JPP Journal of Pharmacy and Pharmacology

JPP 2009, 61: 1243–1248

Received February 27, 2009
Accepted June 22, 2009
DOI 10.1211/jpp/61.09.0015
ISSN 0022-3573

Research Paper

# Inhibition of IgE-induced mast cell activation by ethyl tertiary-butyl ether, a bioethanol-derived fuel oxygenate

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## Abstract

**Objectives** The effect of ethyl tertiary-butyl ether (ETBE), which is widely used as a fuel oxygenate commonly produced from bioethanol, on immunoglobulin (Ig)E-dependent mast cell activation was investigated.

**Methods** The rat mast cell line RBL2H3 sensitised with monoclonal anti-ovalbumin IgE was challenged with ovalbumin in the presence or absence of ETBE, tert-butanol (TBA), which is the main metabolite of ETBE in humans, and ethanol. Degranulation of RBL2H3 was examined by the release of $\beta$-hexosaminidase. To understand the mechanisms responsible for regulating mast cell function, the effects of ETBE, TBA and ethanol on the levels of intracellular calcium, phosphorylation of Akt (as a marker of phosphatidylinositol 3-kinase) and global tyrosine phosphorylation were also measured as indicators of mast cell activation.

**Key findings** In the presence of ETBE, TBA or ethanol, IgE-induced release of $\beta$-hexosaminidase was decreased. These compounds also attenuated the IgE-mediated increase in the levels of intracellular $Ca^{2+}$, phosphorylation of Akt and global tyrosine phosphorylation in RBL2H3 cells.

**Conclusions** ETBE, TBA and ethanol inhibited mast cell degranulation by inhibiting the increase in intracellular calcium ion concentration and activation of phosphatidylinositol 3-kinase and protein tyrosine kinase activation, suggesting that exposure to ETBE might affect immune responses, particularly in allergic diseases.

**Keywords** degranulation; ethyl tertiary-butyl ether; IgE; mast cell; phosphatidylinositol 3-kinase; RBL2H3

## Introduction

Upon exposure to antigen, immunoglobulin (Ig)E aggregates high-affinity IgE receptors (Fc$\varepsilon$RI) on the surface of mast cells and basophils, and induces release of various mediators such as histamine, cysteinyl leukotrienes and enzymes, including $\beta$-hexosaminidase. These molecules are responsible for the major symptoms of type I allergic reactions in allergic rhinitis, atopic dermatitis and asthma, such as vasodilation, mucus secretion, itching and bronchoconstriction. Aetiological studies have shown that exposure to environmental factors such as artificial chemical compounds may be linked to increases in the incidence and the severity of allergic diseases. Allergic diseases have become more common throughout the world in parallel to the distribution of the various environmental factors.

Ethyl tertiary-butyl ether (ETBE) (Figure 1) is widely used as a fuel oxygenate produced from ethanol based on grain and other biomass feedstocks, so-called 'bioethanol'. Since the compound is volatile, ETBE can be inhaled and absorbed via the airways, and occasionally the digestive organs and skin. The majority of ETBE is metabolised to tertiary-butanol (TBA) by liver microsomal enzymes.[1] Considering the increase in ETBE exposure, concern over the safety of ETBE and its metabolite TBA has been growing. Over the past few decades several biological activities of ETBE and TBA have been investigated. Inhalation of ETBE specifically induced nephropathy in male rats.[2] The protein droplets seen in the affected tissues contained $\alpha$2u-globulin, suggesting that an interaction of ETBE with the molecule might provide a mechanistic basis for nephropathy.[3] A human study showed that inhalation of ETBE slightly impaired pulmonary function.[4] TBA in drinking water was associated with an increased incidence of renal tubule adenomas and carcinomas in male rats.[5] However, there have been few studies demonstrating the effects of ETBE and TBA on allergic responses. In-vitro studies have shown that TBA inhibits IgE-mediated and

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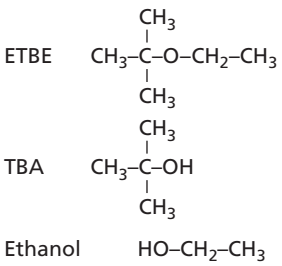


**Figure 1** Structures of ethyl tertiary-butyl ether (ETBE), tert-butanol (TBA) and ethanol

thapsigargin-dependent degranulation of RBL2H3 mast cells,[6] but the mechanism by which it does so remains to be fully elucidated.

In the present study, to clarify whether ETBE and TBA modulate mast cell activation by FcεRI crosslinking, we tested the effects of ETBE and TBA on IgE-dependent degranulation, calcium ion ($Ca^{2+}$) mobilisation, phosphorylation of Akt (as an indicator of phosphatidylinositol 3-kinase (PI3K) activity) and global tyrosine phosphorylation of RBL2H3 mast cells.

## Materials and Methods

### Cells

RBL2H3 cells were supplied by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan. The anti-ovalbumin-IgE (OE-1)-producing hybridoma was established using polyethylene glycol 1500 (Roche Diagnostics, Mannheim, Germany) following the manufacturer's protocol.

### Degranulation of RBL2H3 cells

RBL2H3 cells were maintained in 5% $CO_2$ at 37°C in minimum essential medium (MEM) containing 10% (v/v) fetal bovine serum (FBS), 100 units penicillin and 100 units streptomycin (all from Invitrogen, Tokyo, Japan). The cells were harvested by trypsin (0.25% w/v) (Invitrogen) and EDTA (Wako Pure Chemical Industries Ltd, Osaka, Japan) (0.01% w/v) in phosphate-buffered saline and suspended at $0.25 \times 10^6$ cells/ml in medium containing 50 ng/ml OE-1 monoclonal antibody (mAb) produced by the OE-1 hybridoma. Samples of cell suspension (500 μl) were added to each well of 24-well cluster dishes and cultured for 24 h. The cells were washed with PIPES buffer (25 mmol/l PIPES (pH 7.2), 119 mmol/l NaCl, 5 mmol/l KCl, 5.6 mmol/l glucose, 0.4 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$ and 0.1% (w/v) bovine serum albumin)[7] and stimulated for 20 min with ovalbumin (Sigma-Aldrich, St Louis, MO, USA) at a concentration of 5 μg/ml with various concentrations of ETBE (Sigma-Aldrich), TBA (Sigma-Aldrich) or ethanol (Wako Pure Chemical Industries). In some experiments, the anti-ovalbumin IgE-sensitised cells were pretreated for 15 min with ETBE, TBA or ethanol, and then stimulated by ovalbumin with the compounds. β-hexosaminidase in the cultured supernatant was determined by colorimetric assay using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma-Aldrich). Values for β-hexosaminidase released in the medium were expressed as a percentage of the total β-hexosaminidase determined in the cells lysed in 0.1% Triton X-100.

### Cytotoxicity assay

After treatment of RBL2H3 cells with ETBE or TBA and ovalbumin, cells were washed with prewarmed MEM containing 10% (v/v) FBS, 100 units penicillin and 100 units streptomycin. The cells were further incubated for 4 h with medium containing MTT (Sigma-Aldrich). After removal of the medium, the resultant coloured products were dissolved in DMSO and the absorbance at 562 nm determined.[8]

### Measurement of intracellular calcium ion concentration

Intracellular $Ca^{2+}$ concentrations were measured using the calcium-reactive fluorescence probe fluo-3-AM (Wako Pure Chemical Industries).[9] Briefly, RBL2H3 cell suspensions ($6 \times 10^5$ cells/ml in PIPES buffer) were incubated with MEM containing 10% (v/v) FBS, 100 units penicillin, 100 units streptomycin, anti-ovalbumin IgE and 4 μmol/l fluo-3-AM for 30 min at 37°C and then washed twice with and resuspended in PIPES buffer. The cells were treated with ETBE, TBA or ethanol for 15 min, and then stimulated with ovalbumin and the compounds at 0 min and monitored for fluorescence (FL1) for 140 s by FACScaliver (BD Bioscience, San Jose, CA, USA).

### Immunoblotting

After stimulation, the cells in 60 mm dishes were lysed in 0.18 ml ice-cold lysis buffer (20 mmol/l PIPES, pH 8.0, 1% (v/v) Triton X-100, 1 mmol/l EDTA, 50 mmol/l NaF, 2.5 mmol/l *p*-nitrophenyl phosphate, 1 mmol/l $Na_3VO_4$, 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). Phosphorylation of Akt and total-Akt was detected by immunoblotting using polyclonal antibodies for phospho-Akt (Ser473) and Akt (Cell Signaling Technology, Beverly, MA, USA), respectively. Global tyrosine phosphorylation was detected using monoclonal antibodies for phosphorylated tyrosine (BioLegend, San Diego, CA, USA).

### Statistical analysis

The Kruskal–Wallis test was used to identify any differences among the groups, followed by Dunn's post-hoc test for multiple comparisons. The Mann–Whitney *U* test was used to make comparisons between two groups.

## Results

### Effects of ETBE, TBA and ethanol on anti-ovalbumin-specific IgE-dependent degranulation of RBL2H3 cells

To clarify whether ETBE, TBA and ethanol modulate mast cell activation, we tested their effects on the IgE-mediated degranulation response of RBL2H3 cells, measured by release of β-hexosaminidase. Addition of ETBE simultaneously with ovalbumin to IgE-sensitised RBL2H3 cells did not affect the

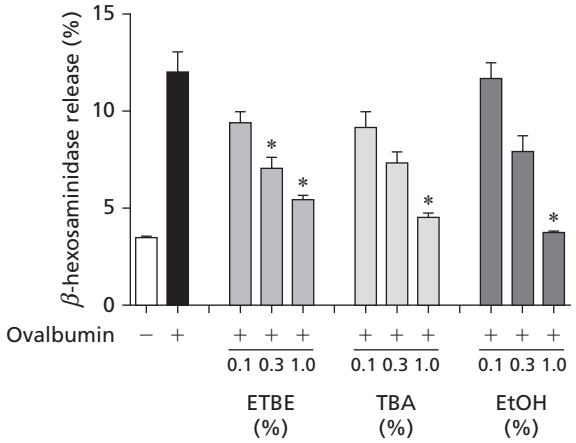


**Figure 2** Effect of ethyl tertiary-butyl ether, tert-butanol and ethanol on the release of $\beta$-hexosaminidase from RBL2H3 cells. RBL2H3 cells were pre-incubated with anti-ovalbumin IgE for 24 h and the indicated concentrations of ethyl tertiary-butyl ether (ETBE), tert-butanol (TBA) or ethanol (EtOH). The cells were then stimulated with ovalbumin (5 $\mu$g/ml) in the presence or absence of ETBE, TBA and EtOH. The quantity of $\beta$-hexosaminidase is expressed as the percentage of total $\beta$-hexosaminidase. Bars show means and SEM of six cultures. $^*P < 0.05$ vs absence of compound.

Fc$\varepsilon$RI-mediated degranulation response (data not shown), whereas pretreatment with 0.3% or 1% ETBE, 1% TBA or 1% ethanol significantly decreased Fc$\varepsilon$RI-mediated $\beta$-hexosaminidase release (Figure 2). ETBE, TBA and ethanol alone did not affect the release of $\beta$-hexosaminidase.

The viability of RBL2H3 cells (determined using the MTT assay) was not affected by treatment with ETBE, TBA or ethanol. However, morphological characteristics of apoptosis such as membrane blebbing and cell shrinkage were observed in small numbers of cells treated with 1% ETBE (data not shown). Similarly, methyl tertiary-butyl ether, a structurally related compound, is reported to induce apoptosis of rodent fibroblasts.[10] The highest concentration of ETBE strongly inhibited release of $\beta$-hexosaminidase but has only weak cytotoxicity; thus, the inhibitory effect of this compound at this concentration on degranulation response is not due to cytotoxicity. Moreover, the degranulation response was attenuated at lower concentrations of ETBE (0.1–0.3%), which did not cause cell death. These results suggest that inhibition of degranulation by ETBE, TBA and ethanol was not due to a cytotoxic effect.

### Effects of ETBE, TBA and ethanol on intracellular calcium

To address the question of how mast cell activation is inhibited by ETBE and TBA, we measured intracellular $Ca^{2+}$ concentrations in RBL2H3 cells. As shown in Figure 3, upon antigen stimulation, intracellular $Ca^{2+}$ concentrations increased in 13.1% (upper-right quadrant) of total cells (upper- and lower-right quadrants), whereas only 3.8% of total cells were detected in the upper-right quadrant in vehicle-treated control cells. The intracellular $Ca^{2+}$ concentration in RBL2H3 cells began to increase from 25 s after ovalbumin stimulation, reaching a peak at 40 s. Stimulation-induced increase of the cells in the upper-right quadrant was reduced to 9.1% by ETBE, 11.3% by TBA and 12.2% by ethanol. Statistical analysis of the cytometric data indicated that ETBE, TBA and ethanol significantly attenuated the increase in the number of cells with elevated intracellular $Ca^{2+}$ concentrations (Figure 3). ETBE, TBA and ethanol alone did not affect the intracellular $Ca^{2+}$ concentration.

### Effects of ETBE, TBA and ethanol on phosphorylation of Akt and global tyrosine phosphorylation

Protein tyrosine kinases (PTK) and phosphatidylinositol 3-kinase (PI3K) are responsible for Fc$\varepsilon$RI-mediated $Ca^{2+}$ mobilisation in mast cells.[11,12] Thus, it is presumed that ETBE, TBA and ethanol inhibit Fc$\varepsilon$RI-mediated $Ca^{2+}$ mobilisation by modulation of PTK and PI3K activities. To further address the question of how the compounds inhibit intracellular $Ca^{2+}$ mobilisation, we tested the effects of ETBE, TBA and ethanol on the phosphorylation of Akt, as an indicator of PI3K activity, and global tyrosine phosphorylation. A certain level of phosphorylation of Akt was detected in non-stimulated RBL2H3 cells and was lowered by ETBE, TBA and ethanol treatment (Figure 4). Fc$\varepsilon$RI-mediated activation by antigen increased the phosphorylation of Akt; this increase was inhibited by ETBE, TBA and ethanol.

In accordance with its ability to lower Akt phosphorylation, ETBE, TBA and ethanol lowered the Fc$\varepsilon$RI-mediated increase in global tyrosine phosphorylation (at least two proteins, 100 kDa and 70 kDa, indicated by arrows in Figure 5). The compounds did not affect global tyrosine phosphorylation in resting cells.

## Discussion

We have demonstrated for the first time that ETBE diminishes the release of $\beta$-hexosaminidase from RBL2H3 mast cells activated with anti-ovalbumin IgE and its antigen, ovalbumin. Our results also showed that ETBE inhibited Fc$\varepsilon$RI-mediated PI3K and PTK activation, and $Ca^{2+}$ mobilisation. We also showed that both TBA and ETBE inhibit the intracellular signalling pathways responsible for Fc$\varepsilon$RI-mediated activation of RBL2H3 cells.

Release of chemical mediators such as histamine and serotonin during degranulation of mast cells is an important step in type I allergic reactions and immune responses. Our results show that ETBE inhibited Fc$\varepsilon$RI-mediated degranulation of RBL2H3 cells (Figure 2); this compound may therefore influence immune and allergic reactions. TBA and ethanol also inhibited Fc$\varepsilon$RI-mediated degranulation, consistent with previous reports.[6,13] The three compounds inhibited Fc$\varepsilon$RI-mediated morphological spreading of cells (data not shown), which is considered to be associated with intracellular-signalling-dependent reorganisation of the cytoskeleton and is a critical component of the degranulation process,[14] in addition to $\beta$-hexosaminidase release. Therefore, ETBE, TBA and ethanol were assumed to inhibit mast cell activation by modulating the activities of signalling molecules.

Inhibition of the degranulation of RBL2H3 cells by ETBE was due to attenuation of the increase in intracellular $Ca^{2+}$

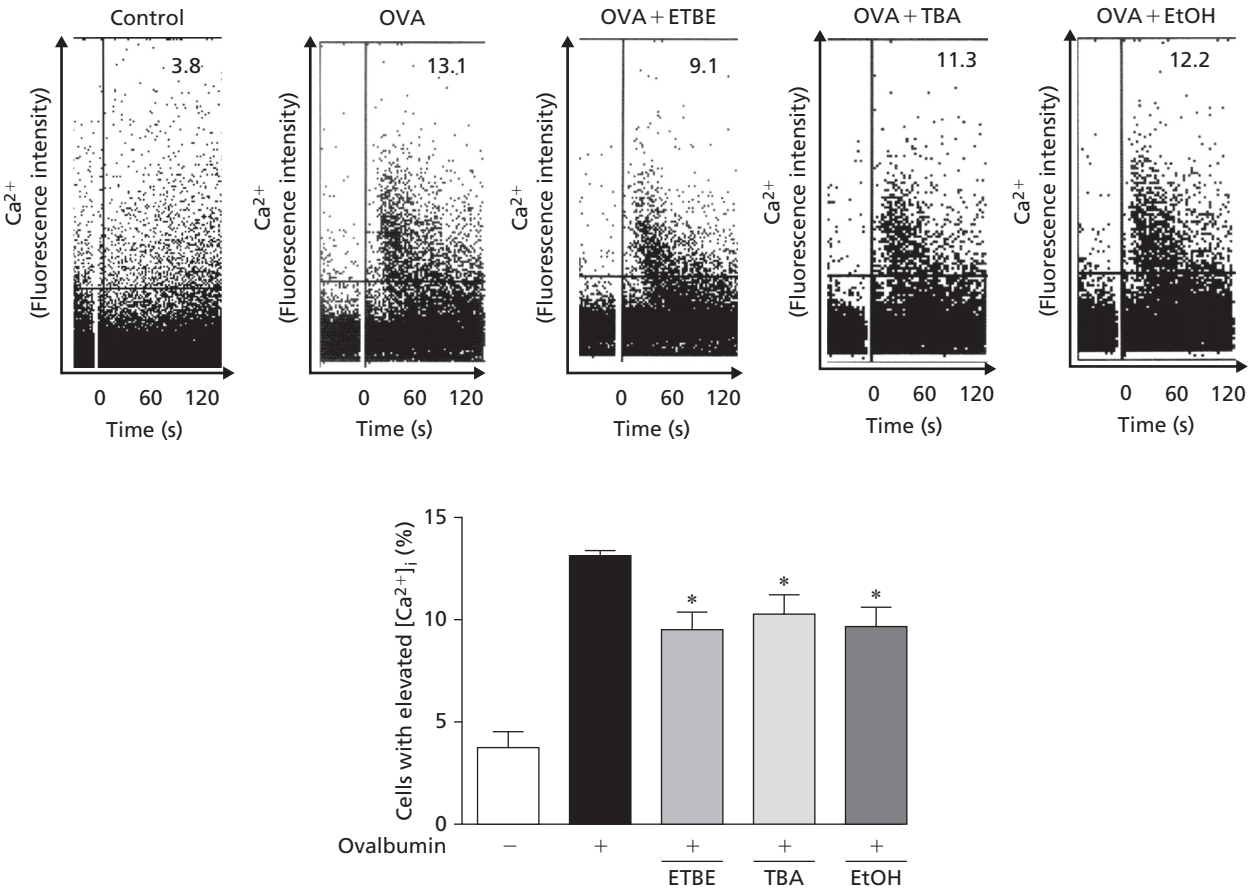


**Figure 3** Effect of ethyl tertiary-butyl ether, tert-butanol and ethanol on the intracellular $Ca^{2+}$ concentration in RBL2H3 cells. RBL2H3 cells were pre-incubated with anti-ovalbumin IgE and fluo-3-AM for 30 min. After 15-min incubation with 0.3% ethyl tertiary-butyl ether (ETBE), tert-butanol (TBA) or ethanol (EtOH), the cells were then stimulated with ovalbumin (OVA; 5 $\mu$g/ml). The number in the upper-right quadrant indicates the percentage of cells with elevated intracellular calcium concentration ($[Ca^{2+}]_i$) calculated by the following equation: cells with elevated $[Ca^{2+}]_i$ (%) = (number of cells in upper-right quadrant) ÷ (number of cells in upper- and lower-right quadrants) × 100. The plots show one representative experiment of three or more performed. The bar graph summarises the flow cytometry results, and shows means and SEM of three or more cultures. *$P < 0.05$ vs absence of compound.

concentration in cells (Figure 3), since an increase in the intracellular $Ca^{2+}$ concentration correlates with the activation of mast cells required for degranulation.[15] From this result and observations from other researchers it can be considered that ETBE (and possibly also TBA and ethanol) have similar inhibitory properties on cell functions as structurally similar volatile anaesthetics such as diethyl ether, halothane and isoflurane. The increase in the intracellular $Ca^{2+}$ concentration crucial for the degranulation response is induced by FcεRI-mediated[15] and muscarinic M1 receptor-mediated stimulation[16] in M1-transfected RBL2H3 cells, following inositol 3,4,5-triphosphate ($IP_3$) production.[17] On the other hand, in neuron-like human SH-SY5Y neuroblastoma cells, the M1-mediated $Ca^{2+}$ increase was inhibited by halothane and isoflurane.[18] It is therefore possible that the inhibitory mechanisms of ETBE on $Ca^{2+}$ increase in RBL2H3 cells is the same as that of volatile anaesthetics on SH-SY5Y cells. Some volatile anaesthetics (50–200 mmol/l ethanol, 3–18.6 mmol/l diethyl ether, 0.25–2 mmol/l halothane, 0.25–2 mmol/l enflurane and 0.3–2 mmol/l isoflurane) inhibit $Ca^{2+}$-activated chloride ion currents induced by stimulation of substance-P in substance-P receptors expressed in *Xenopus* oocytes, which is dependent on $IP_3$-mediated $Ca^{2+}$ release from endoplasmic reticulum.[19] The concentration of ETBE (0.3%, 21.9 mmol/l) that inhibited $Ca^{2+}$ increase in mast cells in the present study is comparable to that of the volatile anaesthetics, particularly structurally similar diethyl ether (18.6 mmol/l), in that report. Moreover, as with volatile anaesthetics, ETBE has a narcotic effect in mice.

We then investigated the effects of ETBE and TBA on PI3K activation and upstream global tyrosine phosphorylation, which are the FcεRI-mediated events responsible for calcium mobilisation. ETBE, TBA and ethanol attentuated the FcεRI-mediated increase in Akt phosphorylation (Figure 4). Our results also demonstrated that ETBE, TBA and, to a lesser extent, ethanol inhibited global tyrosine phosphorylation induced by FcεRI stimulation (Figure 5). The compounds reduced the phosphorylation of the band at about 70 kDa in Figure 5, which is suggested to be Syk by Moriya *et al.*[20] Recently, Yu *et al.*[21] reported that Syk is critical for FcεRI-evoked Gab2/PI3K activation. Together these results suggest that ETBE might affect the critical upstream molecules of

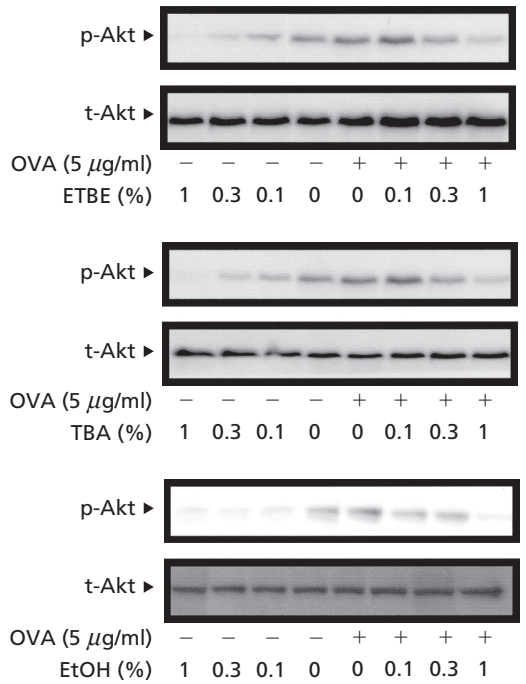


**Figure 4** Effect of ethyl tertiary-butyl ether, tert-butanol and ethanol on the phosphorylation of Akt in RBL2H3 cells. RBL2H3 cells were pre-incubated with anti-ovalbumin (OVA) IgE for 24 h and the indicated concentrations of ethyl tertiary-butyl ether (ETBE), tert-butanol (TBA) or ethanol (EtOH) for 15 min. The cells were then stimulated with OVA in the presence or absence of ETBE, TBA and EtOH for 10 min. Whole cell lysate was applied to SDS-polyacrylamide gel, transferred to PVDF membrane, and immunoblotted with anti-phospho-Akt ($Ser^{473}$) (p-Akt). Antibody against total Akt served as a control for the protein loaded (t-Akt).

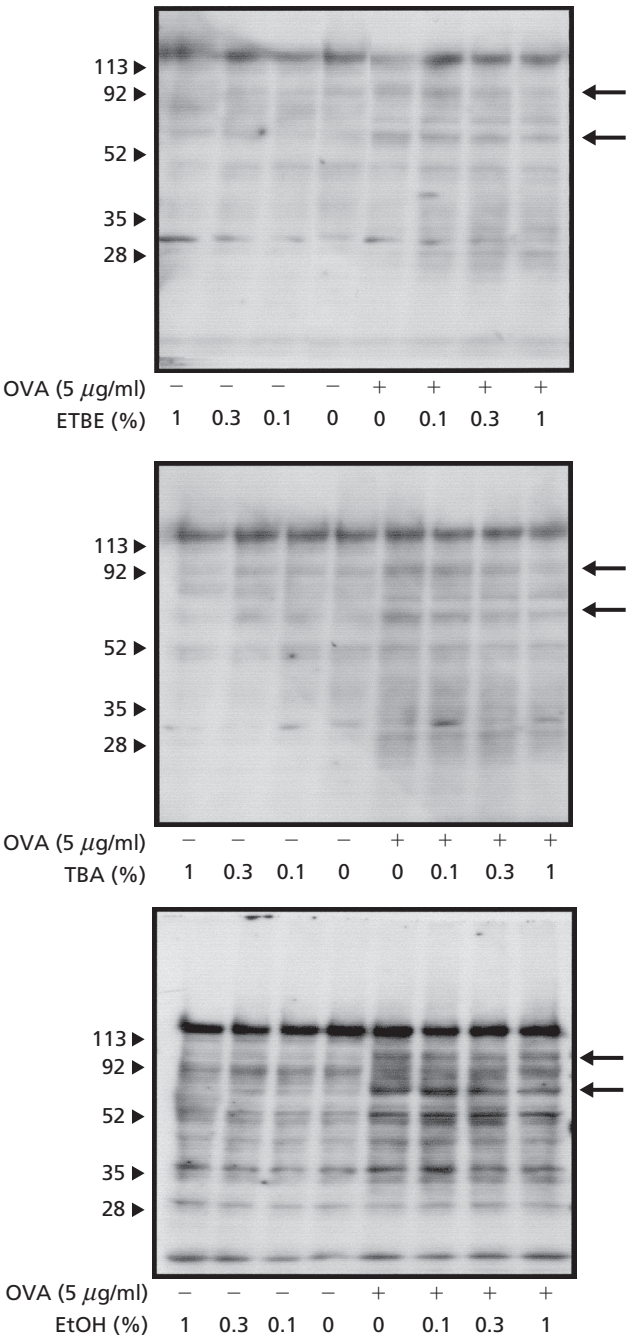


**Figure 5** Effect of ethyl tertiary-butyl ether, tert-butanol and ethanol on global tyrosine phosphorylation in RBL2H3 cells. RBL2H3 cells were pre-incubated with anti-ovalbumin (OVA) IgE for 24 h and the indicated concentrations of ethyl tertiary-butyl ether (ETBE), tert-butanol (TBA) or ethanol (EtOH) for 15 min. The cells were then stimulated with OVA in the presence or absence of ETBE, TBA and EtOH. Whole cell lysates were blotted with the anti-phosphotyrosine antibody PY20. Arrows indicate the proteins whose enhanced tyrosine phosphorylation by FcεRI-mediated stimulation was obviously attenuated by the compounds.

Syk and thus inhibit PI3K and $Ca^{2+}$ increase to attenuate FcεRI-mediated degranulation of RBL2H3 cells. By contrast, Parravicini *et al.*[22] demonstrated that Fyn, not Syk, is required for FcεRI-mediated Gab2/PI3K activation. It is also possible that ETBE affects other tyrosine kinases such as Fyn and the subsequent PI3K/$Ca^{2+}$ pathway to decrease degranulation induced by IgE and antigen. From these observations it seems that ETBE, TBA and ethanol impair early signal transduction from receptors to membrane-bound signalling molecules, and/or other molecules such as PTK to induce degranulation. Furthermore, the volatile anaesthetic halothane, structurally similar to ETBE and diethyl ether, inhibits the signalling molecules located upstream of the phospholipase C (PLC)/$IP_3$/$Ca^{2+}$ pathway (i.e. the early signalling from M1 receptors to PLC),[23] which have roles in M1-dependent degranulation of M1-receptor-transfected RBL2H3 cells.[24] These results also indicate that ETBE and volatile anaesthetics share the ability to inhibit certain early signalling mechanisms generally induced by various kinds of stimulation, including M1 and FcεRI crosslinking.

The high concentration of ETBE used in the present study (1%) is a feasible exposure level, since distributed fuel contains 7% or more ETBE. However, the ETBE concentration in air reaches only about 0.1 part per million even around fuel pumps at filling stations. Only very high concentrations of ETBE were demonstrated to affect mast cell function in our study and to have biological activities in other studies,[2–5] suggesting that the ETBE used commonly in fuel should not have a significant effect on human health.

## Conclusions

Our data demonstrate that ETBE and its metabolite TBA inhibited FcεRI-mediated degranulation through the attenuation of FcεRI-mediated PTK, PI3K activation and calcium mobilisation of RBL2H3 cells *in vitro*. Although low concentrations of ETBE evaporated from fuels might not affect human health, accidental exposure to a high concentration of ETBE is likely to affect allergic diseases in humans such as allergic rhinitis and asthma by modifying mast cell function.

## Declarations

### Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

### Funding

This work was supported by a Grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan.

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