% CO_2 incubator. Cells were detached with trypsin-EDTA solution (Invitrogen, Carlsbad, CA, USA). After washing, the cells were resuspended in fresh medium and used for subsequent experiments.

Measurement of $[Ca^{2+}]i$ Level

5×10^4/mL RBL-2H3 cells were sensitized with 75 μg/L of DNP-specific monoclonal IgE (Sigma: St Louis, Mo, USA) overnight, and washed with Ca^{2+}-free Hanks' Balanced Salt Solution (HBSS, 138 mM NaCl, 5 mM KCl, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 5.6 mM glucose, pH 7.2) for 3 times. Medium was then replaced with Ca^{2+}-free HBSS and loaded with 4 μM Fluo-3 (Dojindo, Kumamoto, Japan) for 1 h at 37°C. After 3 times of washing with Ca^{2+}-free HBSS, Ca^{2+}-free HBSS containing 2-APB (final concentration 100 umol/l) was added to the cells for 15 min when needed[12]. Cells were then stimulated with 20 ng/ml of Dinitrophenyl-bovine serum albumin (DNP-BSA) (Sigma). 2 mM $CaCl_2$ was added when indicated in the experiments. The intracellular calcium ($[Ca^{2+}]i$) levels of RBL-2H3 cells were analyzed by a laser scanning confocal microscopy (LSM710, Carl Zeiss, Göttingen, Germany). The change in fluorescence of fluo-3 was monitored at an excitation wavelength of 488 nm and emission wavelength of 540 nm[13]. The 2-APB we used here was dissolved in dimethylsulfoxide (DMSO) which was subsequently diluted with external standard solution to a final concentration of 100 umol/l. The maximal DMSO concentration used in this study was 0.1%, which had little effect on calcium signals.

Stimulation of RBL-2H3 Cells

2×10^5/mL cells were seeded per well of 24-well plates and sensitized with 75 μg/L of DNP-specific monoclonal IgE overnight. After 3 times of washing with PIPES buffer (119 mM NaCl, 5 mM KCl, 25 mM PIPES, 5.6 mM glucose, 1 mM $CaCl_2$, 0.4 mM $MgCl_2$, and 0.1% BSA, pH 7.2), 2-APB was added for 60 min at 37°C when needed. Cells were then stimulated with 20 ng/ml of DNP-BSA for 30 min at 37°C. The reaction was stopped by cooling in an ice bath for 10 min[14].

Measurement of Degranulation

Degranulation was determined by measurement of the release of the granule marker, β-hexosaminidase[15]. The supernatant was collected by centrifugation at 16,000 × g for 10 min at 4°C. Total cell lysates were obtained by addition of 0.1% Triton X-100. 50 μL of supernatant or total cell lysates was transferred into a 96-well microplate and incubated with 50 μL of substrate (1 mM p-nitrophenyl-N-acetyl-b-D-glucosaminide) (Sigma) in 0.1 M citrate buffer (pH 4.5) (Weijia, China) at 37°C for 1 h. The reaction was stopped by adding 200 μL of stop solution (0.1 M Na_2CO_3/ $NaHCO_3$, pH 10.0). The absorbance was measured with a microplate reader at 405 nm. β-hexosaminidase release (%) = (released β-hexosaminidase/(released β-hexosaminidase + residual β-hexosaminidase)[11,14].

Measurement of IL-4 and TNF-α mRNA Expression by Real-time PCR

Total RNA was extracted from RBL-2H3 cells using a Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA samples were resuspended in diethyl pyrocarbonate (DEPC)-treated water, quantified, and then stored at –80°C until used. First-strand cDNA was synthesized using 0.5 μg of total RNA and oligo-d (T) primer using SYBR PrimeScript RT-PCR Kit (Takara Bio Inc, Otsu, Shiga, Japan). Reverse transcription was carried out at 37°C 15 min, 85°C 5 sec. Synthesized cDNAs were stored at −20°C until required. Quantitative real-time PCR was performed using SYBR Green I dye using the Thermal Cycler Dice Real Time System. The reaction mixture contained 0.4 μM of forward and reverse primers, 50 ng of total cDNA, and 1 × SYBR Premix Ex TaqII in a final volume of 25 μl. The thermal cycle was 95°C for 30 s, and 40 cycles of 95°C for 5s, 60°C for 30s. The oligonucleotide primers were as follows: for rat IL-4, 5'-TCCTTACGGCAACAAGGAAC-3' (forward) and 5'-GTGAGTTCAGACC GCTGACA-3' (reverse); for rat TNF-α, 5'-AACTCGAGTGACAAGCCCGTAG-3' (forward) and 5'-GTACCACCAGTTGG TTGTCTTTGA-3' (reverse). The amount of amplified DNA fragments encoding IL-4 and TNF-α were normalized to that of fragments encoding GAPDH. Results are presented as the "relative expression" in gene expression.

Statistical Analysis

All data are demonstrated as mean±standard error of mean (SEM). Differences between groups of data were explored using one-way ANOVA or Student's paired or unpaired *t* test (two-tailed) as appropriate. Data were analysed

with the statistical package SPSS 13.0 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was regarded as statistically significant.

Results

Effects of 2-APB on Ca^{2+} Signals After Antigen Stimulation

Aggregation of the high-affinity IgE receptor (Fc ε RI) on mast cells is a key step in mediating an allergic response. In order to investigate the Ca^{2+} signals after 2-APB application, we incubated RBL-2H3 cells overnight with DNP-specific IgE molecules and challenged the cells with DNP-BSA in the absence or presence of extracellular Ca^{2+}. As shown in Figure 1A, when DNP-BSA was added to RBL-2H3 cells in the absence of Ca^{2+} in the extracellular medium, a transient increase of intracellular Ca^{2+} was observed, representing release of Ca^{2+} from the intracellular Ca^{2+} stores. Subsequent addition of 2 mM extracellular Ca^{2+} induced a persistent increase in $[Ca^{2+}]i$, which was due to Ca^{2+} influx from the outside. Figure 1B showed the application of 1‰ DMSO did not influence the changes of $[Ca^{2+}]i$ when RBL-2H3 cells were activated. And as shown in Figure 1C, when cells were pre-treatment with 100 umol/L 2-APB in 1‰DMSO 15 minutes before the application of DNP-BSA, no significant increase of $[Ca^{2+}]i$ was observed. After addition of 2 mmol/L Ca^{2+}, still no significant increase of $[Ca^{2+}]i$ was observed.

Effects of 2-APB on Antigen-Induced Degranulation in RBL-2H3 Cells

The secretion of β-hexosaminidase is the hallmark of an allergic reaction[16]. The β-hexosaminidase activity was significantly higher in activated group than in normal control group [(44.8±6.5%) vs (21.3±6.0%)] ($p < 0.05$)]. When treated with 100 μmol/L 2-APB, β-hexosaminidase activity was (30.1±3.4%), which was significantly decreased compared to activated group ($p < 0.05$) (Figure 2).

Effects of 2-APB on IL-4 and TNF-α mRNA Expression

Mast cells are known to produce various cytokines including IL-4 and TNF-α in response to IgE-antigen challenge[13]. In order to investigate the influence of 2-APB on cytokine production, we examined the mRNA expression of IL-4 and TNF-α with or without 2-APB treatment in stimulated RBL-2H3 cells. The mRNA levels for both IL-4 and TNF-α were significantly increased in activated group than in normal control group ($p < 0.05$). After treated with 100 μmol/L 2-APB, the mRNA levels for IL-4 and TNF-α were significantly decreased compared to activated group ($p < 0.05$) (Figures 3 and 4).

Discussion

Our study investigated the effects of 2-APB on antigen-stimulated RBL-2H3 cells. The application of 2-APB suppressed calcium signals, β-hexosaminidase activity as well as mRNA expression of IL-4 and TNF-α, suggesting an inhibitory effect of 2-APB on RBL-2H3 cell activation.

Calcium plays an important role in the degranulation process for activated mast cells. When the Fc ε RI is stimulated, the cytosolic Ca^{2+} is increased in a biphase feature. As shown in Figure 1A, as DNP-BSA was added, the FceRI crosslinking is activated, leading to a transient increase of intracellular Ca^{2+} levels in the absence of extracellular Ca^{2+}. This elevation of Ca^{2+} is due to release of Ca^{2+} from stores in the endoplasmic reticulum induced by inositol 1,4,5-trisphosphate (IP3)[2,17]. But this Ca^{2+} elevation is a transient change, which is not enough for pro-

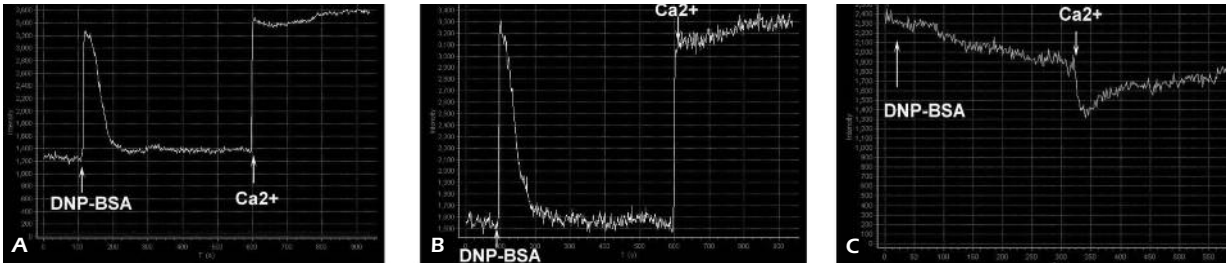

Figure 1. Intracellular Ca^{2+} concentration measured by means of fluorescent indicator Fluo-3. ***A,*** Changes of $[Ca^{2+}]i$ when RBL-2H3 cells were activated. ***B,*** The application of 1‰DMSO did not influence the changes of $[Ca^{2+}]i$ when RBL-2H3 cells were activated. ***C,*** Pre-treatment with 100 μmol/L 2-APB in 1‰ DMSO inhibited Ca^{2+} influx.

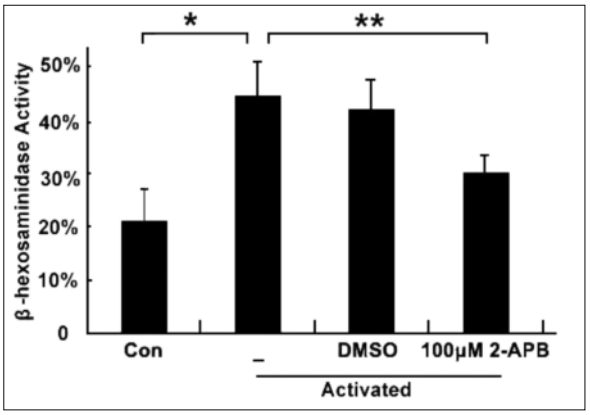

Figure 1. 2-APB treatment suppressed the activity of β-hexosaminidase. The β-hexosaminidase activity was significantly higher in activated group than in control group (*$p < 0.05$); When treated with 100 μmol/L 2-APB, β-hexosaminidase activity was significantly decreased compared to activated group (**$p < 0.05$).

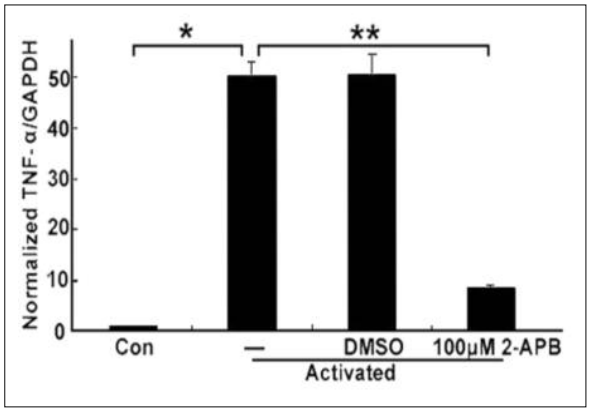

Figure 4. mRNA expression of TNF-α detected by Real-time-PCR. The mRNA levels of TNF-α was significantly increased in activated group than in normal control group (*$p < 0.05$). When treated with 100 μmol/L 2-APB, the mRNA levels for TNF-α was significantly decreased compared to activated group (**$p < 0.05$).

longed reaction. When extracellular Ca^{2+} is added, a sustained elevation of intracellular Ca^{2+} levels is observed (Figure 1A), because depletion of the Ca^{2+} stores activates influx of external Ca^{2+}[17]. For nonexcitable cells such as mast cells, the main routes of Ca^{2+} entry are SOCs or Ca^{2+}-permeable non-selective cation channels[18].

2-APB is a pharmacological blocker extensively used for calcium signals. As shown in Figure 1C, after application of 100 μM 2-APB, the elevation of intracellular Ca^{2+} was suppressed in the absence or presence of extracellular Ca^{2+}. This may be caused by at lease two different mechanisms, including IP3 receptor(IP3R) inhibition and blockade

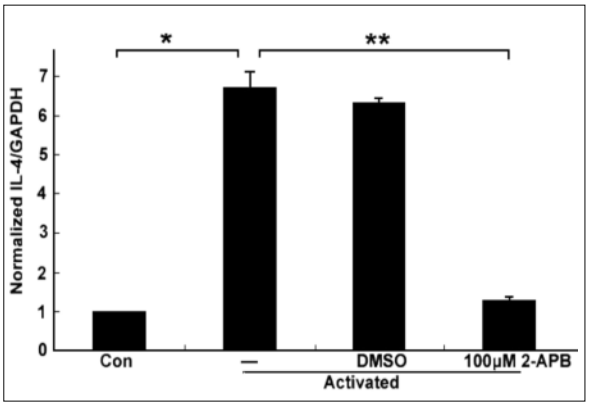

Figure 3. mRNA expression of IL-4 detected by Realtime-PCR. The mRNA levels of IL-4 was significantly increased in activated group than in normal control group (*$p < 0.05$). When treated with 100 μmol/L 2-APB, the mRNA levels for IL-4 was significantly decreased compared to activated group (**$p < 0.05$).

extracellular Ca^{2+} influx mediated by SOCs or Ca^{2+}-permeable non-selective cation channels. These are two independent mechanisms because as reported by Iwasaki et al[19], the activity of IP3Rs is not essential to the generation of SOC-mediated $Ca(^{2+})$ entry. The concentration we used here is 100 μM, because it is reported that the inhibitory effects of 2-APB is in a dose-dependent manner, and a concentration ≤ 50 μM of 2-APB did not inhibit the peak of intracellular Ca^{2+} levels, but only reduced the AUC_{350}; and 100 μM of 2-APB significantly reduced both the AUC_{350} and the Ca^{2+} peak in bovine neutrophils[12,20].

The secretion of β-hexosaminidase is the hallmark of mast cell degranulation[16], and IL-4 and TNF-α are two typical cytokines after mast cells activation. In this study, the addition of antigen to IgE-sensitized RBL-2H3 cells resulted in an increase in β-hexosaminidase activity as well as mRNA expression of IL-4 and TNF-α, and application of 100 μM 2-APB resulted in either reduced β-hexosaminidase activity or IL-4 and TNF-α mRNA expression. These results are consistent with findings of previous research. As a Ca^{2+} signal inhibitor, 2-APB has been reported to suppress reactive oxygen species (ROS) production and matrix metalloproteinase 9 (MMP-9) release after stimulation with platelet-activating factor (PAF)in bovine neutrophils[12]. And *in vivo* study in an allergic rhinitis mice model has found that intranasal administration with 2-APB resulted in improvement of allergic symptoms, decreased infiltration of eosinophils and small lymphocytes in nasal mucosa, reduced production of leukotriene C4 (LTC4), ovalbumin-

specific IgE, and IL-4 in nasal lavage fluid[21]. Taken together, 2-APB may be a useful tool in the resolution of allergic inflammation.

In conclusion, 2-APB inhibits FcεRI-induced degranulation and synthesis of inflammatory cytokines such as IL-4 and TNF-α in rat basophilic leukemia RBL-2H3 cells. Since 2-APB acts on multiple steps in $[Ca^{2+}]i$ supply, further studies are needed to determine the precise source of the $[Ca^{2+}]i$ that is actually involved in the activation process of mast cells. However, our findings suggest that 2-APB is a potential therapeutic strategy for the control of allergy disorders.

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