PMA-induced dissociation of Ku86 from the promoter causes transcriptional up-regulation of histamine H₁ receptor

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Histamine H₁ receptor (H1R) gene is up-regulated in patients with allergic rhinitis, and its expression level strongly correlates with the severity of symptoms. However, the mechanism underlying this remains unknown. Here we report the mechanism of H1R gene up-regulation. The luciferase assay revealed the existence of two promoter regions, A and B1. Two AP-1 and one Ets-1 bound to region A, while Ku86, Ku70, and PARP-1 bound to region B1. Ku86 was responsible for DNA binding and poly(ADP-ribosyl)ated in response to phorbol-12-myristate-13-acetate stimulation, inducing its dissociation from region B1 that is crucial for promoter activity. Knockdown of Ku86 gene enhanced up-regulation of H1R gene expression. Experiments using inhibitors for MEK and PARP-1 indicate that regions A and B1 are downstream regulatory elements of the PKCδ/ERK/PARP-1 signaling pathway. Data suggest a novel mechanism for the up-regulation of H1R gene expression.

Histamine is a well-known biogenic amine that involves in the regulation of many physiological functions in peripheral tissues and the central nervous system. However, accumulating evidence suggests that histamine and its 4 different receptors, named from histamine H₁ receptor (H1R) to histamine H₄ receptor represent a complex system of immunomodulation with distinct effects dependent on receptor subtypes and their differential expression¹. Among these subtypes, typical immediate hypersensitivity responses of allergic reactions, such as redness, itching, and swelling, are mediated by the activation of H1R. H1R mRNA expression has been reported to increase in epithelial, endothelial, and neural cells of the nasal mucosa in patients with occupational rhinitis^{2,3}. The up-regulation of H1R gene expression was also observed in patients with allergic rhinitis^{4,5}, and H1R binding in the nasal mucosa was reported to increase during the development of nasal allergies⁶.

We previously demonstrated that repeated intranasal application of toluene-2,4-diisocyanate (TDI) in guinea pigs and rats increased the release of histamine from mast cells due to neurogenic inflammation, and led to the development of nasal hypersensitivity^{7,8}. We also reported that H1R gene expression is up-regulated at both the mRNA and protein levels in the nasal mucosa of TDI-sensitized rats^{9,10}. Prophylactic treatment with H₁ anti-histamines suppressed TDI-induced up-regulation of H1R gene expression and alleviated nasal symptoms in TDI-sensitized rats¹¹. Recently, we found that the H1R expression level strongly correlated with the severity of allergic symptoms in rat models and patients with pollinosis^{11,12}. Compounds that suppress the up-regulation of H1R gene expression can alleviate allergy symptoms^{9,13–17}. These data, taken together with the finding that the strength of H1R signaling depends on the H1R expression level¹⁷ indicates that H1R gene is allergy-sensitive, i.e., the H1R expression level affects the severity of allergy symptoms. Therefore, understanding the molecular mechanism of the up-regulation of H1R gene expression may be important for the development of new effective anti-allergy medications. However, the mechanism of the up-regulation of H1R gene expression in response to histamine remains unknown.

We previously reported that histamine stimulation increased H1R at both mRNA and protein level via the activation of the H1R in HeLa cells expressing H1R endogenously¹⁹. Stimulation with phorbol 12-myristate 13-acetate (PMA) also up-regulated H1R gene expression in HeLa cells. Histamine- and PMA-induced

up-regulation of H1R gene expression was suppressed by rottlerin, a PKC δ selective inhibitor, indicating that the up-regulation of H1R gene expression is PKC δ dependent. Further studies showed that both histamine- and PMA-induced up-regulation of H1R gene expression involved common downstream signaling mediators of PKC δ .

Recently, we investigated the molecular mechanism of histamine- and PMA-induced up-regulation of H1R gene expression in HeLa cells and found that the PKC δ /ERK/poly(ADP-ribose) polymerase-1 (PARP-1) signaling pathway was involved in histamine- and PMA-induced up-regulation of H1R gene expression in HeLa cells²⁰.

In the present study, we investigated the molecular mechanism of the up-regulation of H1R gene expression in HeLa cells. Our results indicate that two promoter regions are responsible for the up-regulation of H1R gene expression. We identified the transcription factors that bound to these promoter regions and present the mechanistic implications of these transcription factors on H1R promoter activity. We also propose a “positive feedback circuit” mechanism that may regulate H1R signaling via histamine stimulation. In the presence of histamine, the up-regulation of H1R gene expression increased, which in turn caused an increase in the H1R protein levels. The increase in H1R protein induces a cell to become increasingly sensitive to histamine, which in turn further increases H1R gene expression, ultimately exacerbating the allergic response.

Results

Identification of the region responsible for PMA-induced promoter activity in the H1R gene. We previously demonstrated that the 2.1-kb DNA fragment from the upstream regulatory region of the H1R gene (designated as p2029) expressed histamine- or PMA-induced promoter activity in HeLa cells^{19,20}. To identify the functional H1R promoter region and elucidate the regulatory elements of the H1R gene, promoter activity of serial deletion mutants (MTs) based on the p2029 construct were investigated (Fig. 1). According to our recent study, histamine and PMA induced H1R gene up-regulation via common signaling pathway and PMA is a stronger stimulus than histamine²⁰. Therefore, we used PMA as a stimulus for

promoter analysis in this study. As shown in Fig. 1, significant decreases in luciferase activity were observed with p960 and p44 constructs compared with p1137 and p221 constructs. This indicates that the sequences from −1137 to −960 (designated as region A) and from −221 to −44 (designated as region B) are responsible for PMA-induced promoter activity of the H1R gene.

Identification of the transcription factor bound to the minimum sequence responsible for promoter activity in region A of the H1R promoter. PKC δ is involved in histamine- or PMA-induced up-regulation of H1R gene expression in HeLa cells²⁰. Bioinformatic analysis of the H1R promoter revealed one putative NF- κ B binding site located between −1058/−1049 and two putative AP-1 binding sites located between −1025/−1018 and −1005/−995, respectively, in region A (Fig. 2A). NF- κ B and AP-1 are well-known downstream transcription factors of PKC signaling. The luciferase assay using WT and MT oligonucleotides showed that all three binding sites found in region A are necessary for promoter activity (Fig. 2B). EMSA and the supershift assay using anti-c-Fos and anti-c-Jun antibodies demonstrated that AP-1 (comprising c-Fos and c-Jun) bound to both putative AP-1 sites (Fig. 2C and D). To confirm the necessity of two AP-1 transcription factor binding sites, promoter activity in response to the expression of serial nucleotide deletions between the two AP-1 binding sites was measured. The deletion of 6 or 9 nucleotides enhanced H1R promoter activity, whereas that of 12 nucleotides resulted in complete loss of promoter activity due to steric hindrance (Fig. 2E). As shown in Fig. 2F, a sequence-specific band (arrow) was observed in EMSA using the oligonucleotide of the putative NF- κ B binding site. However, this band was not affected by the addition of the unlabeled NF- κ B consensus (CON) oligonucleotide (Supplementary Fig. S1A) but was affected by the addition of unlabeled Ets-1 CON oligonucleotide (Fig. 2F). The binding of Ets-1 to the putative NF- κ B binding site was also confirmed by the supershift assay using the anti-Ets-1 antibody (Supplementary Fig. S1B). These data suggest that Ets-1 binds to the putative NF- κ B binding site found in region A.

Figure 1 | Identification of the region responsible for PMA-induced promoter activity in the H1R gene. Serial deletion MTs were constructed as described in Supplementary methods using the primary construct p2029 (2.1-kb DNA fragment from the upstream regulatory region of the H1R gene). HeLa cells were cotransfected with these MT plasmids and pRL-MPK. After 5 h, cells were serum starved for 24 h and then treated with 100 nM of PMA for 10 h. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System. Data are presented as mean $\pm$ S.E.M. ($n = 9$). *, $p < 0.01$ (t-test).

Figure 2 | Identification of the transcription factor bound to the minimum sequence responsible for promoter activity in region A. (A) Upper panel: Schematic diagram of region A, which contains one putative NF-κB binding site (NF-κBa) and two putative Ap-1 binding sites (AP-1a and AP-1b). Mutations are indicated by lowercase letters. Lower panel; Nucleotide sequences of WT and MT oligonucleotides for the three putative transcription factor binding sites. (B) Luciferase reporter assay. Data are presented as mean $\pm$ S.E.M. ($n = 8$). *, $p < 0.01$ vs p1137. (C and D) EMSA of AP-1a (C) and AP-1b (D). The nuclear extract (3 μ g protein) was incubated with 32 P-labeled probe bearing AP-1a (C) and Ap-1b (D), putative AP-1 binding sites. For competition studies, 200-fold molar excess of unlabeled WT (lane 3), MT (lane 4), or Ap-1 CON sequence (lane 5) oligonucleotides were used. For the supershift assay, the nuclear extract was incubated with 32 P-labeled probe with or without anti-c-Fos (lane 6) or anti-c-Jun (lane 7) antibody. The positions of the sequence-specific DNA–protein complex and the shifted band are shown as an arrow and arrowhead, respectively. (E) The effect of deletion between the two AP-1 binding sites on H1R promoter activity. Upper panel: Schematic diagram of serial deletion MTs. Lower panel: Luciferase assay. Data are presented as mean $\pm$ S.E.M. ($n = 6$). *, $p < 0.01$ vs p960. (F) Ets-1 but not NF-κB binds to the NF-κBa site. The nuclear extract (3 μ g protein) was incubated with 32 P-labeled probe bearing NF-κBa binding site with or without 10–200-fold molar excess of unlabeled WT (lanes 3–5), MT (lanes 6–8), or Ets-1 CON sequence (lanes 9–11) oligonucleotides. The position of the sequence-specific DNA–protein complex is shown as an arrow.

Identification of the transcription factor bound to the minimum sequence responsible for promoter activity in region B of the H1R promoter. Bioinformatic analysis of the H1R promoter revealed two putative NF- κ B binding sites and one putative AP-1 binding site

(Fig. 3A) in region B; however, the luciferase assay revealed that none of these sites were responsible for H1R promoter activity (Fig. 3B). Therefore, we performed promoter analysis using serial deletion MT plasmids. As shown in Fig. 3C, deletions from -221 to

Figure 3 | Identification of Ku86 as a protein that binds to region B1 of the promoter. (A) Upper panel: Schematic diagram of region B, which contains two putative NF- κ B binding sites (NF- κ Bb and NF- κ Bc) and one putative AP-1 binding site (AP-1c). Mutations are indicated by lowercase letters. Lower panel: Nucleotide sequences of WT and MT oligonucleotides for the three putative transcription factor binding sites. (B) Luciferase reporter assay. Data are presented as mean $\pm$ S.E.M. ($n = 9$). *, $p < 0.01$ vs p221. (C) Identification of the minimum sequence responsible for promoter activity in region B. Data are presented as mean $\pm$ S.E.M. ($n = 8$). *, $p < 0.01$ vs p221. (D) DNA affinity chromatography. The nuclear extract (5 mg protein) was incubated with annealed biotinylated oligonucleotides bearing the region B1 for 30 min at 25°C. Beads were collected and boiled with 1 $\times$ SDS-sample buffer. Proteins specifically bound to DNA were visualized by Coomassie blue R250 and identified by MS/MS. (E) Nucleotide sequences for oligonucleotides of region B1. Mutations are indicated by lowercase letters. (F and G) EMSA. The nuclear extract was incubated with 32 P-labeled WT probe bearing the WT oligonucleotide sequence. For the supershift assay, the nuclear extract was incubated with 32 P-labeled WT probe with anti-Ku70, anti-Ku86, or anti-PARP-1 antibody. In G, the nuclear extract prepared from HeLa cells with or without PMA stimulation was incubated with 32 P-labeled WT (lanes 1, 2, 5, and 6), MT1 (lanes 3 and 7), or MT2 (lanes 4 and 8) probes. For the supershift assay, anti-Ku86 antibody was used. The positions of the sequence-specific DNA-protein complex and the shifted band are shown as an arrow and arrowhead, respectively.

–65 did not significantly change promoter activity compared to that in the p221 construct. Significant decrease in promoter activity was observed in p44. These results indicate that the sequence from –65 to –44 (designated as region B1) was sufficient to express H1R promoter activity. To identify the transcription factor bound to the –65/–44 sequence, we performed DNA affinity chromatography using a resin immobilized with the region B1 oligonucleotide. SDS-PAGE analysis revealed that three proteins bound to the affinity resin (Fig. 3D). These proteins were identified as PARP-1, Ku86, and Ku70 by MS/MS. A 32 P-labeled probe was developed using the same oligonucleotide (shown in Fig. 3E, WT) to perform EMSA and the supershift assay. A very faint band was observed (arrow), and this band shifted when the anti-Ku86 antibody was added (Fig. 3F, arrowhead). Since a CON sequence for the specific binding of the Ku protein has been proposed²¹, we developed two MT oligonucleotides, designated as MT1 and MT2, (Fig. 3E) and examined the sequence specificity using these MT oligonucleotides. A more dense band was observed with the nuclear extract prepared from PMA-unstimulated HeLa cells than with the nuclear extract prepared from PMA-stimulated HeLa cells (Fig. 3G, lanes 1 and 2). The density of this band was enhanced when a 32 P-labeled MT1 oligonucleotide was used as a probe (Fig. 3G, lane 3). However, this band disappeared when a 32 P-labeled MT2 oligonucleotide was used as a probe (Fig. 3G, lane 4). Similar results were observed in the supershift

assay using the anti-Ku86 antibody (Fig. 3G, lanes 5–8). These results suggest that Ku86 protein bound to DNA. Furthermore, the MT1 oligonucleotide can stably bind to Ku86, but the MT2 oligonucleotide cannot.

Effect of Ku86 binding to region B1 on H1R promoter activity. EMSA revealed that MT1 and MT2 have opposite effects on the binding of Ku86 to region B1. We investigated the effect of these mutations on H1R promoter activity. The p1137-MT1 construct, a p1137 construct with MT1 mutation, showed promoter activity similar to the p960 construct, a construct in which region A was deleted (Fig. 4A and B), and the p65-MT1 construct showed promoter activity similar to the p44 construct, a construct lacking region B1 (Fig. 4A and C). These results suggest that stable binding of Ku86 to DNA inhibits H1R promoter activity. On the other hand, the p65-MT2 construct did not show any change in promoter activity compared to p65, suggesting that the dissociation of Ku86 from DNA induces H1R promoter activity (Fig. 4A and D). To confirm this, the ChIP assay using anti-Ku86 antibody were performed. It has been reported that H1R has constitutive activity and histamine signaling is active without stimuli^{22,23}. Therefore, HeLa cells pretreated with a PKC δ inhibitor, rottlerin, to completely inhibit endogenous histamine signaling were used as controls. As shown in Fig. 5A, Ku86 bound to region B1 in the absence of PMA stimulation and

Figure 4 | Effect of Ku86 binding to region B1 on H1R promoter activity. (A) Schematic representation of MT constructs used for the luciferase reporter assay. A and B1 represent region A and the region B1 of the promoter, respectively. ○, WT promoter; ⊙, MT1 promoter; and ⊗, MT2 promoter. (B–D) Effect of PMA on the H1R promoter activity. HeLa cells were cotransfected with the MT plasmid and pRL-MPK. After 5 h, cells were serum-starved for 24 h and then treated with 100 nM of PMA for 10 h. (E) Effect of histamine on the H1R promoter activity. Cells were treated with 100 μ M histamine for 10 h. Data are presented as mean $\pm$ S.E.M. [$n = 5$ (B), 6 (C and D), and 8 (E)]. **, $p < 0.05$ vs p1137 (B) or p65 (C and D). In E, **, $p < 0.05$ vs. p1137; #, $p < 0.05$ vs. p65.

Figure 5 | Dissociation of Ku86 from the promoter in response to stimulation activates the H1R promoter activity. (A) Chip assay. HeLa cells were serum-starved for 24 h and stimulated with or without 100 nM of PMA or 100 μ M of histamine for the times indicated. DNA-protein complexes were immunoprecipitated with anti-Ku86 and PCR was performed to amplify the region B1 promoter. For the control, HeLa cells were pretreated with 10 μ M of rottlerin 1 h before PMA stimulation to completely eliminate H1R signaling²⁰. For input, 1% of the extract was used. (B-D) Knockdown study. HeLa cells were reverse-transfected with 10 μ M Ku86-specific siRNA (represented as siRNA) or 10 μ M control siRNA (control). Forty-eight hours after transfection, the cells were stimulated for 24 h followed by treatment with histamine (100 μ M). Expression of Ku86 mRNA (B) and protein (C) was analyzed. In B, total RNA was prepared from 3 individual dishes. Lanes 1, 2, and 3: control; lanes 4, 5, and 6: Ku86-specific siRNA. In D, the H1R mRNA levels were determined by real-time quantitative RT-PCR. Data are presented as the means $\pm$ S.E.M. (n = 3). *, $p < 0.05$ versus control, histamine (-); #, $p < 0.05$ versus control, histamine (+). (E) PMA stimulation induces dissociation of Ku86 from PARP-1. Immunoprecipitation assay was conducted as described in Methods. Lanes 1 and 5, marker; lanes 2 and 6, input (1% of the extract); lanes 3 and 4, the extract incubated with anti-PARP-1 antibody and immunoprecipitated proteins were SDS-PAGE separated and analyzed with anti-Ku86 antibody. Lanes 7 and 8, the extract incubated with anti-Ku86 antibody and immunoprecipitated proteins analyzed with anti-PARP-1 antibody. Lanes 3 and 7, control; lanes 4 and 8, PMA stimulation. For the control, HeLa cells were pretreated with 10 μ M of rottlerin 1 h before PMA stimulation. Arrows indicate Ku86 (left panel) and PARP-1 (right panel). (F) PMA stimulation induces PARylation of Ku86 by PARP-1. Left panel: HeLa cells were serum-starved for 24 h and stimulated with or without 100 nM of PMA for the time indicated. Immunoprecipitated proteins by anti-poly(ADP-ribose) antibody were analyzed with anti-Ku86/Ku70 antibodies. Right panel: Quantification of PARylation of Ku86 and Ku70. Densitometric analysis was performed using ImageJ software.

dissociated from the region upon stimulation. Next, we examined the effect of knockdown of Ku86 expression using a specific Ku86 siRNA. In Ku86 knockdown cells, Ku86 expression was confirmed by RT-PCR analysis (Fig. 5B) and immunoblot analysis (Fig. 5C). Without PMA stimulation, significant H1R mRNA elevation was observed in the Ku86 knockdown cells compared with the control cells (Fig. 5D). Significant increase in H1R mRNA level was also observed in PMA-stimulated cells. These data suggest that the Ku86 expression level affects the H1R mRNA expression level.

Region A and B1 are downstream regulatory elements of the PKC δ /ERK/PARP-1 signaling pathway. We previously demonstrated the involvement of the PKC δ /ERK/PARP-1 signaling pathway in

histamine- and PMA-induced up-regulation of H1R gene expression in HeLa cells²⁰. Thus, for this study, we investigated the interaction between Ku86 and PARP-1 by immunoprecipitation using anti-PARP-1 and anti-Ku86 antibodies. Without PMA stimulation, Ku86 was immunoprecipitated by anti-PARP-1 antibody, and PARP-1 in turn was immunoprecipitated by Ku86 (Fig. 5E, lanes 3 and 7). However, after stimulation with PMA, this interaction was reduced (Fig. 5E, lanes 4 and 8). These results indicate that Ku86 interacts with PARP-1 in the absence of stimulation and dissociates from PARP-1 upon stimulation. It was reported that Ku86 was poly(ADP-ribose)ylated (PARylated) by PARP-1, which reduced its DNA binding activity²⁴. Therefore, we, next, investigated PARylation of Ku86 by PARP-1 in response to PMA stimulation. As shown in Fig. 5F,

immunoprecipitation study using anti-poly(ADP-ribose) antibody showed that Ku86 but not Ku70 was PARylated after stimulation with PMA.

We have also reported that U0126 (a MEK inhibitor) and DPQ (a PARP-1 inhibitor) suppressed PMA-induced up-regulation of H1R gene expression²⁰. The suppressive effect of these inhibitors was confirmed by the luciferase assay using p2029 as the reporter plasmid. Furthermore the effects of U0126 and DPQ on H1R promoter activity using the MT constructs containing two PMA-responsive regions were investigated. As reported previously, promoter activity for p1137 containing regions A and B1 was inhibited by U0126 (Fig. 6A, $IC_{50} = 0.69 \mu\text{M}$) and DPQ (Fig. 6D, $IC_{50} = 59 \mu\text{M}$) in a dose dependent manner. Furthermore, promoter activity for the p1137-MT1 construct, which contained WT region A and inactivated MT region B1, was inhibited by U0126 and DPQ in a dose dependent manner (Fig. 6B, $IC_{50} = 0.97 \mu\text{M}$ and E, $IC_{50} = 51 \mu\text{M}$). Similar inhibition by U0126 and DPQ was observed when p65, which has only the functional region B1, was used as the reporter plasmid (Fig. 6C, $IC_{50} = 0.43 \mu\text{M}$ and F, $IC_{50} = 64 \mu\text{M}$). These results indicate that both regions A and B1 are downstream regulatory elements of the PKC δ /ERK/PARP-1 signaling pathway. The dose of U0126 and DPQ we used in this study seems to be high. However, similar concentration of U0126 was used for HeLa cells in literature, for example, Ren and Guo used up to $25 \mu\text{M}$ U0126 and Lim et al used $20 \mu\text{M}$ U0126^{25,26}. So, we think $10 \mu\text{M}$ of U0126 is not so high. As shown in Supplementary Fig. S2, treatment with high concentration of DPQ showed cytotoxicity, which approximately 20% of cells died

after the cells were treated with 20 to $150 \mu\text{M}$ DPQ. This might affect the promoter activity. However, IC_{50} value for cell cytotoxicity estimated from the Supplementary Fig. S2 (over $150 \mu\text{M}$) is much higher than that obtained from the promoter assay ($50\text{--}60 \mu\text{M}$ Fig. 6). In addition, as we reported, DPQ suppressed PMA-induced up-regulation of H1R gene expression in a dose-dependent manner²⁰ suggesting that DPQ inhibits the H1R signal pathway. From these evidences, it is likely that reduced promoter activity was not due to cell death but reflects direct inhibition of the pathway by DPQ. After HeLa cells were stimulated with PMA, the p1137-MT1 construct, which has only the functional WT region B1, exhibited promoter activity similar to the p960 construct, which has a missing region A. (Fig. 4B). We previously demonstrated that histamine and PMA up-regulate H1R gene expression in HeLa cells¹⁹. Therefore, we investigated the effect of histamine stimulation on H1R promoter activity in regions A and B1. As shown in Fig. 4E, promoter activity in region B1 (p65) was significantly higher than that in region A (p1137-MT1). These results suggest that region B1 is more sensitive to histamine stimulation than region A.

Discussion

We previously demonstrated that the PKC δ /ERK/PARP-1 signaling pathway is involved in histamine- and PMA-induced up-regulation of H1R gene expression in HeLa cells²⁰. H1R mRNA expression was increased in epithelial, endothelial, and neural cells of the nasal mucosa in patients with occupational rhinitis^{2,3}. The up-regulation of H1R gene was also observed in patients with allergic rhinitis^{4,5}.

Figure 6 | Effect of MEK inhibitor (A–C) and PARP-1 inhibitor (D–F) on H1R promoter activity. HeLa cells were serum-starved for 24 h and then treated with or without 0.05– $10 \mu\text{M}$ of U0126, a MEK inhibitor (A–C), or 20– $150 \mu\text{M}$ of DPQ, a PARP-1 inhibitor (D–F), 1 h before stimulation with 100 nM of PMA for 10 h. A and B1 represent region A and B1 of the H1R promoter, respectively. ○, WT promoter; ⊙, MT1 promoter. Data are presented as mean $\pm$ S.E.M. [$n = 4$ (A, B, and D), 7 (C and F), or 3 (E)]. **, $p < 0.05$ vs. control.

Terada et al reported that H1R mRNA expression increased in human nasal epithelial cells after stimulation with diesel exhaust particles known to cause chronic airway diseases²⁷. They also showed that histamine stimulation induced the production of granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin-8 (IL-8) from human nasal epithelial cells. In addition, Matsubara et al reported the involvement of the H1R- $\text{PKC}\delta$ -ERK signaling pathway in the induction of GM-CSF and IL-8 production from bronchial epithelial cells stimulated by histamine²⁸. Involvement of $\text{PKC}\delta$ was also reported by Masaki et al²⁹. These findings indicate that epithelial cells are one of the candidates responsible for histamine-induced up-regulation of H1R gene expression. As HeLa cells are derived from human cervical epithelial cancer cells, we believe that HeLa cells can be the models for analysis of the molecular mechanism histamine-induced up-regulation of H1R gene expression although these cells are not an orthodox representation of a typical target cell involved in allergic reactions.

In the present study, we investigated and proposed a signaling mechanism of histamine- and PMA-induced up-regulation of H1R gene expression in HeLa cells (Fig. 7). Promoter analysis using serial deletion MT plasmids showed two PMA-responsive regions, regions A and B1. In region A, two AP-1 and one Ets-1 transcription factor binding sites were necessary for H1R promoter activity. Binding of the two AP-1 proteins to the tandemly repeated AP-1 binding sites has been shown to be crucial for promoter activity of the large subunit of herpes simplex virus type 2 ribonucleotide reductase³⁰. Furthermore, the interaction of AP-1 with Ets-1 has been reported³¹. Data from the luciferase assay using MT constructs (Fig. 2B) of the two AP-1 binding sites and serial deletion constructs between the two AP-1 binding sites (Fig. 2E) indicate that binding of AP-1 proteins to both AP-1 binding sites is crucial for H1R promoter activity. H1R promoter activity was inhibited when AP-1 binding was blocked. Furthermore, the binding of one AP-1 protein with Ets-1 could affect the binding of the AP-1-Ets-1 complex and the second AP-1 factor.

Figure 7 | Schematic presentation of the signaling pathway involved in histamine-induced up-regulation of H1R mRNA expression in HeLa cells. Binding of histamine to H1R activates $\text{PKC}\delta$ and translocates $\text{PKC}\delta$ from the cytosol to the Golgi apparatus, where $\text{PKC}\delta$ phosphorylates MEK and ERK. Phosphorylated ERK then activates PARP-1. Activated PARP-1 PARylates Ku86 and induces dissociation of Ku86 from region B1 of the promoter, which activates H1R gene transcription. Activated PARP-1 also PARylates histone and induces decondensation of the chromatin structure, enhances the binding of Ets-1 and AP-1 transcription factors to region A of the promoter, which activates H1R gene transcription.

Therefore, it is likely that two AP-1 proteins first bind to the AP-1 binding sites, after which Ets-1 binds to the AP-1-AP-1 dimer to stimulate H1R promoter activity.

DNA affinity chromatography revealed that Ku86, Ku70, and PARP-1 bind to region B1 (Fig. 3D). Among these proteins, Ku86 was responsible for DNA binding to the experimental oligonucleotide model of the H1R promoter (Fig. 3F). The Ku protein is a heterodimer composed of Ku86 and Ku70 and identified as an auto-antigen by sera of patients with autoimmune disorders³². Ku is a regulatory subunit of the DNA-dependent protein kinase³³ and is involved in the phosphorylation of several proteins, such as Hsp90, p53, and Sp1, in a DNA-dependent manner^{34–36}. Ku is important in both repression and enhancement of gene transcription. For example by binding to the regulatory promoter element, Ku can repress glucocorticoid and Hsp70 expression or enhance glucocorticoid receptor³⁷ and osteocalcin promoter activity³⁸. However, interestingly, with regard to the role of Ku86 in the H1R promoter, it is the dissociation and not the binding to region B1 that induces H1R promoter activity (Figs. 4 and 5A). Ku86 knockdown experiments also indicate the importance of dissociation of Ku86 from the B1 promoter for transcriptional activation of H1R gene (Fig. 5D).

U0126 (a MEK inhibitor) and DPQ (a PARP-1 inhibitor) suppressed promoter activity in regions A and B1 (Fig. 6), indicating that both promoter regions are downstream regulatory elements of the $\text{PKC}\delta$ /ERK/PARP-1 signaling pathway. PARP-1 is a nuclear chromatin-associated protein that catalyzes posttranslational modification of proteins by PARylation. This posttranslational modification of proteins by PARP-1 regulates protein-protein and protein-DNA interactions³⁹. PARP-1 is considered to play a role in the repair mechanism of single-stranded DNA breaks; however, recent studies have indicated that PARP-1 may function as a transcriptional regulator or co-regulator by direct interaction with transcription factors such as YY1, NF- κ B, TFIIIF, Oct-1, B-MYB, AP-2, and the HTLV Tax-1 protein^{40–46}. It was reported that Ku86/Ku70 was PARylated by PARP-1 and PARylation reduces Ku86/Ku70 DNA-binding Activity^{24,47}. Immunoprecipitation study with anti-poly(ADP-ribose) antibody demonstrated PARylation of Ku86 in response to PMA stimulation (Fig. 5F). The addition of ADP-ribose molecules increases the negative charge of Ku86, which may impair the binding of Ku86 to region B1. It has also been reported that PARP-1 itself is activated by various extracellular signals that activate Raf/MEK/ERK phosphorylation. PARylated PARP-1 amplifies ERK signaling, which targets core histone acetylation and gene transcription^{38,48–50}. Thus, it could be possible that acetylation or PARylation of histones induces decondensation of the chromatin structure and activates H1R gene transcription.

As shown in Fig. 4B, PMA-induced H1R promoter activity of the p1137-MT1 construct, containing region A, was similar to that of the p960 construct, containing only region B1. On the other hand, H1R promoter activity of the p65 construct, containing only region B1, was significantly higher than that of the p11370-MT1 construct when HeLa cells were stimulated with histamine (Fig. 4E). The observed difference in sensitivity to histamine stimulation between the two regions of the H1R promoter could explain the mechanism of the “positive feedback circuit,” in which histamine-induced up-regulation of H1R gene expression results in an increase in the H1R protein and makes the cells more sensitive to histamine and exacerbates the allergic response. Briefly, we noted that histamine is a weaker stimulator of H1R gene expression than PMA. Our previous data shows that histamine stimulation of HeLa cells induced transient ERK phosphorylation that peaked after 30 s, while PMA stimulation induced transient ERK phosphorylation for up to 15 min²⁰. While PMA activates both regions A and B1, histamine stimulation mainly caused activation of region B1; however, histamine stimulation of HeLa cells up-regulated c-Fos, a subunit of the AP-1 transcription factor complex (Supplementary Fig. S3). In region A, AP-1

probably functions as a molecular switch. Without stimulation, binding of AP-1 to the AP-1 binding site of the promoter competes with the binding of AP-1 to Ets-1. During histamine stimulation of H1R promoter activity, decreased AP-1 binding to region A is probably a limiting factor because of the binding competition with Ets-1, causing only a partial increase in H1R promoter activity. However, as histamine stimulation continues, a “positive feedback circuit” occurs, which leads to up-regulation of the c-Fos gene expression resulting in an increase in the amount of AP-1 in the cell. As the amount of AP-1 increases, the competitive binding of AP-1 to ETS-1 is masked, and the increased amount of AP-1 binds to region A, resulting in complete promoter activity of H1R, at levels similar to that observed upon PMA stimulation.

In summary, together with our recent results, we propose the mechanism for histamine-induced up-regulation of H1R gene expression in HeLa cells (Fig. 7). Binding of histamine to H1R activates and translocates PKC δ from the cytosol to the Golgi apparatus, where PKC δ phosphorylates MEK and ERK. Phosphorylated ERK activates PARP-1. Activated PARP-1 PARylates Ku86 and induces the dissociation of Ku86/Ku70 from region B1, causing activating H1R gene transcription. Activated PARP-1 also PARylates core histones, induces relaxation of the chromatin structure, enhances binding of Ets-1 and AP-1 transcription factors to region A, and activates H1R gene transcription.

Increase in the H1R mRNA level was reported in the nasal mucosa from patients with pollinosis and TDI-sensitized allergy model rats^{11,12}. Intranasal application of histamine to normal (i.e. TDI-untreated) rats increased the H1R mRNA expression (Supplementary Fig. S4). We identified anti-allergic compounds that suppress the up-regulation of H1R gene expression including epigallocatechin-3-O-gallate (EGCG)¹³. Molecular pharmacological studies revealed that EGCG inhibited PKC δ Tyr³¹¹ phosphorylation that is necessary for enzyme activation (Supplementary Fig. S5). They also suppressed H1R mRNA elevation in TDI-sensitized rats¹³. Intranasal application of histamine also up-regulated IL-4 mRNA¹⁷ and the expression level of H1R was strongly correlated with the expression level of IL-5⁵¹, suggesting crosstalk with H1R signaling and Th2 cytokine signaling in patients with pollinosis and suppression of H1R signaling could inhibit Th2 cytokine signaling. Therefore, suppression of H1R signaling is considered to be crucial for treatment of allergic diseases and our data may provide information about target proteins for suppression of H1R signaling and contribute to develop of new anti-allergy medications.

Methods

Promoter assay. The human H1R reporter plasmid was constructed as previously described¹⁸. The deletion mutant (MT) plasmids p1336, p1137, p960, p914, p398, p221, and p44 were described in Supplementary methods. HeLa cells were cultured in MEM- α medium containing 8% fetal calf serum and 1% antibiotic-antimycotic (Gibco) at 37°C under a humidified atmosphere of 5% CO₂ and 95% air. HeLa cells in 12-well culture plates were co-transfected with the H1R reporter plasmid and the internal control plasmid pRL-MPK in a ratio of 100:1 using the PolyFect transfection reagent (Qiagen) according to the manufacturer's instructions. After 5 h, the medium was replaced with 1 ml of serum-free medium, and the cells were starved for 24 h. Next, the cells were stimulated with 100 nM of PMA or 100 μ M of histamine for 10 h in the same medium. Where appropriate, inhibitors were added 1 h prior to PMA or histamine stimulation. After stimulation, the cells were washed twice with 500 μ l of ice-cold phosphate-buffered saline (PBS) and lysed with 100 μ l of Passive Lysis Buffer (Promega). The lysate was frozen for at least 3 h at -85°C and then thawed at room temperature. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Luminescence was measured using the Infinite M200 microplate reader (Tecan Japan, Kanagawa, Japan).

Electrophoresis mobility shift assay (EMSA). Preparation of nuclear extracts was described in Supplementary methods. The nuclear extract was incubated with ³²P-labeled double-stranded oligonucleotides in EMSA buffer [25 mM HEPES-KOH, pH 7.9, 10% glycerol, 0.5 mM EDTA, 300 mM KCl, 5 mM DTT, 0.2 mM PMSF, and 1.25 μ g/ml poly(dI-dC) (Amersham Pharmacia)] for 20 min at 25°C. For oligonucleotide competition analysis, competitor oligonucleotides (10–200-fold molar excess) were added to the reaction mixture. Protein–DNA complexes and free DNA were separated on 6% nondenaturing polyacrylamide gels in 1× TBE buffer (89 mM Tris,

89 mM boric acid, and 1 mM EDTA) at 4°C and visualized using the Bio Imaging analyzer BAS1500 (FujiFilm, Tokyo, Japan). Antibodies used for the supershift assay are as follows: Ku86(B-1), PARP-1(F-2), Ku-70(E-5), and NF- κ B p65(F-6) (Santa Cruz); c-Jun(60A8), c-Fos(9F6) (Cell Signaling); and Ets-1 (Bio Matrix Research, Tokyo, Japan).

DNA affinity chromatography. The DNA affinity resin was prepared by incubating annealed oligonucleotides (5'-GCTCTAAGGCTTGCCTAACC-biotin-3') and (5'-GGTTAGTGAAGACCTTAGAGC-3') with immobilized NeutrAvidineTM protein (Pierce) for 1 h at 4°C. The nuclear extract was incubated with the resin for 30 min at 25°C and then washed with PBS. Protein bound to the beads was collected by boiling with 1× SDS-sample buffer (62.5 mM Tris HCl, pH 6.8, 10% glycerol, 2% SDS, 0.1% 2-mercaptoethanol, 0.001% BPB) for 3 min. The DNA binding proteins were identified by tandem mass spectrometry (MS/MS) as follows. The DNA binding proteins were separated by SDS-PAGE, digested with trypsin, and then subjected to MS/MS as previously described²². Peptides were analyzed using a nanoflow-HPLC/nanospray ionization MS/MS on an Esquire 3000 ion trap mass spectrometer (Bruker-Daltonics, Bremen). MS/MS data were acquired using DataAnalysis software (Bruker-Daltonics, Bremen), converted to text files listing the mass values, and processed using the MASCOT algorithm (Matrix Science Ltd., London) to assign peptides in the NCBI non-redundant sequence database.

Immunoprecipitation assay. Immunoprecipitation assay was performed using anti-Ku86(B-1) or anti-PARP-1(F-2) antibodies. For poly(ADP-ribosylation) assay, anti-pADPr antibody was used. In order to completely inhibit constitutive H1R signaling^{22,23}, HeLa cells were pretreated with 10 μ M of rottlerin for 1 h before PMA stimulation²⁰. Detailed procedures are described in Supplementary methods.

Chromatin immunoprecipitation (ChIP) assay. Immunoprecipitation of the sheared chromatin was conducted using anti-Ku86 antibody. Oligonucleotides sequences for PCR to amplify a region B (-221/-44) within the H1R promoter were as follows: forward primer: 5'-GGGGTACCGAGGGTCTTTCCACTC-3' and reverse primer: 5'-CCCTATTCCTTTAGAAGCCAGG-3'. PCR conditions were as follows: denaturation at 94°C for 10 min and 35 cycles at 94°C for 30 s, 57.8°C for 30 s, and 72°C for 30 s. A Detailed procedure was described in Supplementary methods.

Real-time quantitative RT-PCR. Total RNA was isolated from HeLa cells and rat nasal mucosa using RNAiso Plus (Takara Bio Inc. Kyoto Japan) according to the manufacturer's instructions. RNA samples were reverse-transcribed to cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). TaqMan primers and the probe were designed using Primer Express (Applied Biosystems). Real-time PCR was conducted using a GeneAmp 7300 sequence detection system (Applied Biosystems). The sequences of the primers and TaqMan probe were as follows: forward primer for H1R, 5'-CAGAGATCAGATGTTAGGTGATAGC-3'; reverse primer for H1R, 5'-AGCGAGCCTCTTCCAAGTAA-3'; TaqMan probe, FAM-CTTCTCTCGAACGGACTCAGATACCACC-TAMRA. To standardize the starting material, the human GAPDH gene (Applied Biosystems) was used, and data were expressed as the ratio of H1R mRNA to GAPDH mRNA. A detailed procedure was described in Supplementary methods.

Small Interfering RNA (siRNA) transfection. HeLa cells were reverse-transfected with 10 μ M Ku86-specific siRNA (Silencer[®] siRNA s14953, Applied Biosystems) or 10 μ M control siRNA (Silencer[®] negative control #1 siRNA, Applied Biosystems) using Lipofectamine RNAiMAX according to the manufacturer's instructions (Invitrogen). Forty-eight hours after transfection, the cells were starved for 24 h and then treated with or without 100 μ M histamine for 3 h. The H1R mRNA expression level was determined by real-time RT-PCR as described above. The Ku86 mRNA expression level was confirmed by RT-PCR. Sequences of the primers used and PCR conditions were as follows: forward primer, 5'-CATATGGTGCGGTGCGGGAAT-AAGGC-3' and reverse primer, 5'-GTCGACCTATATCATGTCCAATAAAT-CGTC-3'; PCR conditions, denaturation at 94°C for 10 min, 36 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 3 min, and extension at 72°C for 7 min.

Animal study. Six-week-old male Brown Norway rats (200–250 g; Japan SLC, Hamamatsu, Japan) were used. They were allowed free access to water and food and were kept in a room at 25 ± 2°C and 55 ± 10% humidity with a 12-h light/dark cycle. Histamine (0.6–3 mg/day/rat) dissolved in PBS was instilled into the nasal cavity bilaterally for 1 week. Rats were sacrificed and the nasal mucosa was removed from nasal septum 4 h after the final administration of histamine, and the H1R mRNA levels were determined by real-time RT-PCR. All procedures involving the animals were conducted in accordance with the Guidelines for Animal Experiments approved by the Ethical Committee for Animal Studies of the School of Medicine of the University of Tokushima.

Statistical analysis. The results are shown as mean ± SEM. Statistical analysis was performed using unpaired *t*-tests or ANOVA with Dunnett's test by GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). *p* < 0.05 was considered statistically significant.

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Author contributions

Hiroyuki Mizuguchi designed the project, wrote the manuscript. Kohei Miyagi, Takuma Terao, Noriko Sakamoto, Yosuke Yamawaki, Tsubasa Adachi, Shohei Ono, and Yohei Sasaki carried out experimental work. Yoshiyuki Yoshimura and Yoshiaki Kitamura analyzed data and participated in data interpretation. Noriaki Takeda supervised the research and wrote the manuscript. Hiroyuki Fukui conceived the project, supervised the research and wrote the manuscript.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

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