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Title: Two olfactory receptors -OR2A4/7 and OR51B5- differentially affect epidermal proliferation and differentiation

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Short title: OR2A4/7 and OR51B5 in human skin cells

Abbreviations: AMPKα1, AMP-activated protein kinase α1; Chk-2, checkpoint kinase 2; CNG, cyclic-nucleotide gated; CHS, cyclohexyl salicylate; HEK293, human embryonic kidney 293; Hsp27, heat shock protein 27; IL, interleukin; ISO, isononyl alcohol; MAPK, mitogen-activated protein kinase; NGS, Next Generation Sequencing analyses; OR, olfactory receptor; RT, reverse transcriptase; scRNA, scrambled RNA; siRNA, small interfering RNA

Abstract

Olfactory receptors (ORs), which belong to the G-protein coupled receptor family, are expressed in various human tissues, including skin. Cells in non-olfactory tissues tend to express more than one individual OR gene, but function and interaction of two or more ORs in the same cell type has only been marginally analyzed. Here, we revealed OR2A4/7 and OR51B5 as two new ORs in human skin cells and identified cyclohexyl salicylate and isononyl alcohol as agonists of these receptors. In cultured human keratinocytes, both odorants induce strong Ca^{2+} signals that are mediated by OR2A4/7 and OR51B5, as demonstrated by receptor knockdown experiments. Activation of both receptors induces a cAMP-dependent pathway. Localization studies and functional characterization of both receptors revealed several differences. OR2A4/7 is expressed in suprabasal keratinocytes and basal melanocytes of the epidermis and influences cytokinesis, cell proliferation, phosphorylation of AKT and Chk-2 and secretion of IL-1. In contrast, OR51B5 is exclusively expressed in suprabasal keratinocytes, supports cell migration and regeneration of keratinocyte monolayers, and influences Hsp27, AMPK1 and p38MAPK phosphorylation and interestingly, IL-6 secretion. These findings underline that different ORs perform diverse functions in cutaneous cells, and thus offering an approach for the modulated treatment of skin diseases and wound repair.

Key Words:

Keratinocytes

HaCaT

Ectopic expression

Wound healing

Proliferation

Introduction

Olfactory receptors (ORs), which belong to the G-protein coupled receptor family and offer a basis for odorant molecule detection, were first described as exclusively expressed in the olfactory epithelium (1). However, within the last two decades many studies revealed that ORs are also expressed in various non-olfactory tissues (2–5). Presently, the function of ectopically expressed ORs is still unclear, but an increasing number of studies identified physiological roles for ectopically expressed ORs in spermatozoa (6–8), prostate epithelial cells (9), enterochromaffin cells of the gut (10), tumor liver cells (11) and human skin cells (12).

The skin is the largest human organ and forms an effective barrier between organism and environment, preventing invasion of pathogens and fending off chemical and physical attacks as well as the unregulated loss of water and solutes (13–16). Moreover, keratinocytes, the major cell type of the epidermis, express a variety of sensory receptors that enable them to react to various environmental stimuli and process information in the skin (17–19). Remarkably, also a wide range of classic CNS neuroreceptors, which are usually necessary

for auditory and visual functions, could be found in the skin, such as opsin receptors and transient receptor potential (TRP) channels. They seems to play an important role for example in permeability barrier homeostasis, but their full function is also still under investigation (20–23).

Previously, we identified a novel sensory receptor in human keratinocytes, the OR2AT4, which can be activated by Sandalore and is involved in human keratinocyte proliferation and migration during wound healing (12).

Interestingly, specific ORs showed high expression in each tissue (2, 12). In contrast to olfactory sensory neurons, cells in non-olfactory tissues tend to express more than one individual OR gene (6, 9–12, 24–26). Nevertheless, the function and interaction of two or more ORs in the same cell type or tissue is only marginally analyzed to date (7, 10). Therefore, we investigated the role of OR2A4/7 and OR51B5 in human skin cells and found similarities but also opposite effects on proliferation and re-epithelialization.

Methods

Cell culture and transfection

Human epidermal keratinocytes from juvenile foreskin (single donor) were purchased from Promocell (Heidelberg, Germany) and cultured in keratinocyte growth medium plus keratinocyte supplements (Promocell, Heidelberg, Germany). HaCaT cells represent an appropriate model for human primary keratinocytes with respect to morphology and growth characteristics (27). Hana3A is a HEK293-derived cell line stably expressing RTP1L, RTP2, REEP1, and Ga_{olf}, which supports the heterologous expression of ORs. HaCaT, Hana3A, and HEK293 cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 100 units per ml of penicillin/streptomycin (Gibco/Invitrogen, Carlsbad, CA).

Calcium imaging experiments

HaCaT cells, HEK293 cells or human primary keratinocytes grown in 35-mm dishes were incubated for 30 minutes at 37 °C with Ringer's solution (pH 7.3) and 3 mM Fura-2-AM (Molecular Probes, Eugene, OR). Calcium imaging experiments were performed as described in Spehr et al. (2003). Details of blocker experiments are given as supplementary information online. For the Ca^{2+} imaging experiments, HEK293 cells were grown in 35-mm cell culture dishes (50% confluence) and transfected with pCI-OR2A4/7 (1 µg) or pcDNA3-OR51B5 (1 µg) and a cofactor mixture of plasmids containing RTP1, RTP2, and REEP1 (28) for 48 hours using a standard calcium-phosphate precipitation technique (29). Sham-transfected controls were only transfected with cofactor mixture.

Wound scratch assay

The regeneration of wounded monolayers of HaCaT cells and human primary keratinocytes was analyzed using an *in vitro* wound scratch assay as described before by Busse at al. (2013). The residual overgrowing gap of the migrating cells at 48 h was measured and expressed relative to the initial scratch area (0 h).

Skin organ cultures

Skin organ cultures were prepared from tissue samples derived from patients undergoing routine diagnostic and therapeutic procedures within the Department of Dermatology, University of Münster. All experiments were approved by the Ethical Committee of the University of Münster and performed as recently described (30). Details of experimental procedures are provided as supplementary information.

Statistical Analysis

The results are presented as the means±SEM, and n is the number of experiments/cells. The level of significance was set as *p=0.05, **p=0.01, ***p=0.001. Details of statistical analysis are provided as supplementary information.

For detail of materials and methods see the supplementary information.

Results

OR2A4/7 and OR51B5 are expressed in HaCaT cells, human primary keratinocytes and human skin. For initial detection of ectopically expressed ORs in skin cells, we performed Next Generation Sequencing analyses (NGS) of HaCaT cells (two setups) and compared it with the online available NGS-dataset from sun-exposed skin (GTEx study). In total, 11 ORs were detected with a FPKM value (=fragments per kilobase of exon per million fragments mapped; (31)) higher than 0.1 (Figure 1a). In this study, we concentrated on OR2A4/7 and OR51B5 for functional characterization and deorphanization because both ORs were expressed in both HaCaT cells and human skin (Figure 1a). Due to the high homology of OR2A4 and or OR2A7 (99%), the combined FPKM values are presented as OR2A4/7**.** In the beginning, also the OR51B4 seems to be of interest for our studies because of its expression in HaCaT cells and human skin and its FPKM values of >0.1. Nevertheless, deorphanization of this receptor was not possible during this study, but is subject of future investigations. The expression of OR2A4/7 and OR51B5 in HaCaT cells was validated by reverse transcriptase PCR (RT-PCR) analysis of HaCaT cDNA. Moreover, expression of both ORs was confirmed in keratinocytes by RT-PCR analysis (Figure 1b). To detect OR2A4/7 and OR51B5 receptor proteins, immunocytochemical stainings of cultured HaCaT cells, primary keratinocytes, and

skin specimens were performed using custom-made antibodies. Both HaCaT cells and primary keratinocytes exhibited obvious OR2A4/7 and OR51B5 receptor protein expression (Figure 1c). In normal skin specimens, OR2A4/7 was detected in suprabasal keratinocytes on the surface in a ring-shaped fashion and in basal melanocytes (Figure 1d; arrows e and f). OR51B5 staining was detectable in suprabasal keratinocytes (intracellular; Figure 1d arrow g) but not in the basal layer of the skin (Figure 1d arrow h). Control experiments were performed without the primary antibodies and no staining was detectable (Figure 1c, d). Moreover antibody specificity was validated by immunocytochemistry (Figure S1).

To identify specific ligands of OR2A4/7 and OR51B5, a mixture of 100 structurally different odorants (Henkel 100) was screened using the calcium imaging technique as previously described (32). Henkel 100 provoked a transient increase in intracellular Ca^{2+} in human embryonic kidney 293 (HEK293) cells transiently transfected with OR2A4/7 (Figure S2a) or OR51B5 plasmids (Figure S2d). Subdividing the odorant mixture revealed cyclohexyl salicylate (CHS; 500 µM) as an odorant ligand for OR2A4/7 (Figure S2b) and isononyl alcohol (ISO; 500 µM) for OR51B5 (Figure S2e). OR2A4/7 and OR51B5 transfected cells were significantly more responsive than mock-transfected cells to the odorants CHS (Figure S2c) and ISO (Figure S2f).

CHS and ISO evoke an increase of intracellular Ca^{2+} in HaCaT cells and human primary keratinocytes by activating the cAMP-adenylate cyclase-dependent signaling pathway. Previous studies of OR2AT4 in HaCaT cells and keratinocytes revealed that Sandalore induced Ca^{2+} signals were significantly sensitized on repetitive stimulation. Repetitive application (30 seconds) of CHS (500 µM; Figure 2a) and ISO (500 µM; Figure 2b) also evoked an increase of intracellular Ca^{2+} in HaCaT cells and primary keratinocytes. In

contrast to Sandalore, sensitization of HaCaT cells or primary keratinocytes was not detectable with either CHS (Figure 2c) or ISO (Figure 2d). Moreover, desensitization was detectable after CHS treatment in keratinocytes (Figure 2d). The signal transduction pathways activated by CHS or ISO were characterized pharmacologically in HaCaT cells. Calcium imaging experiments under EGTA buffered calcium-free extracellular conditions revealed that both the CHS- and ISO-evoked responses of HaCaT cells depended on extracellular calcium (Figure 2e). Moreover, stimulation of HaCaT cells in the presence of the adenylyl cyclase inhibitors SQ-22536 (100 µM; (33)) or MDL-12.330A (40 µM; (34)) showed significantly diminished CHS- and ISO-induced Ca^{2+} signals (Figure 2e). Next, we investigated the contribution of CNG channels (35, 36), which are responsible for Ca^{2+} influx in the cAMP-dependent signaling pathway in olfactory cells. In the presence of the CNG channel inhibitor *L-cis*-Diltiazem (100 µM), CHS- and ISO-induced Ca^{2+} increases were completely abolished (Figure 2e). Furthermore, signaling components classically involved in the olfactory signaling pathway, such as Ga_{olf}, adenylyl cyclase 3, as well as the CNG channel subunit CNGA1, were detected via qRT-PCR and immunocytochemical experiments in HaCaT cells and human primary keratinocytes (Figure S3). The blocker experiments suggested an induction of the cAMP-dependent pathway after CHS and ISO treatment. Thus, we performed a cAMP assay to confirm these changes in intracellular cAMP levels after CHS or ISO stimulation. The results demonstrated that the application of both CHS and ISO significantly increased the cAMP level in HaCaT cells in a sigmoidal dose-dependent manner (Figure 2f and 2g).

OR2A4/7 and OR51B5 are essential for CHS- and ISO-induced Ca^{2+} influx in HaCaT cells. To confirm the importance of OR2A4/7 to the CHS-induced and OR51B5 to the ISO-induced Ca^{2+} increase in HaCaT cells, we reduced the endogenous expression level of both receptors using small interfering RNAs (siRNA). Previously, the siRNA functionality was investigated by either a pmirGLO assay (Figure S4a; for OR2A4/7) or immunocytochemical staining (Figure S4 b; for OR51B5). In calcium imaging experiments, we treated HaCaT cells transfected (48 hours) with siRNA or scrambled RNA (scRNA) against OR2A4/7 (Figure 3a) or OR51B5 (Figure 3c) with CHS or ISO for 30 s. For both receptors, the OR-specific siRNA knockdown significantly decreased Ca^{2+} influx (Figure 3b, d). In contrast to scRNA-transfected cells, HaCaT cells transfected with OR2A4/7-siRNA revealed a 48% decreased amplitude signal. Cells transfected with OR51B5-siRNA exhibited a decreased amplitude signal of 51% (Figure 3d). These results confirm the involvement of OR2A4/7 in the CHS-induced Ca^{2+} increase and OR51B5 in the ISO-induced Ca^{2+} increase in HaCaT cells.

Previous studies in HeLa cells indicated that some GPCRs are involved in cytokinesis. RNAi knockdown of these receptors, including OR2A4/7, increased binucleated HeLa cell formation (3). We therefore investigated whether RNAi knockdown of OR2A4/7 in HaCaT cells also influences cytokinesis. Interestingly, OR2A4/7 knockdown in HaCaT cells increased bi- and multinucleated cell formation (Figure 3e). Quantification of multinucleated cell formation in HaCaT cells transfected with either OR2A4/7-siRNA or OR2A4/7-scRNA revealed that siRNA transfection enhanced incomplete cell separation. Therefore, 54% of cell bodies were multinucleated compared to 2% multinucleated cells in scRNA-transfected cells (Figure 3f).

CHS and ISO influence the physiological functions of HaCaT cells and human primary keratinocytes in different ways. To examine and compare the physiological functions of OR2A4/7 and OR51B5 in HaCaT cells and human primary keratinocytes, we first investigated the effect of both odorants on cell viability using the caspase3/7-glo assay. However, there was no enhancement of apoptosis after treatment of HaCaT cells and primary keratinocytes with either CHS (100 µM; for long-term stimulation higher concentration of CHS seems to elicit unspecific side effects) or ISO (500 µM) for 2 days (Figure 4a). Next, we analyzed the influence of both odorants on cell proliferation using the CyQUANT cell proliferation assay. In contrast, CHS significantly increased cell proliferation of HaCaT cells and primary keratinocytes, whereas ISO stimulation had no impact on cell proliferation (Figure 4b). Because proliferation and cell migration are essential actors in epithelial wound healing (37–40) and Sandalore-activated OR2AT4 leads to increased wound healing of keratinocytes (12), we further investigated whether CHS and ISO stimulation also influence "wound closure" in HaCaT cell and human primary keratinocyte monolayers using an *in vitro* wound scratch assay. Interestingly, CHS treatment (100 µM; 48 hour) of HaCaT cells or human primary keratinocytes did not influence wound healing within 48 hours. After 48 hours, the percentage of the initial wound in CHS-treated HaCaT cells was higher than in 0.1% DMSO-stimulated control cells (Figure 4c). In contrast, exposure to ISO (500 µM; 48 hours) significantly accelerated the regeneration rate of the HaCaT cell and keratinocyte monolayers compared with 0.1% DMSO-stimulated control cells (Figure 4c). In the next step, we assessed the impact of CHS and ISO on human skin organ explants as an *ex vivo* model of epidermal wound healing. We measured the length and size of the epidermal tongues in full-thickness skin explants after treatment with CHS (100 µM), ISO (500 µM) and 0.1% DMSO (control) for 5 days. Remarkably, ISO but not CHS significantly increased the length and area of epidermal tongues (Figure 4d).

Cytokine release and kinase phosphorylation are essential modulators of many cellular processes, including cell proliferation and wound healing (41–44). To determine the signaling factors and pathways that mediate the induction of proliferation and wound healing in HaCaT cells and human primary keratinocytes, we used a Multi-Analyte ELISA array kit and a human Phospho-Kinase Array, respectively. The ELISA measured 12 cytokines in the supernatant of CHS- or ISO-treated HaCaT cells and human primary keratinocytes. CHS treatment led to slight increases of IL-2 and IL-13 in the supernatant of HaCaT cells, IL-1α and GM-CSF in the supernatant of keratinocytes, and IL-6 in the supernatant of both cell types compared to controls (Figure 4e). Interestingly, cytokine release in the supernatants of HaCaT cells and primary keratinocytes was more obvious and the number of released cytokines was higher after treatment with ISO (Figure 4e). In sum, ISO strongly increased IL-2, IL-5, IL-6 and IL-13 in the supernatant of HaCaT cells and IL-6 and IL-12 in that of primary keratinocytes (Figure 4e). Next to cytokines, we analyzed kinase phosphorylation with the help of the human Phospho-Kinase Array kit, which detects the phosphorylation levels of 43 kinase phosphorylation sites that are essential regulators in cellular processes. Taken together, 20 phosphorylation sites were regulated by either CHS or ISO treatment in HaCaT cells (Figure 4f). ISO treatment of HaCaT cells strongly upregulated p38, AMPKa1, EGFR, CREB and Hsp27 and strongly downregulated Hsp60, STAT3, p70S6 kinase and AKT phosphorylation (Figure 4f). In contrast, CHS treatment also leads to up- and downregulation of protein phosphorylation, but less abundantly than ISO. The strongest upregulation of phosphorylation after CHS treatment was detectable for the proteins Hsp60 and AKT, and strongest downregulation was observed for the proteins Chk-2 and Yes (Figure 4f).

Discussion

In the last few years, more and more ectopically expressed neuroreceptors, such as visual and auditory receptors, could be identified, also in the skin. Surely, they are no avatism because several functions of these receptors could be identified, for example regulation of barrier homeostasis. Nevertheless, the broad function spectrum of these ectopic receptors is only marginally analyzed to date (20–23). According to these results, also more ectopically expressed human ORs have been identified, and the functionality of ORs in non-olfactory cell types has become better understood (10–12, 12, 24, 26). Nevertheless, the function and interaction of two or more ORs in the same cell type or tissue has rarely been investigated (7, 10). Several ORs were identified in skin cells (12), but also in these cells the function of all ORs and the interaction between two or more ORs remain unknown.

In the present study, we identified the expression of 11 ORs in HaCaT cells and in human skin via NGS analyses. Because OR2A4/7 and OR51B5 were expressed in HaCaT cells and in human skin, we concentrated on these ORs for deorphanization and functional characterization. The expression of both receptors was validated by RT-PCR and at the protein level by immunocytochemistry. Both receptors were detectable in primary human keratinocytes, HaCaT cells, and healthy human skin biopsies. Interestingly, immunocytochemical stainings of the skin revealed the first common features and differences of OR2A4/7 and OR51B5. Both receptors are expressed in suprabasal keratinocytes of the skin but only OR2A4/7 is detectable in the basal layer of the skin.

Deorphanization studies using recombinant OR2A4/7 or OR51B5 identified receptor ligands. As part of this, CHS was identified as specific activator of OR2A4/7. The odorant CHS is an ester of salicylic acid. It emits a strong, sweet-flower aroma, is mostly stable and substantive on hair, fabric and skin, and it is present in hygiene products such as soap (http://chemical.kao.com/global/products/B0010576_glen.html). ISO was identified as specific activator of OR51B5. It is a clear liquid and a member of the branched chain saturated alcohols. It is often used as an ingredient in cosmetics, deodorants and perfumes (45). Because skin comes into frequent contact with these odorants, it is necessary to identify their impact on our skin.

CHS and ISO, the agonists of OR2A4/7 and OR51B5, respectively also induced Ca^{2+} signals in HaCaT cells and human primary keratinocytes. In contrast to Sandalore, neither CHS nor ISO sensitized HaCaT cells or primary keratinocytes (12). The activation of OR2A4/7 and OR51B5 by CHS and ISO was verified by siRNA experiments, which significantly reduced CHS- and ISO-induced Ca^{2+} signals in HaCaT cells.

Additionally, siRNA knockdown experiments of OR2A4/7 in HaCaT cells revealed an increase of binucleated cells, which is a general readout for cytokinesis failure (46, 47). Previous studies of HeLa cells claim that GPCRs, including OR2A4/7, participate in cytokine signaling (3). Our results underline this statement and uncover a new function of OR2A4/7 in keratinocytes.

CHS and ISO stimulation increased cAMP levels in HaCaT cells in a concentration-dependent manner, hinting that CHS and ISO induce a cAMP-dependent signaling cascade in skin cells, similar to Sandalore (12). Using different inhibitors of components participating in the cAMP-dependent signaling cascade (33, 34), we underlined that the CHS- and ISO-evoked intracellular Ca^{2+} increases are mediated via a cAMP-dependent signaling pathway. The adenylyl cyclase inhibitors MDL-12330A and SQ-22536 and the CNG channel inhibitor *L-cis*-Dilitazem significantly blocked the Ca^{2+} increase of both odorants, but in ISO-stimulated HaCaT cells the block was more effective. This difference between OR2A4/7 and OR51B5 underlines that different ORs in the same cell type have overlapping and different functions and use different signaling cascades. In line with these results, HaCaT cells and keratinocytes express CNG subunits (12, 48). We also detected several classical olfactory signaling components, including $G\alpha_{olf}$, adenylyl cyclase 3 and CNGA1, via RT-PCR and immunocytochemical staining of HaCaT cells and primary keratinocytes.

Cell survival, proliferation and migration are essential actors in epithelial wound healing (37–40). Moreover, Sandalore-activated OR2AT4 leads to increased phosphorylation of extracellular signal-regulated kinases (ERK1/2) and p38 mitogen-activated protein kinases (p38MAPK), thereby affecting cell proliferation, migration, and wound healing (12). Therefore, the question arises of which physiological functions are carried out by OR2A4/7 and OR51B5 in skin cells. Interestingly, stimulation of HaCaT cells and human primary keratinocytes with both odorants had no impact on cellular survival, but proliferation of both cell types was significantly increased after CHS treatment. In line with these results, CHS stimulation of HaCaT cells results in increased AKT phosphorylation and decreased Chk-2 phosphorylation. AKT mediates the proliferation of several cell types. One mechanism is by suppressing Chk2-mediated G2 cell cycle arrest (49, 50). Appropriately, CHS treatment also

increases the amount of IL-1α in keratinocyte supernatant. IL-1 is a cytokine that is released as a first step of hyperproliferative keratinocytes in wound healing processes (12, 42, 51). The amount of IL-2 and IL-13 in CHS-treated HaCaT supernatant and GMC-CSF in supernatant of CHS-treated keratinocyte was also elevated. The hypothesized function of these cytokines in skin cells ranges from proliferation to growth promotion (52, 53) and should be analyzed in detail after CHS-treatment. The OR2A4/7 siRNA knockdown experiments in HaCaT cells revealed a cell cycle defect. Proliferation tests, ELISA and kinase array indicated that OR2A4/7 and CHS play important roles in the proliferation of HaCaT cells and keratinocytes.

In contrast, ISO stimulation of HaCaT cells and keratinocytes had no impact on cell proliferation. Instead, ISO treatment significantly increased the regeneration rate of HaCaT cell and primary keratinocyte monolayers in an *in vitro* scratch assay and supported epidermal wound healing in organ skin cultures. Interestingly, ISO treatment also increased p38MAPK, Hsp27 and AMPKα1 phosphorylation and decreased AKT phosphorylation. The p38MAPK pathway is required for human keratinocyte migration (43, 54, 55). Hsp27 phosphorylation is involved in epithelial migration during corneal epithelial wound healing (56). AMPKα1 is involved in several migration processes by activating p38MAPK (57). Moreover, downregulation of AKT phosphorylation could explain the reduced proliferation of HaCaT cells and keratinocytes after ISO treatment (58).

ISO stimulation also induced the upregulation of cytokines in supernatant, particularly IL-2, IL-4, IL-5, IL-6 and IL-13 in HaCaT cell supernatant and IL-6 and IL-12 in keratinocyte supernatant. IL-6 secretion by keratinocytes promotes keratinocyte migration (59). IL-2, IL-4

and IL-13 influence keratinocytes but are not secreted by keratinocytes themselves (53). A connection between IL-12 or IL-5 and keratinocytes has previously not been shown. Therefore, the influence of ISO treatment on cytokine release from keratinocytes should be analyzed in more detail. In sum, the scratch assay, ELISA and kinase array revealed that OR51B5 and ISO play important roles in HaCaT and keratinocyte migration, thereby influencing wound healing.

In vivo, basal epidermal keratinocytes are responsible for natural wound healing (60). Interestingly, immunostainings of human skin sections showed an exclusive expression of OR51B5 in suprabasal keratinocytes, whereas OR2A4/7 is expressed in suprabasal keratinocytes and basal melanocytes. Interestingly, melanocytes are proposed to be key sensory cells of the human skin (61–63). Therefore, it would be interesting to investigate whether OR2A4/7 stimulation of different skin cells, including melanocytes, has the same effect. Most importantly, with our findings that epidermal homeostasis and repair is differently affected by OR2A4/7 and OR51B5, we showed for the first time that keratinocyte behavior could be differentially modulated by the activation of different ORs. This could be a good approach for the modulated treatment of skin diseases and wound repair. Future experiments with organotypic cultures or transgenic mice of both ORs could be helpful to understand OR function in the skin.

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Authorship

T.Tsai, S.Veitinger, I.Peek, D.Busse, J.Eckhardt, D.Vladimirova, N. Jovancevic, S.Wojick, J.Altmüller, and J.Cheret performed the research.

T.Tsai, G.Gisselmann, and H.Hatt designend research study.

S. Ständer, T. Luger, R.Paus and H. Hatt contributed essential reagents or tools.

T.Tsai, S.Veitinger and G.Gisselmann analyzed the data.

T. Tsai wrote the paper.

Conflict of Interest

The authors state no conflict of interest.

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Legends for illustration

Figure 1: OR2A4/7 and OR51B5 are expressed in HaCaT cells, human keratinocytes and human skin. (a) NGS data of HaCaT cells (n=2 cultures) and sun-exposed skin (= GTEx study) revealed 11 ORs expressed higher than FPKM 0.1. (b) Representative RT-PCR analysis of OR2A4/7 and OR51B5 confirmed expression of both receptors in HaCaT cells and primary keratinocytes. (+) cDNA; (-) mRNA; (M) marker. Numbers, length of fragments (bp). (c) Immunocytochemical staining of HaCaT cells and primary keratinocytes with α-OR2A4/7 or α-OR51B5 antibody (red). DAPI (blue) was used to visualize cell nuclei. Bar=20 µm. (d) Immunohistochemical staining of human healthy skin specimen with α-OR2A4/7 and α-OR51B5 antibody (green). Bar=50 µm; e: keratinocyte (ring shaped); f: basal melanocyte; g: keratinocyte (intracellular); h: basal layer (negative). Control stainings were performed without primary antibodies.

Figure 2: CHS and ISO induce Ca^{2+}-signals in HaCaT cells and primary keratinocytes that depend on cAMP-signaling cascade. Representative calcium imaging traces of repetitively (30 s) stimulated HaCaT cells and keratinocytes with 500 µM CHS (a) and 500 µM ISO (b). (c and d) Mean amplitude level of CHS- or ISO-induced Ca^{2+} signals in HaCaT cells (CHS: n=150 cells; ISO: n=19 cells) and keratinocytes (CHS: n=41 cells; ISO: n=28 cells). (e) Quantification of CHS- and ISO-induced Ca^{2+} signals of HaCaT cells in blocker measurements relative to control measurements with only CHS (500 µM; n=150 cells) or ISO (500 µM; n=258 cells) revealed that Ca^{2+} free extracellular solution (CHS+EGTA, n=260 cells; ISO+EGTA, n=360 cells), adenylyl cyclase inhibitor SQ-22536 (100 nM; CHS+SQ, n=216 cells; ISO+SQ, n=316 cells) and MDL-12.330A (40 µM; CHS+MDL, n=50 cells; ISO+MDL, n=750 cells) and CNG channel blocker *L-cis*-Diltiazem (100 µM; CHS+*L-cis,* n=110 cells; ISO+ *L-cis,* n=50 cells) significantly reduced the CHS- and ISO-induced Ca^{2+} signaling. (f) The influence of several CHS concentrations (0.01mM; 0.01 mM; 0.1 mM; 1 mM; 5 mM; 10mM; 100mM) on cAMP release were tested and underline that CHS induced a dose-dependent and significant increase of cAMP in HaCaT cells compared to 0.1% DMSO (control). cAMP levels were normalized were normalized against forskolin (10 µM: triplet; n =3 experiments). (g) The influence of several ISO concentrations (0.01mM; 0.01 mM; 0.05mM; 0.1 mM; 0.5mM; 1 mM; 5 mM; 10mM; 100mM) on cAMP release were tested and underline that CHS induced a dose-dependent and significant increase of cAMP in HaCaT cells compared to 0.1% DMSO (control). cAMP levels were normalized were normalized against forskolin (10 µM; triplet; n =3 experiments). ISO induced a dose-dependent and significant increase of cAMP in HaCaT cells compared to 0.1% DMSO (control); cAMP levels were normalized were normalized against forskolin (10 µM: triplet; n =3 experiments). Data are shown as the means±SEM. *p<0.05; **p<0.01; ***p<0.001.

Figure 3 OR2A4/7 and OR51B5 are responsible for Ca^{2+} signals in HaCaT cells by CHS and ISO treatment. CHS (a) and ISO (c) induced Ca^{2+} signals in OR2A4/7-siRNA or OR51B5-siRNA (gray line) and OR2A4/7-scRNA or OR51B5-scRNA (black line) transfected HaCaT cells. Bars indicate the duration of stimulation. (b and d) Quantification of relative Ca^{2+} signal amplitudes of OR2A4/7-siRNA (n=18 cells) and OR51B5-siRNA (n=28 cells) as well as OR2A4/7-scRNA (n=16 cells) and OR51B5-scRNA (n=26 cells) transfected HaCaT cells, demonstrating a 48% decreased amplitude in OR2A4/7-siRNA and a 51% decreased amplitude in OR51B5-siRNA transfected cells (black bars). (e) Knockdown of OR2A4/7 in HaCaT cells causes multinucleated cell formation. SiRNA- and scRNA-transfected cells were labeled by GFP-coexpression, and cell nuclei were visualized by DAPI (blue). Scale bars: 20 µm. (f) Quantification of multinucleated HaCaT cells in OR2A4/7-RNAi knockdown experiment. (n=120 transfected cells). Error bars represent the means±SEM. $p^*<0.05$.

Figure 4: CHS increased HaCaT and primary keratinocyte proliferation, whereas ISO influenced cell migration and wound healing. (a) Caspase-Glo® 3/7 assay of long-term stimulated (2 days) HaCaT cells and primary keratinocytes with 100 µM CHS and 500 µM ISO revealed no influence on apoptosis relative to control (0.1% DMSO). n=3 experiments and triplet per experiment. (b) Treatment of CHS (2 days; 100 µM) instead of ISO treatment (2 days, 500 µM) resulted in significantly increased proliferation relative to control (0.1% DMSO). n=3 experiments and triplet per experiment. (c) Representative images and quantification after 48 h of wound scratch assay of HaCaT and primary keratinocytes in the presence of 100 µM CHS and 500 µM ISO; n=3 experiments and 6 pictures per experiment. (d) Skin explants were treated with CHS (100 µM), ISO (500 µM) or control (0.1% DMSO) for 5 days. The length and area of the migratory epidermal tongues

of the edges of the explants were evaluated after fixation and hematoxylin eosin staining; n=2 explants and 6 explant areas were analyzed. (e) HaCaT cells and human primary keratinocytes were stimulated for 2 days with CHS (100 µM), ISO (500 µM) or 0.1% DMSO (control). Subsequently, supernatants were collected from all probes and analyzed via a human common cytokine ELISA kit. Optic density of every probe was measured at 450 nm and presented as relative light units to control (1; dotted line). n=1 experiment. (f) HaCaT cells were cultivated for 24 hours and then treated with CHS (100 µM), ISO (500 µM) or DMSO (0.1%, control) for 5 min at 37 °C. Afterwards, cell lysates were analyzed by a human Phospho-Kinase Array. Semi-quantitative analysis of the spots was measured by densitometry, and the mean (n=2 spots) is presented in the graphs as the phosphorylation relative to the control (1; dotted line). Regulation was defined as twofold upregulation of phosphorylation or halved phosphorylation in comparison to the control. Error bars represent the mean ±SEM. p*<0.05; p**<0.01.

a

OR	HaCaT (first setup)	HaCaT (second setup)	Sun-exposed skin (GTEx study)
OR1F1	0.5	0.3	-
OR2A4/7	0.4	0.3	0.17
OR2B6	0.6	-	0.13
OR2W3	0.6	-	0.058
OR4C6	1.5	1.1	-
OR7D2	0.2	0.4	0.085
OR10A3	0.3	-	0.008
OR10A6	0.3	-	0.056
OR13A1	-	0.2	0.12
OR51B4	0.3	0.3	0.17
OR51B5	0.5	1.2	0.17