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Abstract

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Keywords

Animals; Bradykinin; Bradykinin B2 Receptor Antagonists; Cells, Cultured; HEK293 Cells; Humans; Mast Cells; Nerve Tissue Proteins; Peptides; Rats; Receptors, G-Protein-Coupled; Receptors, Neuropeptide

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Antibiotics have been used for the treatment of microbial infections since the early 1900s, but emergence of multidrug-resistant strains of microbes poses a tremendous public health concern globally (1). Thus, there is an urgent need to develop novel therapy for the treatment of infections caused by antibiotic-resistant organisms. Antimicrobial peptides, also known as host defense peptides (HDPs), represent an evolutionarily ancient mechanism of innate immunity found in both animal and plant kingdoms (2–4). These amphipathic peptides provide protection against a variety of organisms, including antibiotic-resistant bacteria, fungi, and parasites, via two pathways, one involving the direct killing of microbes and the other via the activation of immune cells (3, 5). Mast cells (MC) are granule-containing immune cells that are widely distributed in tissues such as the skin and mucosal tissues that interact with the environment. Although MC are best known for their roles in allergic and hypersensitivity diseases, they act as sentinel cells that sense microbial pathogens to initiate protective innate and adaptive immune responses via the recruitment of circulating leukocytes and lymphocytes (6–12). The well-characterized HDPs cathelicidin LL-37, human β-defensins, retrocyclins, and protegrins activate human MC via a G protein–coupled receptor (GPCR) known as MRGPRX2 (13–15). Thus, HDPs that harness MC's immunomodulatory property in addition to their antimicrobial activity may serve as novel targets for the treatment of infections caused by antibiotic-resistant organisms.

A small angiogenic amphipathic peptide (AG-30) was identified from a screen of a human library of angiogenic factors (16). It has direct antibacterial activity, induces growth of endothelial cells, and augments angiogenesis (16). Because AG-30 is easily degraded by proteolysis, a modified version of the peptide was generated by replacing several of its neutral amino acids with cationic amino acids, resulting in a new peptide known as AG-30/5C (17). Compared with the original AG-30 peptide, AG-30/5C displays greater antimicrobial activity and shows enhanced ability to induce endothelial cell migration, angiogenesis, and wound healing (17). Interestingly, topical application of AG-30/5C on a mouse diabetic wound-healing model infected with methicillin-resistant *Staphylococcus aureus* results in clearance of the microbe and promotes accelerated wound healing (17). This in vivo effect of AG-30/5C likely reflects its ability to kill microbes directly, to harness MC's immunomodulatory property, to promote angiogenesis, and to induce keratinocyte migration and proliferation (17–19). Although AG-30/5C induces mediator release in human MC via signaling pathways involving G protein and phospholipase C, the possibility that it does so via a cell surface GPCR has not been determined (19).

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Abbreviations used in this article: FDA, Food and Drug Administration; GPCR, G protein–coupled receptor; HDP, host defense peptide; MC, mast cell; MRGPRX2, Mas-related GPCR X2; NIH, National Institutes of Health; PTx, pertussis toxin; RBL-MRGPRX2, RBL-2H3 cell stably expressing MRGPRX2; rhSCF, recombinant human stem cell factor; shRNA, short hairpin RNA; Tango, transcriptional activation following arrestin translocation; 7TMR, seven-transmembrane receptor.

GPCRs are also known as seven-transmembrane receptors (7TMRs) because their structures are characterized by the presence of seven α helices traversing the plasma membrane. In addition to G proteins, most agonists for 7TMRs activate an additional signaling pathway that involves the recruitment of adapter proteins known as β-arrestins. This pathway was initially characterized for its role in GPCR desensitization (uncoupling of the G protein from the cognate receptor), endocytosis, and internalization (20). However, it also serves an important role in G protein–independent downstream signaling for cell migration, growth, and differentiation (21, 22). Agonists of 7TMRs that preferentially activate G proteins and β-arrestins are known as G protein biased and β-arrestin biased, respectively. However, agonists that activate both pathways are known as balanced agonists.

MRGPRX is a family of primate-specific GPCRs and contains four members (23–25). MRGPRX2 is expressed predominantly on human MC, whereas MRGPRX3 and MRGPRX4 are present on human keratinocytes (18, 26, 27). A diverse group of cationic ligands that include compound 48/80, HDPs, and neuropeptides activate human MC via MRGPRX2 and likely contribute to host defense and chronic inflammatory diseases (26, 28–30). A number of U.S. Food and Drug Administration (FDA)–approved peptidergic drugs also induce MC degranulation via MRGPRX2, resulting in injection-site pseudoallergic drug reactions (31). Lansu et al. (32) recently used a novel high-throughput β-arrestin activation assay, known as transcriptional activation following arrestin translocation (Tango), to screen ∼6000 small molecules for MRGPRX2 activation. A surprising observation from these studies was that, although compound 48/80 activates β-arrestin–dependent gene expression, many of the FDA-approved drugs that induce pseudoallergic drug reactions do not (31, 32). These findings raise the interesting possibility that if AG-30/5C activates human MC via MRGPRX2, it may act either as a balanced or biased agonist for the receptor. The stilbenoid resveratrol activates sirtuin 1 (sirt1) to inhibit IgE-mediated MC degranulation (33). A recent screen of the National Institutes of Health (NIH) Clinical Collection library led to the identification of resveratrol as an inhibitor of MRGPRX2-mediated Tango (34). However, the possibility that resveratrol inhibits AG-30/5C-induced MC degranulation has not been determined.

The purpose of the current study was to determine if AG-30/5C activates human MC via MRGPRX2 and to test if it utilizes G protein, β-arrestin, or both signaling pathways. We used the well-established MRGPRX2 agonist compound 48/80 for comparison. Icatibant, a bradykinin B_2 receptor antagonist used for the treatment of hereditary angioedema, promotes MC degranulation via MRGPRX2 and causes injection-site pseudoallergic drug reaction in nearly every patient (31, 35). However, because Icatibant does not activate β-arrestin–mediated gene expression, it was used as an additional control (32). The data presented in this article demonstrate that both AG-30/5C and Icatibant serve as G protein–biased agonists for MRGPRX2 with clinical implications for MC-mediated host defense and pseudoallergy.

Materials and Methods

Materials

All cell culture and Lipofectamine 2000 transfection reagents were purchased from Invitrogen (Carlsbad, CA); recombinant human stem cell factor cytokine (rhSCF) was from PeproTech (Rocky Hill, NJ); p-nitrophenyl-*N*-acetyl-β-D-glucosamine was from Sigma-Aldrich (St. Louis, MO); PE-conjugated anti-MRGPRX2 Ab was from BioLegend (San Diego, CA); polyclonal MRGPRX2 Ab was purchased from Novus Biologicals (Littleton, CO); PE-conjugated anti-FLAG Ab, HRP-labeled goat anti-rabbit IgG and β-actin Abs were obtained from Cell Signaling Technology (Danvers, MA); SuperSignal West Pico Maximum Sensitivity Substrate was from Thermo Scientific (Rockford, IL); compound 48/80 and Icatibant were from AnaSpec (Fremont, CA); nateglinide was from Tocris Bioscience (Bristol, U.K.); and AG-30/5C was from Peptides International (Louisville, KY), respectively. MRGPRX2-Tango (Addgene no. 66440) and MRGPRX4-Tango (Addgene no. 66442) plasmids were gifts from Dr. Bryan Roth (32).

Cell culture

The human MC line LAD2 was maintained in complete StemPro-34 medium supplemented with L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 100 ng/ml rhSCF. Hemidepletion was performed weekly with media containing rhSCF (100 ng/ml) (36). Rat basophilic leukemia (RBL-2H3) cells were maintained as monolayer cultures in DMEM supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml). RBL-2H3 cells stably expressing MRGPRX2 (RBL-MRGPRX2) were maintained similarly in the presence of 1 mg/ml G418 (37). HTLA (HEK-293T cells stably expressing a β-arrestin2–tobacco etch virus fusion gene) cells were cultured in DMEM medium supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), hygromycin (200 μg/ml), puromycin (5 μg/ml), and G418 (500 μg/ml) (32, 34).

Lentivirus-mediated knockdown of MRGPRX2 in LAD2 cells

MRGPRX2-targeted Mission short hairpin RNA (shRNA) lentiviral plasmids were purchased from Sigma. A nontarget vector was used as a control. Lentivirus generation was performed according to the manufacturer's manual. Cell transduction was conducted by mixing 1.5 ml of viral supernatant with 3.5 ml of LAD2 (5×10^6) cells. Eight hours postinfection, medium was changed to virus-free complete medium, and antibiotic (puromycin, 2 μg/ml; Sigma) was added 16 h later (14, 38). Cells were analyzed for MRGPRX2 knockdown by Western blotting and subsequently used for degranulation experiments.

Generation of HTLA cells stably expressing MRGPRX2 and MRGPRX4

HTLA cells (0.8×10^6 cells per well) were plated in a six-well plate in antibiotic-free medium (DMEM, 10% FBS, and L-glutamine). The following day, the cells were transfected with MRGPRX2-Tango or MRGPRX4-Tango plasmids using the Lipofectamine 2000 DNA transfection reagent (32). Briefly, DNA (2 μg) was mixed with Lipofectamine 2000 (12 μl) in Opti-MEM and incubated with cells for 6 h at 37°C. Transfection reagent was removed, and cells were incubated overnight in antibiotic-free medium. Receptor expression was determined by flow cytometry using PE-conjugated anti-FLAG Ab. Transfected HTLA cells were serially diluted such that a single cell was present in 200 μl of medium and were cultured in a 96-well plate. Cells from each well were analyzed by flow cytometry, and one population derived from a single transfected cell was used for the current study.

Transcriptional activation following arrestin translocation (Tango) assay

HTLA cells stably expressing either MRGPRX2 or MRGPRX4 were plated (50,000 cells per well) on a poly-L-lysine–coated, 96-well, white, clear-bottom cell culture plate in triplicates in 160 μl of antibiotic-free medium. After 6 h at 37°C, the medium was aspirated, and cells were incubated with MRGPRX2 or MRGPRX4 agonists in 160 μl of antibiotic-free medium for an additional 16 h at 37°C. The ligands were aspirated, Bright-Glo solution (100 μl) was added to each well, and relative luminescence unit was measured in a Thermo Labsystems Luminoskan Ascent 392 Microplate Luminometer (32).

Calcium mobilization

Most of the Ca^{2+} mobilization studies in HTLA cells were conducted using a high-throughput FLIPR Tetra (Molecular Devices). Cells were plated (10^4 cells per well) on a collagen-coated, black-wall, clear-bottom plate (no. 3764; Corning) and incubated in a 37°C incubator for 24 h. Cells were loaded with Calcium 6 dye (Molecular Devices) for 2 h at 37°C. Assay plate was allowed to equilibrate to room temperature for 1 h prior to assay. Compound 48/80 (1.0 and 30 μg/ml), AG-30/5C (1.0 and 10 μM), and Icatibant (30 μg/ml) were added to their respective wells and imaged every 1 s for 5 min (31). Ca^{2+} mobilization studies in RBL-MRGPRX2 cells were conducted using a Hitachi F-2700 Fluorescence Spectrophotometer.

Cells were cultured in the absence or presence of pertussis toxin (PTx) (100 ng/ml, 16 h) and loaded with 1 mM Indo-1 acetoxymethyl ester for 30 min at room temperature. Cells were then washed and suspended in HEPES-buffered saline containing 0.1% BSA and then stimulated with compound 48/80 (1.0 μg/ml), AG-30/5C (1.0 μM), and Icatibant (20 μg/ml). Calcium mobilization was determined in excitation wavelength of 355 nm and an emission wavelength of 410 nm (12).

Degranulation

LAD2 cells were washed in HEPES buffer containing 0.1% BSA and seeded at 10^4 cells per well in a 96-well plate in 45 μl of HEPES buffer containing 0.1% BSA. RBL-2H3 and RBL-MRGPRX2 cells (5×10^4 cells per well) were seeded in a 96-well plate and cultured overnight in the presence and absence of PTx. Cells were washed twice in HEPES buffer containing 0.1% BSA and suspended in 45 μl of buffer. For resveratrol pretreatment, RBL-MRGPRX2 cells were suspended in 45 μl of buffer containing resveratrol (100 μM) and incubated for 5 min. Untreated and treated LAD2/RBL-MRGPRX2 cells were stimulated with compound 48/80, AG-30/5C, or Icatibant at 37°C for 30 min. For total β-hexosaminidase release, unstimulated cells were lysed in 50 μl of 0.1% Triton X-100. Aliquots (20 μl) of supernatant or cell lysates were incubated with 20 μl of 1 mM p-nitrophenyl-*N*-acetyl-β-D-glucosamine for 1 h at 37°C. The reaction was stopped by adding 250 μl of a 0.1 M Na_2CO_3/0.1 M $NaHCO_3$ buffer, and absorbance was measured at 405 nm (39).

Flow cytometry

RBL-MRGPRX2 cells (control, compound 48/80–, or AG-30/5C–treated) were washed and suspended in FACS buffer (PBS containing 2% FCS and 0.02% sodium azide). Cells (0.5×10^6) were incubated with the PE-conjugated anti-MRGPRX2 Ab for 1 h at 4°C in the dark. Transfected HTLA cells (HTLA-MRGPRX2, HTLA-MRGPRX4) were incubated with PE-conjugated anti-FLAG Ab similarly. Cells were washed in FACS buffer, fixed in 1.5% paraformaldehyde, and acquired using a BD LSR II flow cytometer (San Jose, CA) (28, 40). The resulting data were analyzed using WinList software, version 8.

Statistical analysis

Data shown are mean ± SEM values derived from at least three independent experiments. Statistical significance was determined by nonparametric *t* test and one- or two-way ANOVA. Error bars represent mean ± SD from three independent experiments. Significant differences were set at $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$ and analyzed by GraphPad Prism version 5.01.

Results

AG-30/5C induces MC degranulation via MRGPRX2

We used a human MC line (LAD2) that endogenously expresses MRGPRX2 and found that AG-30/5C induced significant degranulation at a concentration of 0.01 μM ($p \leq 0.05$), with an EC_{50} value <0.1 μM and reaching a maximal response at 1.0–1.5 μM (Fig. 1A). To determine if MRGPRX2 contributes to AG-30/5C–induced degranulation, we used lentiviral shRNA to silence the expression of MRGPRX2. Using Western blotting, we confirmed >90% MRGPRX2 knockdown (Fig. 1B), and this resulted in significantly ($p \leq 0.01$) reduced AG-30/5C–induced degranulation when compared with nontargeted shRNA pretreatment (Fig. 1C). To further confirm the role of MRGPRX2 in MC degranulation in response to AG-30/5C, we used RBL-MRGPRX2 (28). As shown in Fig. 1D, AG-30/5C induced dose-dependent (0.1–1.5 μM) degranulation in RBL-MRGPRX2 cells, but native RBL-2H3 cells did not respond to AG-30/5C. These findings demonstrate that AG-30/5C activates human MC via MRGPRX2.

PTx inhibits AG-30/5C–induced MC degranulation but not Ca^{2+} mobilization

We previously showed that PTx inhibits MC degranulation in response to HDP human β-defensin 3 without affecting Ca^{2+} mobilization (19). We therefore tested the effect of PTx on degranulation and Ca^{2+} mobilization to the well-established MRGPRX2 agonists compound 48/80 and AG-30/5C. Pretreatment of RBL-MRGPRX2 cells with PTx (100 ng/ml, 16 h) abolished degranulation in response to compound 48/80 (1 μg/ml)

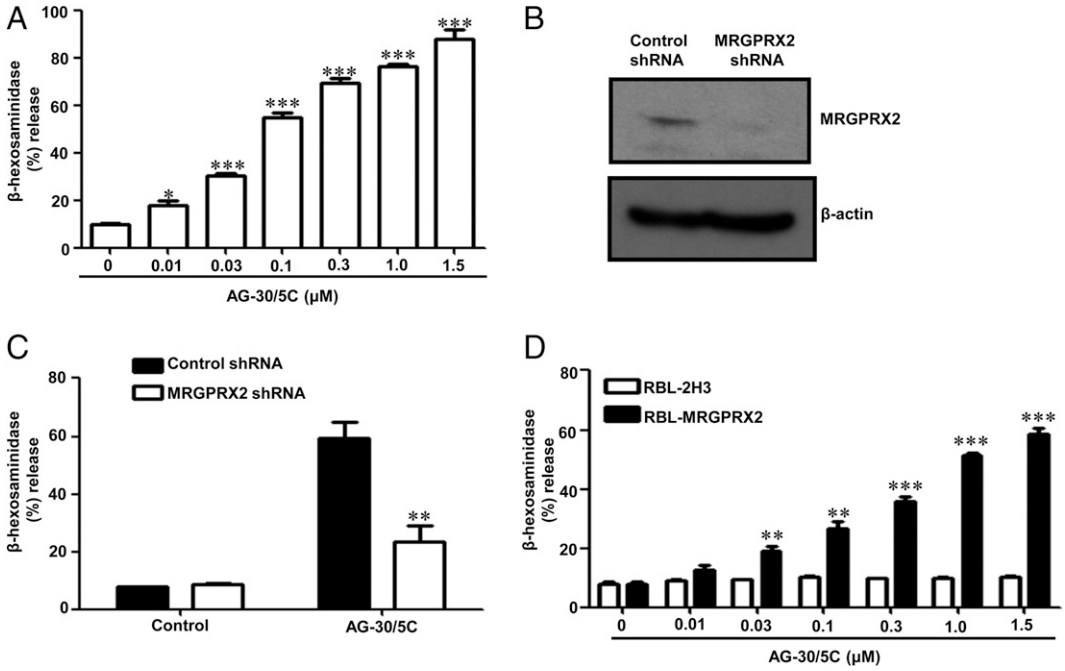

FIGURE 1. AG-30/5C induces MC degranulation via MRGPRX2. (**A**) LAD2 cells were stimulated with different concentrations of AG-30/5C (0.01–1.5 μM) for 30 min. Percent degranulation (β-hexosaminidase release) was determined. (**B**) LAD2 cells were stably transduced with scrambled control shRNA or lentivirus shRNA targeting human MRGPRX2. Western blotting was performed to determine MRGPRX2 expression. β-actin was used as a loading control. (**C**) Control and MRGPRX2 knockdown LAD2 cells were stimulated with AG-30/5C (1 μM), and degranulation was determined. (**D**) RBL-2H3 and RBL-MRGPRX2 were stimulated with different concentrations of AG-30/5C (0.01–1.5 μM), and percent degranulation was determined. All data are expressed as a mean ± SEM of three experiments. Statistical significance was determined by nonparametric *t* test and two-way ANOVA ($p < 0.05$). Western blotting data presented are representative of three similar experiments. $***p < 0.001$, $**p < 0.01$, $*p < 0.05$.

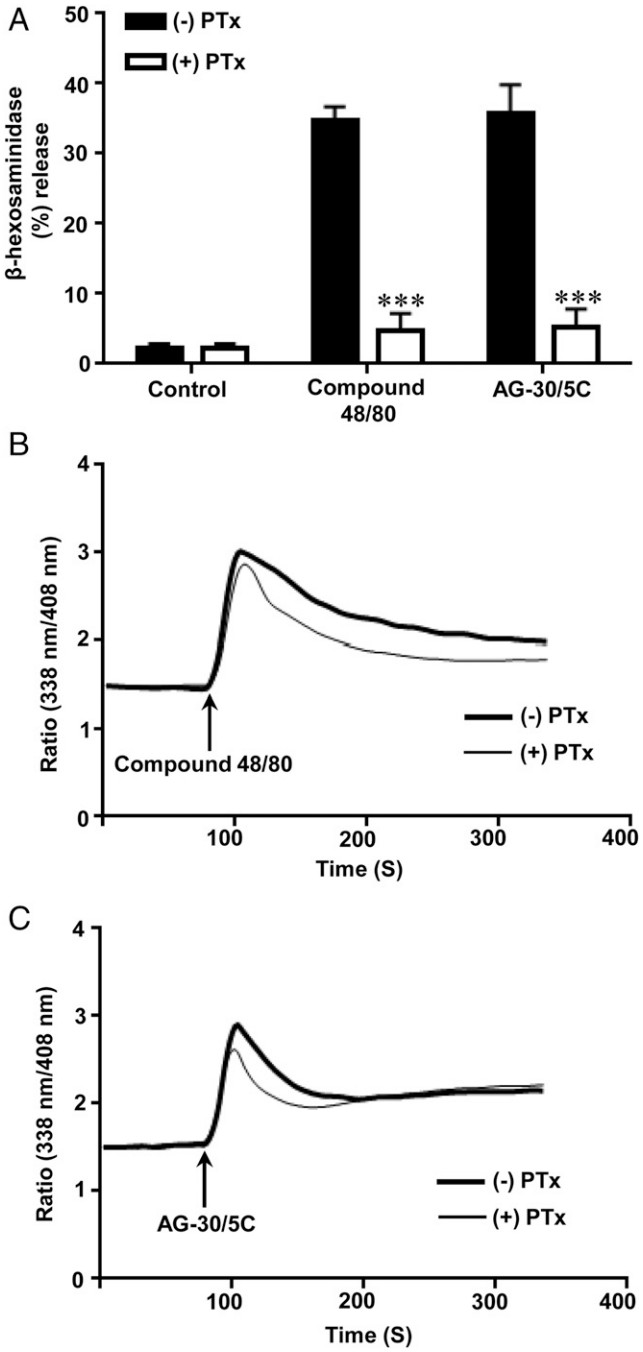

FIGURE 2. PTx inhibits AG-30/5C– and compound 48/80–induced MC degranulation but not calcium mobilization. (**A**) RBL-MRGPRX2 cells were cultured in the absence (−PTx) or presence (+PTx) of PTx (100 ng/ml, 16 h). Cells were stimulated with compound 48/80 (1 μg/ml) or AG-30/5C (1 μM), and percent degranulation was determined. (**B** and **C**) RBL-MRGPRX2 cells were cultured in the absence or presence of PTx, loaded with Indo-1 (1 μM, 30 min), and stimulated with either compound 48/80 (1 μg/ml) or AG-30/5C (1 μM), as indicated by arrows, and time course of calcium mobilization was determined. Representative traces of three similar experiments are shown. All data points for degranulation are expressed as mean ± SEM of three experiments. Statistical significance was determined by nonparametric *t* test and two-way ANOVA. ***$p < 0.001$.

and AG-30/5C (1 μM) ($p \leq 0.001$; Fig. 2A). However, PTx had little to no effect on calcium mobilization to both agonists (Fig. 2B, 2C). These findings suggest that MRGPRX2-mediated MC degranulation via diverse ligands (compound 48/80 and AG-30/5C) requires the interaction of a Gαi-independent Ca^{2+} influx.

AG-30/5C acts as a G protein–biased agonist for MRGPRX2 and MRGPRX4

To determine if AG-30/5C activates β-arrestin–dependent signaling in addition to G protein, we used a newly developed assay that allows for the measurement of both pathways in the same cell line. The β-arrestin activation assay involves transcriptional gene activation; the design of this assay is shown in Fig. 3A and 3B. HTLA cells stably expressing FLAG-tagged MRGPRX2 were incubated with PE-conjugated anti-FLAG Ab to demonstrate cell surface receptor expression by flow cytometry (Fig. 3C). For the β-arrestin–mediated gene expression study, cells were exposed to buffer (control) or comparable concentrations of compound 48/80 (1 μg/ml) and AG-30/5C (1 μM), which induce robust MC degranulation (27) (Figs. 1, 2). At these concentrations, compound 48/80 showed significantly ($p \leq 0.05$) higher β-arrestin–mediated gene expression when compared with AG-30/5C (Fig. 3D) but induced comparable levels of Ca^{2+} mobilization (Fig. 3E). At higher concentrations, compound 48/80 (30 μg/ml) induced ∼75-fold increase in β-arrestin–mediated gene expression when compared with buffer control. However, AG-30/5C (10 μM) induced a weak response, which was <10% of that observed with compound 48/80 (Fig. 3D). Despite this difference in β-arrestin–mediated gene expression, both compound 48/80 and AG-30/5C induced similar peaks in Ca^{2+} mobilization (Fig. 3E). However, compound 48/80 induced a more sustained Ca^{2+} response than AG-30/5C did. These findings demonstrate that, unlike compound 48/80, AG-30/5C serves as a G protein–biased agonist for MRGPRX2.

AG-30/5C activates human keratinocytes via MRGPRX3 and MRGPRX4 (18). Because validated plasmid is available only for MRGPRX4, we sought to determine if it also serves as a G protein–biased agonist for this receptor. We generated HTLA cells stably expressing FLAG-tagged MRGPRX4-Tango and confirmed cell surface receptor expression by flow cytometry (Fig. 4A). Kroeze et al. (34) recently showed that the K_{ATP}-channel blocker nateglinide serves as a balanced agonist for MRGPRX4, activating both G protein– and β-arrestin–mediated signaling pathways. Therefore, to validate the transfection system, we first confirmed that nateglinide induces β-arrestin–mediated gene expression (Fig. 4B) and causes an increase in Ca^{2+} mobilization (Fig. 4C). Compound 48/80, a balanced agonist for MRGPRX2 (Fig. 3), did not activate MRGPRX4 either for β-arrestin (Tango) or G protein (Ca^{2+} mobilization) (Fig. 4B, 4C). By contrast, AG-30/5C caused Ca^{2+} mobilization in HTLA cells expressing MRGPRX4 but did not activate Tango (Fig. 4B, 4C). These findings suggest that AG-30/5C performs its biological function by acting as a G protein–biased agonist for MRGPRX2 in MC and MRGPRX4 in keratinocytes.

AG-30/5C does not cause MRGPRX2 downregulation, but compound 48/80 does

For most GPCRs, β-arrestin recruitment is associated with receptor internalization (41). Given that compound 48/80 caused robust β-arrestin activation and that AG-30/5C did not, we hypothesized that these compounds would have different effects on receptor internalization. To test this possibility, RBL-MRGPRX2 cells were incubated with compound 48/80 or AG-30/5C (16 h), and cell surface receptor expression was determined by flow cytometry. Compound 48/80 pretreatment caused significant ($p \leq 0.05$) reduction in cell surface MRGPRX2 expression when compared with untreated control (Fig. 5A). By contrast, AG-30/5C pretreatment had no significant effect on cell surface MRGPRX2 expression. A representative overlay histogram is shown in Fig. 5B. We then sought to determine if this difference in cell surface receptor

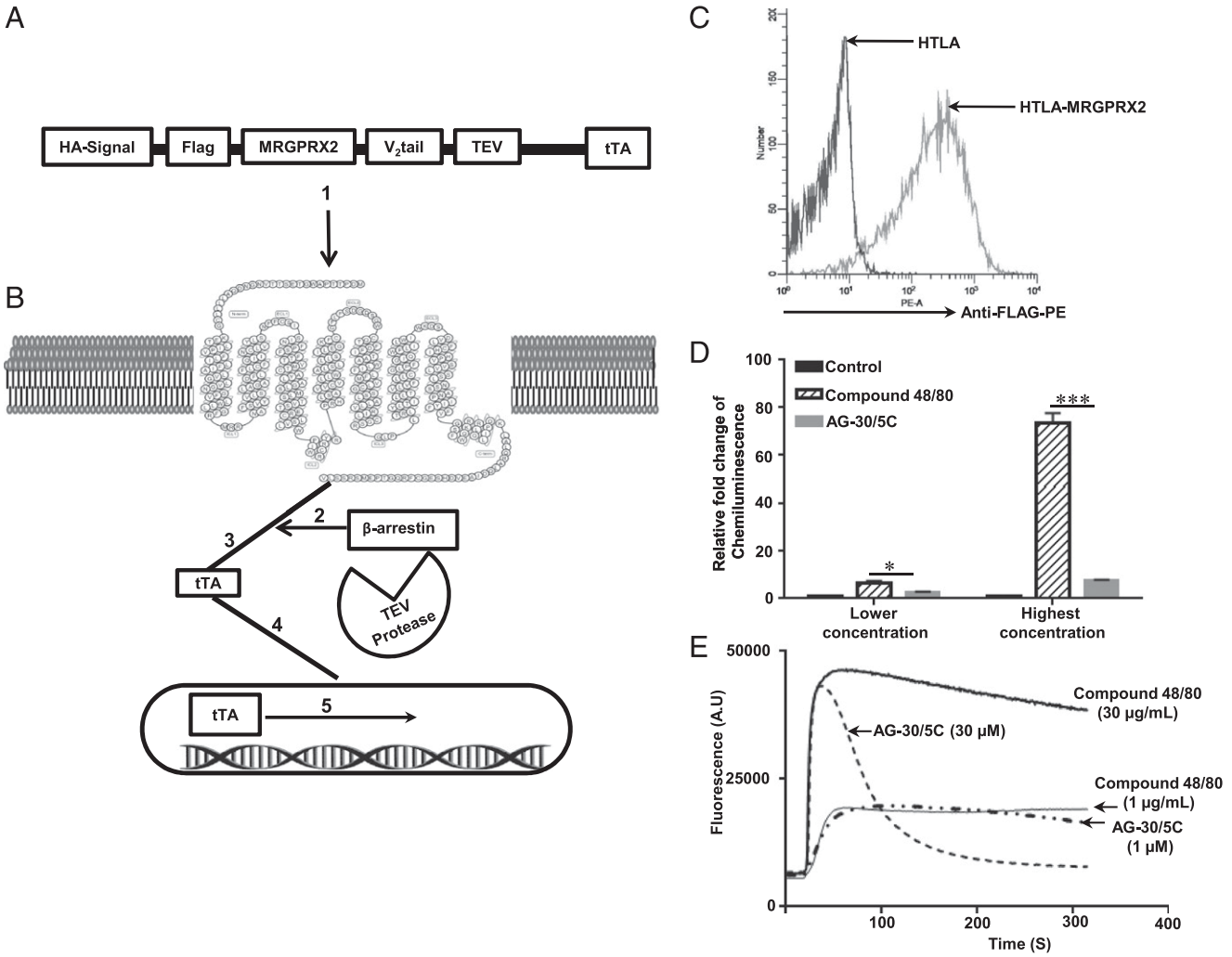

FIGURE 3. Compound 48/80 is a balanced agonist, whereas AG-30/5C is a β-arrestin biased agonist for MRGPRX2. (**A**) Modular design of Tango constructs. (**B**) Upon activation (**1**), β-arrestin is recruited to the C terminus of the receptor (**2**). This is followed by cleavage of the GPCR fusion protein at the tobacco etch virus (TEV) protease cleavage site (**3**). Cleavage results in the release of the tTA transcription factor (**4**), which, after transport to the nucleus, activates transcription of the luciferase reporter gene (**5**). (**C**) Untransfected (HTLA) and stably transfected HTLA-MRGPRX2-Tango (HTLA-MRGPRX2) cells were incubated with anti-FLAG-PE Ab, and cell surface receptor expression was determined by flow cytometry. A representative overlay histogram of MRGPRX2 expression is shown. (**D**) HTLA-MRGPRX2 cells were exposed to compound 48/80 (1.0 or 30 μg/ml) and AG-30/5C (1.0 and 10 μM), respectively, and β-arrestin–mediated gene expression was determined. (**E**) Representative Ca^{2+} measurement traces for compound 48/80 (1 and 30 μg/ml) and AG-30/5C (1 and 10 μM) are shown. All data points for the β-arrestin activation assay are expressed as a mean ± SEM of three experiments. Statistical significance was determined by nonparametric *t* test and two-way ANOVA. ***$p < 0.001$, *$p < 0.05$.

expression correlates with a similar difference in MC degranulation. Consistent with β-arrestin activation (Fig. 3D) and receptor internalization (Fig. 5A), overnight preincubation of LAD2 cells with compound 48/80 (1.0 μg/ml) resulted in complete inhibition of degranulation in response to the same ligand or AG-30/5C (Fig. 5C). However, preincubation with AG-30/5C (1 μM) did not lead to substantial inhibition of degranulation in response to subsequent stimulation by compound 48/80 or AG-30/5C (Fig. 5C).

Resveratrol inhibits both G protein– and β-arrestin–mediated MRGPRX2 signaling

A screen of the NIH Clinical Collection library led to the identification of resveratrol as an inhibitor of constitutive MRGPRX2-mediated Tango (34). However, we did not observe a significant constitutive MRGPRX2 activation in stably transfected HTLA cells. We therefore sought to determine if it inhibits agonist-induced response. Pretreatment of HTLA cells stably expressing MRGPRX2 resulted in significant ($p \leq 0.01$ and $p \leq 0.001$) inhibition of compound 48/80–induced β-arrestin gene expression (Fig. 6A). As AG-30/5C had a minimal effect on β-arrestin signaling, resveratrol pretreatment has no effect on Tango for this agonist (Fig. 6A). To determine if resveratrol inhibits agonist-induced G protein–mediated response, we used RBL-MRGPRX2 and LAD2 cells and determined the effects of resveratrol on degranulation in response to compound 48/80 and AG-30/5C. Preincubation of both cell types with resveratrol (100 μM) significantly ($p \leq 0.05$, 0.01, and 0.001) reduced degranulation in response to both compound 48/80 and AG-30/5C (Fig. 6B, 6C).

Icatibant is a G protein biased agonist for MRGPRX2; inhibition of degranulation by resveratrol

Icatibant, a bradykinin B_2 receptor antagonist used for the treatment of hereditary angioedema, promotes MC degranulation via MRGPRX2 and causes injection-site pseudoallergic drug reaction in nearly every patient (31, 35). To determine the role of the Gαi family of G proteins on MC activation by Icatibant, RBL-MRGPRX2 cells were incubated with PTx, and the effect of

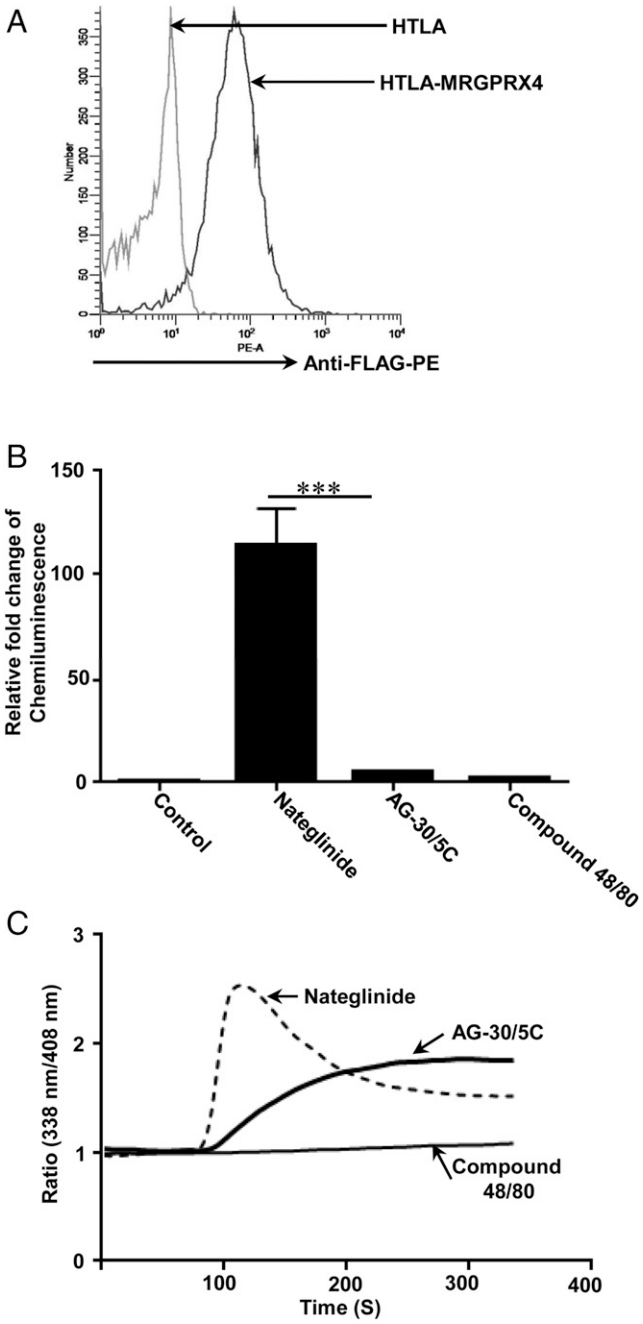

FIGURE 4. Nateglinide is a balanced agonist, whereas AG-30/5C is a G protein biased agonist for MRGPRX4. (**A**) Untransfected (HTLA) and stably transfected HTLA-MRGPRX4-Tango (HTLA-MRGPRX4) cells were incubated with anti-FLAG-PE Ab, and receptor expression was determined by flow cytometry. A representative overlay histogram of MRGPRX4 expression is shown. (**B**) Effects of nateglinide (30 μM), AG-30/5C (10 μM), and compound 48/80 (30 μg/ml) on β-arrestin–mediated gene expression were determined as described in Fig. 3. (**C**) Representative Ca^{2+} measurement traces in response to high concentrations of nateglinide (30 μM), AG-30/5C (10 μM), and compound 48/80 (30 μg/ml) are shown. All data points for the Tango assay are expressed as a mean ± SEM of three experiments. Statistical significance was determined by nonparametric *t* test and two-way ANOVA. ***$p < 0.001$.

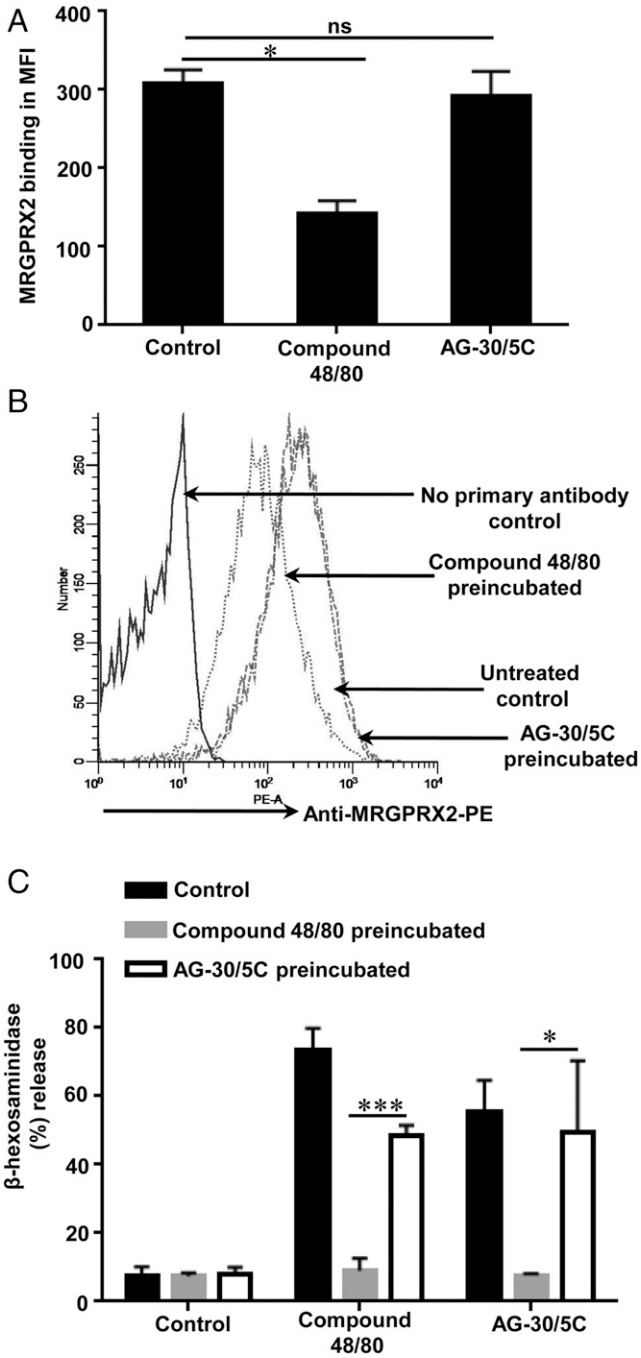

FIGURE 5. Compound 48/80 causes MRGPRX2 internalization and reduced MC degranulation, whereas AG-30/5C does not. (**A**) RBL-MRGPRX2 cells (0.5×10^6) were cultured in the absence (unstimulated) or presence of compound 48/80 (1 μg/ml) or AG-30/5C (1 μM) for 16 h, and cell surface expression of MRGPRX2 was determined by flow cytometry using anti-MRGPRX2-PE Ab. The data are presented as mean fluorescent intensity (MFI) of three experiments. (**B**) A representative histogram of cell surface MRGPRX2 receptor expression is shown. (**C**) LAD2 cells were cultured in the absence (control) or presence of compound 48/80 (1 μg/ml) or AG-30/5C (1 μM) for 16 h, washed, and plated in a 96-well plate (10,000 cells per well). Cells were stimulated with compound 48/80 (1.0 μg/ml) and AG-30/5C (1 μM), respectively, for 30 min, and percent degranulation was determined by β-hexosaminidase release assay. All the points are expressed as a mean ± SEM of three experiments. Statistical significance was determined by nonparametric *t* test and two-way ANOVA. ***$p < 0.001$, *$p < 0.05$.

Icatibant on degranulation and Ca^{2+} mobilization was determined. As shown in Fig. 7A and 7B, PTx completely blocked Icatibant-induced degranulation but not Ca^{2+} mobilization. This finding is similar to what was observed with compound 48/80 and AG-30/5C (Fig. 2). Using RBL-MRGPRX2 cells and LAD2 cells, we found that resveratrol caused significant ($p \leq 0.05$ and $p \leq 0.001$) inhibition of Icatibant-induced degranulation (Fig. 7C, 7D). We sought to determine if Icatibant also acts as a

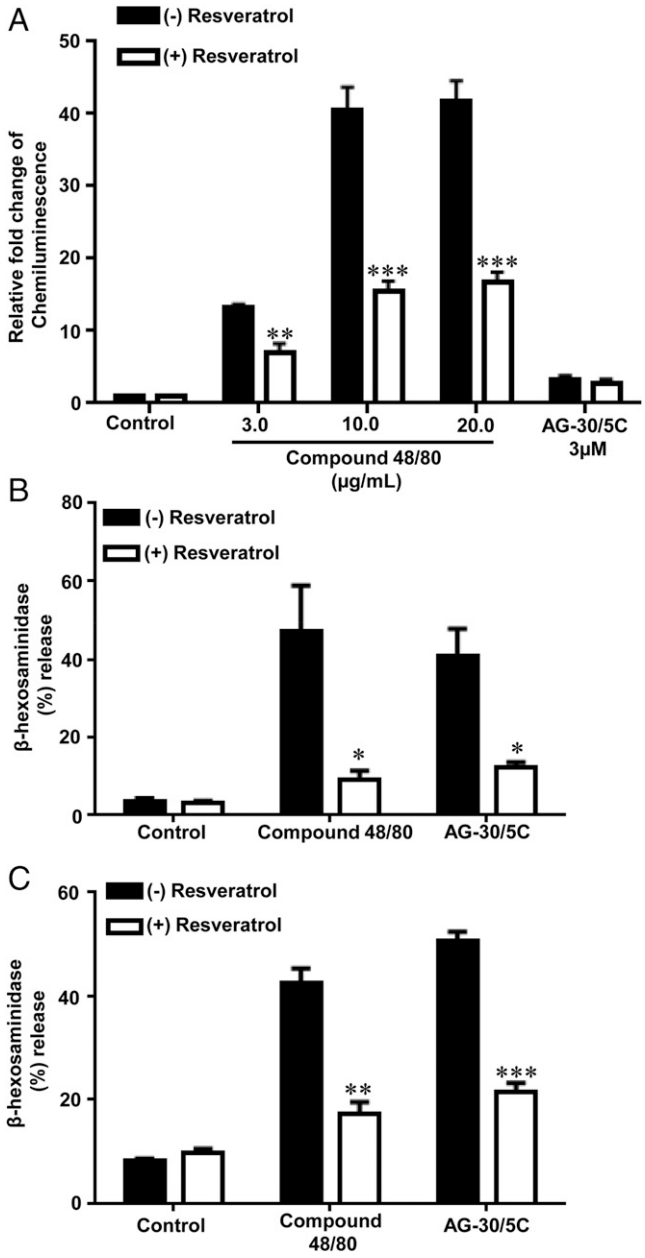

FIGURE 6. Resveratrol inhibits β-arrestin–mediated gene expression and MC degranulation. (**A**) HTLA cells stably expressing MRGPRX2-Tango were incubated with resveratrol (100 μM, 5 min), followed by overnight stimulation with compound 48/80 (3.0, 10, and 20 μg/ml) or AG-30/5C (10 μM), respectively, and relative luminescence was determined. (**B**) RBL-MRGPRX2 cells and (**C**) LAD2 cells were either left untreated (control) or incubated with resveratrol (100 μM, 5 min) and stimulated with compound 48/80 (1 μg/ml) or AG-30/5C (1 μM) for 30 min, and percent degranulation was determined. Statistical significance was determined by nonparametric *t* test and two-way ANOVA. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

biased ligand for MRGPRX2. Tango assay showed Icatibant induced minimal or no β-arrestin signaling compared with compound 48/80 (Fig. 7E). However, it induced Ca^{2+} mobilization, although the peak was much shorter than compound 48/80 (Figs. 7F, 8).

Discussion

AG-30/5C is a synthetic angiogenic HDP that promotes new blood vessel formation, induces human keratinocyte proliferation, effectively clears microbial infection, and induces wound healing in mice (17). The ability of AG-30/5C to harness MC's immunomodulatory property likely contributes to its host defense, but the mechanism via which it activates MC remains unknown (19). The data presented in this article clearly demonstrate that AG-30/5C induces MC degranulation via MRGPRX2. In addition, we demonstrate that AG-30/5C serves as a G protein–biased agonist for MRGPRX2 (MC) and MRGPRX4 (keratinocyte), with important consequences for host defense and wound healing. Furthermore, Icatibant also acts as a G protein–biased agonist for MRGPRX2, and the inhibition of MC degranulation by resveratrol has important clinical implications for modulating pseudoallergic drug reactions.

Kanazawa et al. (19) recently showed that AG-30/5C induces MC degranulation and cytokine generation via signaling pathways that require the activation of a PTx-sensitive G protein. They proposed that AG-30/5C activates MC through a nonselective membrane receptor or via direct activation of G proteins. The data presented in this article with shRNA-silenced LAD2 cells and transfected RBL-2H3 cells clearly demonstrate that AG-30/5C activates MC via MRGPRX2. MC play an important role in innate immunity by causing increased vascular permeability and by initiating the recruitment of neutrophils to the sites of infection (6–8, 10, 42–44). In addition, MC orchestrate the development of adaptive immunity and play an important role in wound healing (11, 12, 45–47). Thus, at sites of microbial infection, mediators released from MC promote migration of dendritic cells, which are subsequently increased in draining lymph nodes (48–50). Furthermore, MC-derived histamine directly modulates dendritic cell activation to enhance Ag presentation to T cells (51). Interestingly, compound 48/80 and the HDP LL-37, which activate human MC via MRGPRX2, are safe and effective vaccine adjuvants in mice (27, 47, 52, 53). Thus, AG-30/5C likely contributes to host defense by harnessing MC's innate and adaptive immune functions via the activation of MRGPRX2.

It is well documented that G protein–independent, β-arrestin–mediated signaling for many GPCRs involves phosphorylation of serine/threonine residues at the C terminus by G protein–coupled receptor kinases (20, 41, 54). Functionally, this is associated with receptor desensitization and β-arrestin–mediated internalization. Interestingly, recent studies with MRGPRX2 using an HDP as an agonist (LL-37) demonstrated that the receptor is resistant to phosphorylation, desensitization, and internalization (14). These findings are consistent with the data presented in this study showing that AG-30/5C functions as a G protein–biased agonist for MRGPRX2 without activating β-arrestin–mediated gene expression. Furthermore, preincubation of cells with AG-30/5C did not induce receptor internalization, nor was there a decrease in MC degranulation in response to compound 48/80 or AG-30/5C. An important clinical implication of these findings is that the lack of functional desensitization of MRGPRX2 by AG-30/5C likely enhances its potential therapeutic efficacy as an immunomodulator in host defense.

In addition to MC activation via MRGPRX2, AG-30/5C induces keratinocyte migration and proliferation via MRGPRX3 and MRGPRX4 (18). However, it is unclear if AG-30/5C also acts as a G protein–biased ligand for MRGPRX3 and MRGPRX4. Our studies with a validated MRGPRX4-Tango system demonstrated that a known MRGPRX4 agonist, nateglinide, induced >100-fold increase in β-arrestin–mediated gene expression. However, AG-30/5C at concentrations that induce robust keratinocyte migration and proliferation (18) induced little to no β-arrestin response. These findings suggest that the immunomodulatory and wound-healing properties of AG-30/5C reflect its ability to serve as a G protein–biased agonist for MRGPRX2 in MC and MRGPRX3/MRGPRX4 in keratinocytes.

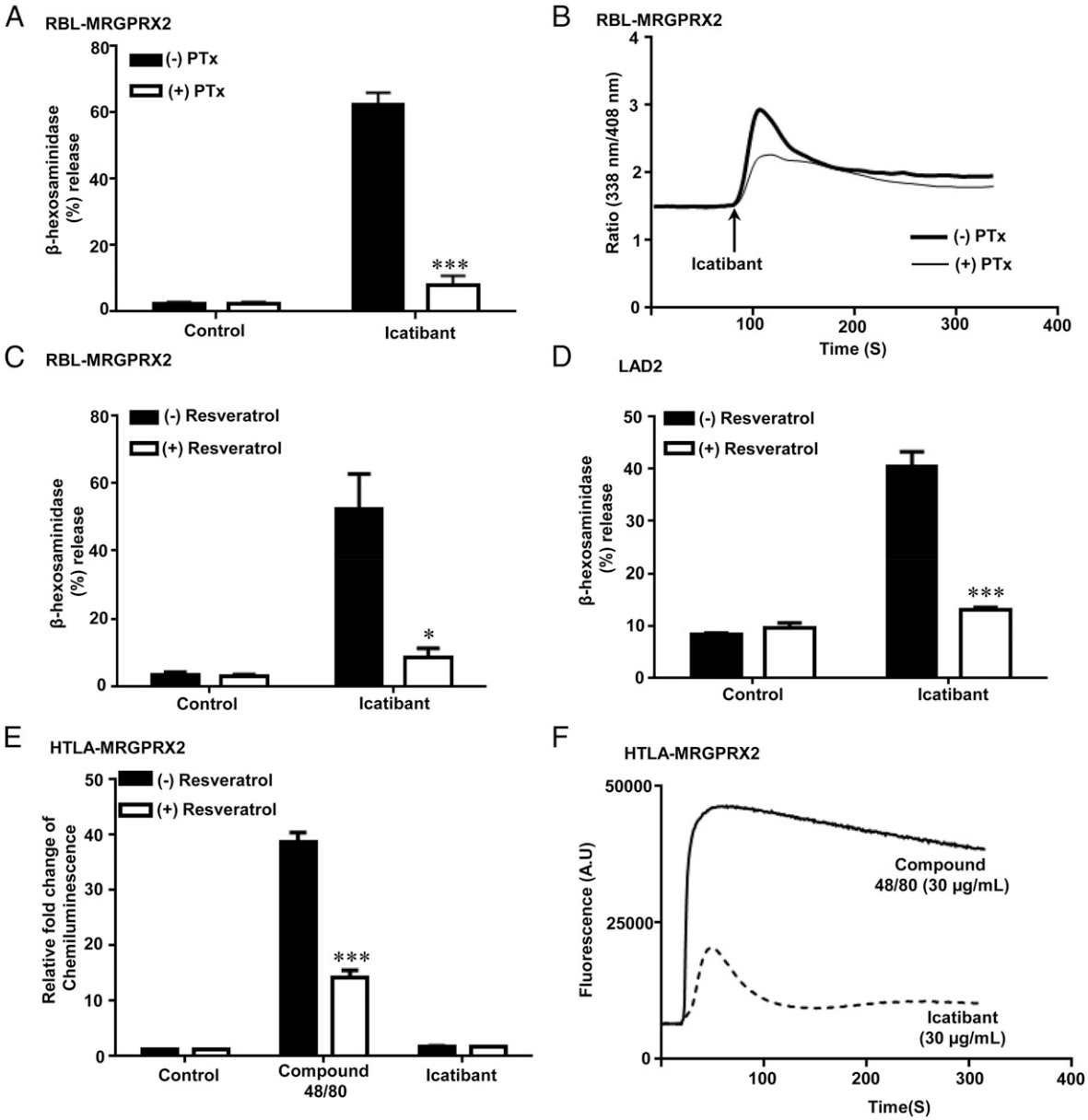

FIGURE 7. Icatibant is a G protein biased agonist for MRGPRX2 and inhibition of degranulation by resveratrol. (**A** and **B**) RBL-MRGPRX2 cells were cultured in the absence or presence of PTx (100 ng/ml, 16 h), and percent degranulation and Ca^{2+} mobilization in response to Icatibant (20 μg/ml) was determined as described for compound 48/80 and AG-30/5C (Fig. 2). (**C**) RBL-MRGPRX2 and (**D**) LAD2 cells were incubated with resveratrol (100 μM, 5 min), stimulated with Icatibant (20 μg/ml) for 30 min, and percent degranulation was determined. (**E**) HTLA cells expressing MRGPRX2 (HTLA-MRGPRX2) were exposed to medium (control) or resveratrol (100 μM, 5 min), followed by overnight stimulation with compound 48/80 (10 μg/ml) or Icatibant (30 μg/ml), and chemiluminescence was measured. (**F**) Representative Ca^{2+} traces for compound 48/80 (30 μg/ml) and Icatibant (30 μg/ml) in HTLA-MRGPRX2 cells are shown. Statistical significance was determined by nonparametric *t* test and two-way ANOVA. $***p < 0.001$, $*p < 0.05$.

In addition to host defense, MRGPRX2 contributes to pseudoallergy in response to a variety of FDA-approved cationic drugs. It has been previously reported that Icatibant does not activate β-arrestin–mediated gene expression in HTLA cells expressing MRGPRX2-Tango (32). Our study confirmed these findings and demonstrated that, similar to AG-30/5C, it also serves as a G protein–biased agonist for MRGPRX2. Thus, unlike the situation of AG-30/5C, lack of desensitization of Icatibant-induced degranulation will likely exacerbate pseudoallergic reaction. However, the finding in the current study showed that resveratrol inhibits Icatibant-induced MC degranulation, suggesting that it may be used to modulate pathologic effects of MRGPRX2 activation.

The molecular mechanism by which compound 48/80, AG-30/5C, and Icatibant activate MRGPRX2 via shared (G protein) and distinct (β-arrestin) signaling pathways is not clear. Based on studies with other GPCRs, it is likely that biased ligands induce a unique receptor conformation, activating a particular signaling pathway. Thus, for arginine-vasopressin-type-2 receptor, transmembrane helix 6 and the third intracellular loop are associated with selective G protein signaling, whereas transmembrane helix 7 is required for selective β-arrestin recruitment (55). For β_2-adrenergic receptor, an unbiased ligand's binding to the receptor primarily shifts the equilibrium toward the G protein–specific active state of helix 6, whereas β-arrestin–biased ligands predominantly regulate the conformational states of helix 7 (56). It is

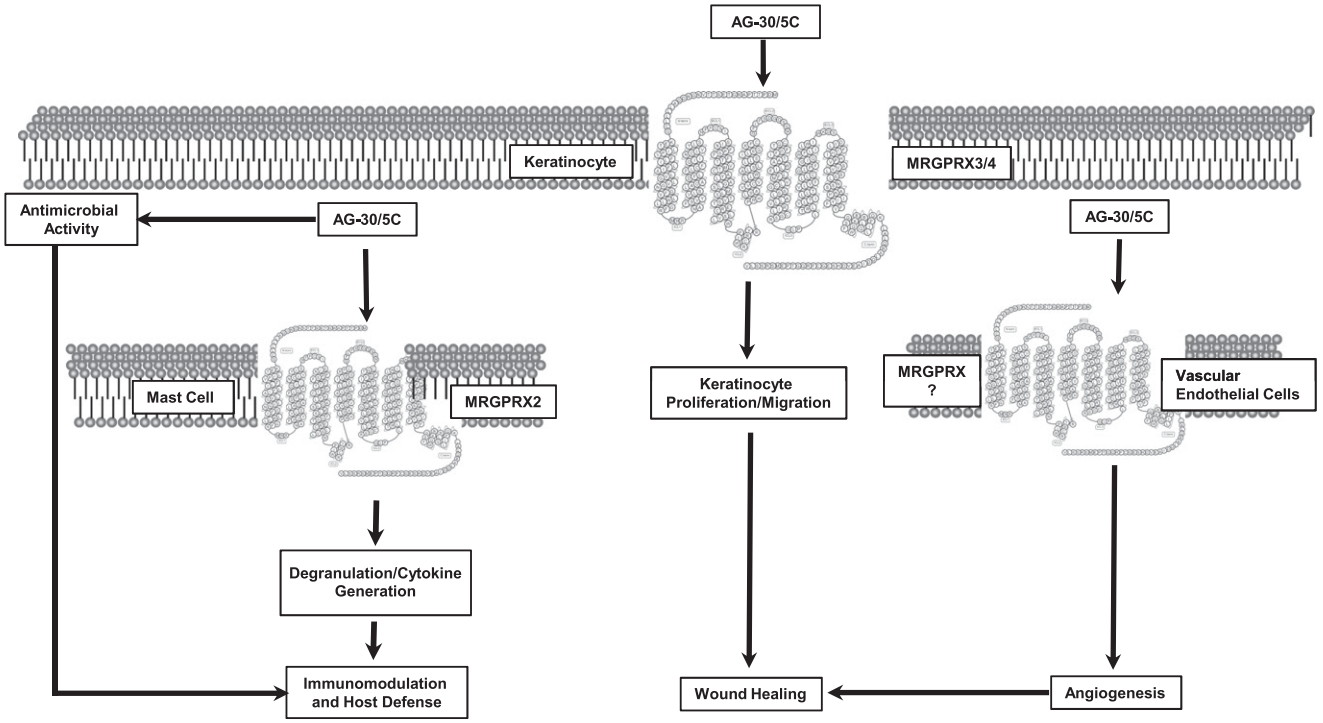

FIGURE 8. Model for the possible mode of action of AG-30/5C on host defense and wound healing. We propose that, in addition to its direct antimicrobial activity, AG-30/5C contributed to host defense via MC degranulation through MRGPRX2. AG-30/5C contributes to wound healing via its action on vascular endothelial cells, keratinocyte proliferation, and migration through MRGPRX3 and MRGPRX4.

therefore possible that compound 48/80, AG-30/5C, and Icatibant induce different conformations of MRGPRX2 to activate distinct signaling pathways. Resveratrol was identified as an antagonist of constitutive activation of MRGPRX2-mediated Tango in HTLA cells (34). However, we did not observe a significant constitutive MRGPRX2 activation, but we found that it effectively blocked both G protein (degranulation) and β-arrestin (Tango) activation. These findings suggest that resveratrol inhibits MRGPRX2 function by preventing ligand–receptor interaction. This possibility will be the subject of future investigation.

In conclusion, we have shown that a peptide identified from a human library of angiogenic factors (AG-30) and structurally modified to enhance both direct antimicrobial and wound-healing properties (AG-30/5C) serves as a novel G protein–biased ligand for MRGPRX2 (MC) and MRGPRX4 (keratinocytes). Interestingly, topical application of AG-30/5C in a diabetic wound-healing model with methicillin-resistant *S. aureus* infection results in clearance of the microbe and promotes accelerated wound healing (17). This effect of AG-30/5C likely reflects its direct antimicrobial activity and its ability to induce angiogenesis and to promote keratinocyte migration and proliferation via MRGPRX3 and MRGPRX4, in addition to harnessing MC's immunomodulatory property via MRGPRX2 (Fig. 8). In addition to MC, MRGPRX2 is expressed in the dorsal root ganglia (57). Thus, it is possible that topical application of AG-30/5C at infected wounds may sensitize the subject to the perception of pain. However, we believe that the potential benefit of AG-30/5C on healing antibiotic-resistant infected wounds outweighs this potential risk.

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Disclosures

The authors have no financial conflicts of interest.

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