Appendix S1

Agents

The TP receptor antagonist (Z)-7-[(1R,3S,4S,5S)-3-[[(2R)-2-cyclopentyl-2-hydroxyacetyl]amino]-6,6-dimethyl-4-bicyclo[3.1.1]heptanyl]hept-5-enoic acid (ONO-3708; Ono Pharmaceutical Co. Ltd., Osaka, Japan) (21, 22) was dissolved in tap water and administered orally in a volume of 0.1 ml per 10 g body weight using a feeding needle. ONO-3708 was administered 1 h prior to observations of spontaneous scratching or pruritogen injections. The proteinase-activated receptor 2 (PAR2) agonist SLIGRL-NH_2 (Ser-Leu-Ile- Gly-Arg-Leu-NH_2; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in physiological saline and injected intradermally into the rostral back in a volume of 50 µl per site using a 19-gauge needle.

For *in vitro* experiments, the TXSyn inhibitor sodium ozagrel (Wako Pure Chemical Ind., Osaka, Japan) was dissolved in culture medium and applied 5 min prior to the application of SLIGRL-NH_2. Non-specific IgG (prepared by the authors) and the anti-PAR2 antibody targeting the second extracellular loop of PAR2 (designed by the authors and manufactured by Sigma-Aldrich) were dissolved in culture medium and applied 10 min prior to the application of SLIGRL-NH_2.

Primary cultures of mouse keratinocytes

The skin was removed from neonatal mice and washed with phosphate-buffered saline (PBS) containing 100 units/ml penicillin (Nacalai Tesque, Kyoto, Japan) and 100 µg/ml streptomycin (Nacalai Tesque). The washed skin was treated with 0.05% collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) dissolved in serum-free MCDB 153 medium (Sigma-Aldrich) containing 0.67% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Dojindo, Kumamoto, Japan), 0.12% sodium bicarbonate (Wako Pure Chemical Ind.), 0.01% penicillin G (Meiji Seika Pharma Co., Ltd., Tokyo, Japan), and 0.006% kanamycin (Wako Pure Chemical Ind.) at 4°C overnight. The epidermal sheets were removed from the dermis and the isolated keratinocytes were cultured in keratinocyte growth medium (CnT-Prime medium; CELLnTEC Advanced Cell Systems AG, Bern, Switzerland) in the 24-well plates and experiments were conducted when cells reached 80–90% confluence in each well.

Behavioral experiments

Hair was removed from the rostral back of healthy control mice 2 days prior to the experiments for an intradermal injection of SLIGRL-NH_2. For the evaluation of spontaneous scratching, the mouse hair was not removed. The animals were housed individually in acrylic cages composed of 4 equally sized compartments ($13 \times 9 \times 35$ cm) for at least 1 h to allow them to acclimate. Following the acclimation period, the spontaneous scratching behavior of the mice was recorded using a digital video camera (HDC-TM25; Panasonic Co., Osaka, Japan) for 1 h without the presence of personnel in the observation room. To observe evoked scratching, the animals were given an intradermal injection after a 1-h acclimation period and immediately returned to the same compartments. The behavior of these mice was also recorded using a digital video camera for 1 h without the presence of personnel in the observation room. Playback of the video was used to determine hind-paw scratching as an index of itching (23). Hind paw scratching toward any region of the body was recorded in mice with dermatitis, while in SLIGRL-NH_2-injected mice, hind paw scratching aimed at the injection site was recorded. A series of several scratching movements of approximately 1 s was considered as a single bout of scratching.

Real-time PCR

Animals were euthanized by transcardiac infusion of PBS under sodium pentobarbital (80 mg/kg) anesthesia. The skin on the rostral back was isolated and stored at −80°C until assayed. Total RNA was extracted from the tissue samples using TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA) and then treated with DNase I (Takara Bio Inc., Otsu, Japan) in 50 µl of reaction buffer. Total RNA (1 µg) was reverse-transcribed in a 20-µl reaction mixture containing 50 pmol oligo $(dT)_{16}$ primer, 1 µl of ReverScript III (Wako Pure Chemical Ind.), 10 pmol of each deoxyribonucleotide (Takara Bio Inc.), and 20 U RNase inhibitor (Toyobo Co. Ltd., Osaka, Japan) at 37°C for 1 h. Quantitative PCR using an aliquot (2.5 µl) of the cDNA and specific primers was performed with a Mx3000PTM real-time PCR system (Agilent Technologies, Inc., Santa Clara, CA, USA). The sequences of the primers used are as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CCAAGCTCATCCATGACAAC-3′ (sense) and 5′-TTACTCCTTGGAGGCCATGT-3′ (antisense); TXSyn, 5′- CCCTGTCCTCTTCTGAGTGC-3′ (sense) and 5′-GCCTCTGCTGTGAACCTTTC-3′ (antisense). The mRNA expression levels of GAPDH and TXSyn were analyzed using Mx Pro QPCR software (Agilent Technologies, Inc.). PCR conditions consisted of 40 cycles of denaturation (30 s at 95°C), primer annealing (30 s at 55°C), and elongation (30 s at 72°C). The relative amount of TXSyn mRNA in each sample was first normalized to the level of GAPDH mRNA and then normalized toTXSyn mRNA expression levels in healthy controls.

Reverse transcription and PCR

Primary cultures of mouse keratinocytes were washed with PBS. Total RNA extraction and cDNA preparation were performed as described in the previous section. An aliquot (1 µl) of the cDNA was mixed with a PCR mix containing 1.5 mM $MgCl_2$, 1 × Green GoTaq® Flexi buffer, 2.5 U GoTaq® DNA Polymerase (Promega CO., Madison, WI, USA), and 50 pmol of the sense and antisense primer. The primer sequences used for reverse transcription (RT)-PCR are as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CCAAGCTCATCCATGACAAC-3′ (sense) and 5′-TTACTCCTTGGAGGCCATGT-3′ (antisense); TXSyn, 5′- CCCTGTCCTCTTCTGAGTGC-3′ (sense) and 5′-GCCTCTGCTGTGAACCTTTC-3′ (antisense); PAR2, 5′- TTTTCCCTCATCCTCGATCA-3′ (sense) and 5′-AAGCCACAAATACAGGCAGT-3′ (antisense).