Effect of glucosamine and related compounds on the degranulation of mast cells and ear swelling induced by dinitrofluorobenzene in mice

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Abstract

Aims: Glucosamine has been safely used to relieve osteoarthritis in humans, but the precise mechanism underlying its efficacy is still unclear. In this study, we investigated the direct effects of glucosamine and related compounds on mast cell mediated inflammation using cultured mast cells and an animal model.

Main methods: Dinitrophenyl (DNP)-IgE sensitized rat basophilic leukemia RBL-2H3 cells were treated with glucosamine-HCl (GlcN-HCl), N-acetylglucosamine (GlcNAc), chitin oligomer or chitosan oligomer. Cells were stimulated by DNP-BSA to induce degranulation and released β-hexosaminedase was determined colorimetrically to measure the degree of degranulation. Dinitrofluorobenzene (DNFB) sensitized BALB/c mice were administrated orally with 1 or 0.1 mg GlcN-HCl or GlcNAc for 6 days. One h after the final administration, mice was challenged by DNFB to induce ear swelling.

Key findings: GlcN-HCl significantly inhibited the antigen-induced degranulation of RBL-2H3 cells at higher than 0.01 mg/mL for 24 h-treatment while GlcNAc, a chitin oligomer and a chitosan oligomer had no effect. GlcN-HCl also suppressed intracellular calcium mobilization. GlcN-HCl and GlcNAc significantly suppressed the antigen-induced up-regulation of TNF-α and IL-6 mRNA. Ear swelling and histamine levels of plasma and ear in DNFB-treated mice were significantly suppressed by oral administration of GlcN-HCl or GlcNAc (0.1 and 1 mg) for 6 days.

Significance: Our results strongly suggest that GlcN-HCl and GlcNAc have anti-inflammatory effects in vivo by suppressing the activation of mast cells.

Keywords: glucosamine; mast cells; degranulation; inflammation; osteoarthritis

Introduction

Glucosamine, a naturally occurring amino-monosaccharide, is an essential component of glycosaminoglycans in almost all human tissues, and glycosaminoglycans are highly concentrated in connective and cartilage tissues (Laverty et al. 2005). Glucosamine and related compounds, which are used as dietary supplements, appear to be safe and are widely marketed for pain relief of osteoarthritis. In fact, numerous studies have shown that glucosamine supplements provide symptomatic relief for osteoarthritis and can normalize cartilage metabolism (Kim et al. 2005). However, clinical trials have yielded conflicting results and the precise mechanism of the effects of glucosamine on arthritis is still obscure. Recent studies raise questions about the positive effects of glucosamine sulfate on pain and structural changes associated with osteoarthritis (Felson and Mcalindon 2000; Mcalindon et al. 2004; Felson 2008). One speculative mechanism for the efficacy of glucosamine is its direct effect on inflammatory reactions. There have been several reports about the potential immunoregulatory ability of glucosamine (Forchhammer et al. 2003; Meininger et al. 2000). Glucosamine inhibits IL-1β-induced NF-κB activation and the production of proinflammatory cytokines in human chondrocytes (Gouze et al. 2002; Shikhman et al. 2001), and suppresses the release of cytokines from T cells by disturbing the functions of antigen presenting cells (APCs) and by inhibiting CD3-induced T cell proliferation (Zhang et al. 2005).

Mast cells are commonly found at sites of contact with the outside environment and they play pivotal roles in inflammation and immediate-type allergic reactions. Degranulation of mast cells causes the secretion of biologically active substances including histamine, eicosanoids, proteolytic enzymes, cytokines and chemokines. Cytokines released after degranulation, such as interleukin-6 (IL-6), IL-8 and tumor necrosis factor-alpha (TNF-α), induce the late-phase allergic reactions and allergic inflammation via the recruitment of

immune cells to the inflamed site (Feldmann et al. 1996). It is well known that the degranulation of mast cells is induced by multivalent antigen-IgE crosslinking and the aggregation of high affinity IgE receptor I (Kabu et al. 2006). The aggregation of high affinity IgE receptor I triggers intracellular signaling pathways, such as the phosphorylation of protein kinases and the influx of intracellular Ca^{2+}. Based on this background, the assay for degranulation of mast cells has been used for screening of anti-inflammatory natural compounds.

Chitin is a polymer of N-acetylglucosamine found abundantly in the exoskeleton of crustaceans and insects and in the cell walls of fungi (Shibata et al. 2000; Georgopapadakou and Tkacz 1995). Chitosan, a polymer of glucosamine, is obtained by the deacetylation of chitin under alkaline conditions. N-acetylglucosamine and glucosamine are industrially produced by the hydrolysis of chitin and chitosan. Glucosamine-related products, such as N-acetylglucosamine, glucosamine, chitin and chitosan, have been used extensively to manage several biological functions. Especially, chitosan possesses many functional properties such as biodegradability, immunological and antibacterial activities, and wound-healing (Kuma et al. 2004) and is used in functional foods and drugs. However, there has been no report concerning the effect of glucosamine and related compounds on the degranulation of mast cells.

The objectives of this study were to investigate the anti-inflammatory activity of glucosamine and related compounds using cultured mast cells and an animal model, and to clarify the mechanism underlying its activity.

Materials and methods

Materials

Glucosamine-HCl (GlcN-HCl), N-acetylglucosamine (GlcNAc), chitin oligomer (2-7mers of GlucNAc) and chitosan oligomer (less than 10mers of GlcN) were kindly provided by Nippon Suisan Kaisha, Ltd. (Tokyo, Japan).

β-Hexosaminidase release assay

Rat basophilic leukemia RBL-2H3 cells (Health Science Resources Bank, Osaka, Japan) were cultured in RPMI-1640 medium containing 10% FBS and supplemented antibiotics (100 unit/mL penicillin and 100 μg/mL streptomycin) at 37℃ in humidified atmosphere in the presence of 5% CO_2. The degree of degranulation of RBL-2H3 cells stimulated by IgE-antigen was determined by the β-hexosaminidase release assay (Nakano et al. 2005). Cells were seeded in 96-well plates (3×10^4 cells/well) with or without 0.45 μg/mL anti-dinitrophenyl (DNP) IgE (Sigma Chemical Co, St. Louis, MO, USA). After overnight incubation, the sensitized or unsensitized cells were washed twice with serum-free RPMI-1640 medium, and then were treated for 4 or 24 h with the indicated concentration of GlcN-HCl, GlcNAc, chitin oligomer or chitosan oligomer dissolved in serum-free RPMI-1640 medium. After washing twice with Tyrode's buffer (1.17 mM NaCl, 5.4 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.6 mM glucose, 25 mM HEPES, 0.1% BSA, pH 7.7), the cells were incubated with 120 μL/well Tyrode's buffer containing 1 μg/mL DNP-BSA (Molecular Probes, Eugene, OR, USA) for 30 min. The supernatants were collected, and cell lysates were obtained in Tyrode's buffer containing 0.1% Triton X-100 (Sigma). Aliquots (50 μL) of each supernatant and cell lysate were incubated with 50 μL 5 mM *p*-nitrophenyl-*N*-acetyl-β-*D*-glucosaminide (Sigma) in 0.1 M citrate buffer (pH 4.5) at 37℃ for 1 h. The reaction was terminated by the addition of 0.1 M $NaHCO_3/Na_2CO_3$ (pH 10.0). *p*-Nitrophenol, the product of the reaction, was detected by optical absorbance at 405 nm. The percentage of β-hexosaminidase released was calculated as follows,

β-hexosaminidase release (%) = supernatant OD value of the stimulated cells / (the cell lysate OD value + supernatant OD value of the stimulated cells).

Measurement of cytosolic Ca^{2+} concentration

Cytosolic Ca^{2+} concentrations were measured using of the fluorescent indicator Fluo-4/AM (Dojindo Laboratories, Kumamoto, Japan) (Kempuraj et al. 2005; Takahashi et al. 1999). RBL-2H3 cells were cultured overnight in 96-well culture plates at 1.5×10^4 cells/well with 0.45 μg/mL anti-DNP IgE. The cells were treated for 4 h with 1 mg/mL of each glucosamine-related compound in RPMI-1640 medium. The treated cells were washed twice and loaded with 4 μM Fluo-4/AM in Tyrode's buffer at 37°C for 1 h. After washing twice with Tyrode's buffer, the cells were stimulated with 1 μg/mL DNP-BSA for 90 sec. Intracellular calcium mobilization was detected at 485 nm excitation wavelength and 535 nm emission wavelength with a Wallac 1420 ARVOSX-FL spectrophotometer (Wallac, Waltham, MA, USA).

Western blot analysis

IgE-sensitized cells were treated with 1 mg/mL GlcN-HCl or GlcNAc for 4 h. The cells were stimulated by 1μg/mL DNP-BSA for 5 min and then cells were lysed by incubation for 30 min on ice with lysis buffer (25 mM Tris buffered saline, 50 mM sodium fluoride, 1 mM sodium orthovanadate and protease inhibitor cocktail (Roche, Basel, Switzerland)) containing 1% Triton X-100. For immunoprecipitation of Lyn, equal amount of protein (200 μg) of cell lysates were incubated with Lyn specific antibodies (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) coupled to protein G-sepharose (Sigma Chemical Co, St. Louis, MO) with slow rotation overnight at 4°C. Each precipitated protein was separated by SDS-PAGE, and were then transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were

blocked with Blocking One-P (Nacarai Tesque, Inc., Kyoto, Japan). Lyn and phosphorylated Lyn were probed with anti-phospho-Lyn antibody (Sigma) and anti-Lyn antibody at 1:1000 dilution for 2 h. For detecting ERK1/2 and phospho-ERK1/2, equal protein of each cell lysates (20 μg) was separated by SDS-PAGE and transferred to PVDF membranes. After blocking, ERK1/2 and phospho-ERK1/2 were probed with anti-ERK1/2 antibody (Cell signaling technology, Inc., Danvers, MA) and anti-phospho-ERK1/2 antibody (Cell signaling technology, Inc) at 1:1000 dilution for 2 h. HRP-anti-rabbit-antibody (R&D Systems, Inc., Minneapolis, MN) was used as the secondary antibody at 1:500 dilutions for 1 h. Detection was performed using Chemi-Lumi One L (Nacalai) and image-analyzer LAS-3000 (FUJIFILM, Tokyo, Japan).

Quantification of TNF-α and IL-6 mRNAs by real-time RT-PCR

IgE-sensitized cells were treated with the indicated concentration of each glucosamine-related compound for 4 h as described above. After washing twice, cells were stimulated with 1 μg/mL DNP-BSA for 30 min at 37℃. Cells were washed sufficiently and total RNAs were extracted using the Sepasol reagent (Nacalai Tesque, Inc. Kyoto, Japan) according to the manufacturer's instructions. RNAs were treated with RNase-free DNase (Invitrogen, Carlsbad, CA, USA) to remove contaminating genomic DNA. After inactivating DNase by heating at 65℃ for 10 min, each RNA was transcribed to cDNA using SuperScript RNase Ⅱ reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with random hexamers at 25℃ for 10 min and then at 42℃ for 50 min. The reactions were stopped by incubation at 70℃ for 15 min and then 6 μL of each mixture, 10 μL iQ SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA, USA) and 2 μL TNF-α, IL-6 or GAPDH gene-specific primers (forward and reverse) were mixed in a final volume of 20 μL. Primers used for the quantification of each gene are listed in Table 1. Primer pairs were selected to yield gene-specific single amplicons

based on the analyses by melting curves and by agarose gel electrophoresis. Real-time quantitative PCR was performed using a DNA Engine Opticon system (Bio-Rad Laboratories). The thermal cycler parameters were as follows: 3 min at 95℃ for one cycle, followed by amplification for 40 cycles with melting for 15 seconds at 95℃ and with annealing and extension for 30 seconds at 60℃. Values were normalized using GAPDH as an endogenous internal standard.

Measurement of DNFB-induced mouse ear swelling

Six-week-old female BALB/c mice were purchased from Japan SLC Inc. (Shizuoka, Japan). The animals were housed at 25℃ with a 12-hour light/dark cycle and were allowed free access to commercial chow (Oriental Yeast Co., LTD., Tokyo, Japan) and tap water. Animal experiments were performed in accordance with the guidelines of Kyoto University for the use and care of laboratory animals.

In vivo anti-allergic activity was tested by the dinitrofluorobenzene (DNFB) -induced contact hypersensitivity reaction (Maeda et al. 2008). The dorsal skin of each mouse was shaved and 100 μL 0.5% DNFB in acetone-soybean oil (4:1) was applied to that area to sensitize the mice. GlcN-HCl or GlcNAc (0.1 or 1 mg/mouse) was administrated orally once a day for 6 days. One h after the final administration, both right and left ears were challenged with 20 μL 0.5% DNFB in acetone-soybean oil (4:1). The thickness of the right ear was measured with a Dial Thickness Gauge (Mitutoyo Co., Kanagawa, Japan) 0, 6 and 24 h after the DNFB challenge. The degree of ear swelling was expressed as the percentage of the thickness of the ear measured before DNFB challenge. Twenty four hour after DNFB treatment, blood was collected and mice were sacrificed under anesthesia. Left ear was fixed by formalin solution, and each paraffin section was stained by hematoxylin and eosin (HE) to observe morphological changes. The amount of TNF-α in the right ear was measured by

ELISA kit (Thermo Scientific, Yokohama, Japan). Histamine in the plasma and the right ear was measured by HPLC with fluorescent detection (Yoshitake et al. 2003).

Statistical analysis

Data are reported as means ± SD. Statistical analyses were performed by paired-t-test or one-way analysis of variance (ANOVA) with Dunnett's test to identify levels of significance between the groups.

Results

Effect of glucosamine and related compounds on the antigen-induced degranulation of RBL-2H3 cells

As an indicator of the degranulation of RBL-2H3 cells, the release of β-hexosaminidase from the cells was measured. Pre-treatment with 1 mg/mL GlcN-HCl for 4 h significantly reduced the release of β-hexosaminidase from sensitized RBL-2H3 cells stimulated with 1 μg/mL DNP-BSA for 30 min (Fig. 1A). The release of β-hexosaminidase from cells treated with 1 mg/mL GlcN-HCl for 4h was inhibited by approximately 50% compared to cells treated with antigen alone. Treatment with GlcN-HCl for 24h significantly inhibited the release of β-hexosaminidase at more than 0.01 mg/mL (Fig.1B). In contrast, GlcNAc, a chitin oligomer or a chitosan oligomer had no effect on the antigen-induced degranulation of mast cells (Fig. 1A and B). GlcN-HCl, GlcNAc, chitin oligomer and chitosan oligomer had no direct effect on the enzyme activity of β-hexosaminidase (data not shown).

Effect of glucosamine and related compounds on the expression of proinflammatory cytokines

We examined whether glucosamine and related compounds could modulate the expression of proinflammatory cytokines (TNF-α and IL-6) induced by antigen in RBL-2H3

cells because those cytokines from mast cells are involved in late-phase allergy. Both GlcN-HCl and GlcNAc significantly suppressed the antigen-induced expression of TNF-α and IL-6 mRNAs more than 60% compared with the control treated with antigen alone, while the chitin oligomer and the chitosan oligomer had no significant effect (Fig. 2A and B).

Effect of glucosamine-HCl and N-acetylglucosamine on intracellular Ca^{2+} mobilization and the activation of intracellular signaling molecules

It is well known that elevation of the intracellular Ca^{2+} concentration triggers the degranulation of mast cells. We evaluated the effects of GlcN-HCl, GlcNAc, chitin oligomer and chitosan oligomer on intracellular Ca^{2+} mobilization in RBL-2H3 cells. The results were similar to the effects on degranulation as described above. RBL-2H3 cells stimulated with 1 μg/mL DNP-BSA had increased intracellular Ca^{2+} levels at 90 sec. Pre-treatment with 1 mg/mL GlcN-HCl for 4 h significantly suppressed the antigen-induced elevation of intracellular Ca^{2+} compared to cells treated with antigen alone (Fig. 3A). GlcNAc, chitin oligomer and chitosan oligomer had no effect on the intracellular calcium mobilization. We examined the effect of GlcN-HCl and GlcNAc on the tyrosine phosphorylation of Lyn which is critical for degranulation. The level of phosphorylated Lyn was increased 2-fold by stimulation with DNP-BSA for 5 min. In GlcN-HCl-treated cells, the DNP-BSA-induced tyrosine phosphorylation of Lyn was significantly lower than in control cells (Fig. 3B). On the other hand, the activation of ERK1/2, which leads to the up-regulation of cytokines gene expression, in both GlcN-HCl and GlcNAc treated cells was significantly reduced as compared with that of the control cells (Fig.3C).

Effect of glucosamine-HCl and N-acetylglucosamine on DNFB-induced ear swelling in BALB/c mice

To investigate anti-inflammatory activity in vivo, the effect of oral administration of GlcN-HCl or GlcNAc on DNFB-induced mouse ear swelling was evaluated. DNFB-induced ear swelling is a model for contact hypersensitivity and is caused by cytokines and infiltrating neutrophils. We examined the effects of dietary GlcN-HCl and GlcNAc on DNFB-induced immediate (6 h) and delayed (24 h) hypersensitivity. Oral administration of GlcN-HCl or GlcNAc for 6 days (0.1 and 1 mg/mouse/day) significantly inhibited DNFB-induced ear swelling in mice at both 6 h and 24 h after the DNFB challenge (Fig. 4A and B).

Swelling of hypodermal tissue and the infiltration of inflammatory cell were observed in the ear section by histochemical analysis of hematoxylin-eosin staining. In the ear of the mice administrated GlcN-HCl or GlcNAc, the pathological changes induced by DNFB treatment were mitigated (Fig.5A). The content of TNF-α in the ear of the mice administrated of GlcN-HCl and GlcNAc tended to be reduced as compared with control mice. Oral administration of GlcN-HCl and GlcNAc significantly reduced the concentration of histamine in both ear and plasma of DNFB-treated mice (Fig.5C and D).

Discussion

In the present study, we demonstrated that GlcN-HCl and GlcNAc have different effects on mast cell activation. GlcN-HCl suppresses the degranulation and up-regulation of inflammatory cytokine gene expression in mast cells while GlcNAc inhibits only the cytokine gene expression. Chitin and chitosan, which are oligomers of GlcNAc and GlcN, respectively, did not affect the antigen-induced reaction of mast cells. Oral administration of GlcN-HCl or GlcNAc (0.1 or 1 mg/day) for 6 days suppressed the ear swelling and histamine levels of plasma and ear in DNFB-treated mice. These results strongly suggested that the anti-inflammatory effects of dietary GlcN-HCl or GlcNAc in vivo are caused by the inhibition of antigen-induced release of chemical mediators and cytokines from mast cells.

Mast cells are mainly involved in the inflammation of type I allergy due to the secretion of chemical mediators such as histamine, leukotriene and inflammatory cytokines (Kabu et al. 2006). It is well understood that antigen-IgE binding initiates intracellular signaling cascades such as the phosphorylation of Lyn kinase which leads to the phosphorylation of mitogen-activated protein kinase (MAPK), the mobilization of intracellular Ca^{2+} and degranulation (Kabu et al. 2006; Gilfilan and Tkaczyk. 2006). In the present study, we focused on the effects of glucosamine and related compounds on the degranulation of mast cells. GlcN-HCl, but not GlcNAc, significantly inhibited the antigen-induced degranulation, intracellular Ca^{2+} influx, and the phosphorylation of Lyn in mast cells. The antigen-induced intracellular signaling in mast cells involves the phosphorylation of mitogen-activated protein kinase (MAPK) followed by the activation of nuclear factor-kappa B (NF-κB). Activated NF-kB up-regulates TNF-α and IL-6 expression. Proinflammatory cytokines are secreted from degranulated mast cells and induce the infiltration of leukocytes and macrophages to inflamed sites. Activated leukocytes and macrophages release inflammatory cytokines and induce cellular dysfunction (Feldmann et al 1996). It has been shown that GlcN and GlcNAc have inhibitory effects on the IL-1-mediated intracellular signaling cascade by reducing the activation of NF-κB in chondrocytes (Gouze et al. 2002; Largo et al. 2003). GlcN suppresses the inflammation induced by the activation of neutrophils and synoviocytes but GlcNAc has no effect (Hua et al. 2002, Hua et al 2007). In the present study, we show that GlcN-HCl inhibits both degranulation and cytokine expression while GlcNAc only suppresses cytokine expression. Our data support that GlcN-HCl inhibits both degranulation and the up-regulation of cytokine genes by suppressing the phosphorylation of Lyn and ERK1/2, and that GlcNAc only affects ERK1/2 phosphorylation, a regulator of cytokine genes.

Oral administration of GlcN-HCl or GlcNAc suppresses immediate (6 h) and delayed (24 h) reactions in DNFB-induced contact allergy model mice. DNFB is used as a contact

allergen to induce ear inflammatory reactions including the degranulation of mast cells (Kabu et al. 2006). It has been reported that GlcN is rapidly absorbed after oral administration (Persiani et al. 2005; Pashkova et al. 2009). The absorption rate of the intact form of GlcNAc is lower than GlcN and appreciable amounts of GlcNAc would be deacetylated during the intestinal absorption (Liu et al. 2008; Capps et al. 1966; Robinson 1968). In the present study, oral administration of GlcN-HCl or GlcNAc significantly reduced the levels of histamine in plasma and ear in DNFB-treated mice. Histamine is released from activated mast cells in mucosal tissue and connective tissue. Our data strongly suggest that GlcN hydrolysed from GlcNAc after oral administration inhibits DNFB-induced degranulation of mast cells in mice.

Mast cells provide a critical cellular link between soluble factors and synovial eruptions and can contribute to the pathogenic mechanisms in the synovium that result in arthritis (Lee et al. 2002; Nigrovic and Lee 2007; Eklund 2007). Thus, mast cells play a very important role in the inflammation of arthritis. A recent study revealed that mast cells are involved in cellular dysfunction via activation induced by immunocomplexes (Complement 3a, Complement 5a receptor and low affinity IgG receptor Ⅲ) (Baumann et al. 2001). This suggests that the immunocomplexes induce the degranulation of mast cells and the migration of neutrophilis by cytokines released from mast cells and finally evoke inflammation in the joint (Lee et al. 2002). GlcN-HCl and GlcNAc would be the potential effective compounds to relieve inflammation caused by the activation of mast cells. On the other hand, there are some reports that suggest glucosamine has no effect for osteoarthritis (MacAlindon et al. 2004; Felson 2008). The conflicts in clinical trials of glucosamine supplements for osteoarthritis might be due to inter-patient variation in the pathogenic mechanism and the pathological process.

Figure legends

Fig. 1. Effect of glucosamine and related compounds on antigen-induced degranulation of RBL-2H3 cells.

DNP-IgE sensitized cells were incubated with the indicated concentrations of GlcN-HCl, GlcNAc, chitin oligomer or chitosan oligomer for 4 h (A) or 24 h (B) and were then stimulated with 1 μg/mL DNP-BSA for 30 min. Released β-hexosaminidase was measured. Values are means ± SD, $n = 4$. * Significantly different from control, $P < 0.05$. Non, non stimulation; Con, stimulation; GlcN, glucosamine-HCl; GlcNAc, N-acetylglucosamine; Chitin, chitin oligomer; Chitosan, chitosan oligomer.

Fig. 2. Effect of glucosamine and related compounds on the mRNA expression of proinflammatory cytokines in RBL-2H3 cells.

Sensitized cells were incubated with 1 mg/mL GlcN-HCl, GlcNAc, chitin oligomer or chitosan oligomer for 4 h and then cells were stimulated with 1 μg/mL DNP-BSA for 30 min. The mRNA expression levels of TNF-α (A) and IL-6 (B) were quantified by real-time RT-PCR. Data are presented as fold induction relative to the unstimulated cells. Values are means ± SD, $n = 4$. * Significantly different from control, $P < 0.05$. Abbrevations are the same as those given for Fig. 1.

Fig. 3 Effect of glucosamine-HCl and N-acetylglucosamine on DNP-BSA induced intracellular Ca^{2+} mobilization and activation of intracellular molecules.

(A) Sensitized cells were loaded with 4 μM Ca^{2+} indicator Fluo-4/AM for 1 h after treatment with 1 mg/mL glucosamine or related compounds and were stimulated by 10 mg/mL DNP-BSA for 90 sec. The fluorescence of Fluo-4 was then measured. Values are means ± SD, $n = 6$. * Significantly different from control, $P < 0.05$. (B, C) Sensitized cells

were incubated with GlcN-HCl or GlcNAc for 4 h and stimulated with DNP-BSA for 5 min. Cell lysate was immunoprecipitated with Lyn specific antibody, and then phosphorylation of Lyn was detected by western blot analysis using anti-phospho-Lyn antibody (B). Phosphorylation of ERK1/2 in the cell lysate was detected using anti-phospho-ERK1/2 antibody (C). Western blotting was performed independent three experiments. Data were analyzed by the density of bands and represented the intensity of the bands of phosphorylated Lyn/normal Lyn (B) and phosphorylated ERK/normal ERK (C), as a relative value for that of the non-stimulated cells. Values were means±SD, n=3. * Significantly different from control, $P < 0.05$. Statistical analyses were performed by paired-t-test. Abbrevations are the same as those given for Fig. 1.

Fig. 4 Effect of glucosamine-HCl and N-acetylglucosamine on DNFB-induced ear swelling in BALB/c mice.

DNFB-sensitized mice were administrated orally with GlcN-HCl or GlcNAc (0.1 or 1 mg) once a day for 6 days. One hour after the final administration, the mice were challenged on the ears with 20 μL 0.5% DNFB in acetone-soybean oil (4:1). Thickness of the ear was measured at 6 h (A) and at 24 h (B) after treatment with DNFB and values are presented as a percentage of the thickness of the ear measured before DNFB challenge. Values are means ± SD, *n*=3. *Significantly different from control, $P < 0.05$. Abbrevations are the same as those given for Fig. 1.

Fig. 5 Effect of glucosamine-HCl and N-acetylglucosamine on DNFB-induced inflammation.

(A) Twenty-four hours after DNFB-challenge, the ear was fixed by formalin solution and its paraffin section was stained by hematoxylin and eosin (HE). Bars indicate 100 μm. (B) The contents of TNF-α in the ear were measured by ELISA kit. Values are means ± SD,

n=3. (C, D) Histamine in ear (C) and plasma (D) was determined by HPLC with spectrofluorometer. Values are means ± SD, *n*=3. *Significantly different from control, $P < 0.05$. Abbrevations are the same as those given for Fig. 1.

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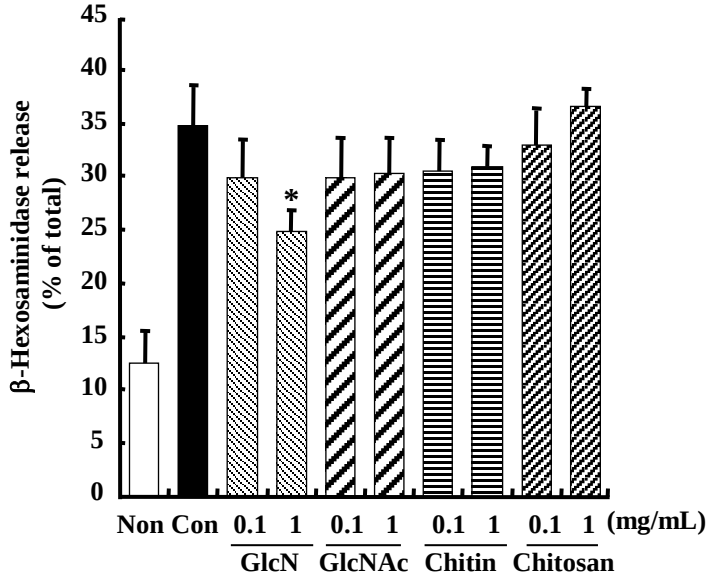
Fig. 1

A. 4 h



B. 24 h

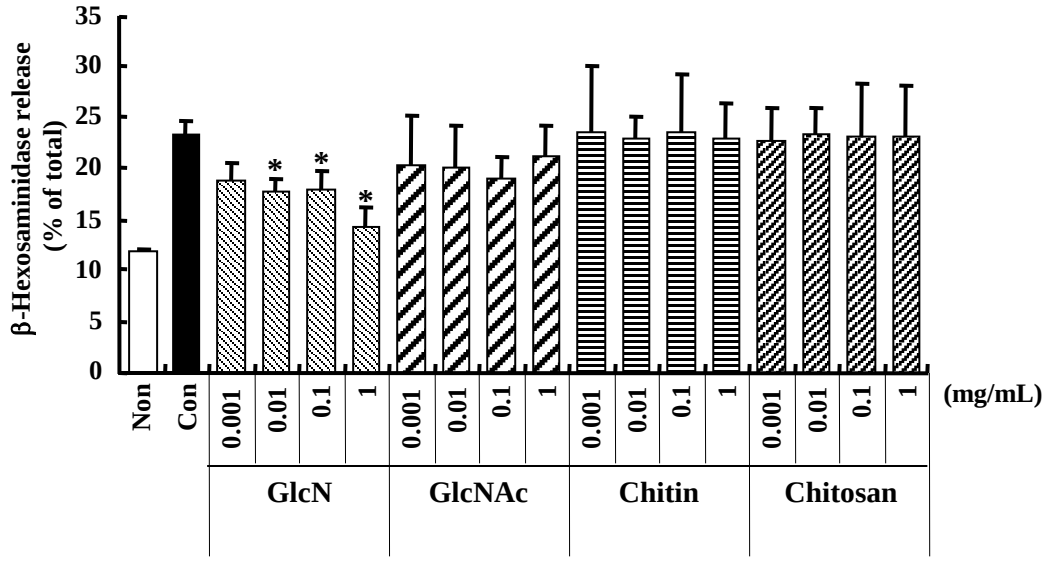


Fig. 2

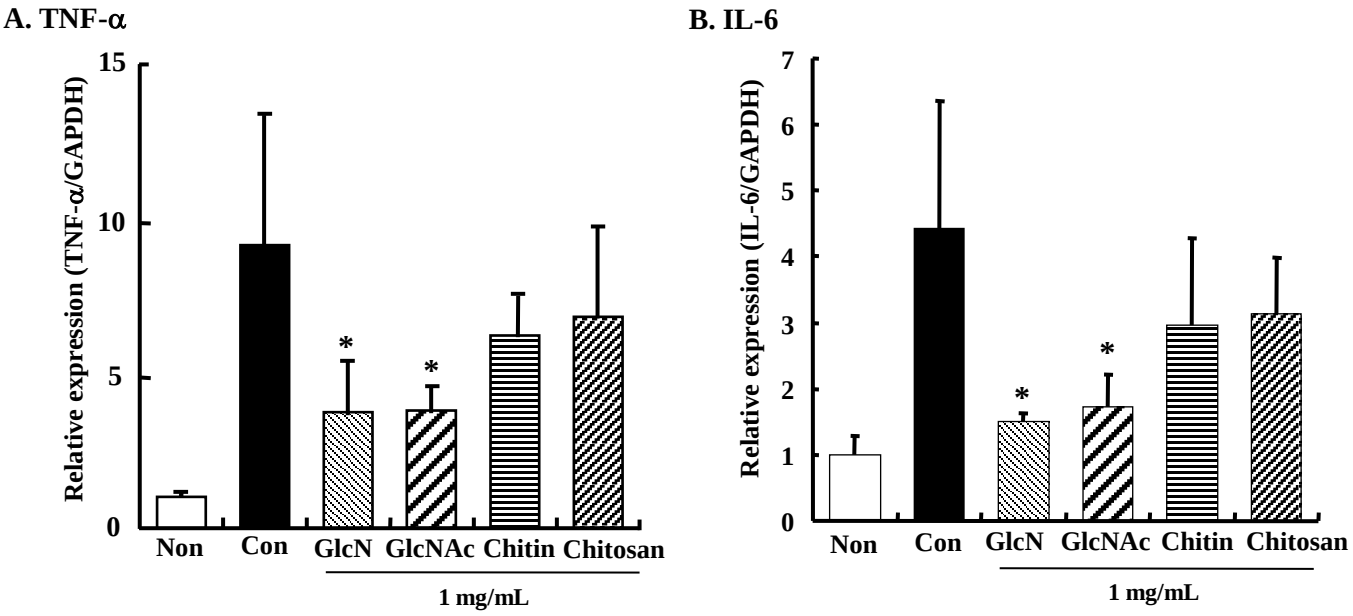

A. TNF-α
Relative expression (TNF-α/GAPDH)
15
10
5
0
*
*
Non
Con
GlcN
GlcNAc
Chitin
Chitosan
1 mg/mL
B. IL-6
Relative expression (IL-6/GAPDH)
7
6
5
4
3
2
1
0
*
*
Non
Con
GlcN
GlcNAc
Chitin
Chitosan
1 mg/mL

Fig. 3

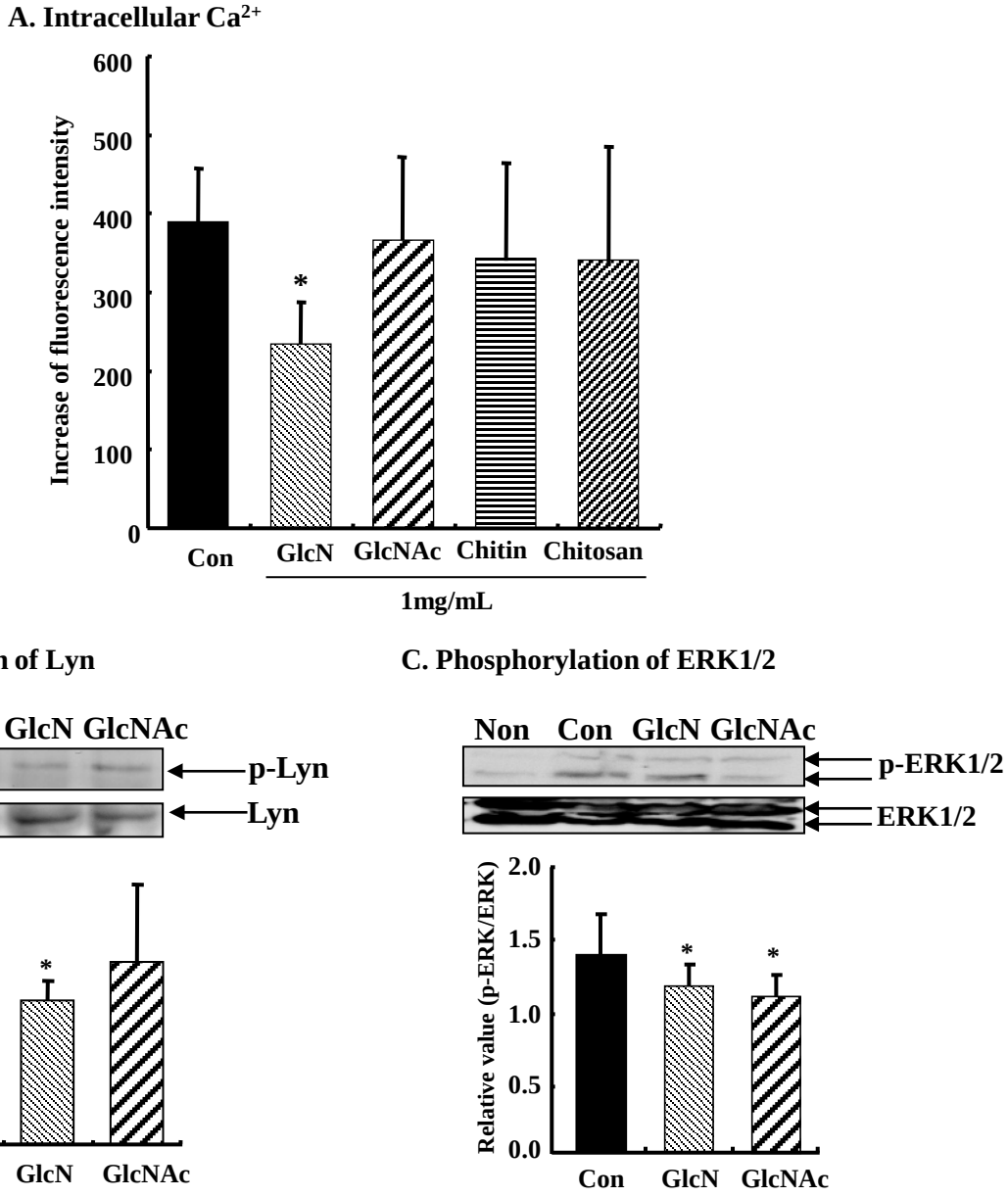
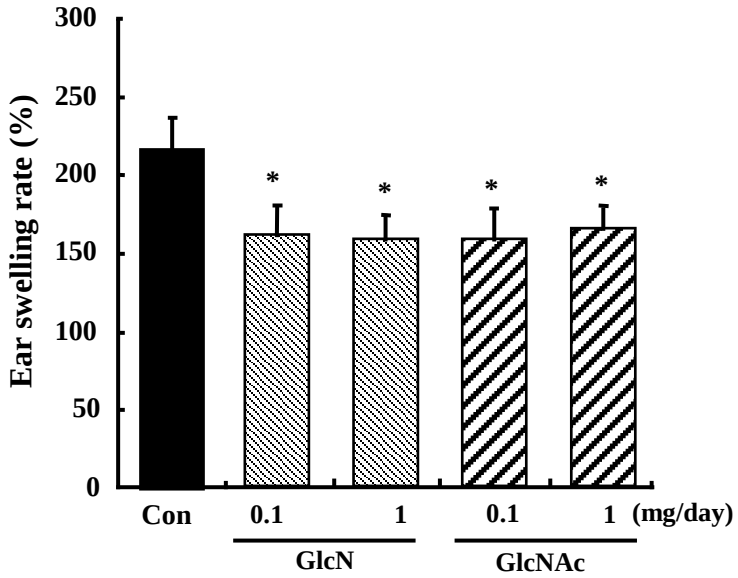

A. Intracellular Ca2+
Increase of fluorescence intensity
600
500
400
300
200
100
0
*
Con
GlcN
GlcNAc
Chitin
Chitosan
1mg/mL
B. Phosphorylation of Lyn
Non
Con
GlcN
GlcNAc
p-Lyn
Lyn
Relative value (p-Lyn/Lyn)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Con
GlcN
GlcNAc
C. Phosphorylation of ERK1/2
Non
Con
GlcN
GlcNAc
p-ERK1/2
ERK1/2
Relative value (p-ERK/ERK)
2.0
1.5
1.0
0.5
0.0
*
*
Con
GlcN
GlcNAc

Fig. 4

A. 6 h



B. 24 h

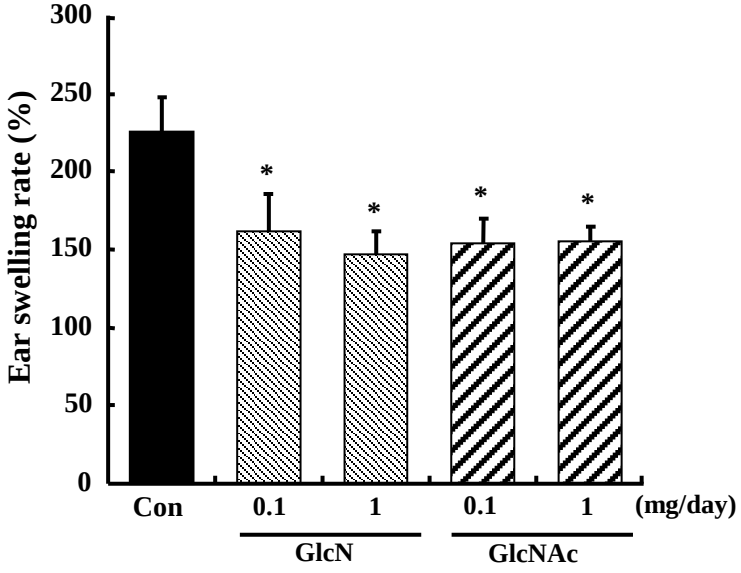
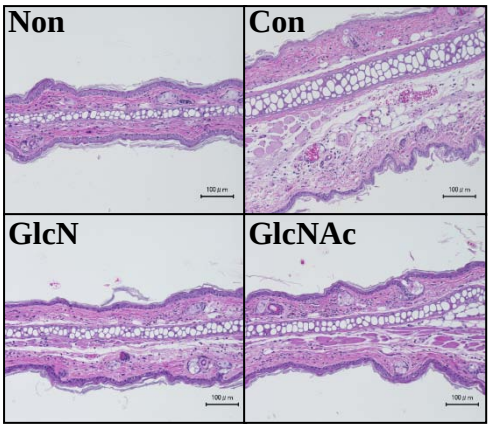
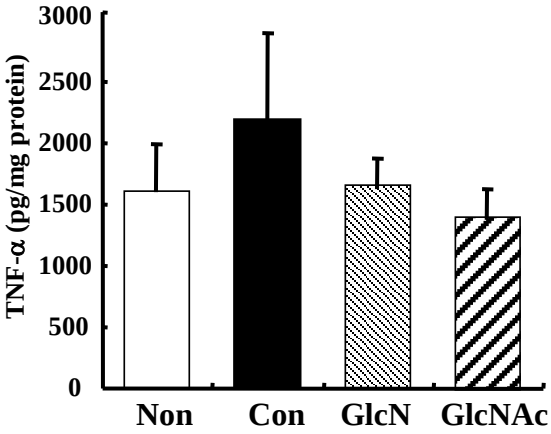


Fig. 5

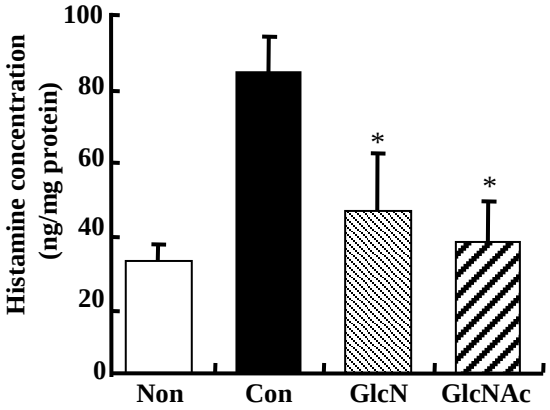
A.



B. TNF-α



C. Histamine in ear



D. Histamine in plasma