

Rebamipide suppresses TNF- α mediated inflammation *in vitro* and attenuates the severity of dermatitis in mice

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Keywords

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Rebamipide is a routine drug for the treatment of gastritis in a clinical setting. Recently, it has been shown to protect against various inflammatory diseases, and has provided a potential therapy for these diseases. However, whether rebamipide has a role in dermatitis remains to be elucidated. Here, we found that rebamipide alleviated the inflammatory reaction induced by tumor necrosis factor- α in RAW264.7, a stable macrophage cell line. Furthermore, rebamipide treatment repressed activation of nuclear factor-kappaB signaling, a well-established inflammatory signaling pathway. Moreover, an oxazolone-induced dermatitis mouse model was established to investigate the role of rebamipide *in vivo*. PBS control group exhibited typical skin inflammation, whereas treatment with rebamipide remarkably attenuated a dermatitis phenotype in this mouse model. The protective role of rebamipide in dermatitis *in vivo* was probably due to its inhibition of nuclear factor-kappaB signaling. Collectively, rebamipide may represent a promising molecular target for the prevention and treatment of inflammatory skin diseases.

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Introduction

Contact dermatitis is an inflammatory skin disorder commonly observed in the clinical setting, and is characterized by pruritic skin lesions and immunodysregulation, as well as disrupted epidermal barrier function. Moreover, it is a well-accepted model system

by which to assess potential therapies for psoriasis and atopic dermatitis [1]. It is reported that tumor necrosis factor (TNF) - α plays a harmful role in various inflammatory diseases [2]. To date, anti-TNF therapy has been developed in clinic for autoimmune inflammatory

Abbreviations

COX-2, cyclooxygenase-2; DLN, draining lymph node; Foxp3, forkhead box P3; IL, interleukin; iNOS, inducible nitric oxide synthase; NF- κ B, nuclear factor-kappaB; TNF- α , tumor necrosis factor α ; Treg, regulatory T cell; VEH, vehicle; WT, wild-type.

diseases such as psoriasis, and has been proved to be effective in these patients [3,4].

Rebamipide is a commonly accepted gastroprotective agent that has been used in clinic for years [5]. It is known to be a multifunctional molecule, and play a critical role in various physiological and disease processes such as gastric ulcer [6], gastric cancer [7], hypersensitivity [8] and primary Sjögren's syndrome [9]. To date, different groups have identified the anti-inflammatory function of rebamipide [10–12]. Rebamipide is known to protect corneal epithelial cells from the TNF- α -induced disruption of barrier function [13]. Recently, it was reported that rebamipide suppresses bladder inflammation in a rat cystitis model [11]. In addition, rebamipide is found to attenuate collagen-induced arthritis, an inflammatory arthritis mediated dominantly by TNF- α [12]. The nuclear factor-kappaB (NF- κ B) signaling pathway is a well-established pathway for inflammation, including dermatitis [14,15]. It has been shown that rebamipide interferes with inflammatory signaling pathways including the NF- κ B pathway [7], but it is not known whether rebamipide plays a role in inflammatory skin diseases. Herein, we examined the antagonistic function of rebamipide in TNF- α -mediated inflammation *in vitro*, and determined the potential therapeutic function of rebamipide in inflammatory skin diseases using oxazolone-induced contact dermatitis models.

Results

Rebamipide suppressed TNF- α -mediated inflammatory reaction in RAW264.7 cells

It is well established that macrophages play a critical role in dermatitis [16]. To examine the therapeutic function of rebamipide in the inflammatory reaction of macrophages, a stable macrophage cell line, RAW264.7, was cultured. The cells were then stimulated with 10 ng·mL⁻¹ TNF- α – a dominant inflammatory mediator that plays an indispensable role in inflammation, including dermatitis – in the presence or absence of 5 mM rebamipide for indicated time points. As shown in Fig. 1(A–D), rebamipide significantly abolished the levels of inflammatory biomarkers in the presence of TNF- α . It is known that inducible nitric oxide synthase (iNOS) is a critical inflammatory mediator in dermatitis [17]. To examine the expression profiling of TNF- α -induced iNOS in the presence or absence of rebamipide, western blotting was performed and Fig. 1(E) shows that rebamipide largely impaired the expression of iNOS in the RAW264.7 macrophage cell line. Moreover, the Greiss experiment was per-

formed and the amount of nitrite promoted by TNF- α was suppressed by rebamipide in a dose-dependent manner (Fig. 1F). Furthermore, levels of interleukin (IL) -1 and IL-6 in conditional medium of the indicated groups were measured through ELISA. As shown in Fig. 1(G,H), both inflammatory mediators were greatly antagonized by the additional administration of rebamipide.

Rebamipide improved skin inflammation in a mouse contact dermatitis model

Rebamipide is reported to be protective in mouse arthritis models [12]. To evaluate whether rebamipide also plays a therapeutic role in inflammatory skin diseases, an oxazolone-induced dermatitis mouse model was established and treated with PBS or rebamipide, as described in the Materials and Methods. Ear tissue was collected from various groups and histological analysis was performed. Ear thickness from all the groups was measured, and the rebamipide treatment group showed much less promotion of ear thickness than the PBS control group (Fig. 2A). It is known that inflammatory cells are accumulated in the skin of dermatitis [18]. Moreover, rebamipide has been reported to antagonize macrophages and T lymphocytes in various diseases [12,19]. To investigate whether rebamipide prevents against infiltration by T lymphocytes and macrophages, immunohistochemistry was performed for CD4 and CD68. As shown in Fig. 2(A), rebamipide dramatically antagonized the accumulation of T lymphocytes and macrophages in this dermatitis model. Histological assessment of ear samples showed that thickening of the epithelium and infiltration of inflammatory cells were also inhibited in the rebamipide treatment group (Fig. 2B,C). Furthermore, RNA was isolated and collected from ear tissues of the indicated groups, then assayed by real-time PCR. The results determined that levels of inflammatory parameters, including IL-1 β , IL-6 and iNOS, were significantly decreased in rebamipide treatment group (Fig. 3A–C). Sera were collected from both the rebamipide and PBS treatment groups, and circulating levels of IL-1 β as well as IL-6 were assayed through ELISA. As revealed in (Fig. 3E,F), levels of both inflammatory cytokines induced in the oxazolone-induced dermatitis model were remarkably diminished through additional treatment with rebamipide. Total protein was also collected from ears in both the PBS and rebamipide treatment groups ($n = 3$, respectively), and western blotting was performed to assess the iNOS level. As shown in (Fig. 3H), rebamipide treatment markedly reduced the iNOS protein level in dermatitis.

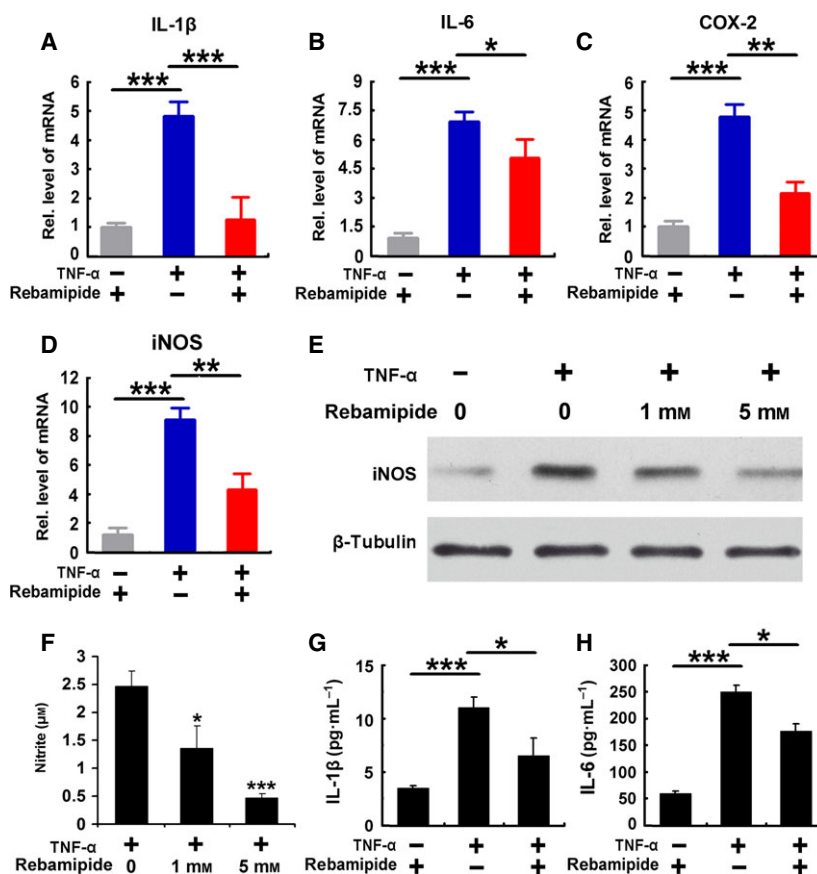


Fig. 1. Rebamipide attenuated skin inflammation in a TNF- α -induced inflammatory reaction in RAW264.7 cells. (A–D) Relative levels of IL-1 β , IL-6, COX-2 and iNOS in RAW264.7 cells treated by mere rebamipide, or stimulated by TNF- α with or without treatment of rebamipide, assayed by real-time RT-PCR. (E) Expression of iNOS in PBS and rebamipide treatment group following TNF- α stimulation, assayed by western blotting. Protein extracts were collected from RAW264.7 cells 24 h after TNF- α stimulation, in indicated treatment groups, and western blotting was performed. (F) Levels of nitrite in the PBS and rebamipide treatment groups following TNF- α stimulation, assayed by Greiss experiment. Conditional media were collected from indicated treatment groups of RAW264.7 cells, and the Greiss experiment was performed. (G,H) Expression levels of IL-1 β and IL-6 in TNF- α -stimulated RAW264.7 cells were significantly diminished by rebamipide, as measured by ELISA. Conditional media were collected from both treatment groups of RAW264.7 cells, and ELISA was performed for IL-1 β and IL-6. The values are the mean $\pm$ SD. * P < 0.05, ** P < 0.01 and *** P < 0.005 vs. PBS treatment groups.

Rebamipide-attenuated inflammatory reaction in the draining lymph node of a mouse contact dermatitis model

Draining lymph node (DLN) is known to be critical for testing the severity of inflammation in various diseases [20]. To further investigate the role of rebamipide, cervical DLNs were isolated from rebamipide- and PBS treated oxazolone-induced dermatitis models, as described in Materials and Methods. As shown in Fig. 4 (A), rebamipide treatment remarkably reduced DLN size, which suggested attenuation of skin inflammation. The DLN samples were also isolated and assessed via real-time PCR, and the result revealed that gene expression levels of inflammatory cytokines

in DLN from the mouse dermatitis model, including IL-1 β , IL-6, cyclooxygenase-2 (COX-2) and iNOS, were dramatically diminished by rebamipide treatment (Fig. 4B–E). iNOS is known to mediate the inflammatory reaction in oxazolone-induced dermatitis mouse models. To determine the effect of rebamipide on the induction of iNOS in DLN of skin inflammation, western blotting assay for iNOS was then performed. Figure 4(F) indicates that iNOS expression was greatly suppressed in DLN homogenates in the rebamipide treatment group when compared with PBS controls. Regulatory T cells are a well-established repressor of inflammation. In the current study, DLNs were harvested from dermatitis models, followed by real-time PCR (Fig. 4G), as well as flow cytometry (Fig. 4H) to

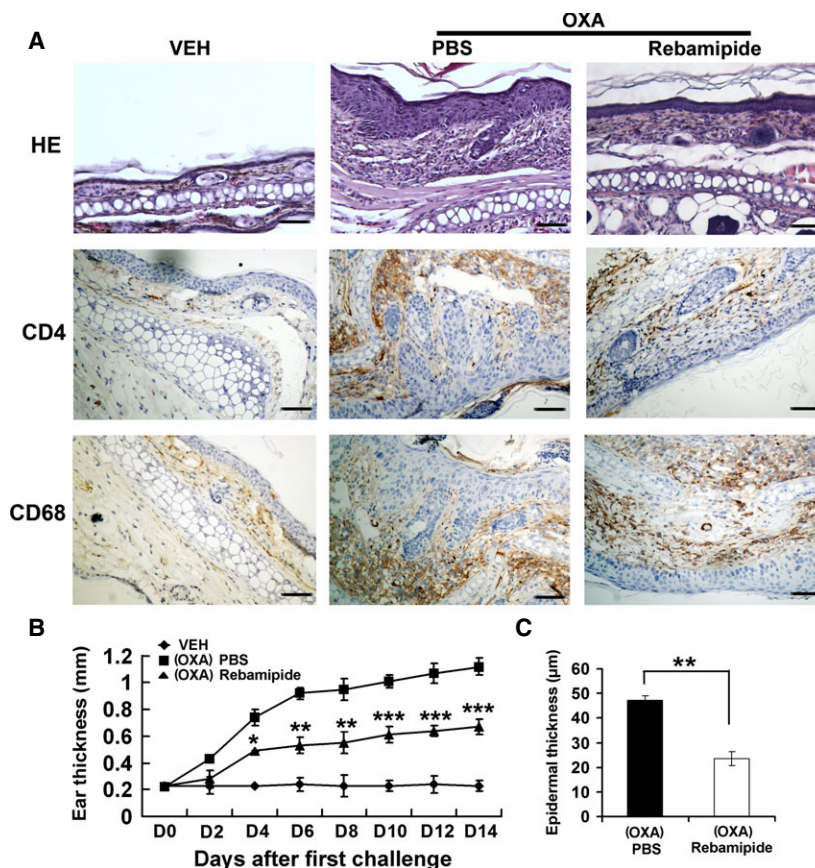


Fig. 2. Rebamipide-attenuated skin inflammation in an oxazolone-induced dermatitis model. (A) Rebamipide treatment inhibited the increase in ear thickness, and antagonized infiltration of macrophages as well as T lymphocytes in oxazolone-induced dermatitis, detected by H&E staining and immunohistochemistry for CD68 as well as CD4. Scale bar = 50 μ m. (B) Ear thickness was measured every other day for 14 days and compared between PBS and rebamipide treatment groups ($n = 7$, respectively). (C) Rebamipide treatment attenuated thickening of epithelium and infiltration of inflammatory cells in a dermatitis model, as measured by histology. Each experiment was repeated in triplicate and the values are given as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$ vs. PBS treatment group. OXA, oxazolone; PBS; VEH, vehicle.

compare CD4 + Foxp3 + regulatory T-cell (Treg) development in both the PBS and rebamipide treatment groups. The result revealed that the Treg/total T-cell ratio was remarkably higher in the rebamipide treatment group, suggesting that rebamipide may alleviate skin inflammation by enhancing Treg development in DLNs.

Rebamipide inhibited the expression and activity of NF- κ B signaling both *in vitro* and in mouse dermatitis models

It is known that rebamipide antagonizes NF- κ B signaling pathway activity in many inflammatory situations [7]. Moreover, NF- κ B signaling is reported to have a detrimental role in various skin inflammatory diseases [21]. In this study, we investigated whether rebamipide

affected NF- κ B signaling in macrophage and dermatitis mouse models. To demonstrate the potential effect of rebamipide on TNF- α -mediated NF- κ B signaling, RAW264.7 cells were cultured and stimulated with 10 ng·mL⁻¹ TNF- α , in the presence or absence of 5 mM rebamipide. At the indicated time points, protein was collected and western blotting was performed for the phosphorylation of I κ B, a crucial parameter for activation of NF- κ B signaling. As shown in Fig. 5 (A), rebamipide administration greatly diminished activation of the NF- κ B signaling pathway. RNA extracts were collected from cultured RAW264.7 cells, which were treated with 10 ng·mL⁻¹ TNF- α for 24 h, with or without additional use of 5 mM rebamipide, and the NF- κ B2 level was measured using real-time PCR. As revealed in Fig. 5(B), the TNF- α -induced NF- κ B2 level was significantly inhibited by rebamipide. Fur-

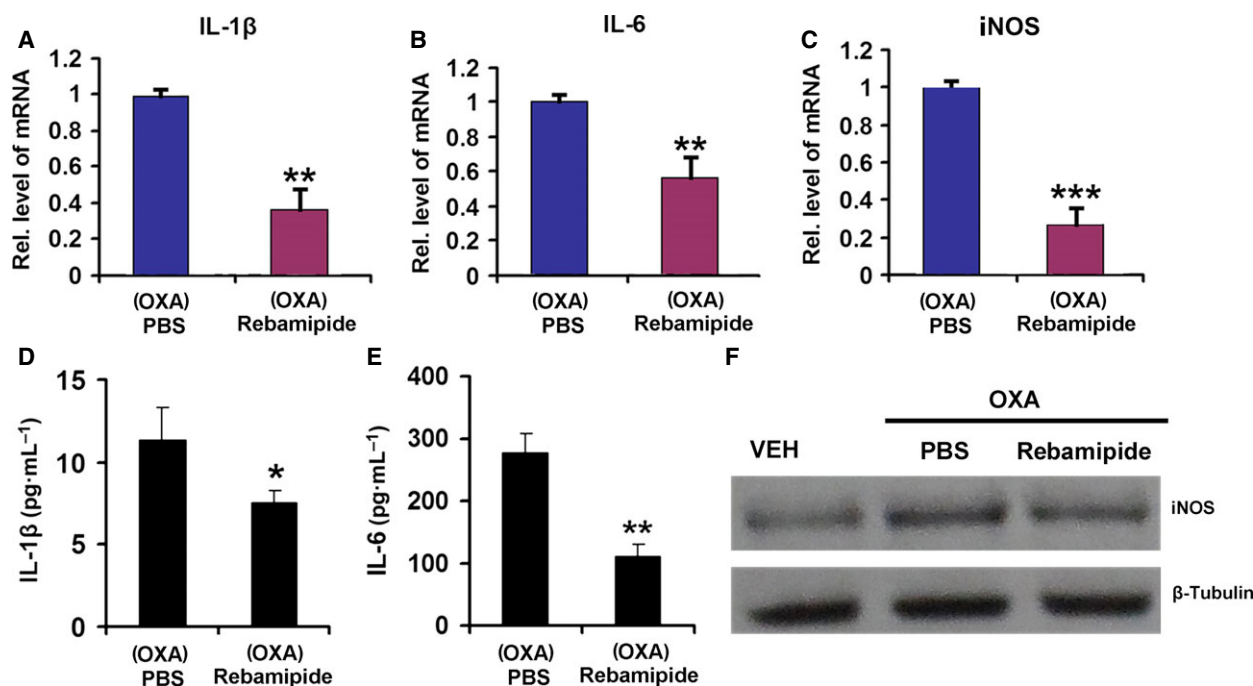


Fig. 3. Rebamipide suppressed elevation of inflammatory cytokine levels in skin and serum of oxazolone-induced dermatitis model. (A–C) Expression levels of IL-1 β , IL-6 and iNOS in the ear tissue of PBS and rebamipide-treated dermatitis models, assayed by real-time PCR. (D–E) Serum levels of IL-1 β and IL-6 in PBS and rebamipide-treated dermatitis models, as measured by ELISA. Sera were collected from mice of each experimental dermatitis group and ELISA was performed for IL-1 β as well as IL-6. (F) Expression of iNOS in ear extracts of PBS and rebamipide-treated mice in dermatitis models, assayed by western blotting. Each experiment was repeated in triplicate, and the values are the mean $\pm$ SD. * P < 0.05, ** P < 0.01 and *** P < 0.005 vs. PBS treatment group.

thermore, a reporter gene assay was performed for NF- κ B signaling, and Fig. 5(C) indicates that rebamipide inhibited TNF- α induction of the NF- κ B signaling pathway in a dose-dependent manner. In addition, RAW264.7 cells were cultured as mentioned above for 24 h, and cell immunostaining for NF- κ B p65 was performed. As shown in Fig. 5(D), rebamipide treatment greatly impaired TNF- α -mediated nuclear translocation of NF- κ B p65. Collectively, this set of experiments implied that rebamipide might inhibit the inflammatory reaction of macrophages through repression of the NF- κ B signaling pathway.

Discussion

Rebamipide is known to be therapeutic in a mouse inflammatory arthritis model [12]. In addition, rebamipide was found to antagonize TNF function [22,23]. Recently, it was reported that rebamipide plays a protective role in inflammatory arthritis mouse models, which prompted us to investigate whether rebamipide plays a role in dermatitis.

It is well accepted that macrophages play a pivotal role in skin inflammation, and serve as one of the pre-

dominant targets for therapy in dermatitis. TNF- α is a well-established inflammatory mediator and inducer. Studies have reported that antagonization of TNF- α function alleviated skin inflammation in various skin inflammatory diseases [24,25]. Anti-TNF agents have been used in therapy for psoriasis [26,27]. Furthermore, IL-1 β , IL-6, COX-2 and iNOS are critical biomarkers for inflammation, which can be induced by TNF- α . In this study, we used RAW264.7 cell, and showed that the TNF-mediated inflammatory reaction was dramatically abolished through administration of rebamipide, as indicated by the diminished expression of the above-mentioned inflammatory-associated molecules.

The oxazolone-induced skin inflammation mouse model is well accepted for studying dermatitis. In this study, this model was established and rebamipide therapy dramatically attenuated the severity of skin inflammation. Infiltration of inflammatory cells is one feature of dermatitis [18]. Here, we found that rebamipide repressed the accumulation of T lymphocytes and macrophages in the oxazolone-induced dermatitis model. DLN analysis is another parameter for local inflammation, and expression levels of inflammation-

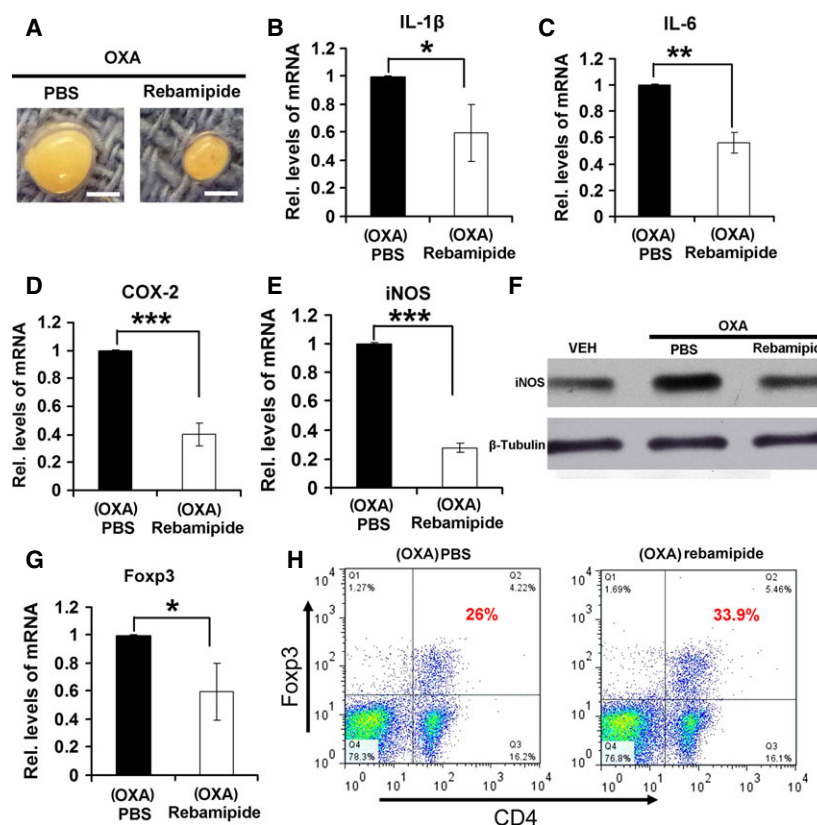


Fig. 4. Rebamipide repressed the inflammatory reaction in the DLN of oxazolone-induced dermatitis. (A) Rebamipide treatment dramatically reduced DLN size in an oxazolone-induced dermatitis mouse model. The DLN samples were collected and representative pictures are shown. Scale bar = 2 mm. (B–E) Gene expression of inflammatory cytokines in DLN of dermatitis model, including IL-1 β , IL-6, COX-2 and iNOS, was significantly diminished by rebamipide treatment in comparison with PBS as assayed by real-time PCR. Total RNA extracts were collected from each treatment group of dermatitis mouse models and real-time PCR was performed. (F) Expression of iNOS in DLN samples of PBS and rebamipide-treated mice in dermatitis models, measured by western blotting. (G) The gene level of Foxp3 was remarkably higher in the rebamipide treatment group than in the PBS group. DLNs were harvested from dermatitis models and real-time PCR was performed for Foxp3. (H) Treg development in both rebamipide- and PBS-treated groups, as detected by flow cytometry. DLNs were collected and cells were isolated for flow cytometry. Numbers in red in the upper right quadrant represent the Foxp3⁺ population as a percentage of CD4⁺ cells. Each experiment was repeated in triplicate, and the values are the mean $\pm$ SD. * P < 0.05, ** P < 0.01 and *** P < 0.005 vs. PBS treatment group.

associated molecules are commonly tested. In this study, levels of inflammation-associated molecules, including IL-1 β , IL-6, COX-2, as well as iNOS, were remarkably diminished in DLNs from the rebamipide treatment group compared with the PBS control group. It is known that Treg differentiation implies anti-inflammatory potential [22,28], and rebamipide was reported to elevate Treg differentiation in mouse arthritis models. Here, the administration of rebamipide noticeably increased the ratio of Treg in DLN of an oxazolone-induced dermatitis mouse model. Taken together, this set of experiments suggested that rebamipide plays a protective role in skin inflammation.

NF- κ B signaling is one of the critical pathways for the TNF- α -induced inflammatory reaction and plays a

pivotal role in skin inflammation [29–31]. It has been reported that rebamipide inhibited TNF- α -mediated activation of NF- κ B signaling in several disease models in mice [23,32,33]. In this study, we found that treatment of rebamipide effectively suppressed the expression and activity of the NF- κ B signaling pathway, and affected the levels of its downstream molecules in dermatitis models. This finding suggests that the protective role of rebamipide in dermatitis may be through inhibiting activation of NF- κ B signaling pathway.

Taken together, our results show that rebamipide plays a critical role in the progression of dermatitis, and might provide a potential treatment instrument in inflammatory skin diseases in clinic.

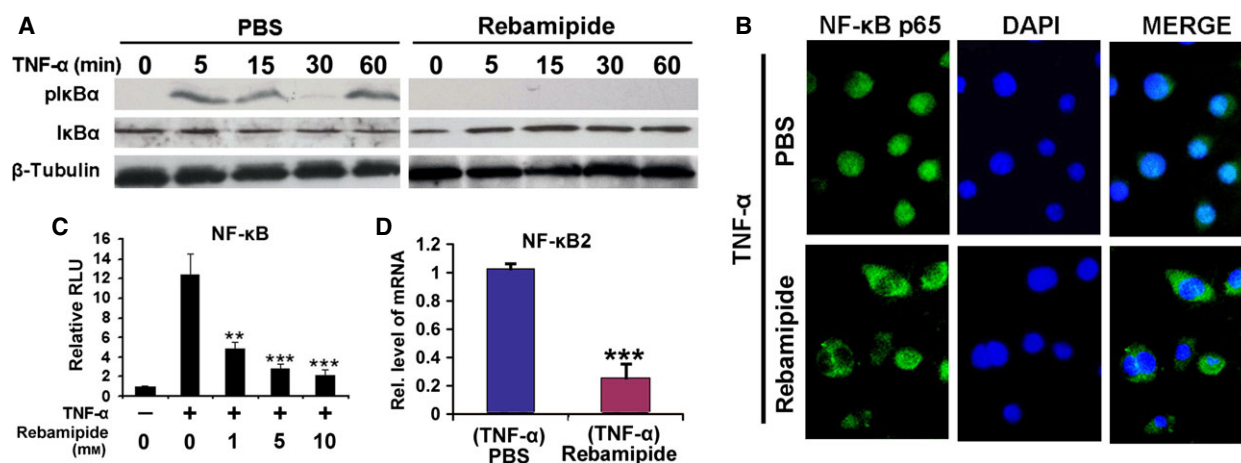


Fig. 5. Rebamipide inhibited TNF- α -mediated augmentation of NF- κ B signaling in RAW264.7 cells. (A) TNF- α -induced phosphorylation of I κ B was remarkably diminished by additional treatment with rebamipide, as measured by western blotting. RAW cells were cultured for the indicated time points with PBS or rebamipide treatment, protein was then collected and western blotting was performed for phosphorylated I κ B. (B) RNA extracts were collected from cultured RAW264.7 cells treated with 10 ng·mL⁻¹ TNF- α for 24 h, with or without additional use of 5 mM rebamipide, and the NF- κ B2 level was measured using real-time PCR. The TNF- α -induced NF- κ B2 level was significantly inhibited by rebamipide. (C) Rebamipide inhibited TNF- α induction of the NF- κ B signaling pathway in a dose-dependent manner as shown by a reporter gene assay. (D) Rebamipide treatment greatly impaired TNF- α -mediated nuclear translocation of NF- κ B p65, as assayed by cell immunostaining. Collectively, this set of experiments implied that rebamipide might inhibit the inflammatory reaction of macrophages through repression of the NF- κ B signaling pathway. ** $P < 0.01$ and *** $P < 0.005$ vs. PBS treatment control group following stimulation of TNF- α .

Materials and methods

Mice

All animal studies were performed in accordance with institutional guidelines and approval by the Institutional Animal Care and Use Committee of Shandong University. Three-month old wild-type BL6/C57 male mice used for these experiments were purchased from Jackson Laboratories (Sacramento, CA, USA).

Chemical-induced dermatitis mouse models

An oxazolone-induced contact dermatitis model was established, as previously described with minor modifications [21]. Mice were first sensitized by a single application of 50 μ L of 1.5% oxazolone (Sigma, St Louis, MO, USA) in ethanol on the dorsal skin 1 week before challenge. The experiments were then performed as follows.

To test the therapeutic function of rebamipide for contact dermatitis, the inner and outer surfaces of the right ears of mice from each experimental group were challenged with 20 μ L of 1% oxazolone in a mixture of acetone and olive oil (4 : 1); the left ears were treated with acetone and olive oil (4 : 1) only as the vehicle control (VEH) every 2 days for 14 days. PBS or rebamipide (6 mg·kg⁻¹ body weight) was delivered through intraperitoneal injection every 2 days and ear thickness was measured every other day using a micrometer (Mitutoyo, Kanagawa, Japan).

Afterwards, ear lobes were collected from both treatment groups for further analysis. Seven mice were used for each group of all this set of experiments.

Real-time RT-PCR

Ear tissue was collected from each indicated experimental group, and total RNA was extracted by using an RNeasy kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed by using a RT-for-PCR kit (Qiagen) following the manufacturer's protocol. Reactions were performed in a 20 μ L SYBR Green PCR volume in a 96-well optical reaction plate formatted in the 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Sequence-specific primers were synthesized as follows: 5'-CTTCCTACCCCAATTTCCAAT-3' and 5'-GCCACTCCTTCTGTGACTCCAG-3' for IL-6; 5'-AATCTCACAGCAGCACATCA-3' and 5'-AAGGTGCTCATGTCTCATC-3' for IL-1 β ; 5'-AATGCTGACTATGGCTACAAAA-3' and 5'-AAAAGTATGCGTGAAGTGCTG-3' for COX-2; 5'-TACAAGCTGGCTGGTGGGGA-3' and 5'-GTCCGGGTCTCAGGACCTT-3' for NF- κ B2; 5'-ACAGGAGGGGTTAAAGCTGC-3' and 5'-TTGTCTCCAAGGGAACAGG-3' for iNOS; 5'-AGAACATCATCCCTGCATCC-3' and 5'-AGTTGCTGTTGAAGTCGC-3' for GAPDH. The presence of a single specific PCR product was verified by melting curve analysis for each molecule, and the experiments were repeated for three times.

Western blotting

In this study, ear as well as lymph node extracts from each indicated group were homogenized, and the proteins were collected. Thereafter, proteins were resolved on one 10% SDS/polyacrylamide gel and then electroblotted onto a nitrocellulose membrane. Samples were blocked in 5% non-fat dry milk in Tris buffer/saline/Tween 20 (10 mM Tris/HCl, pH 8.0; with 150 mM NaCl and 0.5% Tween 20). Then the blots were incubated with polyclonal anti-(phosphorylated I κ B- α) or anti-iNOS (diluted 1 : 1000) serum for 1 h. The blots were then washed, horseradish peroxidase-conjugated secondary antibody in 1:2000 dilution was added, and bound antibody was detected by using an enhanced chemiluminescence system.

Histology

Mouse ear specimens from each experimental group were collected, fixed in 10% formalin, then dehydrated, followed by clearance with dimethylbenzene. Thereafter, the specimens were embedded in olefin. At least four consecutive sections 6 μ m thick were cut from sagittal planes, then stained with H&E for routine histological analysis. The epidermal thickness was determined by OSTEOMEASURE software (OsteoMetrics, Inc., Decatur, GA, USA).

Immunohistochemistry

Mouse ear tissue from the indicated experimental groups was collected, prepared and incubated with polyclonal anti-CD4 or anti-CD68 serum (1 : 100 dilution; Santa Cruz Biotechnology) at 4 °C overnight, followed by incubation with a horseradish peroxidase-conjugated secondary antibody for 60 min at room temperature. The signal was detected by using the Vector Elite ABC Kit (Vectastain; Vector).

Flow cytometry

In this study, single-cell suspensions from the cervical DLN of the indicated groups were collected through isolating lymph node cells by crushing the nodes then filtering through 70- μ m strainers (BD Biosciences). PerCP-Cy5-conjugated anti-CD4 Ig was utilized for surface staining. For intracellular staining of Foxp3, cells were fixed and a commercial Foxp3 staining kit (eBiosciences) was used for permeabilization. Thereafter, the cells were stained with Alexa Fluor 647-conjugated anti-Foxp3 Ig (BD Biosciences).

Immunofluorescence staining of NF- κ B p65

RAW264.7 cells were cultured on coverslips, stimulated with 10 ng-mL⁻¹ TNF- α in the presence or absence of 5 mM rebamipide for 1 h. Immunofluorescence staining of NF- κ B p65 was then performed on these cells, as described

previously and examined using a confocal fluorescence microscope system [14,15].

Reporter gene assay

To examine whether rebamipide inhibits TNF-mediated transactivation of NF- κ B-dependent reporter genes, a reporter gene assay was performed, as described previously with modifications. RAW264.7 cells grown to ~50% confluence were transfected with 1 μ g of the p6XNF- κ B-Luc reporter plasmid and 1 μ g of the pSVGal plasmid (internal control), using Lipofectamine 2000 reagent (Invitrogen). Forty-eight hours following transfection, the cells were starved overnight and then stimulated for 6 h with 10 ng-mL⁻¹ TNF- α in the presence or absence of various doses of rebamipide. Luciferase activity was then measured as reported previously [34].

Nitrite production assay

To examine whether rebamipide inhibits TNF- α -mediated inflammatory reaction in RAW264.7 cells, a Greiss assay was performed as reported previously [14,15]. In brief, RAW264.7 cells were cultured in 96-well flat-bottom plates (1×10^5 cells-well⁻¹) with 10 ng-mL⁻¹ TNF- α (R&D systems) for 24 h in the absence or presence of various amounts of rebamipide. Cell culture supernatants were harvested and tested for NO via the Griess reaction through using a commercial kit.

Assays for IL-1 β and IL-6 levels both *in vitro* and *in vivo*

Supernatant from cultured RAW264.7 cells and serum from the dermatitis mouse model were collected, and IL-1 β as well as IL-6 were assayed using an ELISA, as reported previously [15]. IL-1 β and IL-6 were assayed by using a commercial kit (eBioscience), according to the manufacturer's instructions.

Statistical analysis

The data in this study were presented as mean $\pm$ SD. The SPSS v. 17.0 (SPSS Inc, Chicago, IL, USA) was used for standard statistical analysis including one-way ANOVA as well as Student's *t*-test. A *P*-value < 0.05 was regarded as significant.

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Author contributions

Study concept and design: Yunpeng Zhao and Ruifeng Li. Acquisition of data: Yunpeng Zhao, Weiwei Li, Yan Wang and Ke Liang. Analysis and interpretation of data: Weiwei Li, Weiyuan Ma, Peng Gao, Xiangling Xu, Yan Wang and Ke Liang. Statistical analysis: Weiwei Li and Yunpeng Zhao. Drafting of the manuscript: Weiwei Li, Yunpeng Zhao, Ruifeng Li, Xiangling Xu and Peng Gao.

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