Details about cultivation of human immortalized keratinocytes

Human immortalized keratinocytes are cultivated in Keratinocyte SFM supplemented with rhEGF, BPE and 1 % v/v penicillin/streptomycin at 5 % CO_2 and 37 °C. Cells were split when they reached a confluency of 80 %. Passage 3 or 4 were used. Cells were plated in 20 Petri dishes (10 cm^2), 1 x 10^6 cells/ per dish. One part (10 dishes) was cultivated for 3 days in 10 mL of the above mentioned medium for adherence and served as control. Cells in the remaining 10 dishes were incubated with 1.95 µM betulin (10 µL of the 1.95 mM stock solution in DMSO) for 8 hrs prior to removal of the medium. The concentration of 1.95 µM of betulin was used, as this concentration has shown effects in our previous studies on the molecular wound healing effect [1]. In each case medium was removed on day 4 and cells were incubated with 3 ml of trypsin (0.05 %) at 37°C. After 5 min cells were washed by adding 7 mL medium. The suspension was transferred into 15 mL falcon tubes and centrifuged for 5 min at 4°C and 1.200 rpm, respectively. The supernatant was withdrawn and the remaining cell pellets were washed and then frozen at -20°C in the falcon tubes until use.

Lipid extraction

Extraction solvents were cooled down for at least 20 min on ice before their addition to samples. Lipid extraction was performed with IPA:H_2O (90:10 v/v) as described previously [2]. Briefly, a 5% (v/v) SPLASH Lipidomix solution was prepared in MeOH and then 50 µL of the diluted solution was added to the cell pellet. Next, 4.95 mL of IPA:H_2O (90:10 v/v) were added. Samples were vortexed (30 s), sonicated (2 min) and vortexed again (30 s) to disrupt the pellet. Incubation on ice was continued on a shaker for 1h (500 rpm, 60 min). Samples were centrifuged (3500 rcf, 10 min) and the supernatant was transferred to 15 mL falcon tubes. Samples were dried in an evaporator (Genevac EZ-2; Warminster, Pennsylvania, USA) for 10 hours under nitrogen protection. Dried extract was resuspended in 100 µL of methanol containing odd-chained lipid standards LPC 17:1 and PC 17:0-20:4 at 500 ng/mL and 125 ng/mL, respectively. Sonication (2 min) and vortexing (30 s) were applied to ensure that lipids were not stuck to the surface of the extraction container. Then, samples were centrifuged (3500 rcf, 10 min) and the supernatant was transferred to vials for LC-MS measurements. Quality control (QC) sample was prepared by pooling together 15 µL aliquot of each sample. Samples were measured randomly and QC samples were run at the beginning, at the end and every fifth sample during the sequence.

References

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