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Rifampicin Alleviates Atopic Dermatitis-Like Response *in vivo* and *in vitro*

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Abstract

Atopic dermatitis (AD) is a common inflammatory skin disorder mediated by inflammatory cells, such as macrophages and mast cells. Rifampicin is mainly used for the treatment of tuberculosis. Recently, it was reported that rifampicin has anti-inflammatory and immune-suppressive activities. In this study, we investigated the effect of rifampicin on atopic dermatitis *in vivo* and *in vitro*. AD was induced by treatment with 2, 4-dinitrochlorobenzene (DNCB) in NC/Nga mice. A subset of mice was then treated with rifampicin by oral administration. The severity score and scratching behavior were alleviated in the rifampicin-treated group. Serum immunoglobulin E (IgE) and interleukin-4 (IL-4) levels were also ameliorated in mice treated with rifampicin. We next examined whether rifampicin has anti-atopic activity via suppression of mast cell activation. Rifampicin suppressed the release of β-hexosaminidase and histamine from human mast cell (HMC)-1 cultures stimulated with compound 48/80. Treatment with rifampicin also inhibited secretion of inflammatory mediators, such tumor necrosis factor-α (TNF-α) and prostaglandin D_2 (PGD_2), in mast cells activated by compound 48/80. The mRNA expression of cyclooxygenase 2 (COX-2) was reduced in the cells treated with rifampicin in a concentration-dependent manner. These results suggest that rifampicin can be used to treat atopic dermatitis.

Key Words: Rifampicin, NC/Nga mice, Mast cell, Atopic dermatitis

INTRODUCTION

Atopic dermatitis (AD) is an inflammatory skin disorder associated with epidermal hyper-reactivity to allergens such as dust mites, pollen, and mold spores (Leung *et al*., 2004).

AD is related to immunoglobulin E (IgE) overproduction and is well characterized by increased inflammatory infiltration that leads to elevated serum levels of IgE (Bergmann *et al*., 1998; Chang and Shiung, 2006). Mast cells are effector cells in the inflammatory activity associated with allergic disorders, including asthma and AD. Histamine released from mast cells is a major mediator leading to hypersensitivity, and remains the most characterized and potent vasoactive mediator near allergic lesions (Petersen *et al*., 1996). With the release of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin-4 (IL-4), IL-8, and IL-13, mast cells induce allergic inflammation (Kalesnikoff and Galli, 2008). In general, using medications of adequate potency, mainly corticosteroids, is conventional in the treatment of the vast majority of AD patients (Sidbury and Hanifin, 2000). However, the long-term use of steroids is not desirable because of the systemic adverse effects, including skin atrophy, secondary infection, and acne (Furue *et al*., 2003). Therefore, alternative therapeutic agents are required for the treatment of AD.

Rifampicin is mainly used to treat mycobacterial infections, such as Hansen's disease (leprosy) and tuberculosis (Eule *et al*., 1974; Loeffler, 1999). Rifampicin inhibits bacterial RNA synthesis by inhibiting bacterial DNA-dependent RNA polymerase (Wehrli *et al*., 1968; di Mauro *et al*., 1969; Tsai and Saunders, 1973). Recently, it has been reported that rifampicin exhibits anti-inflammatory effects as well as plays a role in relieving neuropathic pain and in immune modulation (Bellahsene and Forsgren, 1980; Kim *et al*., 2009; Wang *et al*., 2013). Rifampicin was also reported to possess therapeutic effects against psoriasis in clinical practice (Tsankov and Angelova, 2003). However, the anti-allergic effects of rifampicin are not clear. In this study, an atopic *in vivo* and *in vitro* model was used to assess the effects of rifampicin therapy for AD. To this end, we investigated the effect of rifampicin on AD using NC/Nga mice, which are genetically predisposed to develop an AD-like skin disease (Matsuda *et al*., 1997; Tsudzuki *et al*., 1997). Furthermore, we examined the effect of rifampicin

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on degranulation, secretion of TNF-α and prostaglandin D_2 (PGD_2), and mRNA expression of cyclooxygenase 2 (COX-2) in human mast cell (HMC)-1 cultures.

MATERIALS AND METHODS

Reagents

Rifampicin, compound 48/80, disodium cromoglycate (cromolyn), p-nitrophenyl-N-acetyl-β-D-glucosaminide [PN-$(GlcNAc)_2$], thiazolyl blue tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), toluidine blue dye, $CaCl_2$, $MgCl_2$, $NaHCO_3$, and glucose were purchased from Sigma-Aldrich Korea (Yongin, Korea). The histamine enzyme-linked immunosorbent assay (ELISA) kit was obtained from IBL International GmbH (Hamburg, Germany). We purchased the human PGD_2 ELISA kit from Cusabio (Wuhan, China) and the TNF-α ELISA kit from eBioscience (San Diego, USA). The total RNA isolation kit was purchased from GeneAll (Seoul, Korea). Fluo-3 AM was obtained from Molecular Probes (Eugene, OR, USA).

Animals

Four-week-old male NC/Nga mice were purchased from Central Laboratory Animal, Inc (Woomyundong, Seoul, Korea). They were housed under standard conditions of ambient temperature (23 ± 2°C) and humidity (55 ± 10%), with free access to chow pellets and water for one week before the start of the experiments. The experimental groups included 5-7 animals per drug and dose. Animals were treated and maintained according to the Animal Care and Use Guidelines of Sahmyook University, Korea.

AD induction and treatment with rifampicin

The dorsal surface of each mouse was shaved with animal clippers before the experiments. The dorsal region was treated with 2, 4-dinitrochlorobenzene (DNCB) to induce AD (Jung *et al*., 2014). After induction of AD, mice were treated with rifampicin by oral administration (Fig. 1A). Dexamethasone was used as a control. Mice were randomly assigned to one of five groups at the start of the experiment (n=5-7): Group A, normal control; Group B, AD control; Group C, rifampicin treatment (25 mg/kg); Group D, rifampicin treatment (50 mg/kg); Group E, dexamethasone treatment (1 mg/kg).

Skin severity score and scratching behavior

Mice were photographed before and after rifampicin treatment. The skin severity test was evaluated once a day after completing treatment as previously described (Suto *et al*., 1999). Briefly, the development of (1) erythema/hemorrhage, (2) dryness/scaling, (3) edema, (4) erosion/excoriation, and (5) lichenification was scored as 0 (none), 1 (mild), 2 (moderate), or 3 (severe). The sum of the individual scores was taken as the dermatitis score, ranging from 0 to 15. Scratching behavior was recorded on video as previously described (Umeda *et al*., 2006; Chung *et al*., 2012). The scratching number was counted as the number of times a mouse scratched the dorsal skin lesion within a period of 30 min. The number of scratches in the non-treatment group was regarded as the baseline scratching behavior before the start of treatment.

Blood collection for serologic tests

Mice were killed 1, 3, or 7 days after treatment with rifampicin, and cardiac blood was collected for the measurement of serum IgE and IL-4 levels. Blood was separated by centrifugation at 3,000 *g* for 10 min at 4°C, and the serum was stored at -80°C until use.

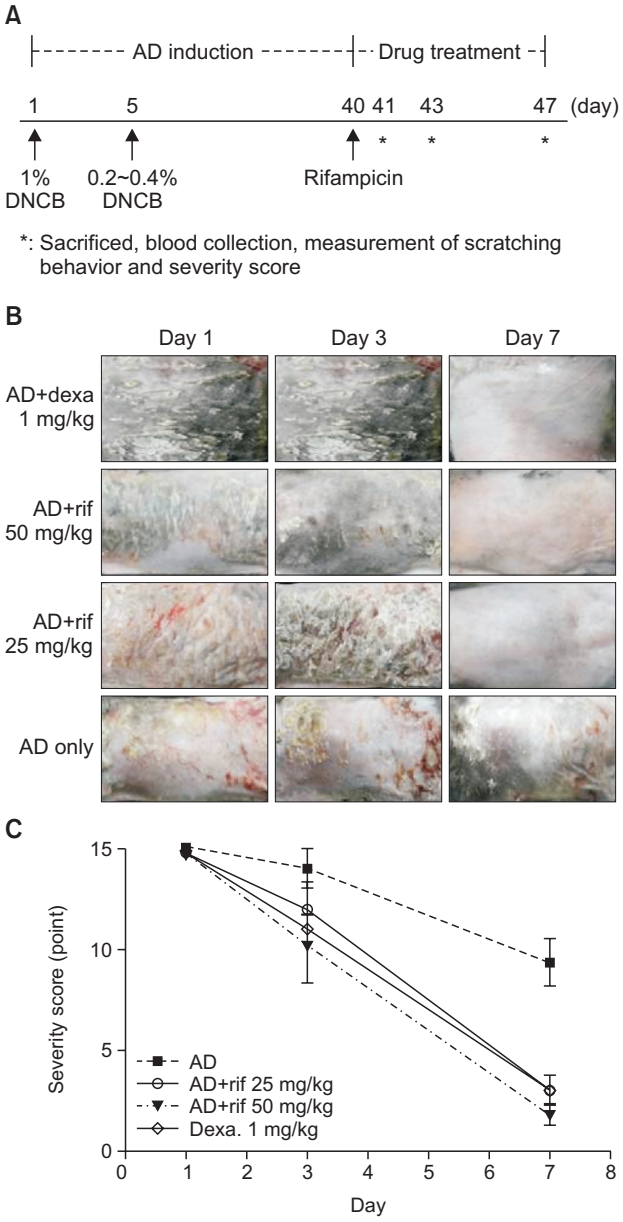

Fig. 1. The effect of rifampicin on AD-induced NC/Nga mice (n=5-7). The mice were observed 7 days after oral administration of rifampicin. Dexamethasone (1 mg/kg) was used as a positive control. (A) The scheme of the animal experiment. (B) Image of the lesions from AD-induced mice at day 1 and day 7 after rifampicin treatment. (C) Rifampicin significantly mitigated five symptoms: erythema/darkening, edema/population, excoriations, lichenification/prurigo, and dryness 7 days after treatment. The improvement in skin lesions was evaluated based on the skin severity score. **p*<0.05, ***p*<0.01, when compared with the AD-induced and untreated mice.

Cell culture and treatment

The HMC-1 cell line was kindly provided by Prof. Jang (Col-

lege of Korean Medicine, Kyung Hee University, Korea). HMC-1 cells were cultured in Dulbecco's modified Eagle medium (DMEM, Welgene, Seoul, Korea) with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% CO_2 with 95% humidity. The cells were treated with the indicated concentrations of rifampicin for the indicated times and were then stimulated with compound 48/80 (10 μg/ml) for 30 min. The cell culture supernatants and cell lysates were used for ELISA and reverse-transcription polymerase chain reaction (RT-PCR), respectively.

Measurement of cell viability

The trypan blue exclusion method and MTT assay were used to determine viable and dead cells to test the cytotoxicity of rifampicin. Cell viability was evaluated by the colorimetric MTT assay. Cells were seeded in 96-well plates (2×10^5 cells/well) and incubated for 24 h. After treatment with rifampicin, 20 μl of MTT solution (5 mg/ml) was added for 4 h at 37°C. Subsequently, the medium was removed, and 100 μl DMSO was added to extract formazan. The plate was shaken for 15 min. A microplate reader was used to read the absorbance at 540 nm.

β-Hexosaminidase (β-HEX) secretion assay

Degranulation of HMC-1 cells was measured by β-HEX assay. The amount of β-HEX released into the medium was determined as described previously (Schwartz *et al*., 1979). HMC-1 cells were grown in 24-well plates for one day. After washing twice with Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.1 mM $MgCl_2$, 11.9 mM $NaHCO_3$, 0.6 mM NaH_2PO_4, 5.6 mM glucose, pH 7.2), the cells were pretreated with different concentrations of rifampicin (6.25, 12.5, 25, or 50 μM) for 30 min at 37°C. Cromolyn, which is a mast cell stabilizer, was used as a positive control. Next, the cells were treated with compound 48/80 (10 μg/ml) for 30 min at 37°C. The supernatant (50 μL) was transferred to a 96-well plate and incubated with 50 μL of 5 mM PN-$(GlcNAc)_2$ for 1 h at 37°C. The assay was terminated by the addition of stop solution (200 μL/well of 0.1 M Na_2CO_3/$NaHCO_3$ pH 10.2). The absorbance was measured with a microplate reader at 405 nm (Schwartz and Austen, 1980).

Histamine release assay

HMC-1 cells (5×10^5 cells/well) were incubated for 12 h at 37°C. The cells were treated with various concentrations of rifampicin for 2 h and then treated with compound 48/80 (10 μg/ml) for 30 min. Cromolyn was used as a positive control. The cell culture supernatants were collected, centrifuged at 400 g for 5 min at 4°C, and then used for the measurement of histamine release using an ELISA kit. The assay was performed according to the manufacturer's instructions.

Intracellular calcium

To measure intracellular calcium levels, HMC-1 cells were pre-incubated with 5 μM Fluo-3 AM for 30 min at 37°C and then washed with Tyrode's buffer. The cells were treated with rifampicin for 30 min at 37°C and then stimulated with compound 48/80. After 10 min, intracellular calcium levels were analyzed using a fluorescence microscope (Olympus CKX41, Olympus Corp., Tokyo, Japan). Cromolyn was used as a positive control.

Table 1. RT-PCR primer sequences for the genes used in this study

Target gene	Primer sequence
COX-2	Forward: 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3'
	Reverse: 5'-AGATCATCTCTGCCTGAGTATCTT-3'
GAPDH	Forward: 5'-CAAAAGGGTCATCATCTC-3'
	Reverse: 5'-CCTGCTTCACCACCTTCTTG-3'

Measurement of PGD_2 and TNF-α production

HMC-1 cells (1×10^6 cells/well) were incubated for 12 h at 37°C. The cells were treated with various concentrations of rifampicin for 2 h and then with compound 48/80 (10 μg/ml) for 30 min. The cells were washed with PBS and incubated for 18 h. The supernatants were collected, centrifuged at 400 g for 5 min at 4°C, and used for PGD_2 and TNF-α ELISA tests, according to the manufacture's protocol.

RT-PCR

RT-PCR was performed as previously described (Kwon *et al*., 2016). HMC-1 cells (1×10^6/well) were seeded in plates. The cells were pretreated with various concentrations of rifampicin at 37°C for 2 h. After washing, the cells were stimulated with compound 48/80 (10 μg/ml) for 4 h. Extraction of total RNA was carried out with an RNA isolation kit (GeneAll), and cDNA was synthesized using an RT premix (Bioneer, Seoul, Korea). RT-PCR was used to determine COX-2 mRNA expression levels. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a standard control. The PCR reaction was performed with a Bio-Rad PCR system (Thermal cycler, Bio-Rad, USA). After PCR, the products were electrophoresed on 1% agarose gels containing ethidium bromide. The COX-2 band signal was normalized to that of GAPDH. Table 1 shows the primer sequences used in this study.

Measurement of IgE and cytokines

Mouse serum and cell culture supernatants were assayed for IgE, IL-4, and TNF-α using ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions, and as described previously (Lim *et al*., 2015; Park *et al*., 2016).

Statistical analysis

All data were analyzed with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical comparisons of data were performed by using Student's *t*-tests. A value of $p<0.05$ or $p<0.01$ indicated statistical significance.

RESULTS

Rifampicin improves AD in the NC/Nga mouse model.

To investigate the effect of rifampicin on AD, the NC/Nga mice were orally administered the drug. The skin lesions of the mice were observed until 7 days after the treatment. Rifampicin improved AD-like symptoms, which was comparable to the effect of dexamethasone (Fig. 1B). The lesions were also evaluated with severity scores and scratching behavior assessments. As shown in Fig. 1C, the severity score significantly decreased in the rifampicin-treated group after 7

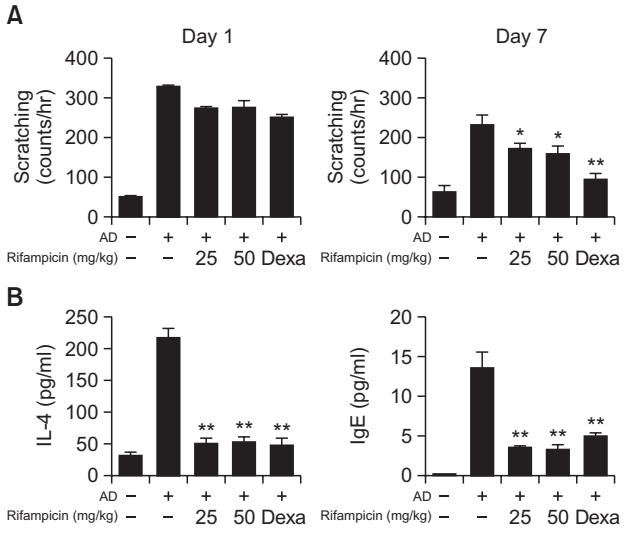

Fig. 2. The effect of rifampicin on scratching behavior, serum IgE, and IL-4 levels in AD-induced NC/Nga mice. The AD-induced mice were observed 7 days after oral rifampicin administration. Dexamethasone (1 mg/kg) was used as a positive control. (A) Scratching behavior and (B) serum IL-4 and IgE levels were measured at the indicated times. Experimental values are given as means ± SEM (n=5). **p*<0.05, ***p*<0.01, when compared with the AD-induced and untreated mice.

days compared to that of the non-treated group. To determine whether the effect of rifampicin was dose dependent, two different doses (25 and 50 mg/kg) were administered to the AD-induced mice. As we expected, rifampicin reduced the skin severity score in a dose-dependent manner. We next investigated the effects of rifampicin on scratching behavior in NC/Nga mice. The treatment with rifampicin significantly reduced scratching behavior in the AD mice on day 7 after treatment compared to that of the untreated AD mice (Fig. 2A).

As it has been reported that AD is an IgE-mediated type I hypersensitivity reaction and its major characteristic is the hyperproduction of IgE (Chang and Shiung, 2006), total serum IgE levels were measured in the presence or absence of rifampicin and the values were compared with those of the controls. As shown in Fig. 2B, when AD was induced, the IgE levels were higher than those of the non-treated control group, and the levels in the AD mice treated with rifampicin were significantly attenuated on day 7 after treatment. IL-4 levels were also increased in the AD-induced mice, and treatment with rifampicin alleviated this increase (Fig. 2B). Consistent with the results of the skin severity score and scratching behavior assessments, rifampicin exhibited a strong inhibitory effect on IgE and IL-4 secretion in the AD mouse model.

Effect of rifampicin on β-HEX and histamine release from HMC-1 cells

Granules, such as those containing histamine, derived from mast cells are important mediators of itching and vasodilation. Therefore, the effect of rifampicin on the degranulation of mast cells using the HMC-1 cell line was investigated. An MTT assay was performed to examine the effect of rifampicin on cell viability. HMC-1 cells were treated with various concentrations of rifampicin. Rifampicin did not alter cell viability even at 50 μM, a concentration greater than that routinely used in this study, which showed its inhibitory effect on pro-inflammatory cytokine production (data not shown).

β-HEX has been used as a marker of degranulation of mast cells (Schwartz *et al*., 1979; Wang *et al*., 2012). We evaluated the effect of rifampicin on compound 48/80-induced β-HEX release from HMC-1 cells. Although compound 48/80 stimulated HMC-1 cells to release β-HEX, rifampicin significantly inhibited the secretion of β-HEX induced by compound 48/80 in a concentration-dependent manner (Fig. 3A). We next assessed the ability of rifampicin to affect compound 48/80-induced histamine release from HMC-1 cells. Cromolyn was used as a positive control because it is a mast cell stabilizer. The HMC-1 cells released increased levels of histamine when stimulated with compound 48/80. As we expected, rifampicin inhibited compound 48/80-induced histamine release in a concentration-dependent manner (Fig. 3A). Histamine release from mast cells is correlated with increased intracellular calcium concentrations (Pearce, 1985). Therefore, we assessed the effect of rifampicin on intracellular calcium levels in HMC-1 cells activated with compound 48/80. The results show that, while intracellular calcium was increased in compound 48/80-stimulated HMC-1, calcium levels were decreased in HMC-1 cells pretreated with rifampicin (Fig. 3B). These results demonstrate that rifampicin has anti-histamine activity via an inhibitory effect on mast cell degranulation.

Effect of rifampicin on the secretion of inflammatory mediators in HMC-1 cells

PGD2 and TNF-α are important mediators in allergic inflammation (Gordon *et al*., 1990). COX-2-derived PGD_2 is known to mediate AD (Fournier *et al*., 1997). To investigate the anti-inflammatory effects of rifampicin, cell culture media were used for PGD_2 and TNF-α ELISAs. Treatment of rifampicin attenuated the secretion of PGD2 and TNF-α in HMC-1 stimulated with compound 48/80 (Fig. 4A, 4B). COX-2 is known to be associated with inflammatory reactions. The mRNA expression of COX-2 was determined by RT-PCR (Fig. 4C). The results show that rifampicin inhibited COX-2 expression in compound 48/80-activated HMC-1 cells, suggesting that rifampicin inhibits compound 48/80-induced PGD_2 secretion via suppression of COX-2 expression in mast cells.

DISCUSSION

Our study showed a novel anti-AD activity of the existing drug, rifampicin. During our study for novel anti-AD agents, we found that rifampicin possessed anti-AD activity in a mouse model. Our results showed that rifampicin effectively reduced the characteristic features of AD, such as elevated serum IgE and IL-4 levels (Fig. 1, 2). The mice treated with rifampicin did not show evidence of skin disease 7 days after oral administration of rifampicin, which was comparable to the outcome in the dexamethasone-treated group (positive control group). The reduction in the severity of symptoms and IgE production suggest that rifampicin is effective for the treatment of AD in a dose-dependent manner.

Furthermore, we assessed whether rifampicin has anti-allergic activity using an *in vitro* model in this study. In the 1970s, researchers discovered that rifampicin suppressed both humoral and cellular immunity *in vitro* and *in vivo* (Paunescu, 1970; Nilsson, 1971). Recent studies have again brought

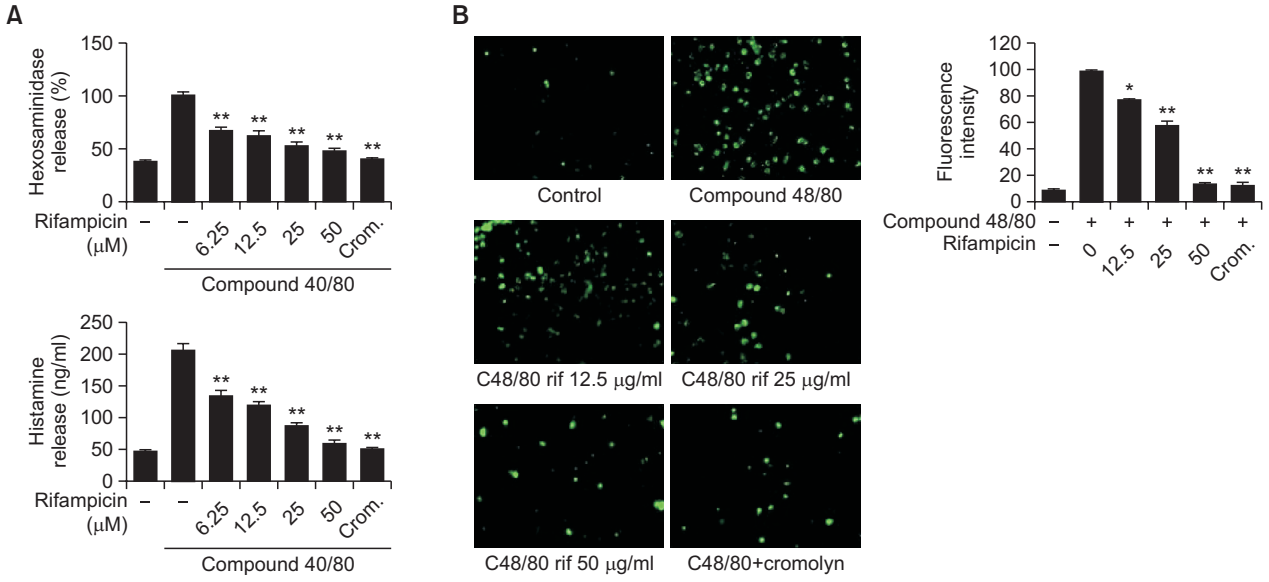

Fig. 3. Effect of rifampicin on degranulation of mast cells. The HMC-1 cells were stimulated with compound 48/80 (10 μg/ml) and then treated with rifampicin (6.25 μM to 50 μM). Cromolyn (100 μM) was used as a positive control. (A) β-hexosaminidase and histamine release, (B) intracellular calcium levels were assessed in the HMC-1 cells. **p*<0.05, ***p*<0.01, compared with the C48/80-treated group.

the immunomodulating ability of rifampicin into the spotlight (Mlambo and Sigola, 2003; Tsankov and Angelova, 2003; Ziglam *et al*., 2004). Our study showed that rifampicin has anti-allergic activity. β-HEX is stored in secretory granules of mast cells and basophils, and is released along with histamine when mast cells are activated (Wang *et al*., 2012). Rifampicin reduced both β-HEX and histamine secretion, which are biomarker of allergic reactions (Fig. 3). Intracellular calcium, as well as IgE, activates mast cells, and histamine release is affected by intracellular calcium levels (Pearce, 1985; Eisenhut and Wallace, 2011). Stimulation with compound 48/80 increased the intracellular calcium levels in HMC-1 cells. When the cells were pretreated with rifampicin, however, the intracellular calcium levels decreased (Fig. 3). Thus, it seems that rifampicin suppresses the degranulation of mast cells by controlling intracellular calcium concentrations, suggesting that rifampicin can be used as a new anti-allergic agent.

The early-phase of an allergic reaction occurs within minutes after allergen exposure, whereas the late-phase reaction occurs hours later and involves secretion of cytokines, such as TNF-α and IL-4. Proinflammatory cytokines have been shown to play a critical role in late-phase hypersensitivity reactions (Wang *et al*., 2012). The present study showed that rifampicin also decreased the production of TNF-α in mast cells (Fig. 4).

In a recent study, it was demonstrated that COX-2 plays important roles in mast cell-mediated inflammation (Kim *et al*., 2005). PGD_2 is a marker of mast cell activation by allergens in bronchial asthma, and it is a major cyclooxygenase metabolite generated by mast cells (Bochenek *et al*., 2004). The expression of COX-2 and PGD_2 is related to mast cell-mediated chronic inflammation. Our study showed that their expressions were also decreased by rifampicin pretreatment (Fig. 4). Decreasing the release of these cytokines and proinflammatory mediators is a good therapeutic approach.

In conclusion, we demonstrated that rifampicin suppressed the release of allergic mediators, including β-HEX, histamine,

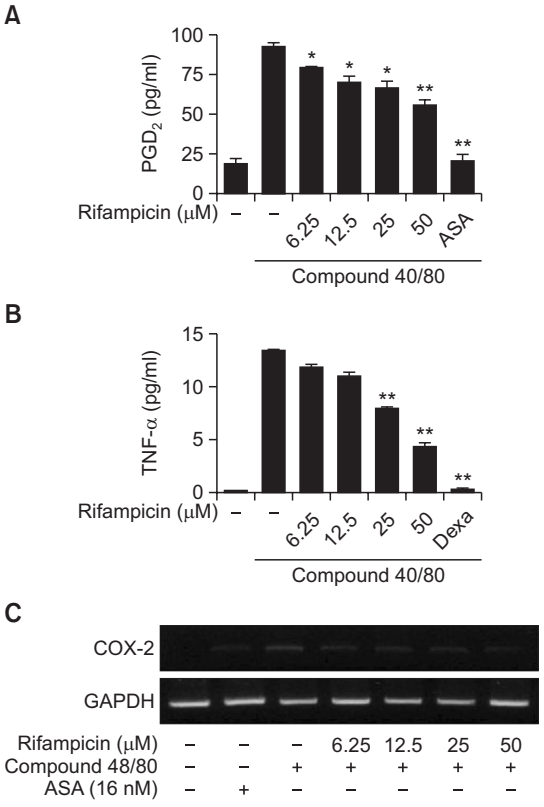

Fig. 4. Effect of rifampicin on anti-inflammatory mediator expression in mast cells. The HMC-1 cells were stimulated with compound 48/80 (10 μg/ml) and then treated with rifampicin (6.25 to 50 μM). Acetylsalicylic acid (ASA: 16 nM) or dexamethasone (Dex; 2 nM) were used as positive controls. The production of (A) PGD^2 and (B) TNF-α was assessed in the cells. (C) The expression of COX-2 mRNA was also measured in the cells. **p*<0.05, **p<0.01, compared with the C48/80-treated group.

PGD_2, and the proinflammatory cytokines, TNF-α and COX-2. And it enables cell membrane to become stable. Rifampicin could be used as a treatment to alleviate allergic symptoms.

Drug repositioning is the application of known drugs and compounds to treat new diseases, and is the process of identifying new uses for drugs outside the scope of their original medical indication. Traditional drug discovery approaches have largely failed to deliver on promises of improved productivity, despite large increases in funding (Ashburn and Thor, 2004; Sleigh and Barton, 2010). By utilizing existing drugs, drug repositioning can provide a faster and cheaper approach than traditional drug development because the repositioned drug has already passed many regulatory requirements, its safety is known, and the risk of failure due to unacceptable side effects is reduced.

Rifampicin has been used for the treatment of mycobacterial infection for many years. Our study revealed a novel anti-AD activity of rifampicin. The effect of rifampicin on AD was comparable to that of dexamethasone, which is a well-known anti-inflammatory agent, suggesting that rifampicin is an alternative medication for the management of AD. Understanding the relationships between each step involved in the anti-AD activities and the compounds targeting them is pivotal to study the molecular mechanisms underlying the cellular responses. Our future studies will establish the biochemical and molecular mechanism of rifampicin's anti-AD activity. Nevertheless, our current findings suggest that rifampicin it effective, and it could also be used to develop future, potent therapeutic reagents for the treatment of AD.

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