



Naturally Occurring Imidazole Peptides, Carnosine and Anserine Inhibit the Degranulation of Mast Cells and Basophils by Modulating Intracellular Signaling

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Abstract

Imidazole peptides possess multiple functions, including antioxidant effects, although their biological activities are largely unclear. Their production in humans and animals suggests their physiological roles and validates their safety for pharmaceutical or supplemental use. This study investigated the *in vitro* anti-anaphylactic potential of two histidine-containing dipeptides, carnosine and anserine in mast cells and basophils. Carnosine and anserine reduced mast cell degranulation elicited by anti-ovalbumin monoclonal IgE and ovalbumin or ionomycin in rat basophilic leukemia RBL2H3 cells without affecting cell viability. In contrast, interleukin-4 production following stimulation was enhanced in the presence of carnosine. Carnosine and anserine strongly inhibited Akt phosphorylation and moderately inhibited ERK phosphorylation. However, these peptides enhanced the increase in phosphorylated JNK levels upon IgE stimulation. The phosphorylation levels of p38 were not affected by carnosine or anserine. To determine the effect of carnosine on basophils, we established a method for detecting IgE-dependent activation in primary cultured mouse splenic basophils via CD63 expression for the first time. Carnosine treatment significantly reduced the increase in CD63 surface expression, a marker of degranulation, in mouse splenic basophils stimulated with anti-IgE. Flow cytometry analysis revealed that mean fluorescence intensities for non-stimulated, anti-IgE-stimulated, and carnosine-pre-treated/anti-IgE-stimulated basophils were 3853 ± 320 , 5548 ± 282 , and 3853 ± 203 , respectively. The findings indicate carnosine and anserine suppress mast cell and basophil IgE-dependent degranulation. The proposed mechanism of the inhibitory effect is the suppression of the activation of phosphoinositide 3-kinase–Akt. Carnosine and anserine may exert anti-anaphylactic effects under physiological and pathological conditions and serve as safe potential drug candidates.

Keywords Anaphylaxis · Basophils · Carnosine · IgE · Kinase · Mast cells

Introduction

Anaphylaxis is an acute, and potentially life-threatening phenomenon induced by food allergens, insect stings, and drugs. Symptoms of anaphylaxis can vary, ranging from mild, such as urticaria and diarrhea, to severe, including dyspnea and anaphylactic shock, characterized by circulatory failure (Oettgen 2023). Preventive steroid treatments and oral immunotherapy to desensitize the patient to the allergen, as well as symptomatic antihistamine treatments, are commonly beneficial for patients (Wallace 2023). However, these therapies may cause side effects, prompting some patients to seek alternative therapies (Anderson and Dinulos 2009). Notably, Hughes et al. (2007) found that 45% of children

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with atopic dermatitis opted for alternative medicine due to concerns over the side effects associated with steroid use.

Recent studies have elucidated numerous critical cell types and mediators involved in anaphylaxis (Bruhns and Chollet-Martin 2021; González de Olano et al. 2023). Antibodies such as IgE and IgG trigger the activation of mast cells, macrophages, neutrophils, and basophils and cause allergic reactions in mice. Histamine, released by these cells, serves as a primary mediator inducing symptoms such as vasodilation, increased vascular permeability, and itchiness associated with allergic response and anaphylaxis (Patel and Mohiuddin, 2023). In addition to histamine, platelet-activating factors and complement components also can cause anaphylaxis (Bruhns and Chollet-Martin 2021). However, IgE-induced mast cell and basophil activation and the release of histamine from cells play major roles in this phenomenon (Stevens et al. 2023). Owing to this reason, an *in vitro* mast cell model of rat basophilic leukemia (RBL2H3) is often selected for research on anti-anaphylactic compounds (Baran et al. 2023). In contrast, the method to measure the activation of mouse primary basophils by detecting the expression of a degranulation marker, CD63, has not been established. Therefore, the development of such a method is warranted, along with the identification of anti-allergic compounds.

Carnosine and anserine (a methylated derivative of carnosine) are naturally occurring imidazole peptides found in various mammalian and avian tissues (Boldyrev et al. 2013). These peptides are thought to exert antioxidant effects and help in recovery from fatigue. These peptides positively affect various diseases in humans and animals. Carnosine intake improves the Gilliam Autism Rating Scale (covering total score, behavior, socialization, and communication subscales) and the Receptive One-Word Picture Vocabulary test in patients with autism spectrum disorders (Chez et al. 2002) and reduced HbA1C levels in glucose disorders (Peng et al. 2020) in humans. In rats, imidazole peptides have been shown to have anticonvulsant effects in penicillin-induced epilepsy (Kozan et al. 2008). Moreover, the presence of these peptides in the body proves their safe use (Boldyrev et al. 2013); thus, these peptides are widely sold as health foods. Therefore, elucidating the potential of imidazole peptides as anti-anaphylactic agents is worthwhile. In addition, the presence of extremely high concentrations (millimolar levels) of imidazole peptides and/or their derivatives, such as acetyl-carnosine, in skeletal and cardiac muscles implies that they have important original physiological and pathophysiological roles (Boldyrev et al. 2013).

To this end, we explored the inhibitory effects of carnosine and anserine on the degranulation response in a mast cell model of rat basophilic leukemia RBL2H3 cells stimulated with IgE and allergens. In addition, the inhibitory mechanism was partly revealed by analyzing intracellular signaling molecules. Finally, we established a method to

measure primary cultured basophil activation based on the increase in CD63 expression for the first time and unveiled the inhibitory effect of carnosine on IgE-dependent activation of mouse splenic basophils.

Materials and Methods

Animals and Cell Lines

BALB/c mice (male, 8–14 weeks old) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The mice were housed in a controlled environment and provided with standard feed and water *ad libitum*. All animal experiments were approved by the Animal Ethics Committee of Kobe Pharmaceutical University. RBL2H3 cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University, Sendai, Japan. Hybridoma-producing anti-ovalbumin (OVA) monoclonal IgE (OE-1) was established as previously reported (Yamaki and Yoshino 2009).

Degranulation Assay and Cytokine Measurement

RBL2H3 cells (2.5×10^5 cells/well) were suspended in RPMI1640 medium supplemented with 10% fetal bovine serum, antibiotics, and 1 $\mu\text{g}/\text{mL}$ OE-1 and then seeded in a 24-well plate (Corning, Corning, NY, USA). After 18 h, OE-1-sensitized RBL2H3 cells were pretreated with pH-adjusted (7.0 to 7.4) PIPES buffer containing the indicated concentrations of carnosine (3–50 mM, Fujifilm Wako Pure Chemical Co., Osaka, Japan) or anserine (Toronto Research Chemicals Inc., Toronto, ON, Canada) for 20 min. The cells were then stimulated with OVA (Merck KGaA, Darmstadt, Germany) in the presence or absence of the test compounds for 20 min. The supernatants were mixed with *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide (Merck KGaA) and incubated at 37 °C for 2 h. The absorbance of the resultant yellow solution was measured at 405 nm after the addition of bicarbonate buffer. In some cases, ionomycin 3 μM was used for stimulation of non-sensitized RBL2H3 cells.

For interleukin (IL)-4 production measurement, sensitized RBL2H3 cells were pre-incubated with carnosine in the medium and then stimulated with OVA and carnosine for 4 h. The IL-4 concentration in the culture supernatants was measured using an ELISA kit (DIAclone SAS, Besançon cedex, France).

Western Blotting

The phosphorylation and total protein expression levels of Akt, ERK, JNK, and p38 were determined using western blotting. Similar to the measurement of degranulation,

OE-1-sensitized RBL2H3 cells (1×10^6 cells/35 mm dish) were pretreated with carnosine for 20 min or anserine for 15 min and then stimulated with OVA for 20 min. Cell lysates were loaded onto a 12% polyacrylamide gel containing sodium dodecyl sulfate and electrophoresed. The proteins in the gels were blotted onto PVDF membranes. The membranes were blocked with skimmed milk according to the manufacturer's instructions. Primary antibodies against phospho-Akt, Akt, phospho-ERK, ERK, phospho-JNK, JNK, phospho-p38, and p38 were purchased from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase-labeled anti-rabbit IgG secondary antibody (Cell Signaling Technology) and EZWest LumiOne (ATTO Co., Tokyo, Japan) were used to detect chemiluminescence using a LAS4000 imaging system (General Electric Company, Boston, MA, USA).

Cell Viability (MTT) Assay

After an adequate period of stimulation, the culture medium was removed, and the cells were further cultured with a medium containing 0.5 mg/mL MTT (Merck KGAA) for 4 h. The MTT-containing medium was then removed and the resultant violet products were dissolved in dimethyl sulfoxide (Fujifilm Wako Pure Chemical Co.). The absorbance of the solution was measured at 550 nm.

Basophil Activation Test in a Mouse Primary Cell Culture

Splenocyte suspensions were obtained from BALB/c mice. Erythrocytes in the suspension were lysed and the aliquots of the resultant leukocytes were used for the subsequent experiment. Leukocytes (5×10^6 cells) were suspended in the RPMI1640 medium supplemented with 10% fetal bovine serum and antibiotics and seeded in a 1.5 mL tube. Cells were preincubated with or without 50 mM carnosine for 15 min at 37 °C and stimulated with goat anti-mouse IgE (5 µg/mL, Southern Biotech, Birmingham, AL, USA) in the presence or absence of the imidazole peptide for 20 min. After stimulation, the cells were washed with ice-cold PBS and stained with FITC-anti-CD63, PE-anti-CD200R3, and PECy7-anti-CD49b (Biolegend Inc., San Diego, CA, USA) for 30 min on ice to detect basophil activation. The cells were washed and analyzed on BD FACSAria™ III (BD Bioscience).

Statistical Analysis

Dunnett's post hoc test was performed for comparisons among multiple groups when the *p*-value for one-way analysis of variance was less than 0.05. The Mann-Whitney

U-test was used for comparisons between any two groups. A *p*-value of <0.05 was considered statistically significant.

Results

Inhibition of IgE-Dependent Degranulation of RBL2H3 Cells by Carnosine and Anserine

In the in vitro experiments, RBL2H3 cells, a commonly used mast cell line, sensitized with anti-OVA IgE OE-1, showed degranulation upon stimulation with OVA (Fig. 1a). Carnosine, at concentrations of 12 mM or higher, significantly inhibited IgE-dependent degranulation in a dose-dependent manner (Fig. 1a). The carnosine concentration did not affect the formation of MTT formazan (Fig. 1b), suggesting that the dipeptide was not cytotoxic. Anserine inhibited IgE-induced degranulation at concentrations similar to those of carnosine (Fig. 1c), without exhibiting cytotoxicity (Fig. 1d).

In addition to IgE-mediated degranulation, various chemicals, including drugs (e.g., morphine and taxol) and toxins (e.g., mastoparan and ionomycin), induce mast cell degranulation, resulting in a pseudo-anaphylactic response (Hammond 2023). Carnosine and anserine decreased ionomycin-induced degranulation of RBL2H3 cells (Fig. 1e and g, respectively) without showing cytotoxicity (Fig. 1f and h, respectively), suggesting that the peptides attenuated mast cell degranulation induced by various stimuli.

In the absence of stimulation, carnosine did not induce degranulation (data not shown).

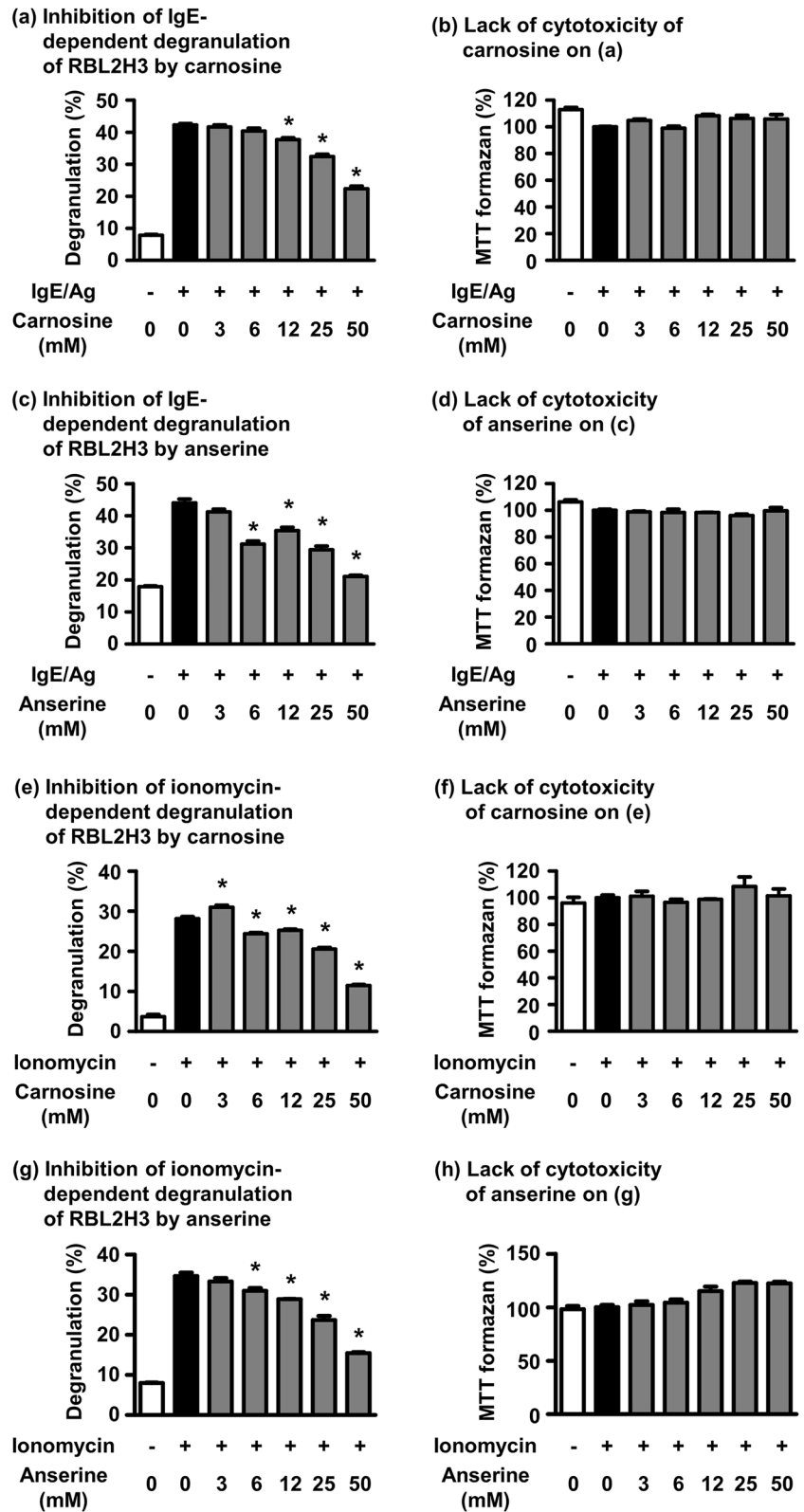
Lack of Effects of Alanine, a Representative Essential Amino Acid, on IgE-Induced Degranulation

To investigate whether the addition of amino acids inhibits mast cell degranulation, the effect of 50 mM alanine (Fig. 2a) was compared with that of anserine. In contrast to the significant inhibitory effect of anserine (Fig. 2b), alanine failed to affect degranulation induced by IgE and allergens (Fig. 2b). The inhibitory effects of carnosine (Fig. 2c) and anserine (Fig. 2d) on IgE-dependent degranulation were not substituted by the addition of alanine, suggesting distinctive inhibitory effects of imidazole dipeptides among the amino acids and peptides.

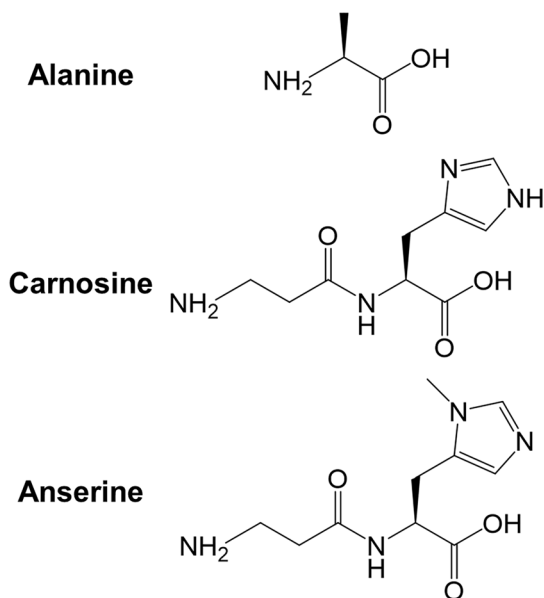
Enhancement of IgE-Induced IL-4 Production by Carnosine

Four hours after antigen addition to IgE-sensitized RBL2H3 cells, production of the cytokine IL-4 was induced (Fig. 3a). Co-treatment with carnosine significantly enhanced IL-4 production in a dose-dependent manner (Fig. 3a) without affecting cell viability (Fig. 3b).

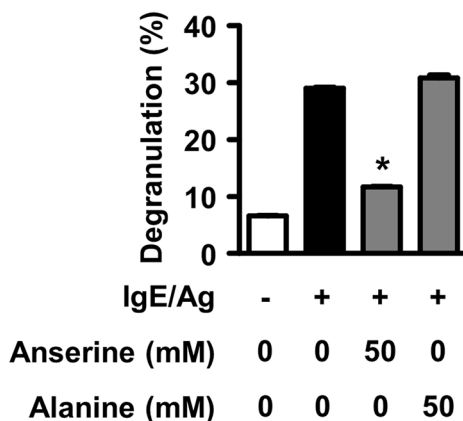
Fig. 1 Inhibitory effects of carnosine and anserine on degranulation of RBL2H3 cells. RBL2H3 cells were sensitized with anti-ovalbumin (OVA) monoclonal IgE OE-1. After 20 h of incubation, cells were cultured with carnosine (a, b) or anserine (c, d) for 20 min. The cells were then stimulated with OVA in the presence of the peptides for 20 min. β -Hexosaminidase activity in the supernatant (a, c) was measured, and cell viability was assayed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2 H-tetrazolium bromide (MTT) analysis (b, d). Non-sensitized RBL2H3 cells were pre-incubated with carnosine or anserine for 20 min and then stimulated with ionomycin in the presence of the peptides for 20 min to determine β -hexosaminidase release (e, g) and cell viability (f, h). Bars show the mean \pm SEM of the three cultures. * $p < 0.05$ vs. OVA (IgE/Ag) or ionomycin alone, one-way analysis of variance, followed by Dunnett's multiple comparison test. Similar results were obtained from two independent experiments



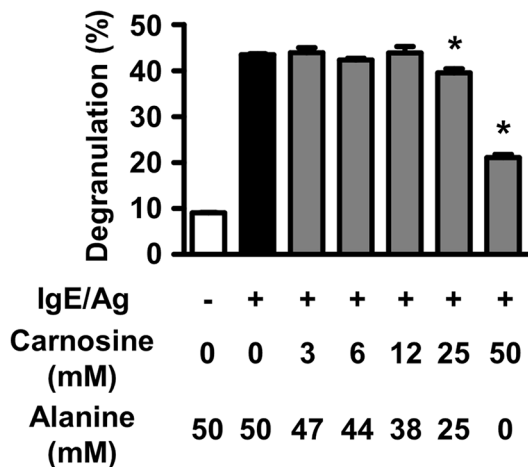
(a) Chemical structures of carnosine, anserine, and alanine



(b) Lack of the effect of alanine on IgE-dependent degranulation of RBL2H3



(c) Co-treatment of carnosine and alanine



(d) Co-treatment of anserine and alanine

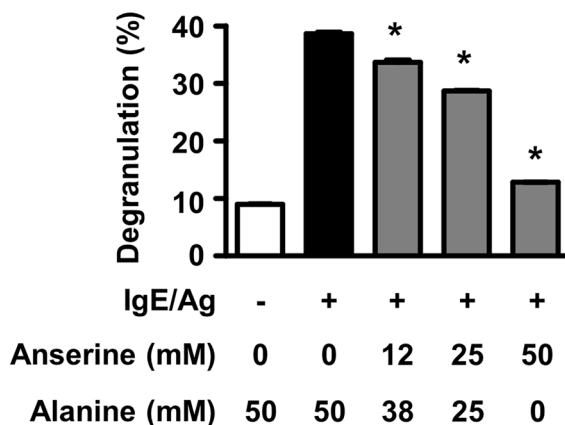


Fig. 2 Comparison of the effects of imidazole peptides and essential amino acid alanine on degranulation of IgE-stimulated RBL2H3 cells. The structural formulas of alanine, carnosine, and anserine are shown in (a). OE-1-sensitized RBL2H3 cells were cultured with anserine or alanine (b), carnosine and alanine (c), anserine and alanine (d) for 20 min. Cells were stimulated with ovalbumin (OVA) in

the presence of amino acids or peptides for 20 min. β -hexosaminidase activity in the supernatant was measured. Bars show the mean + SEM of the three cultures. * $p < 0.05$ vs. OVA (IgE/Ag) alone (b), * $p < 0.05$ vs. OVA (IgA/Ag) and alanine 50 mM (c, d) one-way analysis of variance followed by Dunnett’s multiple comparison test. Similar results were obtained from two independent experiments

Both Inhibitory and Enhancing Effects of Carnosine and Anserine on IgE-Dependent Activation of Intracellular Signaling Molecules

Next, we investigated the signaling molecules affected by carnosine and anserine in IgE-stimulated RBL2H3 cells.

Phosphorylation of Akt, an indicator of the PI3K activity responsible for the degranulation response (Ching et al. 2001; Nishida et al. 2011), was induced by IgE-mediated activation (Fig. 4a). Carnosine and anserine at 25 mM or higher diminished the IgE-dependent increase in Akt phosphorylation (Fig. 4a). In contrast, the IgE-dependent

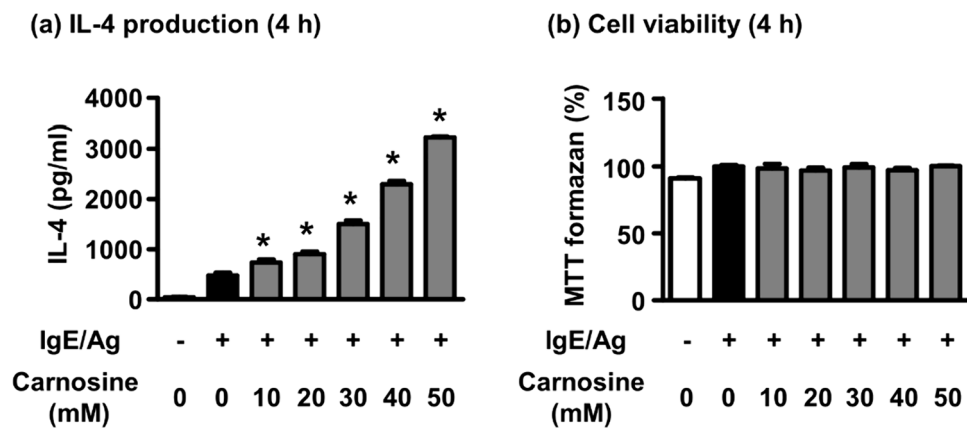


Fig. 3 Enhancing effects of carnosine on interleukin (IL)-4 production of IgE-stimulated RBL2H3 cells. OE-1-sensitized RBL2H3 cells were cultured with carnosine for 20 min. The cells were then stimulated with ovalbumin (OVA) in the presence of the peptide for 4 h. IL-4 concentrations in the culture supernatants were measured (a), and cell viability was assayed using 3-(4,5-dimethylthiazol-2-yl)-2,5-di-

phenyl-2 H-tetrazolium bromide (MTT) analysis (b). Bars show the mean + SEM of the three cultures. * $p < 0.05$ vs. OVA (IgE/Ag) alone, one-way analysis of variance, followed by Dunnett's multiple comparison test. Similar results were obtained from two independent experiments

phosphorylation of JNK, a signaling molecule that plays a role in cytokine production (Kim et al. 2019), was further increased by carnosine and anserine (Fig. 4b). ERK phosphorylation upon IgE and allergen stimulation was moderately attenuated by the imidazole peptides (Fig. 4c), whereas p38 phosphorylation was not affected (Fig. 4d).

Inhibitory Effects of Carnosine on IgE-Dependent Basophil Activation

Despite the importance of basophils in various allergic events (Poto et al. 2023), the method to detect the degranulation of the cells is limited, especially in mice. Increased expression of CD63 in mouse mastocytoma P815 cell line upon stimulation with IgE and antigens has been reported (Furuno et al. 1996) but the experimental protocol has not been used for further analyses. In the present study, to determine the effect of carnosine on basophil activation, we established a method to detect the increase in CD63 expression by anti-IgE stimulation in mouse splenic basophils. CD200R3⁺CD49b⁺ cells after gating with FSC and SSC are considered mouse basophils (Zellweger et al. 2018). According to this gating strategy, we measured the increase in the expression of the degranulation marker, CD63, on basophils by anti-IgE stimulation and its inhibition by carnosine (Fig. 5). The blue dots in the dot plots in Fig. 5 represent the gated population comprising basophils. IgE-dependent stimulation induced by anti-IgE increased CD63 expression (Fig. 5, middle histogram) compared to the non-stimulated control (Fig. 5, upper histogram), approximately 1.5 times higher in terms of the mean fluorescence intensity (MFI, Fig. 5 column graph). Carnosine at 50 mM almost

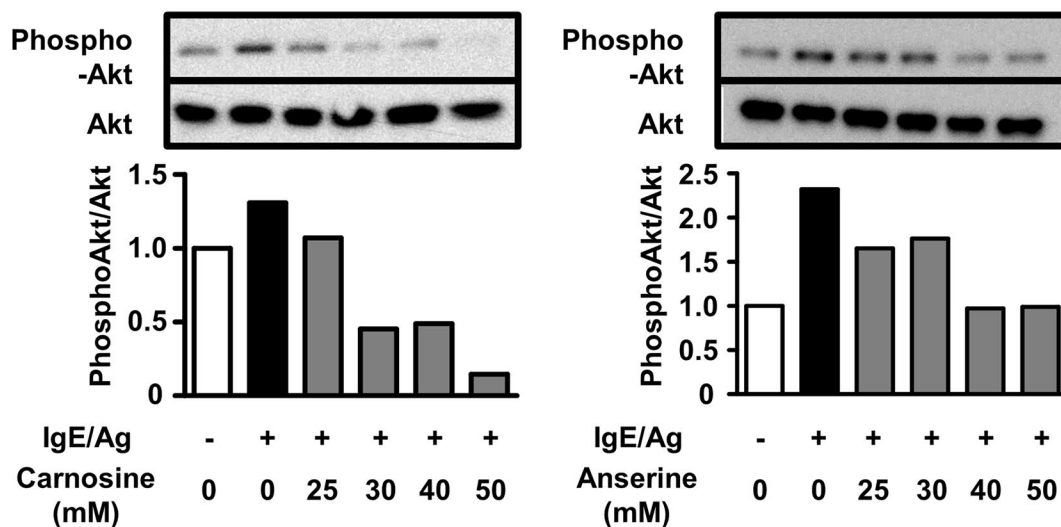
completely suppressed the increase in expression induced by anti-IgE (Fig. 5, lower histogram and column graph).

Discussion

Our results indicated that carnosine and anserine inhibited the degranulation response in RBL2H3 cells by blunting the PI3K-Akt signaling pathway. Moreover, we demonstrated for the first time that IgE-dependent activation of mouse primary basophils was attenuated by carnosine. These findings may accelerate the application of imidazole peptides as anti-anaphylactic health foods and improve our understanding of the roles of endogenous carnosine and anserine.

RBL2H3 cells have been widely used as a mast cell model to examine the inhibitory effects of various compounds on degranulation and screen their anti-anaphylactic efficacy in vitro. In this study, carnosine and anserine were shown to inhibit the physiological stimulation-induced degranulation of RBL2H3 cells (Fig. 1). This observation strongly suggests the potential inhibitory ability of these peptides against anaphylaxis, consistent with previous reports. Carnosine attenuates the degranulation response of rat peritoneal mast cells induced by compound 48/80 (Akhalya et al. 2006). Acetylcarnosine, an intrinsic derivative of carnosine, attenuates the degranulation response induced by oxidants through its antioxidative effects (Liu et al. 2021). Inhibition of IgE-dependent increase of CD63 expression in mouse primary splenic basophils by carnosine (Fig. 5) showed its ability to suppress basophil degranulation and supported the observations obtained in experiments using RBL2H3. These results strongly suggest the anti-anaphylactic potential of

(a) Inhibition of IgE-dependent increase of phospho-Akt level by imidazole peptides



(b) Augmentation of IgE-dependent increase of phospho-JNK level by imidazole peptides

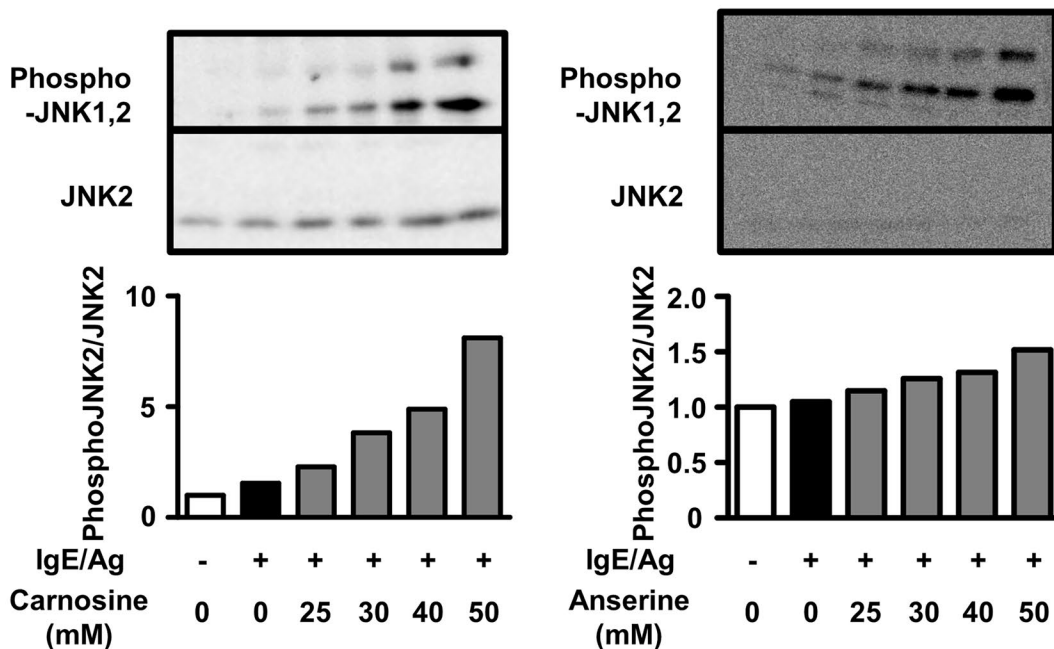


Fig. 4 Various effects of carnosine and anserine on increased phosphorylation levels of Akt, ERK, JNK, and p38 in IgE-stimulated RBL2H3 cells. OE-1-sensitized RBL2H3 cells were cultured in the presence of carnosine for 20 min or anserine for 15 min. The cells were then stimulated with ovalbumin (OVA) in the presence of pep-

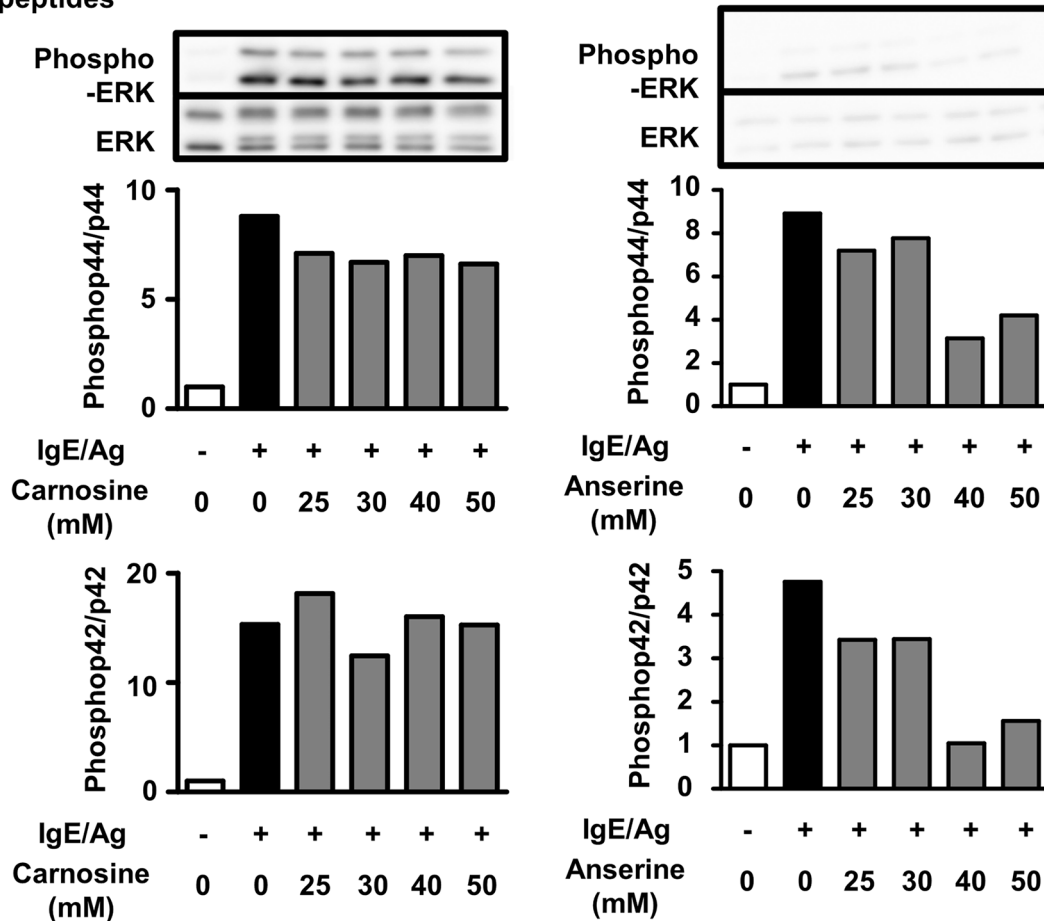
tides for 20 min. Cell lysates were electrophoresed and transferred onto PVDF membrane. The phosphorylation and total levels of Akt (a), ERK (b), JNK (c), and p38 (d) were determined using western blotting. The bars indicate densitometric values representative of two or more independent experiments

imidazole peptides by modulating the functions of mast cells and basophils.

Our analysis further explored the inhibitory mechanism by which the inhibitory effect in RBL2H3 cells

was mediated through the blunting of the activation of the PI3K-Akt pathway (Fig. 4a), which participates in degranulation mechanisms (Ching et al. 2001; Nishida et al. 2011). Although PI3K inhibitors have not yet been

(c) Scarce inhibition of IgE-dependent increase of phospho-ERK level by imidazole peptides



(d) Lack of inhibition of IgE-dependent increase of phospho-p38 level by imidazole peptides

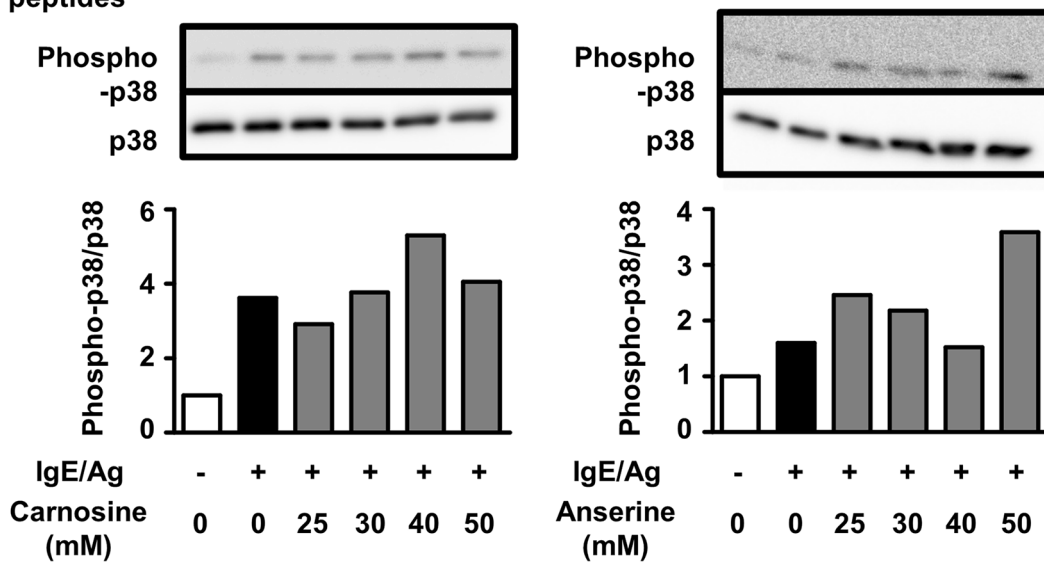
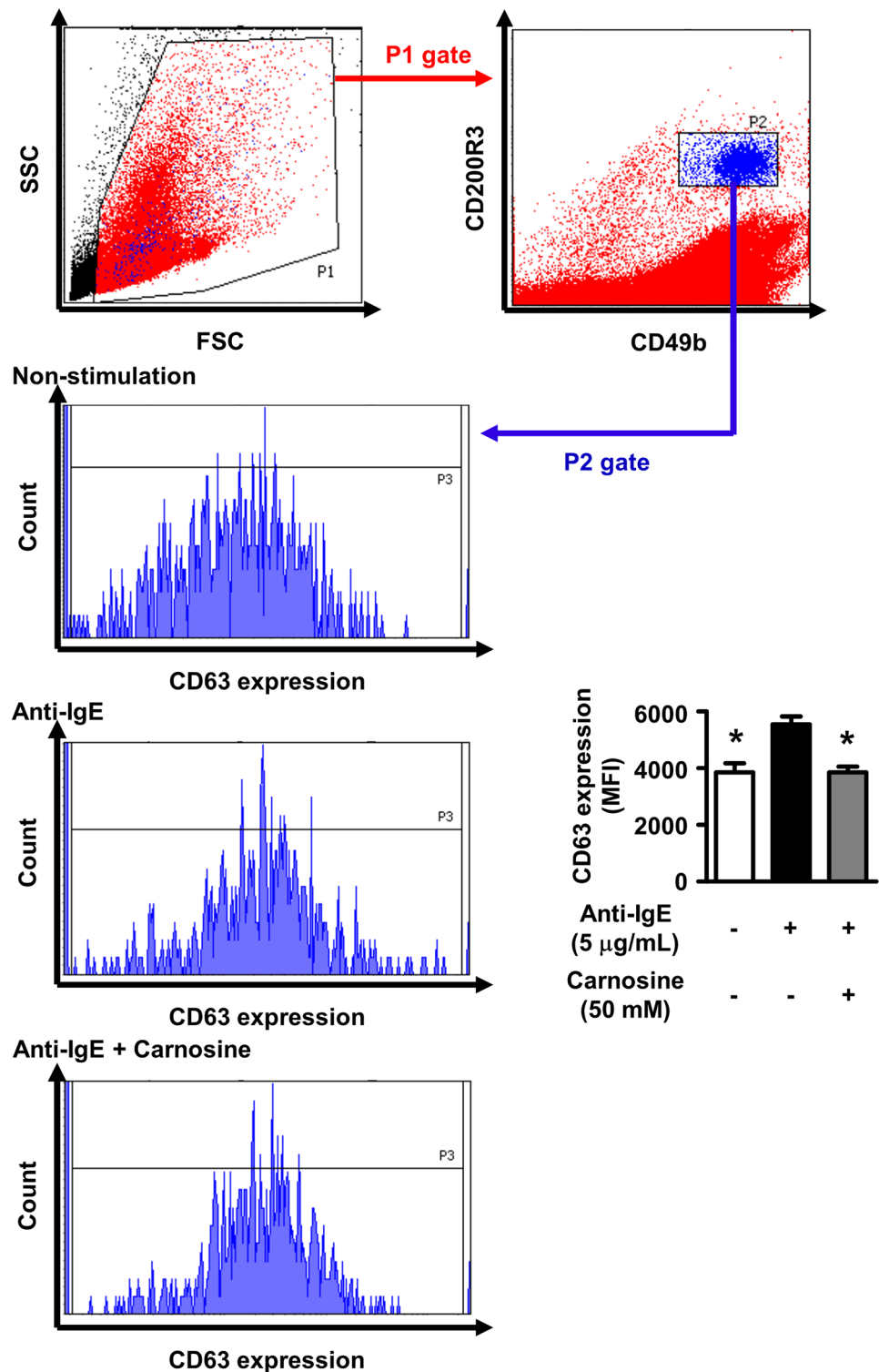


Fig. 4 (continued)

Fig. 5 Inhibition of IgE-dependent increase in CD63 expression in mouse primary splenic basophils by carnosine. Spleen tissues were obtained from BALB/c mice and cell suspensions were pretreated with carnosine at 50 mM for 15 min. Subsequently, cells were stimulated with anti-IgE (5 μ g/mL) in the presence or absence of carnosine for 20 min. After washing, CD63 expression on CD200R3⁺CD49b⁺ basophils was analyzed. Dot plots show the gating strategies used to distinguish basophils. Histograms show representative data of CD63 expression in each treatment. Bars in the column graph represent the mean \pm SEM of quadruplicated cultures. * $p < 0.05$ vs. anti-IgE alone, one-way analysis of variance followed by Dunnett's multiple comparison test. Similar results were obtained from two independent experiments



used for anti-allergic therapy in clinical settings, evidence for the potential of PI3K inhibitors as anti-allergic agents has been reported in many studies (Saw and Arora 2016; Ma et al. 2021). Carnosine significantly reduced the basal phosphorylation of Akt in unstimulated U87 glioblastoma, reported previously (Oppermann et al. 2019). In contrast,

carnosine does not affect the PI3K pathway in platelet-derived growth factor-induced rat vascular smooth muscle cells (Hwang et al. 2020). The discrepancy in the effects of carnosine on the PI3K pathway among the experiments may have been caused by differences in the conditions and cell types used in these reports.

In contrast to its inhibitory effect on degranulation (Fig. 1), carnosine increased the production of the Th2 cytokine IL-4 (Fig. 3a) in RBL2H3 cells, which exerted two opposing effects: anti-allergic and pro-allergic. IL-4 acts as an anti-allergic factor that negatively regulates mast cell function to attenuate IL-4-dependent inflammation (Sherman 2001). In contrast, IL-4 acts as a pro-allergic factor that induces the class switching of B cells to produce IgE to promote allergic predisposition (Maspero et al. 2022). However, the importance of IL-4 derived from mast cells for allergic predisposition, including enhancement of IgE production, remains unclear, since other cells, especially Th2 cells, also produce IL-4 under allergic conditions (Burchett et al. 2022). Simultaneously, with an increase in IL-4 production (Fig. 3a), carnosine increased JNK phosphorylation (Fig. 4b). This observation is consistent with a previous report indicating that the JNK inhibitor, SP600125, down-regulates IgE-induced IL-4 production in RBL2H3 cells (Kim et al. 2019). Thus, co-administration of a JNK inhibitor or other strategies to reduce the enhancement of IL-4 production by mast cells might lead to a safer use of carnosine as an anti-anaphylactic drug.

High concentrations of imidazole peptides in skeletal muscle cells (millimolar range) and cardiac muscles (millimolar range, including carnosine derivatives) imply their important role in these areas (Boldyrev et al. 2013). Mast cells are distributed in these locations and regulate various functions. Mast cells enhance skeletal muscle regeneration (Duchesne et al. 2013) but also induce inflammation to destroy the tissue (Tu and Li 2023). Similarly, mast cells have a dichotomous role in protecting against or accelerating cardiovascular diseases (Varricchi et al. 2020). Carnosine and anserine may contribute to these phenomena by modulating mast cell function. Recently, Sicklinger et al. (2021) reported the presence of basophils in the heart after myocardial infarction. Infiltrated basophil cells contribute to the repairing of the myocardium through the production of IL-4 and IL-13, which change macrophages from an inflammatory type to a reparative type. Augmentation of IL-4 production of mast cells/basophils by carnosine, as observed in RBL2H3 cells (Fig. 3), may contribute to its beneficial effect on cardiovascular diseases (Varicchi et al. 2020).

One limitation of this study is the lack of results defining the direct target molecules of imidazole peptides. Whether imidazole peptides affect the surface or intracellular molecules is an important issue. The relationships between the previously demonstrated functions of the peptides, such as antioxidant effects and modulatory functions of signaling molecules, will be further elucidated.

In conclusion, our data suggest the possible anti-anaphylactic effects of carnosine and anserine by inhibiting mast cell degranulation through the dampening of the PI3K-Akt pathway. Carnosine inhibited degranulation in mouse

primary splenic basophils. The ability of imidazole peptides to inhibit mast cell/basophil degranulation, and increase IL-4 production, suggests their physiological and pathological roles in the skeletal muscle and cardiovascular functions, as well as anaphylactic responses. Further analyses will support the use of carnosine and anserine as health foods to prevent anaphylaxis and modulate physiological functions and pathological conditions in the skeletal muscles and heart.

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Author Contributions Conception and design: KY, YK, KI, HF, KO, MO. Development of methodology: KY, NK, AS. Acquisition of data: KY, NK, N.N, HY, AS, SK. Analysis and interpretation of data: KY, YK, KO, M.O. Writing, review, and/or revision of the manuscript: KY, YK, KI, HF, KO, MO.

Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Competing interest KY received research grants from Japan Eco System Co., Ltd.

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