Journal of Dermatological Science xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

Letter to the Editor

Bepotastine besilate downregulates the expression of nerve elongation factors in normal human epidermal keratinocytes

H_1-antihistamines are used as the first choice of medication for pruritus and inflammation resulting from allergic diseases such as urticaria. Second-generation H_1-antihistamines (*e.g.* bepotastine) hardly penetrate the blood-brain barrier, and thus exhibit only mild sedative effects or are non-sedating [1,2]. These agents have both antihistamine and antiallergic effects, such as suppressing the release of chemical mediators by stabilizing mast cells [3]. Recently, it was reported that some second-generation H_1-antihistamines have beneficial effects on pruritus in atopic dermatitis (AD) patients [4]. Cutaneous hyperinnervation is partly involved in itch sensitization in the periphery, and is mainly caused by an imbalance between nerve elongation factors (NEFs) and nerve repulsion factors (NRFs) produced by keratinocytes [5]. Nerve growth factor (NGF) is a major mediator of cutaneous innervation density, and local NGF concentrations are higher in skin with AD lesions [5]. Artemin (gene: *ARTN*) is also involved in cutaneous nerve sprouting and hypersensitivity to warm sensations in AD [5,6]. Moreover, levels of NRFs, such as Semaphorin 3A (Sema3A) and anosmin-1 (gene: *KAL-1*) are decreased in atopic skin [5]. However, there is no evidence of any direct or indirect effect of H_1-antihistamines on expression of these axon guidance molecules in human keratinocytes. Our preliminary results showed that expression of NEFs was more effectively reduced by bepotastine than other second-generation H_1-antihistamines. Here, we assessed the effects of second-generation H_1-antihistamine, especially bepotastine, on expression of NEFs in normal human epidermal keratinocytes (NHEKs).

NHEKs derived from healthy adult epidermis were purchased from Lonza (Basel, Switzerland). Cells were cultured in KBM-Gold (Lonza) containing $CaCl_2$ (0.15 mM) and KGM-Gold SingleQuots™ (Lonza) at 37 °C with 5% CO_2. Bepotastine besilate (bepotastine) was purchased from Wako Pure Chemical (Osaka, Japan). Transcription levels of axon guidance molecules were analyzed by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) as described previously [S1]. Primers for each gene are listed in Table S1. The cells were lysed in M-PER mammalian protein extraction reagent and protease inhibitor cocktail (Thermo Scientific, Waltham MA, USA), then NGF and artemin levels in cell lysate were measured using the enzyme-linked immunosorbent assay (ELISA) kit for human β-NGF (Raybiotech, Norcross GA, USA) or human artemin (Abcam, Cambridge, UK), respectively. Nuclear lysates were obtained using NE-PER nuclear extraction reagents containing a protease- and phosphatase-inhibitor cocktail (Thermo Scientific). The method of western blotting is described in Supplementary materials and Methods. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla CA, USA). In all analyses, $P < 0.05$ was deemed significant.

Histamine stimulates allergic reactions by activating NF-κB *via* histamine H_1 receptor (H_1R) [1]. In addition, it has been reported to enhance the transcription activity of AP-1 through H_1R-mediated activation of the MEK/ERK pathway, thereby inducing NGF production [7]. First, we examined the effect of bepotastine on expression of *NEFs* in the presence of histamine. *NGF* mRNA was upregulated in the presence of histamine (1.57-fold, $P < 0.001$), whereas bepotastine inhibited the histamine-mediated upregulation of *NGF* mRNA (0.56-fold, $P < 0.001$) (Fig. 1a). Histamine did not affect the level of *ARTN* mRNA (0.99-fold) (Fig. 1b). Although c-Fos was phosphorylated in the presence of histamine, bepotastine inhibited the phosphorylation of c-Fos (Fig. 1c). These results demonstrate that (i) histamine upregulates the NGF expression followed by activation of AP-1 *via* H_1R, (ii) bepotastine blocks the binding of histamine to H_1R, and (iii) bepotastine suppresses the histamine-mediated upregulation of NGF expression. In addition, we showed the downregulation of *NEFs* mRNA in the absence of histamine. Therefore, the mechanism by which bepotastine inhibits expression of *NEFs* in the absence of histamine was investigated by assessing the effects of inhibitors of AP-1 and NF-κB. NHEKs were pre-incubated with the AP-1 inhibitor T-5224 (AdooQ Bioscience, Irvine CA, USA) or the IKKβ inhibitor IMD-0354 (Sigma-Aldrich, St. Louis MO, USA), then incubated with 50 μM bepotastine. After 2 h, bepotastine significantly suppressed *NGF* mRNA compared with vehicle (0.39-fold, $P < 0.001$), a suppression completely abolished by pre-incubation with T-5224 (Fig. 1d). Similarly, expression of *ARTN* mRNA was reduced by bepotastine (0.63-fold, $P < 0.001$), but this effect was not inhibited by pre-incubation with T-5224 (Fig. 1e). Notably, IMD-0354 significantly suppressed the bepotastine-mediated *NGF* and *ARTN* downregulation (Fig. 1d and e). These results indicate that the modulation of *NGF* expression by bepotastine have been mediated by AP-1- and NF-κB-dependent mechanism *via* H_1R. In addition, *ARTN* expression in unstimulated NHEKs may be partially involved in the NF-kB pathway.

Next, we examined the dose-dependent effects of bepotastine on expression of *NEF* mRNAs. Bepotastine dose-dependently decreased both *NGF* and *ARTN* mRNA expression in NHEKs (Fig. 2a and b). Similar results were obtained using other second-generation H_1-antihistamines including olopatadine and fexofenadine (Fig. S1). ELISA showed that the concentrations of NGF proteins were dose-dependently decreased by 10 and 50 μM bepotastine (Fig. 2c), whereas bepotastine did not affect the protein levels of artemin (Fig. 2d). We further examined the effects of H_1-antihistamines on expressions of *NRFs* in NHEKs. None of the H_1-antihistamines used in this study dose-dependently increased *Sema3A* mRNA expression in NHEKs (Fig. S2). In

https://doi.org/10.1016/j.jdermsci.2018.04.012

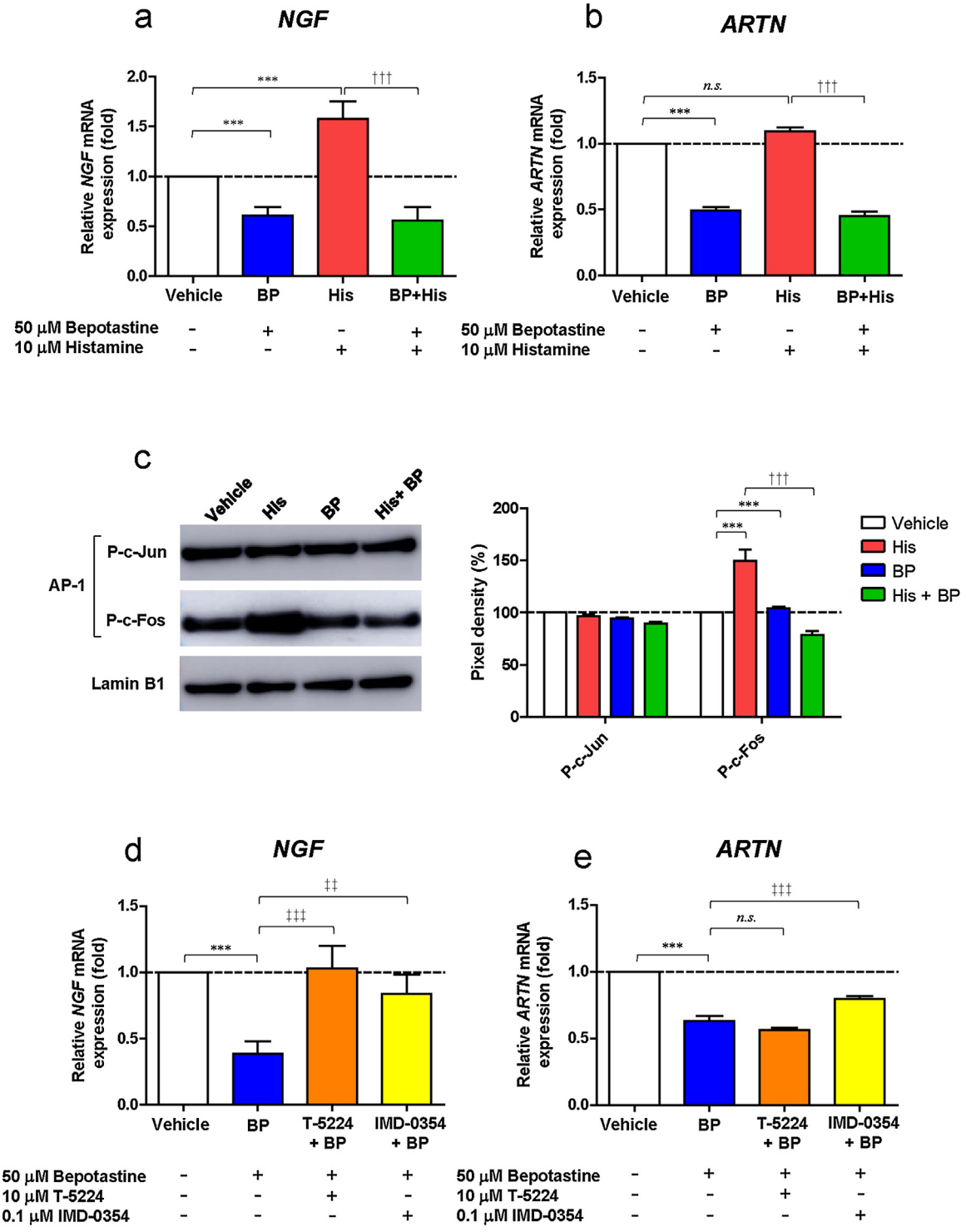

Fig. 1. Bepotastine suppresses the expression of *NEFs* mRNA in NHEKs.
(a–c) NHEKs were pre-incubated with 50 μM bepotastine for 1 h, then treated with 10 μM histamine. After 2 h, total RNA was isolated and the levels of *NGF* (a) and *ARTN* (b) mRNAs were quantified by qRT-PCR. Nuclear lysates were isolated and extracts (5 μg) were subjected to SDS-PAGE on 10% polyacrylamide gels followed by western blotting (c). Western blots were densitometrically analyzed using NIH Image J software. (d, e) NHEKs were pre-incubated with 10 μM T-5224 (AP-1 inhibitor) or 0.1 μM IMD-0354 (IKKβ inhibitor) for 1 h, followed by incubation with 50 μM bepotastine. After 2 h, total RNA was isolated and the levels of *NGF* (d) and *ARTN* (e) mRNAs were analyzed by qRT-PCR. All data were normalized relative to the *RPS18* gene. qRT-PCR data are expressed as (expression level in the stimulated group)/(expression level in the vehicle group). $^{***}P < 0.001$ (*vs.* vehicle, medium alone); $^{†††}P < 0.001$ (*vs.* histamine alone); $^{‡‡‡}P < 0.001$ (*vs.* bepotastine alone) (One-way ANOVA, Tukey's multiple comparison test). The results are presented as mean ± SD of three independent experiments. BP: bepotastine, His: histamine.

contrast, olopatadine, chlorpheniramine, and diphenhydramine dose-dependently increased *KAL-1* mRNA expression in NHEKs (Fig. S2). Bepotastine and other second-generation H_1-antihistamines exhibited no marked cytotoxicity on NHEKs (Fig. S3).

The doses of H_1-antihistamines in some animal experiments were higher than those normally used clinically for humans [8,9]. Based on our results, bepotastine at high concentrations (10–50 μM) were more effective than at low (1 μM) *in vitro*. It

Please cite this article in press as: Y. Kamata, et al., Bepotastine besilate downregulates the expression of nerve elongation factors in normal human epidermal keratinocytes, J Dermatol Sci (2018), https://doi.org/10.1016/j.jdermsci.2018.04.012