SUPPLEMENTARY MATERIAL

Supplementary materials and methods

Materials

Carvacrol, 2-Aminoethoxydiphenyl borate (2-APB), arachidonic acid (AA), testosterone, linoleic acid, and N-arachidonoylethanolamine (anandamide, AEA) were obtained from Sigma-Aldrich whereas AMG 9810 and HC067047 were from Tocris Bioscience (Bristol, UK). Ruthenium red was purcheased from Research Biochemicals International (Natick, MA, USA).

Cell culturing

Human immortalized SZ95 sebocytes, originated from human facial sebaceous glands (Zouboulis et al. 1999), were cultured in Sebomed® Basal Medium (Merck-Millipore, Berlin, Germany) supplemented with 10% fetal bovine serum (Life Technologies Corporation, Carlsbad, CA, USA), 1 mM $CaCl_2$, 5 ng/ml human recombinant epidermal growth factor (Sigma-Aldrich), 50 IU/ml penicillin and 50 μg/ml streptomycin (both from PPA Laboratories, Pasching, Austria). The medium was changed every other day, and cells were sub-cultured at 60-70% confluence. Human keratinocytes were isolated from skin samples of healthy individuals undergoing dermatosurgery following written informed consent adhering to Helsinki guidelines, and after obtaining Institutional Research Ethics Committee's and Government Office for Hajdú-Bihar County's permission (protocol No.: DE OEC RKEB/IKEB 3721-2012). Primary keratinocytes were handled and cultured as described previously (Szöllősi et al. 2018).

Immunocytochemistry

SZ95 sebocytes, seeded and cultured on sterile coverslips in 24-well plates, were fixed in ice-cold acetone (VWR, Radnor, PA, USA) for 5 min and then permeabilized by 0.1% Triton-X-100 in phosphate-buffered saline (PBS; 115 mM NaCl, 20 mM Na_2PO_4, pH 7.4; all from Sigma-Aldrich) for 10 min. After washing in PBS and blocking in 1% bovine serum albumin (BSA, Sigma-Aldrich) in PBS for 30 min, cells were incubated with the TRPV3 (AbCam, Cambridge, UK) specific primary antibodies (produced in rabbit; dilution 1:200 in blocking solution; overnight incubation at 4°C). For fluorescence staining, slides were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat IgG-specific secondary antibody (Life Technologies) for 60 min (dilution 1:200), and the nuclei of cells were visualized using 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA). Cells were examined on a Nikon Eclipse E600 fluorescent microscope (Nikon, Tokyo, Japan). As negative control, the primary antibody was omitted from the procedure.

Immunohistochemistry

The immunohistochemical investigation of TRPV3 was performed on formalin fixed paraffin embedded, skin samples rich in sebaceous glands, all diagnosed as trichilemmal cyst in Kenézy County Hospital (Debrecen, Hungary). Serial 4 μm thick sections were cut from paraffin blocks. Heat-induced antigen retrieval was performed on formalin fixed samples. EDTA buffer (Reanal, Hungary, 1 mM, pH 8.0) was applied in microwave oven for 15 min to unmask epitope of TRPV3. Endogenous peroxidase activity was blocked with 3% H_2O_2 (Sigma-Aldrich) for 10 minutes. After blocking, tissue sections were incubated with a specific rabbit primary antibody against TRPV3 (Alomone, Jerusalem, Israel, 1:500, 60 min). TRPV3 expression was detected with HRP labelled Polymer Anti Rabbit EnVision+ system (Dako, Glostrup, Denmark) following the manufacturers protocol TRPV3 staining was visualized with 3,3'-Diaminobenzidine (DAB, DAKO, Golstrup, Denmark), nuclei were counterstained with hematoxylin (Sigma-Aldrich) and tissue sections were finally mounted in permanent mounting medium (Histolab, Göteborg, Sweden). Negative controls were obtained by omitting the primary antibody.

Determination of intracellular lipids

For quantitative measurement of lipid content, cells (20,000 cells/well) were cultured in 96-well black-well/clear-bottom plates (Greiner Bio-One, Frickenhausen, Germany) in quadruplicates and were treated with compounds for 24 hours. Subsequently, supernatants were discarded and 100 μl of a 1 μg/ml Nile Red (Sigma-Aldrich) solution in PBS was added to every well. Fluorescence was measured on a FlexStation 3 multi-mode microplate reader (Molecular Devices, San Francisco, CA). Results are expressed as percentages of the relative fluorescence units in comparison with the controls using 485 nm excitation and 565 nm emission wavelengths for neutral lipids (Alestas et al. 2006; Dobrosi et al. 2008).

Determination of viability

Viability was determined by measuring the conversion of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) to formazan by mitochondrial dehydrogenases. Cells were plated in 96-well plates (in 20,000 cells/well density) in quadruplicates and were treated with compounds for 24-hours. Cells were then incubated with 0.5 mg/ml MTT for 3 hours, subsequently colorimetric analysis of the concentration of formazan crystals took place according to the manufacturer's instruction.

Fluorescent Ca^{2+}-measurement

SZ95 sebocytes were seeded in 96-well black-well/clear-bottom plates (Greiner Bio-One) at a density of 20,000 cells/well. Cells were washed once with 1% BSA and 2.5 mM probenecid (both from Sigma-Aldrich) containing Hank's solution (136.8 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 0.81 mM $MgSO_4$, 1.26 mM $CaCl_2$, 5.56 mM glucose, 4.17 mM

$NaHCO_3$, pH 7.2, all from Sigma-Aldrich), and loaded with 1 μM Fluo-4 AM (Life Technologies) dissolved in Hank's solution (100 μl/well) at 37°C for 30 min, and were then washed three times with Hank's solution (100 μl/well). The plates were then placed in a FlexStation 3 multi-mode microplate reader (Molecular Devices), and changes in cytoplasmic Ca^{2+} concentration (reflected by changes in fluorescence at 490 nm excitation and 520 nm emission wavelengths) induced by various TRPV3 agonist in various conditions were monitored. Experiments were performed in multiple wells and data were obtained as F/F_0, where F_0 is the average fluorescence of the baseline (before compound application) and F is the actual fluorescence.

RNA isolation, reverse transcription, quantitative "real-time" PCR (Q-PCR)

Total RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's protocol, and the isolated RNA was quality-checked by Nanodrop-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). 2 μg of total RNA were then reverse-transcribed into cDNA by using the High Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's protocol. PCR amplification was performed by using the TaqMan primers and probes (assay ID-s: Hs00376854_m1 for TRPV3, Hs00985639_m1 for IL-6, Hs00174103_m1 for IL-8, Hs00174092_m1 for IL-1α, Hs00174128_m1 for TNFα, Hs00234592_m1 for PPARγ and Hs00942766_s1 for NRIP1) using the TaqMan universal PCR master mix protocol (Life Technologies). As internal controls, transcripts of peptidyl-prolyl isomerase A (Cylophylin A, PPIA) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined (assay ID-s: Hs99999904_m1 for PPIA and Hs99999905_m1 for GAPDH). The amount of the transcripts was normalized to those of the housekeeping gene using the ΔCT method.

Western blotting

Cells were harvested and homogenized in detergent mixture (50 mM TRIS HCl, 150 mM NaCl, 1% Triton X-100, 1% Igepal CA 630, 0.5% sodium deoxicholate), containing protease inhibitor cocktail (1:100, all from Sigma-Aldrich). After ultrasonic homogenization, the protein content of the resulting samples was determined with a BCA protein assay kit (Pierce, Rockford, IL, USA). Protein samples (30 μg/well) were subjected to SDS-PAGE (10% Mini Protean TGX gels, Bio-Rad, Hercules, CA, USA), and transferred to nitrocellulose membranes, by using Trans-Blot® Turbo™ Nitrocellulose Transfer Packs and Trans Blot Turbo System (both from Bio-Rad). Membranes were probed with the rabbit primary antibody against human TRPV3 (1: 200 dilution, Alomone Labs, Jerusalem, Israel), in PBS containing 1% dry milk and 0.1% Tween-20) overnight at 4°C. As a secondary antibody, horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:1000, Sigma) was used and the immunoreactive bands were visualized by a SuperSignal West Pico or Femto Chemiluminescent Substrate-Enhanced Chemiluminescence kits (Pierce) using a Kodak Gel Logic 1500 Imaging System (Kodak, Tokyo, Japan). To assess equal loading, the membranes were re-probed by using rabbit-anti-tubulin antibody (1:1000, AbCam).

RNA interference

Cells were transfected at ≈70% confluence with specific Stealth RNAi oligonucleotides (40 nM, Invitrogen) against TRPV3 (ID: HSS175965), using Lipofectamine RNAiMax Transfection Reagent (Invitrogen). For controls, Stealth RNAi Negative Control Med GC Duplexes (scrambled RNA construct; Invitrogen) were employed. The efficacy of RNAi-driven ‘‘knock-down’’ was evaluated 48 hours after transfection by Western blotting and Q-PCR as described previously above.

Determination of cytokine release (ELISA)

Supernatants were collected from sebocytes exposed to various treatments. While RNA was isolated from the cells as described above, the supernatants were analyzed for human IL-1α, IL-1β, IL-6, IL-8/CXCL-8 and TNFα using commercially available ELISA kits (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer’s protocols. In brief, plates were coated with capture antibody diluted in coating buffer (0.1 M Na_2CO_3, pH 9.5 with 10 N NaOH) and incubated overnight at 4°C. Then plates were incubated with assay diluent (10% fetal bovine serum in PBS) at room temperature (RT) for 1 hour, while standard and sample dilutions were prepared in assay diluent. Concentration standards and samples were added into appropriate wells and incubated for 2 hrs at RT. After 2 hours, working detector (detection antibody + SAv-HRP reagent) was added to each well, and incubated for 1 hour at RT. After every step, plates were washed with wash buffer (0.05% Tween-20 in PBS). After washing, substrate solution (tetramethylbenzidine and hydrogen peroxide in citrate-buffer, pH 5.0) was added to every well for 30 minutes in the dark, followed by stop solution (2 N H_2SO_4). Absorbance was read at 405 nm within 30 minutes of stopping reaction. The amount of cytokines in pg/ml was calculated from calibration curve created by serial dilution of interleukine standards. The experiments were repeated 3 times using supernatants of independent cultures. TNFα was not detected in the supernatants (data not shown).

Determination of cellular differentiation by flow cytometry

SZ95 cells were cutured in 6-well plates and exposed to various treatments for 24 hrs. Then, cells were stained with Nile Red (1 µg/ml Nile Red solution in PBS) for 30 minutes. Following staining, cells were harvested and subjected to flow cytometry in a NovoCyte Flow Cytometer (ACEA Biosciences, San Diego, CA, USA) using a 488 nm extinction laser. Side scatter and fluorescence characteristic for Nile Red via FL-2 channel were detected simultaneously and data were analyzed using FlowJo (FlowJo LLC, Ashland, OR, USA) software.

Data and statistical analysis

Unless it is stated otherwise, all data are presented as mean±SEM of the indicated sample size where n represents the number of independent biological repeats. Data analysis and plotting were carried out using Origin 9.0 (OriginLab Corporation, Northampton, MA, USA). Comparison of

means was done by two-tailed Student's t-test or, in case of multiple groups, by One-Way ANOVA followed by either Dunnett or Bonferroni *post hoc* tests, as appropriate. All statistical analyses were performed using IBM SPSS Statistics 23.0 (IBM, Armonk, NY, USA).

Supplementary references

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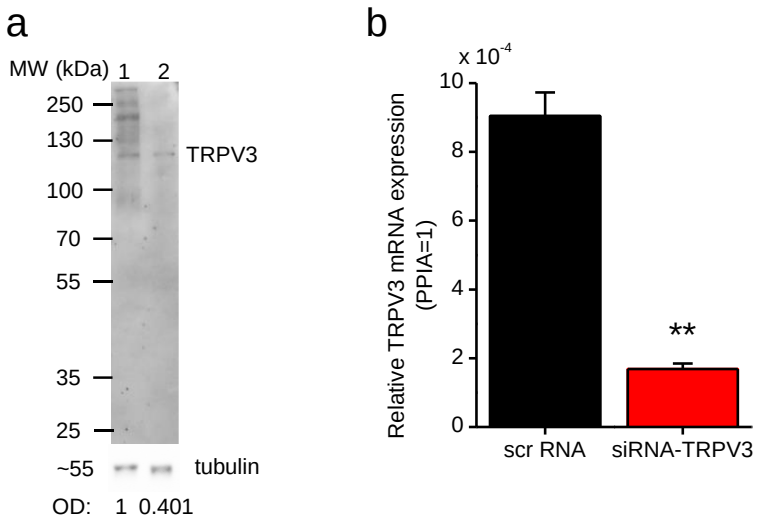
Zouboulis CC, Seltmann H, Neitzel H, Orfanos CE. Establishment and characterization of an immortalized human sebaceous gland cell line (SZ95). J. Invest. Dermatol. 1999;113(6):1011–20

Supplementary figures

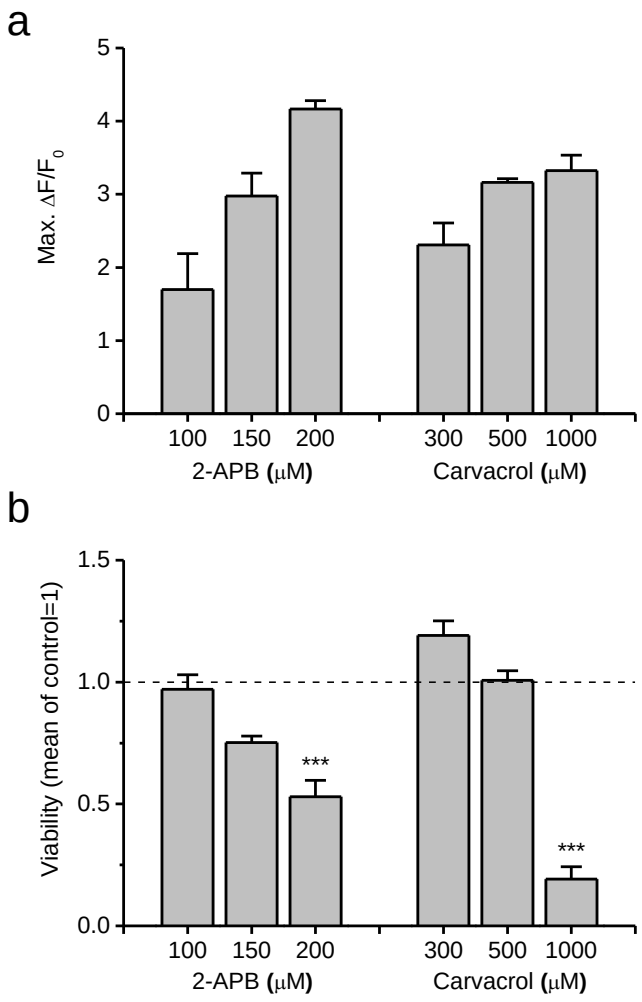
Supplementary Figure S1



Supplementary Figure S1. RNAi-mediated silencing of TRPV3 expression in human sebocytes

SZ95 sebocytes were transfected with either scrambled RNA (scr RNA) or siRNA targeting TRPV3 (siRNA-TRPV3) as detailed in the 'Supplementary materials and methods' section. Expression of TRPV3 was checked 48 hours after transfection. a, Western blot analysis of sebocytes transfected with scrambled RNA (lane 1) or siRNA specifically targeting TRPV3 (lane 2) indicates a marked decrease of TRPV3 protein expression at 48 hours after transfection Tubulin stained on the same membrane served as a loading control to asses equal amount of total protein loaded to the PAGE. OD: relative optical density of the TRPV3 bands normalized to the corresponding tubulin bands. OD of scrambled RNA transfected sebocytes = 1. b, Quantitative analysis of TRPV3 transcripts by Q-PCR in scrambled RNA (scr RNA) and TRPV3-specific siRNA (siRNA-TRPV3) transfected sebocytes reveals a significant decrease of TRPV3 gene expression at 48 hour after transfection. N=3 independently transfected samples, **$p<0.01$ between the scr RNA and siRNA-TRPV3 transfected samples as determined by Student's t-test for independent samples. In other experiments, pharmacological treatments of scr RNA and siRNA-TRPV3-transfected cells were started 48 hours after transfection.

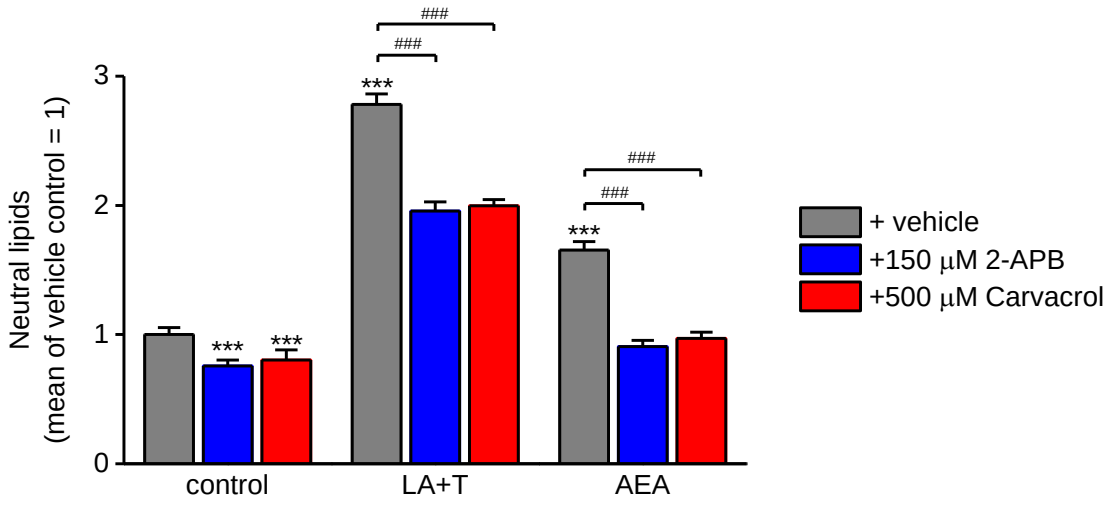
Supplementary Figure S2



Supplementary Figure S2. Concentration dependent effect of TRPV3 agonists on cytoplasmic Ca^{2+} concentration and viability of SZ95 sebocytes

a. Statistics on the amplitude of cytoplasmic Ca^{2+} signals evoked by acute application of TRPV3 agonists in the indicated concentrations. N=3 in each group. b. Effect of the TRPV3 agonists on the viability of SZ95 cells as measured by MTT assay. N=7-8 in each group, ***$p<0.001$ compared to the vehicle treated control group as determined by One-way ANOVA and Dunnett *post hoc* test. TRPV3 agonists were applied in the same concentrations as on panel a for 24 hours prior the assay. Although high concentrations significantly reduced the MTT signal indicating declined living cell number, lower concentrations of the agonists did not decreased cellular viability after 24 hours. In other experiments, these effective but non-toxic concentrations (150 μM 2-APB and 500 μM carvacrol) were tested on lipid synthesis and cellular differentiation (24 hr-treatments) and inflammatory cytokine expression and release (6 hr-treatments).

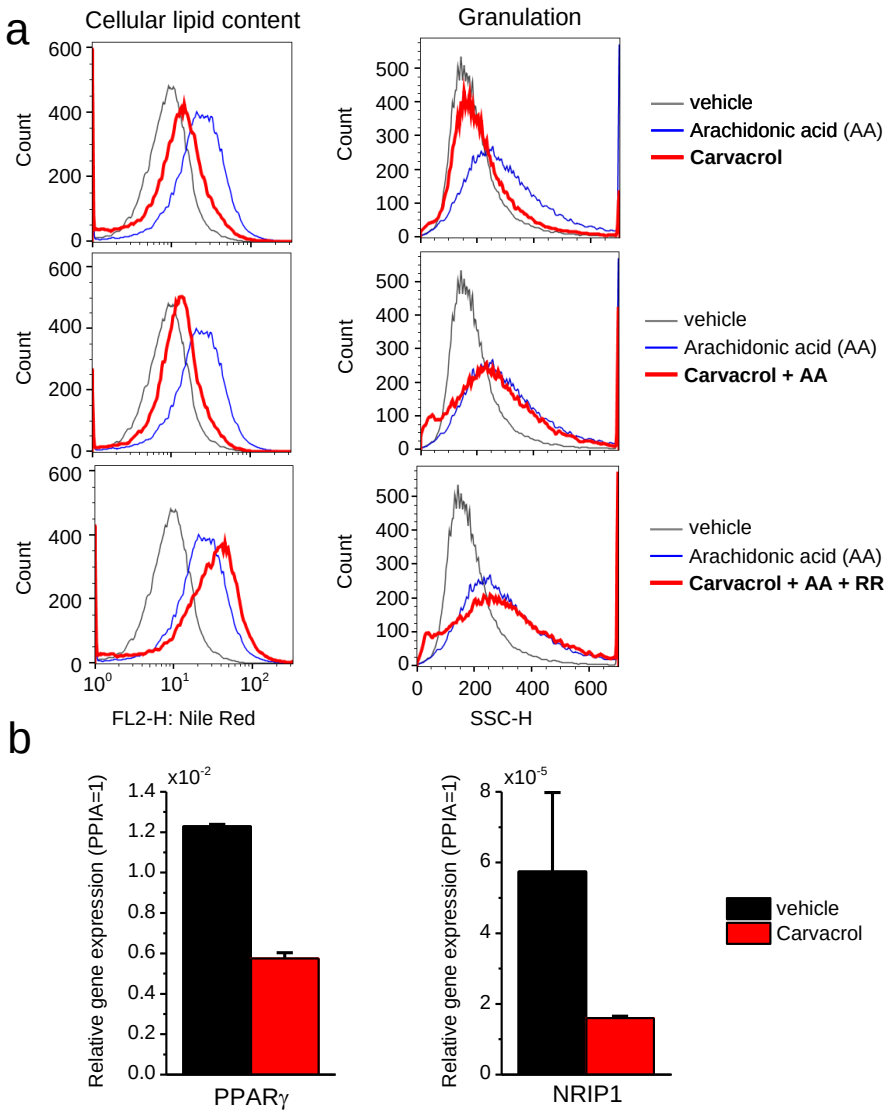
Supplementary Figure S3



Supplementary Figure S3. Effect of TRPV3 agonists on lipid synthesis of human sebocytes

2-APB and Carvacrol were tested on the basal and induced lipogenesis of SZ95 cells. Lipogenesis was induced either by the combination of 100 µM linoleic acid and 1 µM testosterone (LA+T) (Makrantonaki and Zouboulis 2007) or by the endocannabinoid anandamide (arachidonoylethanolamine, AEA, 30 µM) (Dobrosi et al. 2008). The presence of TRPV3 agonists significantly decreased both basal and induced lipid synthesis. N≥4 in each group, ***$p<0.001$ compared to the control and $^{###}p<0.001$ between the indicated groups as determined by One-way ANOVA and Bonferroni *post hoc* test.

Supplementary Figure S4



Supplementary Figure S4. Effect of TRPV3 agonist carvacrol on cellular differentiation of human sebocytes

a. SZ95 sebocytes were treated with vehicle, arachidonic acid (AA, 100 µM), carvacrol (500 µM), ruthenium red (RR, 10 µM) and combinations of the compounds as indicated in the figure for 48 hrs. Cells were stained with Nile red solution then harvested and subjected for flow cytometry as described in the Supplementary materials and methods. Fluorescence of Nile red staining and side scatter (SSC) were detected in parallel to assess cellular lipid content and granularity, respectively. AA induced a marked differentiation of the cells indicated by increased lipid accumulation and granulation of SZ95 cells. Carvacrol increased cellular lipid content only moderately, however it inhibited the AA induced cellular lipid accumulation. These effects of carvacrol were abolished by the TRPV3 blocker RR suggesting that activation of TRPV3 inhibits AA induced lipid

accumulation. In contrast, neither basal nor AA-induced granulation of sebocytes were influenced by carvacrol, suggesting that TRPV3 activation primarily affects lipid synthesis, and it has no effect on other processes of differentiation. b. Relative expression of peroxisome proliferator-activated receptor γ (PPARγ) and nuclear receptor interacting protein 1 (NRIP1) positively regulating sebaceous lipid synthesis during sebocytes' differentiation was determined by QPCR after treating cells with 500 μM carvacrol or vehicle for 24 hrs. Carvacrol markedly downregulated the expression of genes regulating lipid synthesis. N=3 independent determinations.