Appendix S1

MATERIALS AND METHODS

The investigations were performed on skin-punch biopsies of inpatients with psoriasis in the Department of Dermatology, Venerology and Allergology in Silesian Piast Wroclaw Medical University, Poland. All patients provided written informed consent prior to any study procedures. The study was performed according to good clinical and laboratory practices in accordance with the principles of the Declaration of Helsinki and was approved by the local Bioethics Committee at Wroclaw Medical University (KB-669/2011; Wroclaw, Poland).

The control group comprised 20 healthy patients who underwent routinely scheduled plastic surgery in the Plastic Surgery Ward of the Department of Dermatology, Venerology and Allergology. A short clinical interview was performed with the surgeon and, after the exclusion of systemic disorders or any local therapies, subjects were included in the control group.

Clinical data

A total of 20 patients with psoriasis were included in the study, who had not received any local or systemic therapies for at least 3 months prior entering the study. Patients with any other cutaneous or systemic diseases that might interfere with the study results were excluded. Psoriasis severity was measured using the Psoriasis Area Severity Index (PASI). Itch intensity was estimated using a visual analogue scale (VAS) score. The study included 6 men without itch (VAS ≤ 3; mean VAS 2.0 ± 0.5 points) and 10 men and 4 women with itch (VAS ≥ 3; mean VAS: 3.9 ± 2.7 points), who declared generalized itch of the skin. The patients with psoriasis were also asked about the impact of itch on their psychophysiological quality of life. All procedures were performed in accordance with the recommendations of Psychodermatology Section of the Polish Dermatological Society.

Skin biopsies and tissue collection

All patients with psoriasis underwent 4 5-mm skin-punch biopsies (Kai Industries, Seki, Japan) from non-lesional (2 biopsies) and lesional skin (2 biopsies) under local anaesthetic with 1% lidocaine solution. Biopsies were divided and immediately transferred to RNAlater reagent (Qiagen, Valencia, CA, USA) and fixed in fresh 4% paraformaldehyde (PFA) solution in 0.1 M phosphatase buffer saline (PBS) without Ca2+ and Mg2+ pH 7.4 for 12 h at 4°C, respectively. After fixation biopsies were transferred to 30% sucrose (POCH, Gliwice, Poland) solution in 133 mM Sörensen buffer and covered with optimum cutting temperature medium (OCT, Cell Path, Newtown, UK). Frozen skin sections (14 µm thick) were sectioned onto poly-L-lysine glass slides (Superfrost Ultra Plus, Thermo Scientific, Darmstadt, Germany).

Tissue immunofluorescence

Frozen sections were hydrated by rinsing 3 times for 5 min in cold PBS without Ca2+ and Mg2+ (rinse scheme was applied in all the subsequent steps) and post-fixed in 4% PFA in 0.1 M PBS for 45 min at 4°C. Sections were then blocked for 1 h in blocking solution (BS): 3% bovine serum albumin (BSA) (LabEmpire, Rzeszów, Poland), 5% normal donkey serum (Jackson Immunoresearch, West Grove, USA), 0.05% Tween 20 (Sigma-Aldrich, Steinheim am Albuch, Germany), 0.02% Triton-×100 (Sigma-Aldrich, Germany) in PBS. Sections were then incubated for 24 h at 4°C with primary antibodies rinsed in BS: rabbit polyclonal anti-human MOR antibody (dilution 1:200, SC-15310, Santa Cruz Biotechnology, Dallas, USA) against epitope mapping external, N-terminus of receptor and rabbit polyclonal anti-human KOR antibody (dilution 1:200, SC-9112, Santa Cruz Biotechnology, USA) mapping of epitope against external, N-terminus of receptor. The next day sections were incubated with secondary antibodies diluted in PBS (dilution 1:500 for all): donkey anti rabbit-AlexaFluor IgG 488 H+L (A-21206, Invitrogen, Waltham, USA) for µ-MOR, donkey anti-rabbit-AlexaFluor IgG 594 H+L (A-21207, Invitrogen, USA) for κ-KOR in dark conditions for 2 h at room temperature (RT). Negative controls were included by omitting the primary antibodies. Finally, sections were mounted in immunofluorescence medium with DAPI (4',6-diamidino-2-phenylindole) (Invitrogen, USA) for keratinocyte nuclei counterstaining.

Immunofluorescence intensity analysis with ImageJ

Semiquantitative fluorescence intensity (FI) analysis was used to determine expression of opioid receptor protein. All fluorescence *.zvi images were transferred to Fiji software (Fiji, ImageJ, National Institute of Health, Bethesda, USA). Five different ROI per group of 20 epidermal keratinocytes were calculated in 2 separate experiments for non-lesional and lesional skin of patients with psoriasis and the control group.

Western blot

Skin biopsies were cut into small pieces and transferred to an Eppendorf probe with 400 µl cold PBS with proteinase cocktail inhibitor (Sigma-Aldrich, Germany) against protein degradation. Homogenization was performed for 3 min on ice and 400 µl 2× radioimmunoprecipitation assay buffer (RIPA: 50 mM Tris-HCl, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% nonyl phenoxypolyethoxylethanol (NP-40), 0.5% sodium deoxycholate (DOC), pH 8.0) was added for tissues lysis, followed by a sonification step and incubation on ice for 30 min. After centrifugation (15 min 10×10^3 g, 4°C) supernatants were collected and protein concentration determined using a Pierce™ BCA Protein Assay Kit (Thermo™ Scientific, Rockford IL, USA). Total skin protein was mixed with Laemlli's loading buffer (4:1), denatured by incubation at 95°C for 5 min. and transferred onto ice. Two sodium dodecyl sulfate-polyacrylamide gel electrophoreses (SDS-PAGE); 10% for µ-MOR and κ-KOR and 12% for β-actin, were prepared and gel electrophoresis was performed for 20 min at 190 V and 60 mA, then 80 mA for 1.5 h (Bio-rad, Irvine, CA, USA).

Thirty µg total protein in a 40 µl volume or 3 µg/40 µl ready-to-use reference material of normal human hippocampus (Clontech Laboratories, Mountain View, CA, USA) and temporal lobe (Abcam, Branford, CT, USA). Protein transfer in the SDS-PAGE gels was confirmed using Coomassie Brilliant Blue (Sigma, USA) staining. Following gel electrophoresis, semi-dry electroblotting at 300 mA and 25 V for 1 h (Bio-rad, USA) transferred separated proteins from gels to polyvinylidene difluoride (PVDF) membranes (0.45 µm, Millipore, USA). The membranes were hydrated by rinsing 3 times for 5 min in Tris-buffered saline with 0.05% Tween 20 (TBST) and incubated with 1% casein in TBST (0.1% Tween-20) at RT for 1 h. After rinsing again in TBST the solutions of primary antibodies (the same antibodies as used for immunofluorescence) in 1% casein dissolved in TBST were prepared for detection of opioid receptors and β-actin (reference protein). The solutions of rabbit polyclonal antibody against µ-MOR (1 µg/ml; 1:2,000), rabbit polyclonal against κ-KOR (1 µg/ml; 1:2,000) and rabbit polyclonal anti-human β-actin (1µg/ml; 1:2,000, Santa Cruz Biotechnology, USA) were applied with overnight incubation at 4°C. Next day secondary goat anti-rabbit antibody conjugated with horseradish peroxidase-HRP was used (1:1,000, Dako, Denmark). Membranes detection was carried out using a chemiluminescence detection kit (SuperSignal™ West Pico Chemiluminescent, Thermo Scientific™, USA) for 30 min in the

dark at RT and 30 min exposure to light-sensitive film (Kodak, Rochester, NY, USA).

Gene expression

All gene expression analysis procedures were performed in accordance with Minimum Information for Publication of Quantitative Real-time PCR Experiments (MIQE) guidelines.

Isolation of total RNA from skin biopsies

Isolation of total RNA from non-lesional and lesional skin biopsies and healthy control group was carried out using RNA Fibrous Tissues Mini Kit (Qiagen, Germany) and Trizol (Ambion, Austin, TX, USA). The 30 mg of skin were cut into small pieces and transferred to 1 ml Trizol (Ambion, USA) for mechanical homogenization until achieving a uniform homogate. The tissue homogenate was centrifuged (12,000× g for 15 min at 4°C), 200 µl chloroform added, and it was incubated for a further 5 min at RT and centrifuged to obtain 3 layers. The upper clear aqueous phase containing RNA was collected in a new tube, then 500 µl 75% ethanol was added and the total sample was placed on silica membranes in new tubes for RNA binding. Next, DN-ase I treatment (10 µl DN-ase I + 80 µl RDD buffer per silica membrane) was prepared and added to the each silica membranes. Next, 2×500 µl RPE buffer was added to the each silica membranes, with a 15 s centrifugation step between each application (10,000× g at 4 °C). Following vaporization, silica membranes were removed to new tubes and 50 µl water added. The RNA samples were additionally conditioned to remove any potential inhibitors of PCR using commercially available One Step PCR Removal Inhibitor Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Analysis of RNA quality and quantity was performed using Nanodrop (Eppendorf, Hamburg, Germany). Only those samples with 260/280 nm absorbance coefficient and range between 1.8 and 2.1 were used in the next steps.

Reverse transcription: cDNA synthesis

The 500 ng of total RNA were reverse-transcribed on cDNA using 1st Strand cDNA Synthesis Kit for RT-PCR with Avian Myeloblast Virus (AMV) reverse transcriptase (Roche, Mannheim, Germany). The reaction was performed in accordance with manufacturer's instructions and conducted in a 20 µl volume (2 µl 10× Reaction Buffer: 4 µl MgCl2 5 mM, 2 µl deoxynucleotide mix 1 mM, 2 µl oligo-p$(dT)_{16}$ primers, 2 µl p(dN)6 primers, RNase inhibitor, AMV Reverse Transcriptase with variable volume of RNA sample and water depending on concentration) per reaction. Single-strand cDNA synthesis was carried out at 25°C for 10 min, 42°C for 60 min, 99°C for 5 min and 10°C for 4 min in a thermocycler (MJ Research, Waltham, USA). All probes were immediately frozen at –20°C.

Primer, probe and experiment design

Primers and probes for ORs were constructed using Roche Assay Design Center and ProbeFinder 2.48 (Roche, Germany) software, based on human reference sequences. Multiplex real-time PCR and detection system for gene expression analysis was performed using universal probe library (UPL) hydrolysis probes. The primers were designed at the border of constitutively expressed exons (expressed in all known human alternative transcripts) to exclude any possible hybridization to gDNA (**STable I**). The stability of proposed housekeeping genes between non-lesional and lesional skin samples were verified using the RealTime ready Human Reference Gene Panel (Roche, Germany) and bioinformatic tool BestKeeper (Germany).

Real-time PCR with UPL probes

RT-PCR was performed using a Roche LightCycler 480 II thermocycler (Roche, Germany). The reaction was carried out using a multiplex system, with *OPRM1* and *OPRK1* as targets and *ACTB* as housekeeping gene in a single 96-reaction plate (Roche, Germany). The reaction was carried out in a 10 µl volume: 5 µl LightCycler UPL ProbeMaster (Roche, Germany), 0.5 µM forward and reverse primers for target (Genomed, Warszawa, Poland) and reference (Roche, Germany): *OPRM1*/*ACTB* and *OPRK1*/*ACTB*, 0.2 µM of universal probe library for target and reference, 1 µl cDNA and molecular biology water (Roche, Germany). Reaction conditions were 10 min pre-incubation at 95°C, 50 cycles of amplification step: denaturation at 95°C for 15 s, elongation at 58°C for 1 min, and detection at 72 oC for 10 s. The last stage involved cooling at 40°C for 10 s. Fluorescence signal detection of and calculations of Ct values were performed using Roche LightCycler480 Software. Relative gene expression was presented using $2^{-\Delta\Delta Ct}$ method.

Statistical analysis and data presentation

Statistical analyses were carried out using Statistica 12.0 Software (Dell Software, USA). A Kolmogorov–Smirnov test was used to document non-parametric characteristics and all data were presented as medians with lower and upper quartiles. A Mann–Whitney *U* test was used to compare control skin and non-lesional and lesional skin with and without itch. Correlation data were analysed using Spearman rank-order correlations test. Values of $p < 0.05$ were considered significant (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Graphs were plotted using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA), and figures were prepared using LibreOffice 5.0 Software (The Document Foundation, Germany).

STable I. Sequences of primer pairs and universal probe library (UPL) probes for human opioid receptors and β-actin housekeeping gene with information about gene regions, exon border and amplification sizes

Gene symbol	Gene bank ID number	Forward (F) and reverse (R) primer sequences (5′-3′)	Universal probe library sequences and fluorescence days	Amplicon size (bp)	Exon border
OPRM1	NM_000914.3	F: GCTACAACAAAATACAGGCAA R: AGGCGAAGATGAAAACACAGA	UPL 62: ACCTGCTG-FAM	106	2/3
OPRK1	NM_000912 .3	F:CCTTGAAGGCAAAGATCATCA R: TGCAAGGAGCACTCAATGAC	UPL 71: CTGGCTGC-FAM	124	3/4
ACTB	NM_001101.3	F: CAACCGCGAGAAGATGAC R: GTCCATCACGATGCCAGT	UPL: TGTACGTTGCTA-VIC	121	3/4