

Pycnogenol® Inhibits Immunoglobulin E-Mediated Allergic Response in Mast Cells

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IgE-dependent mast cell activation is known to be associated with the allergic diseases. Pycnogenol® (PYC) is a standardized extract of the bark of French maritime pine containing bioflavonoids with a potent antioxidant activity. The antiallergic activity of PYC was evaluated using both *in vivo* and *in vitro* experimental models. Oral administration of PYC significantly inhibited anti-dinitrophenyl (DNP) IgE-mediated passive cutaneous anaphylaxis in rats. In an *in vitro* study, PYC dose-dependently reduced histamine release from rat peritoneal mast cells (RPMC) triggered by anti-DNP IgE. PYC inhibited the protein expression and secretion of tumor necrosis factor- α and interleukin-6 in anti-DNP IgE-stimulated RPMC. Moreover, PYC decreased anti-DNP IgE-induced calcium uptake into RPMC. Furthermore, PYC suppressed nuclear factor-kappa B activation. From these results, the clinical use of PYC in the mast cell-mediated immediate-type allergic diseases is proposed. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: mast cells; allergic diseases; Pycnogenol®; histamine; calcium; nuclear factor-kappa B.

INTRODUCTION

Allergic diseases, such as atopic rhinitis, bronchial asthma and urticaria, are prevalent and increasing in frequency. Mast cells, which are constituents of virtually all organs and tissues, are important mediators of inflammatory responses such as allergic diseases and anaphylaxis (Metcalf *et al.*, 1981). Mast cell activation occurs through Fc ϵ RI, which is the high-affinity surface membrane receptor for IgE. The interaction of antigen-bound IgE and Fc ϵ RI on mast cells induces the release of histamine, prostaglandins, leukotrienes and an array of inflammatory cytokines such as tumor necrosis factor (TNF)- α and interleukins (IL)-4, IL-6, IL-8 and IL-13 (Gordon *et al.*, 1990). Consequently, various acute and chronic allergic responses are induced by these mediators.

Pycnogenol® (PYC), isolated from French maritime pine (*Pinus maritima*) bark extract, is a standardized extract composed of a mixture of flavonoids, including procyanidins and phenolic acids (Sharma *et al.*, 2003). PYC is now utilized throughout the world as a nutritional supplement and as a phytochemical remedy for various diseases ranging from chronic inflammation to circulatory dysfunction. Studies that have been done consistently indicate that this flavonoid mixture has tremendous antioxidant capacity as well as the ability to inhibit capillary permeability and inflammation (Packer *et al.*, 1999).

Although Sharma *et al.* (2003) previously demonstrated that PYC inhibits histamine release from the mast cells

triggered by compound 48/80 or ionophore A23187, the direct effect of PYC on IgE-mediated mast cell activation still remains unknown. In the present study, PYC was tested in a murine model of passive cutaneous anaphylaxis. Next the effect of PYC was evaluated on anti-dinitrophenyl (DNP) IgE-induced histamine release from rat peritoneal mast cells (RPMC). In addition, the protein expression and production of TNF- α and IL-6 were studied using RPMC. Finally, the calcium uptake and nuclear factor-kappa B (NF- κ B) activity were investigated to clarify the mechanism by which PYC inhibited mediator release from RPMC.

MATERIALS AND METHODS

Materials. Pycnogenol® (PYC), isolated from the French maritime pine (*Pinus maritima*), was donated from Horphag Asia Pte. Ltd. (19 Loyang way #08-20, Singapore 508724). Anti-DNP IgE, DNP-human serum albumin (HSA), azelastine, anti-actin antibody and HEPES were purchased from Sigma Chemical Co. (St Louis, MO, USA). Percoll solution was purchased from Pharmacia (Uppsala, Sweden).

Animals. Male Sprague–Dawley rats (8 week old, 230–280 g) were purchased from Damool Science (Daejeon, Korea). Animals were housed 3–5 per cage in a laminar air-flow cabinet maintained at $22 \pm 1^\circ\text{C}$ and relative humidity of $55 \pm 10\%$ throughout the study. All animal experiments were performed in compliance with the NIH guidelines for the care and use of laboratory animals (NIH Publication No. 80-23; revised 1978) and were approved by the Institutional Animal Care and Use Committee of Yanbian University School of Medical Sciences.

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Passive cutaneous anaphylaxis (PCA). Anti-DNP IgE-mediated PCA was examined as reported previously (Neel *et al.*, 2004). Rats were sensitized in the dorsal skin by the intradermal injection of 500 ng anti-DNP IgE. After 24 h, each rat received into the penile vein an injection of 100 µg of antigen (DNP-HSA) with 1% Evans blue. PYC [10, 50, 100 mg/kg body weight (BW)] or 10 mg/kg azelastine (reference drug) was administered orally at 1 h before the antigen challenge. After 30 min, the rats were killed by terminal anaesthesia, tissue sections around the intradermal injection site were excised and weighed, followed by extraction of extravasated Evans blue dye by incubation of biopsies in 1 mL formamide at 55 °C for 24 h and measurement of absorbance at 620 nm using a spectrophotometer (Spectra Max Plus, Molecular Devices, CA, USA). Tissue Evans blue concentrations were quantified by interpolation on a standard curve of dye concentrations in the range of 0.01–30 µg/mL.

Preparation of RPMC. RPMC were isolated as described previously (Cochrane and Douglas, 1974). In brief, rats were anesthetized with ether and injected with 10 mL of calcium-free HEPES-Tyrode buffer into the peritoneal cavity, and the abdomen was gently massaged for about 90 s. The peritoneal cavity was opened, and the fluid was aspirated using a Pasteur pipette, and RPMC were purified by using a Percoll density gradient as described in detail elsewhere (Hachisuka *et al.*, 1988). RPMC preparations were at least 95% pure as assessed by toluidine blue staining and at least 98% of these cells were viable as judged by trypan blue exclusion. Purified mast cells (1×10^6 cells/mL) were resuspended in HEPES-Tyrode buffer.

Mast cell viability assay. To test the viability of cells, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was performed as described previously (Yoshimura *et al.*, 2004). Briefly, RPMC (2×10^5 cells/well) were incubated with various concentrations (10–40 µg/mL) of PYC at 37 °C for 2 h. After addition of MTT [100 µg in 100 µL phosphate buffered saline (PBS)], the cells were incubated at 37 °C for 1 h. The crystallized MTT was dissolved and the absorbance was measured at 570 nm with a spectrophotometer.

Assay of histamine release. RPMC were sensitized with 10 µg/mL anti-DNP IgE for 6 h and preincubated with PYC at 37 °C for 10 min prior to the challenge with DNP-HSA (100 ng/mL). Following centrifugation at $150 \times g$ for 10 min, the amount of histamine in the supernatant was determined by the radioenzymatic method (Harvima *et al.*, 1988). The inhibition percentage of histamine release was calculated using the following formula:

$$\text{Inhibition (\%)} = \{1 - (T - B)/(C - N)\} \times 100$$

Control (C): IgE (+), PYC (–); Normal (N): IgE (–), PYC (–); Test (T): IgE (+), PYC (+); Blank (B): IgE (–), PYC (+).

Assay of TNF- α and IL-6 secretion. RPMC were sensitized with 10 µg/mL anti-DNP IgE for 6 h and then stimulated with 100 ng/mL DNP-HSA for 30 min with or without PYC. TNF- α and IL-6 concentrations in the supernatant were determined by using commercial ELISA kits, according to the manufacturer's instructions

(Invitrogen-Biosource Cytokine & Signaling, Camarillo, CA, USA).

Western blotting analysis. RPMC extracts were prepared by a detergent lysis procedure. Protein concentrations were determined using the Bradford reagent (Bio-Rad, Hercules, CA, USA). Samples (30 µg of protein per lane) were loaded on a 12% SDS-PAGE gel. After electrophoresis at 120 V for 90 min, separated proteins were transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA) by the wet transfer method (250 mA, 90 min). The amount of TNF- α and IL-6 was determined using anti-TNF- α and -IL-6 antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Immunodetection was done using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Measurement of ^{45}Ca uptake. The calcium uptake of mast cells was measured according to the method described by Choi *et al.* (2006). Purified mast cells were resuspended in HEPES-Tyrode buffer containing ^{45}Ca (1.5 mCi/mL; 1 Ci = 3.7×10^{10} becquerels; Perkin-Elmer Life Sciences, MA, USA) and incubated at 4 °C for 10 min. Mast cell suspensions were sensitized with 10 µg/mL anti-DNP IgE for 6 h and preincubated with PYC at 37 °C for 10 min prior to the challenge with DNP-HSA (100 ng/mL). The reaction was stopped by the addition of 1 mM lanthanum chloride. The samples were centrifuged three times at $150 \times g$ at 4 °C for 10 min, and then the mast cells were lysed with 10% Triton X-100 and vigorous shaking. Radioactivity of the solution was measured in a scintillation β -counter (Liquid Scintillation Analyzer, A Canberra Company, Australia).

Cytosolic and nuclear protein extractions for analysis of NF- κB . The RPMC were sensitized with 10 µg/mL anti-DNP IgE for 6 h and preincubated with PYC at 37 °C for 10 min prior to the challenge with DNP-HSA (100 ng/mL). The cells were harvested and washed twice with ice-cold PBS. The washed cell pellets were resuspended in 2 volumes of buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 0.5 mM DTT, 5 mM MgCl_2 , 1 mM PMSF and protease inhibitor cocktails). This suspension was centrifuged at $1000 \times g$ for 15 min at 4 °C. The supernatant fraction was incubated on ice for 10 min and centrifuged at $100\,000 \times g$ for 1 h at 4 °C to obtain cytosolic protein extracts. The pellets were washed twice in buffer A and resuspended in buffer B (1.3 M sucrose, 1.0 mM MgCl_2 and 10 mM potassium phosphate buffer, pH 6.8) and pelleted at $1000 \times g$ for 15 min. The pellets were suspended in buffer B with a final sucrose concentration of 2.2 M and centrifuged at $100\,000 \times g$ for 1 h. The resulting nuclear pellets were washed once with a solution containing 0.25 M sucrose, 0.5 mM MgCl_2 and 20 mM Tris-HCl, pH 7.2, and centrifuged at $1000 \times g$ for 10 min. The pellets were solubilized with a solution containing 50 mM Tris-HCl (pH 7.2), 0.3 M sucrose, 150 mM NaCl, 2 mM EDTA, 20% glycerol, 2% Triton X-100, 2 mM PMSF and protease inhibitor cocktails. The mixture was kept on ice for 1 h with gentle stirring and centrifuged at $12\,000 \times g$ for 30 min. The resulting supernatant was used as the soluble nuclear proteins. For western blotting analysis, samples (30 µg of protein per lane) were loaded on a 12% SDS-PAGE gel. After electrophoresis at 120 V for 90 min, separated proteins were transferred to

polyvinylidene difluoride membranes by the wet transfer method (250 mA, 90 min). The NF- κ B activation was determined using anti-NF- κ B p65 antibody (Upstate Biotech, Lake Placid, NY, USA). Immunodetection was done using an enhanced chemiluminescence detection kit.

Statistical analysis. The results obtained were expressed as mean $\pm$ SEM for the number of experiments. Statistical evaluation of the results was performed using one-way ANOVA, followed by Duncan's multiple range tests. Results with $p < 0.05$ were considered statistically significant.

RESULTS

Effect of PYC on PCA

To confirm the anti-allergic effect of PYC in an *in vivo* model, the PCA model was used. Local extravasation was induced by a local injection of anti-DNP IgE followed by an intravenous antigenic challenge. Oral administration of PYC dose-dependently inhibited anti-DNP IgE-mediated PCA (Table 1). Likewise, azelastine showed significant inhibition of PCA at a dose of 10 mg/kg BW.

Effect of PYC on mast cell viability

The MTT conversion assay was used to determine the viability of RPMC exposed to PYC. There were almost 100% viable cells after exposure to 40 μ g/mL of PYC for 2 h (data not shown). Thus, PYC had no cytotoxicity on RPMC.

Effect of PYC on histamine release from RPMC

To investigate how PYC inhibited PCA, the effect of PYC on IgE-induced mast cell activation was evaluated. As shown in Fig. 1, PYC dose-dependently inhibited anti-DNP IgE-mediated histamine release from RPMC (7%, 42% and 54% inhibition at 10, 20 and 40 μ g/mL, respectively). Azelastine also showed a significant inhibitory potency on histamine release at a dose of 40 μ g/mL (64% inhibition). From these results, it is proposed that PYC inhibits the IgE-induced anaphylactic reaction by blocking histamine release from RPMC.

Table 1. Inhibitory effect of Pycnogenol® on anti-DNP IgE-mediated passive cutaneous anaphylaxis in rats

Treatment (mg/kg BW)	Anti-DNP IgE	Amount of Evans blue (μ g/g)	Inhibition (%)
Normal	–	45.3 $\pm$ 3.8	–
Control	+	256.8 $\pm$ 15.7	–
PYC 10	+	174.3 $\pm$ 11.4 ^a	40.32
50	+	148.6 $\pm$ 12.9 ^a	50.90
100	+	117.9 $\pm$ 10.3 ^a	65.61
Azelastine 10	+	100.4 $\pm$ 6.8 ^a	72.97

PYC was administered orally at 1 h prior to the challenge with antigen (DNP-HSA). Each amount of Evans blue represents the mean $\pm$ SEM of five independent experiments.

^a $p < 0.05$, significantly different from the control value. Azelastine (10 mg/kg) was used as a typical antiallergic control drug.

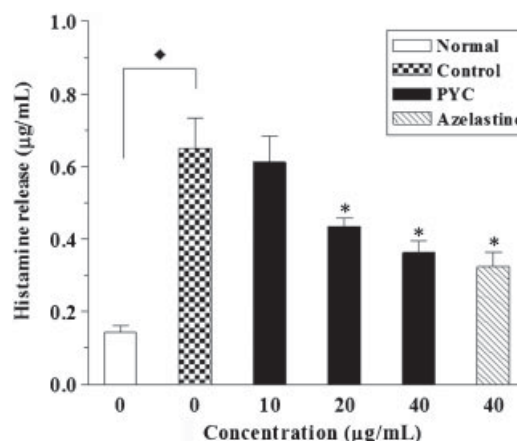


Figure 1. Inhibitory effect of Pycnogenol® (PYC) on anti-DNP IgE-mediated histamine release from rat peritoneal mast cells (RPMC). RPMC were preincubated with PYC at 37 °C for 10 min prior to the incubation with DNP-HSA. Each bar represents the mean $\pm$ SEM of five independent experiments. ♦ $p < 0.05$, when compared between the normal and control value; * $p < 0.05$, significantly different from the control value.

Effect of PYC on anti-DNP IgE-mediated TNF- α and IL-6 expression and secretion

Activation of mast cells also stimulates cytokine release. Thus, we next determined whether PYC could modify the protein expression of cytokines. PYC dose-dependently inhibited the antigen-stimulated protein expression for TNF- α and IL-6 (Fig. 2). To further prove the above results, we tested whether PYC suppressed the secretion of both cytokines in the cells. In agreement with the Western blotting results, a dose-dependent suppression of the production of both cytokines was shown in the presence of PYC together with anti-DNP IgE. PYC significantly inhibited the release of TNF- α and IL-6 at 20 μ g/mL (58% and 40%, inhibition, respectively), and at 40 μ g/mL of PYC, the inhibition was 64% and 45% inhibition, respectively.

Effect of PYC on calcium uptake into RPMC

Calcium uptake has been recognized as one of the chief elements leading to a secretory response of mast cells (McCloskey and Zhang, 2000). Thus, to examine the possible mechanism of PYC on the reduction of mediator release, the levels of calcium uptake were measured. Treatment with PYC alone showed no change in calcium uptake. However, calcium uptake was substantially increased by the stimulation of RPMC with anti-DNP IgE (data not shown). The antigen-elicited calcium uptake was inhibited in a concentration-dependent manner of PYC (Fig. 3). In particular, PYC showed a significant inhibition rate at 20–40 μ g/mL. These results suggest that PYC attenuates mediator release through hindering calcium uptake into RPMC.

Effect of PYC on NF- κ B activity

Western blot analysis revealed that levels of NF- κ B p65 protein in nuclear protein extracts from IgE-stimulated RPMC were increased compared with the levels in the

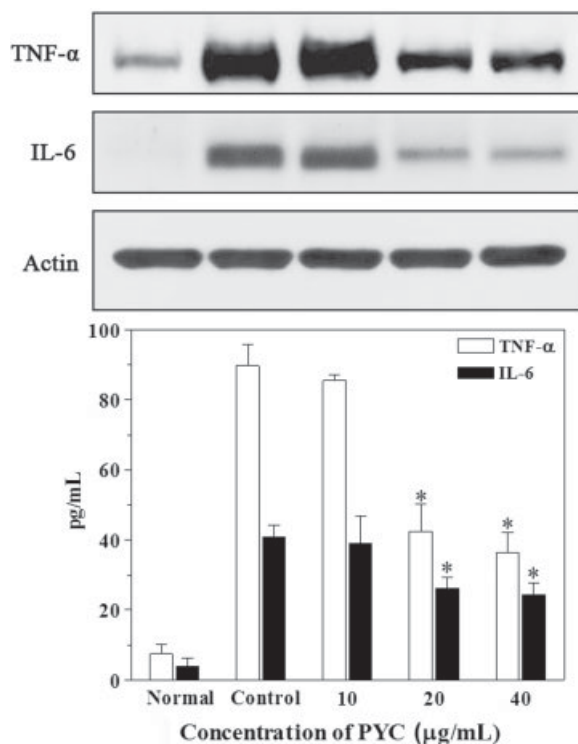


Figure 2. Inhibitory effect of Pycnogenol® (PYC) on the anti-DNP IgE-induced protein expression and production of cytokines (TNF- α and IL-6) in rat peritoneal mast cells (RPMC). RPMC were sensitized with anti-DNP IgE and preincubated with PYC at 37 °C for 10 min before the challenge with DNP-HSA. The protein expression of these cytokines was measured by Western blotting. A representative result is presented from at least five separate experiments with similar results. The level of cytokine in the supernatant was measured by using ELISA. Each bar represents the mean $\pm$ SEM of five independent experiments. * $p < 0.05$, significantly different from the control value.

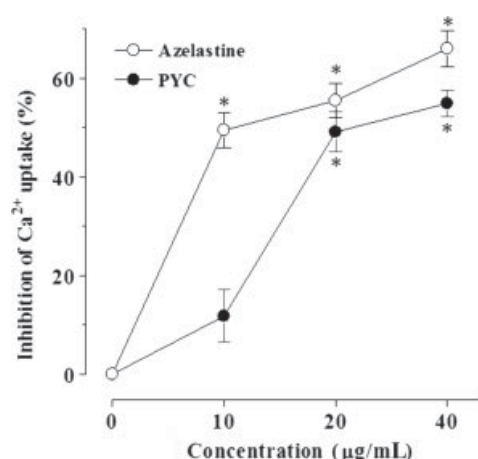


Figure 3. Inhibitory effect of Pycnogenol® (PYC) on anti-DNP IgE-mediated calcium uptake in rat peritoneal mast cells (RPMC). RPMC were sensitized with anti-DNP IgE and preincubated with PYC at 37 °C for 10 min before the challenge with DNP-HSA. Each data value represents the mean $\pm$ SEM of five independent experiments. * $p < 0.05$, significantly different from the control value.

normal group (Fig. 4). The increased NF- κ B p65 levels in nuclear preparations were decreased by the pretreatment of PYC. In contrast, levels of NF- κ B p65 protein in cytosolic protein extracts from IgE-stimulated RPMC were decreased compared with the levels in the normal group (Fig. 4). The decreased NF- κ B p65 levels in

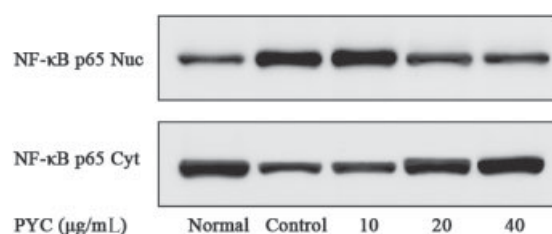


Figure 4. Effect of Pycnogenol® (PYC) on NF- κ B expression in nuclear and cytosolic protein extracts from anti-DNP IgE-stimulated rat peritoneal mast cells (RPMC). NF- κ B p65 expression in nuclear (Nuc) and cytosolic (Cyt) protein extracts from RPMC is shown. A representative result is presented from at least five separate experiments with similar results.

cytosol preparations were increased by the pretreatment of PYC. These results indicate that PYC inhibits NF- κ B activity by preventing translocation of this transcription factor into the nucleus.

DISCUSSION

The PCA animal model used here is a well-established model for evaluating localized mast cell-mediated allergic reactions *in vivo* (Kabu *et al.*, 2006). The present results revealed that PYC effectively suppressed the local allergic reaction in this model in a dose-dependent manner. In view of the inhibitory effect of PYC on PCA *in vivo*, its effect on RPMC triggered by IgE was examined. PYC dose-dependently inhibited antigen-induced histamine release from RPMC, which is in agreement with the *in vivo* effect. Here the question remains whether the tissue concentrations of PYC after administration of 10–100 mg/kg PYC are sufficient to suppress mast cell activation, namely 20–40 μg/mL PYC *in vitro* (Table 1, Fig. 1). However, as pharmacokinetic data in rats are limited, the bioavailability and bioefficacy of PYC remain to be explored. Accordingly, it is inferred that PYC inhibits the immediate-type allergic reaction through interference with the degranulation system.

The mast cell-derived pro-inflammatory cytokines, particularly TNF- α and IL-6, play a key biological role in the allergic reaction. TNF- α has been found to be prestored in the cytoplasmic granules and is released with histamine and other preformed mediators within minutes of antigenic stimulation. This suggests a potential role in the early inflammation (Thomas, 2001). It is also clear that the production of TNF- α has a detrimental role in allergic disorders, as a mediator of the late-phase inflammatory reaction (Gordon *et al.*, 1990). IL-6 is a pleiotropic inflammatory cytokine produced by mast cells, monocytes, macrophages and T cell, and plays a major role in cellular activation (Conti *et al.*, 2002). Moreover, its local accumulation is associated with a PCA reaction (Mican *et al.*, 1992). Therefore, modulation of these cytokines secreted from mast cells can provide a useful therapeutic strategy for allergic inflammatory disease. The present work has demonstrated that PYC inhibits the protein expression and secretion of TNF- α and IL-6 in RPMC stimulated by IgE. From this, it is postulated that an antiallergic effect of PYC is the result of its ability to decrease the level of TNF- α and IL-6 generation from mast cells.

To gain insight into the mechanism of PYC inhibition of mediator release from mast cells, its effects on calcium signaling and NF- κ B activation in RPMC were investigated. As noted, calcium uptake is a key modulatory element in Fc ϵ RI-induced degranulation of mast cells (Janiszewski *et al.*, 1992). As expected from the inhibition of degranulation and cytokine production by PYC, PYC caused a dose-dependent decrease in antigen-evoked influx of extracellular calcium into RPMC. This observation encourages the view that PYC reduces the release of mediators by attenuating calcium uptake into mast cells.

Previous reports have shown that NF- κ B plays a critical role in immune and inflammatory responses (Siebenlist *et al.*, 1994). Activation of NF- κ B has also been observed after Fc ϵ RI-mediated allergic response in mast cells (Klemm and Ruland, 2006). Determination of the NF- κ B protein level in nuclear extracts has revealed that this protein level is substantially increased in IgE-stimulated RPMC, suggesting that NF- κ B is activated. The pretreatment of PYC results in a significant reduction of nuclear NF- κ B level as well as the expression

of cytokines. Because it is known that expression of pro-inflammatory cytokines is regulated through NF- κ B expression (Azzolina *et al.*, 2003), these results strongly suggest that PYC inhibits cytokine production in allergic inflammation by decreasing the activity of NF- κ B.

In conclusion, this study has revealed that PYC attenuates IgE-mediated allergic response by inhibiting the release of histamine and pro-inflammatory cytokines from mast cells; this PYC inhibition of mediator release appears to be involved in the suppression of calcium uptake and NF- κ B activity in mast cells. These reports indicate that PYC may be promising as an adjunctive treatment for mast cell-dependent allergic diseases, while the results presented here need further evaluation in other clinically relevant animal models and in humans.

Acknowledgements

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