



Enhancement of allergic responses in vivo and in vitro by butylated hydroxytoluene

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Abstract

The effect of butylated hydroxytoluene (BHT), which is used widely as an antioxidant, on IgE-dependent allergic responses in vivo and in vitro was investigated. For in vivo study, passive cutaneous anaphylaxis (PCA) was elicited in rats by i.d. injection of anti-DNP IgE and 48 h later by i.v. injection of DNP-HSA. BHT was i.p. given immediately after anti-DNP IgE injection. For in vitro studies, the rat mast cell line RBL2H3 sensitized with monoclonal anti-dinitrophenol (DNP) IgE was challenged with the multivalent antigen DNP-human serum albumin (DNP-HSA) in the presence or absence of BHT. β -Hexosaminidase and histamine released from RBL2H3 cells, as indicators of degranulation of the cells, the concentration of intracellular Ca^{2+} , the level of phosphorylated-Akt, and global tyrosine phosphorylation as indicators of mast cell activation, were measured. The results showed that BHT given to anti-DNP IgE-sensitized rats augmented DNP-specific PCA in a dose-dependent manner. In the presence of BHT, IgE-induced releases of β -hexosaminidase and histamine from RBL2H3 cells were increased. BHT also further elevated IgE-mediated increased concentrations of intracellular Ca^{2+} and the levels of phosphorylated-Akt, but did not affect global tyrosine phosphorylation, in RBL2H3 cells. Moreover, the PI3K inhibitor LY294002 inhibited IgE-dependent degranulation and its enhancement by BHT. These findings indicate that BHT may upregulate PCA by enhancing mast cell degranulation associated with enhancements of intracellular Ca^{2+} concentration and PI3K activation, suggesting that BHT might affect allergic diseases such as allergic rhinitis and asthma.

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Introduction

The type I allergy is induced by the interaction between antigen and antigen-specific IgE and is seen in allergic rhinitis, atopic dermatitis and asthma. This isotype of antibody binds to IgE receptor (Fc ϵ RI) on the surface of mast cells and basophils and upon exposure to the antigen these cells release various mediators such as histamine, serotonin and enzymes including β -hexosaminidase. These molecules play a role in the appearance of major symptoms of allergic diseases such as vasodilation, mucous secretion, itching, and bronchoconstriction.

Recently, a number of studies have suggested that allergic diseases have become more common throughout the world. Etiological studies have demonstrated that exposure to environmental factors such as artificial chemical compounds may be linked to increases in the incidence and the severity of allergic diseases.

Butylated hydroxytoluene (BHT) is widely used as an antioxidant additive in foods, drugs, and cosmetics and thus absorbed by human via many tissues including digestive organs, airways, and skins. Considering the increase in BHT exposure by humans, concern over the safety of BHT has been growing. Over the past few decades, some biological activities of BHT have been investigated. BHT has hepatic toxicity in rats when administered orally (Nakagawa et al., 1984), and the compound acts as a tumor promoter in animals treated with tumor initiators

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including urethane and 3-methylcholanthrene (Witschi et al., 1977; Malkinson et al., 1997). Lung inflammation is induced in mice by intraperitoneal injection of BHT (Adamson et al., 1977; Bauer et al., 2001). In a clinical study, it was demonstrated that BHT exacerbates a chronic urticaria which is partly mediated by type I allergy (Goodman et al., 1990), although the mechanisms of the exacerbation of disease by this compound have not been clarified. Furthermore, there have been few *in vivo* studies demonstrating the effect of BHT on type I allergy in detail. From *in vitro* studies, enhancement of 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-dependent, but not IgE-mediated, degranulation by BHT may depend on promotion of an intracellular Ca^{2+} mobilization in the mast cell line RBL2H3 (Akasaka et al., 1996), but the mechanism by which it does so remains to be fully elucidated.

Therefore, in the present study, to clarify whether BHT modulates allergic responses *in vivo* and *in vitro*, we tested the effect of BHT on passive cutaneous reaction (PCA) in rats and IgE-dependent degranulation, Ca^{2+} mobilization, the level of phosphorylated-Akt and global tyrosine phosphorylation of RBL2H3 cells, respectively.

Methods

Materials. Male Sprague–Dawley rats were purchased from Japan SLC, Inc., Shizuoka, Japan. The animals were maintained in a temperature-controlled environment with free access to standard rodent chow and water. All procedures in animal experiments were approved by the Animal Ethics Committee of Kobe Pharmaceutical University. RBL2H3 cells were supplied by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan.

Induction of PCA. One hundred microliters of diluted cell culture medium containing 4, 8 and 20 ng of dinitrophenol (DNP)-specific IgE (DNP-specific IgE-producing hybridoma was kindly supplied by Dr. Kazutaka Maeyama, Ehime University, Ehime, Japan) was i.d. injected into the backs of rats. The rats were then administered i.p. with 10 ml of PBS containing 1% ethanol with the indicated doses of BHT (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The control rats received the same volume of PBS containing 1% ethanol. Forty-eight hours later, 1 ml of PBS containing 0.5% Evans blue and 1 mg of DNP-HSA was i.v. injected. Thirty minutes later, the blue areas of skin sample were removed and extravasated blue dye was measured by the method used previously (Inagaki et al., 1988) with some modifications. In brief, the rats were anesthetized by ether and euthanized by cutting the carotid artery. Each two-centimeter diameter area of skin was dissolved with 2.8 ml of 1 M KOH at 37 °C overnight. Then, a mixture of 1.032 ml of 0.028 M H_3PO_4 and 6.72 ml of acetone was added to the dissolved skin. After vigorous shaking, the precipitates were removed following centrifugation at 1700×g for 5 min, and the amount of dye extracted was measured at 620 nm. No obvious extravasation of Evan's blue dye was observed in anti-DNP IgE-sensitized, BHT-treated, non-challenged rats. Anti-DNP IgE-sensitized, OVA challenged rats and non-specific IgE-sensitized, DNP-HSA-challenged rats also showed no remarkable responses.

Degranulation of RBL2H3 cells. RBL2H3 cells were maintained in 5% CO_2 at 37 °C in minimum essential medium (Invitrogen Japan K.K., Tokyo, Japan) containing 10% (v/v) fetal bovine serum (Invitrogen Japan K.K.), 100 units of penicillin and 100 units of streptomycin (Invitrogen Japan K.K.). The cells were harvested by trypsin (0.25%, w/v) and EDTA (0.01%, w/v) in phosphate-buffered saline and suspended at 0.25×10^6 cells/ml in medium containing 0.5% (v/v) conditioned culture medium of dinitrophenol (DNP)-specific IgE-producing hybridoma. Five hundred microliters of the cell suspension was added to each well of 24-well cluster dishes and cultured for 24 h. The cells were washed with PIPES buffer (25 mM PIPES (pH 7.2), 119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM

MgCl_2 , 1 mM CaCl_2 and 0.1% (w/v) bovine serum albumin) (Hirasawa et al., 1995) and stimulated for 20 min or for the indicated periods with DNP-human serum albumin (HSA) (Sigma-Aldrich Fine Chemicals, St. Louis, MI, USA) at a concentration of 50 ng/ml with the indicated concentrations of BHT (Wako Pure Chemical Industries, Ltd.). Varying doses of BHT were dissolved in ethanol and diluted with PIPES buffer. β -Hexosaminidase and histamine in the cultured supernatant were determined by the colorimetric assay using *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide (Sigma-Aldrich Fine Chemicals) and by the fluorometric assay using *o*-phthalaldehyde (Sigma-Aldrich Fine Chemicals).

When the effect of LY294002 (Sigma-Aldrich Fine Chemicals), a PI3K inhibitor, on degranulation is examined, the anti-DNP IgE-sensitized cells were pretreated for 15 min with the indicated concentrations of LY294002 and then stimulated by DNP-HSA with the inhibitor.

In case of using ionomycin (Sigma-Aldrich Fine Chemicals), harvested cells were incubated without anti-DNP IgE for 24 h. Then the indicated concentrations of ionomycin were added to the cells simultaneously with BHT.

Cytotoxicity assay for BHT-treated RBL2H3 cells using MTT tetrazolium salt. After treatment of RBL2H3 cells with BHT and DNP-HSA, the cells were washed with the minimum essential medium containing 10% (v/v) fetal bovine serum, 100 units of penicillin and 100 units of streptomycin prewarmed. The cells were further incubated for 4 h with the medium containing MTT (Sigma-Aldrich Fine Chemicals). After removal of the medium, the resultant colored products were dissolved in DMSO, and the absorbance at 562 nm was determined (Mosmann, 1983).

Measurement of intracellular Ca^{2+} concentration. Measurement of intracellular Ca^{2+} concentrations was performed using the calcium-reactive fluorescence probe, fluo-3 (Kao et al., 1989). Briefly, RBL2H3 cell suspensions (6×10^6 cells/ml in PIPES buffer) were incubated with the minimum essential medium containing 10% (v/v) fetal bovine serum, 100 units of penicillin, 100 units of streptomycin, 0.5% (v/v) conditioned culture medium of anti-DNP IgE-producing hybridomas and 4 μM fluo-3-AM (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 30 min at 37 °C and then washed twice with and resuspended in the PIPES buffer. The cells were stimulated with DNP-HSA and the indicated concentrations of BHT at 0 min and monitored fluorescence until 90 s (excitation and emission at 506 and 526 nm, respectively). $[\text{Ca}^{2+}]_i$ was calculated using the equation: $[\text{Ca}^{2+}]_i = K_d(F - F_{\min}) / (F_{\max} - F)$, where K_d is the dissociation constant of Ca^{2+} -fluo-3 complex (450 nM) (Suzuki et al., 2002). $F_{\max}$ represents the maximum fluorescence (obtained by treating cells with 0.1% Triton X-100), and $F_{\min}$ represents the minimum fluorescence (obtained from 0.1% Triton X-100-treated cells in the presence of 5 mM EGTA). F is actual sample fluorescence.

Immunoblotting. After the stimulation, the cells in a 60 mm dish were lysed in 0.18 ml of ice-cold lysis buffer (20 mM PIPES, pH 8.0, 1% (v/v) Triton X-100, 1 mM EDTA, 50 mM NaF, 2.5 mM *p*-nitrophenyl phosphate, 1 mM Na_3VO_4 , 0.02 mg/ml leupeptin and 10% (v/v) glycerol). The proteins in the cell lysate were separated by SDS–PAGE and transferred onto a PVDF membrane (ATTO, Tokyo, Japan). The phosphorylated-Akt and total-Akt were detected by immunoblotting using polyclonal antibodies for phospho-Akt (Ser473) and Akt (Cell Signaling Technology, Beverly, MA, USA), respectively. The relative ratios of phospho-Akt to the corresponding Akt are determined by densitometric analysis. The level of global tyrosine phosphorylation was detected by using monoclonal antibodies for phosphorylated tyrosine (BioLegend, San Diego, CA, USA).

Statistics. To analyze data statistically, ANOVA (Tukey) test and Mann–Whitney *U* test were used for multiple comparisons and unpaired observations, respectively.

Results

Effect of BHT on PCA in rats

The effect of BHT on anti-DNP IgE-dependent anaphylactic reactions *in vivo*, i.e., a model of PCA in rats, was tested. As

shown in Fig. 1, treatment of the animals with BHT significantly enhanced PCA at the dose(s) of 1 and/or 10 mg/kg when medium containing 4, 8, or 20 ng of anti-DNP IgE was injected i.d.

Effect of various doses of BHT on anti-DNP-specific IgE-dependent and ionomycin-dependent degranulation of RBL2H3 cells

To address the question of how BHT enhances type I allergic reaction in vivo, we tested the effect of BHT on IgE-mediated degranulation response of RBL2H3 cells. IgE-dependent mast cell degranulation was accomplished in two phases, IgE-sensitizing and subsequent antigen-challenging. In this study, since we intended to investigate the effect of BHT on the antigen-challenging phase, BHT was added simultaneously

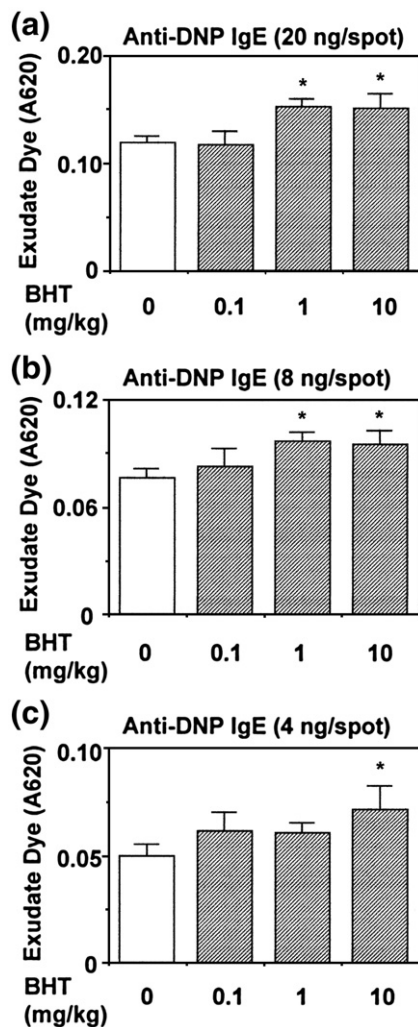


Fig. 1. Enhancement of PCA reactions in rats by BHT. Conditioned culture medium containing 20 ng (a), 8 ng (b) and 4 ng (c) of anti-DNP IgE was i.d. injected into the back of rats. The indicated doses of BHT were i.p. injected immediately after injection of the antibodies. Forty-eight hours later, rats were i.v. challenged with 1 mg of DNP-HSA dissolved in 0.5% of Evans blue dye as described in Methods. Bars show the mean $\pm$ SEM of 4 rats. * p < 0.05 vs. control treatment.

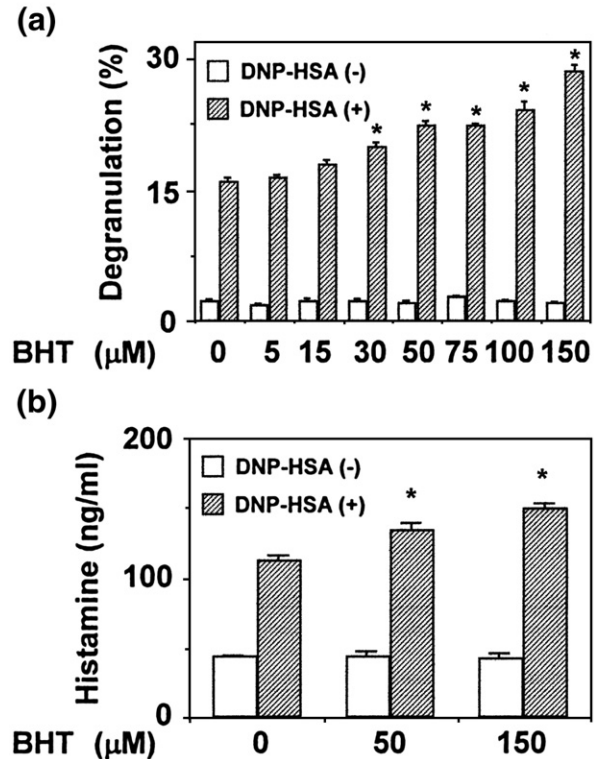


Fig. 2. Effect of BHT on the FcεRI-mediated release of β -hexosaminidase and histamine from RBL2H3 cells. RBL2H3 cells (2.5×10^5 cells/well) were incubated for 24 h in the medium containing anti-DNP IgE. The cells were washed and then incubated with PIPES buffer containing DNP-HSA (50 ng/ml) in the presence or absence of the indicated doses of BHT. After 20 min, the releases of β -hexosaminidase (a) and histamine (b) from the cells were measured. * p < 0.05 vs. absence of BHT. Bars show mean $\pm$ SEM of triplicated cultures of the cells. Data are representative of three experiments.

with DNP-HSA to IgE-sensitized RBL2H3 cells. For the determination of degranulation of RBL2H3 cells, β -hexosaminidase released from the cells was measured. Treatment with BHT increased FcεRI-mediated β -hexosaminidase release in a dose-dependent manner (Fig. 2a). Significant increases in β -hexosaminidase were observed at 30 μ M and higher doses of BHT. The release of the enzyme from RBL2H3 cells treated with 150 μ M of BHT was 60% greater than the control. To confirm the increased effect of BHT on degranulation, the release of histamine, another indicator of degranulation, was also measured. When the cells were treated with 50 and 150 μ M of BHT, significantly enhanced FcεRI-mediated release of histamine was seen (Fig. 2b). BHT itself failed to affect the release of either β -hexosaminidase or histamine from RBL2H3 cells.

The viability of RBL2H3 cells assayed using MTT was not affected by the treatment with 150 μ M of BHT in the presence (without BHT = 1.39 ± 0.0129 (A562) vs. 150 μ M BHT = 1.45 ± 0.0518) or absence (without BHT = 1.41 ± 0.0373 vs. 150 μ M BHT = 1.44 ± 0.00608) of DNP-HSA, suggesting that the enhanced release of the granule components by BHT was not induced by its cytotoxicity. BHT had the obvious cytotoxic effect at the concentration above 300 μ M (data not shown).

The calcium ionophore ionomycin, which bypasses FcεRI signaling, induced degranulation of RBL2H3 cells (Fig. 3). The simultaneous addition of BHT enhanced the ionomycin-induced degranulation of RBL2H3.

Effect of various doses of BHT on the concentration of intracellular Ca^{2+} in RBL2H3 cells

To clarify the mechanism underlying the enhancement of degranulation response by BHT, the concentration of intracellular Ca^{2+} in RBL2H3 cells was measured. The concentration of intracellular Ca^{2+} in RBL2H3 cells began to increase from 30 s after DNP-HSA stimulation, reaching a peak at 60 s (Fig. 4a). BHT itself induced only mild and transient increases in intracellular Ca^{2+} concentration. In the presence of 150 μM of BHT, the concentration of intracellular Ca^{2+} began to increase from 15 s after antigenic challenge, reaching a peak at 50 s. The facilitation of the FcεRI-mediated increase of intracellular Ca^{2+} concentration by BHT was dose-dependent, as shown in Fig. 4b.

Effect of various doses of BHT on the level of phosphorylated-Akt and global tyrosine phosphorylation in RBL2H3 cells

Protein tyrosine kinases and PI3K are responsible for FcεRI-mediated Ca^{2+} mobilization in mast cells (Zhang et al., 2002; Ching et al., 2001). Thus, it is presumed that BHT promotes FcεRI-mediated Ca^{2+} mobilization via modulations of protein tyrosine kinase and PI3K activities. To further address the question of how BHT increases the intracellular Ca^{2+} level, we tested the effects of BHT on the level of phosphorylated-Akt, as an indicator of PI3K activity, and global tyrosine phosphorylation. When RBL2H3 cells were incubated in the presence of BHT, the level of phosphorylated-Akt in the cell began to increase at 2 min, and thereafter increased time-dependently up to 20 min (Fig. 5a). FcεRI-

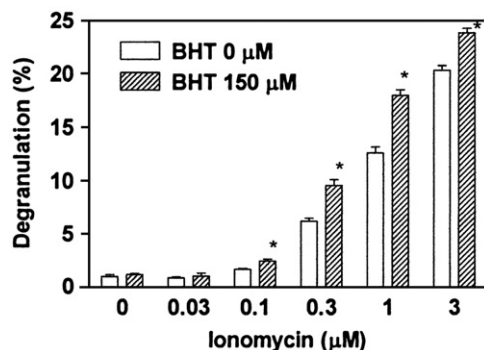


Fig. 3. Effect of BHT on the ionomycin-induced release of β -hexosaminidase from RBL2H3 cells. RBL2H3 cells (2.5×10^5 cells/well) were incubated for 24 h in the medium. The cells were washed and then incubated with PIPES buffer containing the indicated concentrations of ionomycin in the presence or absence of BHT (150 μM). After 20 min, the release of β -hexosaminidase from the cells was measured. * $p < 0.05$ vs. absence of BHT. Bars show mean $\pm$ SEM of triplicated cultures of the cells. Data are representative of three experiments.

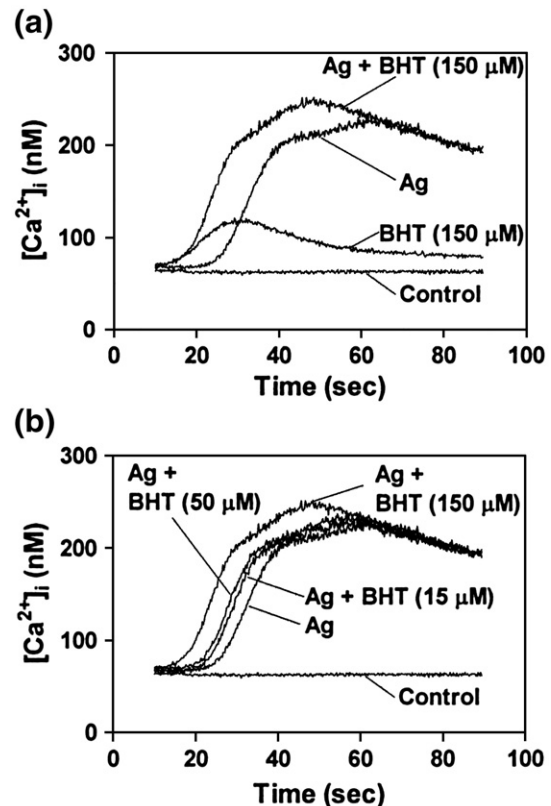


Fig. 4. BHT enhances the FcεRI-mediated increase of the concentration of intracellular Ca^{2+} in RBL2H3 cells. RBL2H3 cells (6×10^6 cells) were incubated in the medium containing anti-DNP IgE and 4 μM fluo-3-AM at 37 °C for 30 min. The cells were then resuspended in the PIPES buffer and stimulated by DNP-HSA (Ag; 50 ng/ml) in the absence or presence of 150 μM (a) or the indicated doses (b) of BHT. Data are representative of three experiments.

mediated activation by antigen also increased the level of phosphorylated-Akt from 2 min after stimulation and the level was further increased until 20 min. The FcεRI-mediated increase of the level of phosphorylated-Akt was facilitated in the presence of BHT all the time we measured. Next, RBL2H3 cells were incubated for 10 min in the presence of various concentrations of BHT and antigen. The level of phosphorylated-Akt was increased by BHT in a concentration-dependent manner up to 150 μM (Fig. 5b), and the FcεRI-mediated increase of the level of phosphorylated-Akt was facilitated by the drug dose-dependently.

In contrast to its ability to induce Akt phosphorylation, BHT failed to affect the level of global tyrosine phosphorylation until 20 min (Fig. 6a). FcεRI-mediated increase of the level of global tyrosine phosphorylation (at least three proteins, 100 kDa, 70 kDa and 40 kDa, indicated by arrows in Fig. 6) was observed from 2 min after stimulation, and the level reached a maximum at 5 min. BHT also failed to affect the FcεRI-mediated increase of the level of global tyrosine phosphorylation until 20 min. Even when RBL2H3 cells were incubated in the presence of various concentrations of BHT up to 150 μM for 5 min, the global tyrosine phosphorylation and FcεRI-mediated increase of the global tyrosine phosphorylation were not affected (Fig. 6b).

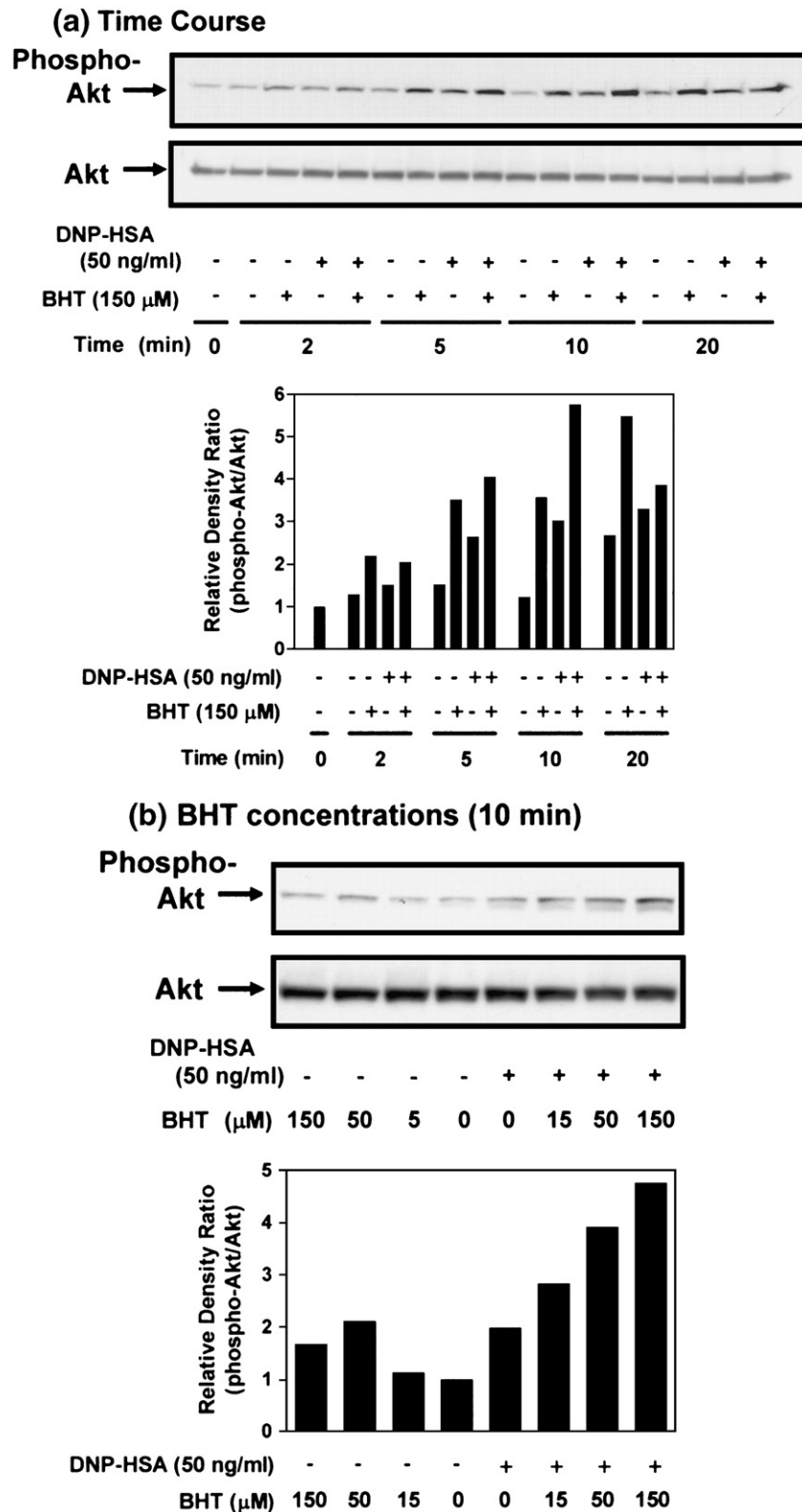


Fig. 5. Effect of BHT on the Fc ϵ RI-mediated phospho-Akt level increase in RBL2H3 cells. RBL2H3 cells (7.5×10^5 cells) were sensitized by anti-DNP IgE for 24 h. The cells were stimulated by DNP-HSA (50 ng/ml) with BHT (150 μ M) for the indicated periods (a) or stimulated by DNP-HSA (50 ng/ml) with the indicated concentrations of BHT for 10 min (b). Phospho-Akt and Akt levels expressed in RBL2H3 were determined by Western blot. The columns in the graphs indicate the relative density ratio of the phospho-Akt to the corresponding Akt as determined by densitometric analysis.

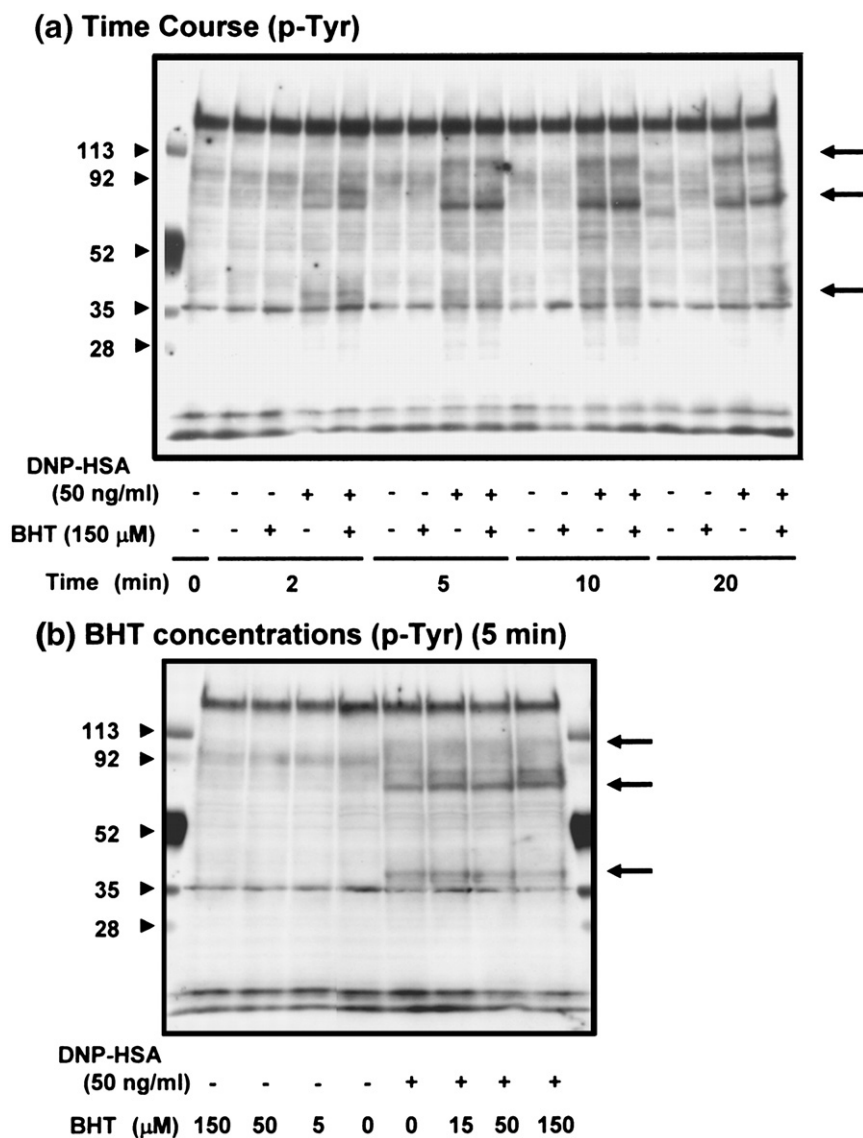


Fig. 6. Effect of BHT on the Fc ϵ RI-mediated global tyrosine phosphorylation in RBL2H3 cells. RBL2H3 cells (7.5×10^5 cells) were sensitized by anti-DNP IgE for 24 h. The cells were stimulated by DNP-HSA (50 ng/ml) with BHT (150 μ M) for the indicated periods (a) or stimulated by DNP-HSA (50 ng/ml) with the indicated concentrations of BHT for 5 min (b). Whole cell lysates were blotted with the anti-phosphotyrosine antibody PY20. Arrows indicate the protein phosphorylated obviously by Fc ϵ RI-mediated stimulation.

Effect of LY294002, a PI3K inhibitor, on anti-DNP-specific IgE-dependent degranulation and its enhancement by BHT of RBL2H3 cells

The involvement of PI3K activation in Fc ϵ RI-mediated degranulation and its enhancement by BHT were investigated pharmacologically by the PI3K inhibitor LY294002. Pretreatment of LY294002 strongly inhibited both IgE-dependent degranulation and its enhancement by BHT in a dose-dependent manner (Fig. 7).

Discussion

In this study, we demonstrated for the first time that PCA, a type I allergic reaction in vivo, mediated by DNP-specific IgE in rats was increased following treatment with BHT. Our results

also showed that treatment with BHT of the mast cell line RBL2H3 sensitized with anti-DNP IgE and then challenged with DNP-HSA resulted in the enhancement of the release of β -hexosaminidase and histamine from the cells. Furthermore, we firstly showed that BHT facilitated Fc ϵ RI-mediated calcium mobilization and PI3K activation.

The augmentation of DNP-specific PCA reactions in rats by BHT (Fig. 1) suggested that the chemical has stimulatory effects on antigen-specific IgE-mediated allergic reactions in vivo. This result supports the finding by Goodman et al. (1990) that patients with chronic urticaria given foods with BHT had significantly greater exacerbation of disease compared to those fed without BHT.

Release of chemical mediators such as histamine, serotonin and leukotrienes by degranulation of mast cells results in type I allergic reactions in vivo. Our results showed that BHT en-

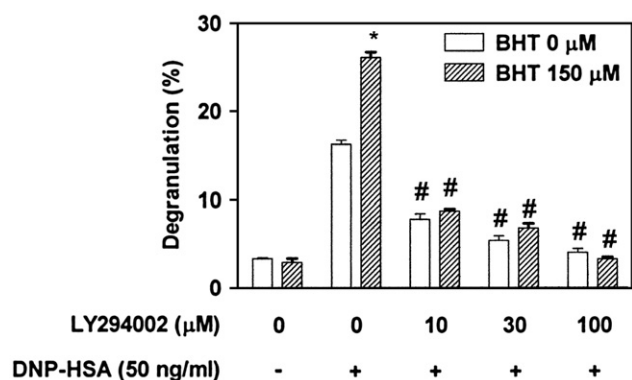


Fig. 7. Effect of LY294002, a PI3K inhibitor, on the FcεRI-mediated release of β-hexosaminidase from RBL2H3 cells and its enhancement by BHT. RBL2H3 cells (2.5×10^5 cells/well) were incubated for 24 h in the medium containing anti-DNP IgE. The cells were washed and then incubated with PIPES buffer containing the indicated concentrations of LY294002. The cells were washed again and then stimulated DNP-HSA (50 ng/ml) in the presence or absence of BHT (150 μM) and LY294002. After 20 min, the release of β-hexosaminidase from the cells was measured. * $p < 0.05$ vs. absence of BHT, # $p < 0.05$ vs. absence of LY294002. Bars show mean $\pm$ SEM of triplicated cultures of the cells. Data are representative of three experiments.

hanced FcεRI-mediated degranulation of RBL2H3 cells (Figs. 2a, b), which was well correlated with the promoting effect of BHT on IgE-dependent PCA reactions in vivo. These observations suggested that BHT might enhance the FcεRI-mediated PCA reaction by exacerbation of mast cell degranulation in vivo.

The enhancement of degranulation of RBL2H3 cells by BHT was supported by the result that the concentration of Ca^{2+} in the cells was increased following treatment with the antioxidant (Fig. 4) since increased intracellular concentrations of Ca^{2+} are correlated with activation of mast cells required for degranulation (Beaven et al., 1984). The enhancement of degranulation of RBL2H3 cells by BHT may be also in part due to the facilitation by the agent of activation of c-Jun amino-terminal kinase (JNK) (Shrivastava and Aggarwal, 1999) and protein kinase C (PKC) (Ruzzene et al., 1991) that are involved in the induction of degranulation of mast cells (Ishizuka et al., 1999; Cho et al., 2004).

It is unlikely that BHT itself caused degranulation of RBL2H3 cells by its direct effect on the cells without interaction between anti-DNP IgE and DNP-HSA since the levels of β-hexosaminidase and histamine were not affected when the antioxidant alone was added to the cells. Marked enhancement by BHT of the release of these mediators and the cell activation was seen only when this chemical was given to RBL2H3 cells sensitized with anti-DNP IgE and then challenged with DNP-HSA. In contrast, the addition of ionomycin, a calcium ionophore (Liu and Hermann, 1978), alone to RBL2H3 cells induced degranulation of the cells (Fig. 3). The mechanism that defines the differences in the effect on degranulation between BHT and ionomycin is unknown. However, BHT has been shown to increase the level of Ca^{2+} in RBL2H3 cells via its inhibition of activation of Ca^{2+} -ATPase in the endoplasmic reticulum (Hughes et al., 2000) that

plays a role in the termination of cell activation by removing activator Ca^{2+} from the cytosol. It was also revealed in this study that BHT increases the intracellular Ca^{2+} level via activation of PI3K-dependent signaling (discussed below). On the other hand, ionomycin augments the intracellular Ca^{2+} level via enhancement of a Ca^{2+} influx into RBL2H3 cells by increasing the membrane permeability of Ca^{2+} (Penner and Neher, 1988) and activation of a Ca^{2+} release-activated calcium channel (CRAC) by depleting intracellular Ca^{2+} (Nakata and Hide, 1998). The result that BHT enhanced ionomycin-induced degranulation (Fig. 3) also supported the consideration that ionomycin and BHT modified degranulation response via different signaling pathways. Therefore, the difference in the effect on the intracellular Ca^{2+} concentration and degranulation between BHT and ionomycin may be in part explained by the mechanism of activation of RBL2H3 cells between these chemicals, although more extensive studies are required for the explanation.

In contrast to our results, Akasaka et al. (1996) showed that BHT failed to affect IgE-mediated degranulation of RBL2H3 cells, although other phenolic antioxidants including 2,5-di(*tert*-butyl)-1,4-hydroquinone (DTBHQ) and 2,5-di(*tert*-amyl)-1,4-hydroquinone (DTAHQ) enhanced the cell degranulation. Interestingly, these studies demonstrated that TPA-induced degranulation of RBL2H3 cells was facilitated by BHT. The difference in the effect of BHT on IgE-dependent degranulation between their and our studies may be in part explained by the difference in timing of treatment with and in doses of BHT used for experiments between their and our groups. In our experiments showing increased degranulation following treatment with 30 μM and higher doses of BHT, RBL2H3 cells were sensitized with anti-DNP IgE for 24 h then washed and challenged by DNP-HSA with 5 to 150 μM of BHT. On the other hand, Akasaka et al. (1996) sensitized RBL2H3 cells with anti-DNP IgE for 1 h then washed and added with 1, 10 and 50 μM of BHT. After the pretreatment of BHT for 10 min, without removing BHT, the cells were challenged by DNP-HSA. The results were unconvincing since 1 and 50 μM of BHT suppressed degranulation while 10 μM of BHT promoted degranulation. Since pretreatment of 50 μM of BHT inhibited, rather than enhanced, subsequent IgE-dependent activation of mast cells, it may be that calcium mobilization by pretreatment of BHT makes the cells hypo-responsive to subsequent calcium mobilizing stimulation by antigen. In contrast, our treatment of the cells with relatively high concentrations of BHT, such as 150 μM, simultaneously with the antigen could induce strong synergistic effects on mobilization of calcium and degranulation response between BHT and FcεRI-mediated stimulation.

We next investigated the effect of BHT on PI3K activation and global tyrosine phosphorylation which are the FcεRI-mediated events responsible for calcium mobilization. Our results showed that BHT increased the level of phosphorylated-Akt obviously and exacerbated FcεRI-mediated increase in the level of phosphorylated-Akt (Figs. 5a, b). Additionally, PI3K activation was essential for FcεRI-mediated degranulation and its enhancement by BHT (Fig. 7). In contrast, our results demonstrated that BHT failed to affect global tyrosine phos-

phorylation regardless of FcεRI stimulation (Fig. 6). Furthermore, BHT could not affect the phosphorylation level of the band at about 70 kDa molecular weight in Figs. 5a and b, which may result from the phosphorylation of Syk, as suggested in a previous report (Moriya et al., 1997). Recently, Parravicini et al. (2002) demonstrated that Fyn, not Syk, is required for FcεRI-mediated Gab2/PI3K activation. More recently, Yu et al. (2006) reported that Syk is critical for FcεRI-evoked Gab2/PI3K activation. Together, these results suggested that BHT might affect the critical downstream molecules of Syk such as Gab2 and PI3K to exacerbate FcεRI-mediated degranulation of RBL2H3 cells (Fig. 8). It is also possible that BHT might affect the Fyn/Gab2/PI3K pathway to increase degranulation induced by IgE and antigen.

It is well known that although low concentration of BHT has antioxidant properties, higher doses can result in oxidative stress. However, as a matter of fact, 150 μM of BHT has both antioxidative (Courtois et al., 2002) and pro-oxidative (Palomba et al., 1999) effects for cultured cells. In the present study, this concentration of BHT enhanced FcεRI-mediated degranulation and PI3K activation. Noteworthy, these two events, inductions of PI3K activation (Frossi et al., 2007) and degranulation (Ohmori et al., 1980), are also induced by oxidative stress in mast cells. These observations might suggest that in the present study 150 μM of BHT enhances FcεRI-mediated PI3K activation and degranulation via its oxidative effect.

Similar to in vitro, oxidative machinery has a role in allergic responses in vivo as suggested by the observation that an antioxidant, chlorophyllin, inhibits PCA reaction in rats (Nagai et al., 1983). Therefore, 10 mg/kg of BHT used in the present study which exacerbated PCA reaction might act as a pro-oxidant in vivo. In fact, i.p. injection of 62.5 mg/kg of BHT, which is not quite different from the doses we used, changes lung metabolism via induction of oxidative stress in mice (Omaye et al., 1977).

ADIs of BHT are set as 0.125 and 0.3 mg/kg by IPCS (International Program on Chemical Safety) and government of Japan, respectively. The used doses of BHT in our experiment in rats (0.1 to 10 mg/kg) which promoted PCA reaction are comparable to the ADIs. According to Williamson et al. (1978), the maximum concentration of BHT in rat serum is 300 μM at 2 h after the i.p. administration of 400 mg/kg of BHT. The concentrations of BHT in serum at day 2, PCA challenge day, are 5 to 8 times as low as at the peak. Thus, the concentration of BHT after injection of 10 mg/kg of BHT i.p. in serum is hard to be considered to increase as much as 30 μM which is the lowest concentration to enhance FcεRI-mediated degranulation of RBL2H3 significantly. There are two possible explanations why similar promoting effects in allergic responses in vivo and in vitro are observed in spite of the difference of BHT concentrations. First, long pre-exposure of BHT might change the condition of rats and bring out the latent ability of low concentration of BHT in vivo at day 2 to enhance FcεRI-mediated allergic response. Second, in contrast to above-mentioned consideration, BHT concentrations in the tissues including skin might over 30 μM temporally within 2 h to 8 h after i.p. injection since Williamson et al. (1978) also reported

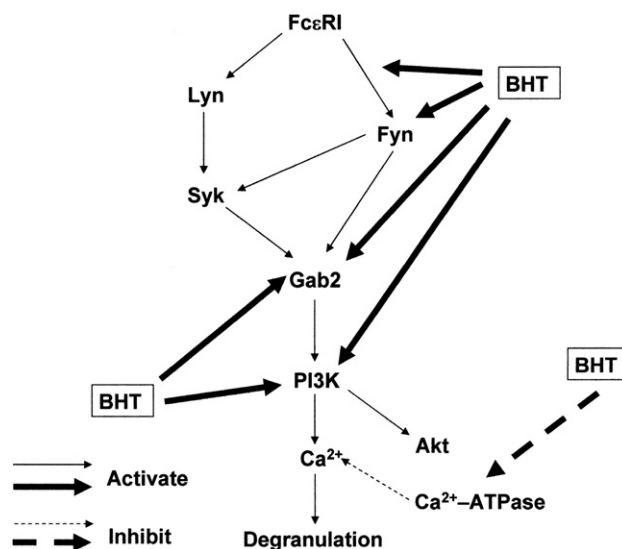


Fig. 8. Schematic diagram of the possible enhancement mechanisms of BHT in the activation of RBL2H3 cells.

that the concentrations of BHT in the tissues such as liver and kidney are assumed higher than in the serum. The effect of the peak concentration of BHT might be maintained until day 2 and observed as an exacerbation of PCA reaction.

In summary, BHT enhances FcεRI-mediated degranulation via promotions of FcεRI-mediated PI3K activation and calcium mobilization of RBL2H3 cells in vitro. Exacerbation of the PCA reaction in rats by BHT might be induced, at least in part, by facilitation of degranulation of mast cells proved by in vitro experiments. Therefore, BHT might have the ability to affect allergic diseases in humans such as allergic rhinitis and asthma.

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