

Opioid receptor delta as a global modulator of skin differentiation and barrier function repair

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Synopsis

OBJECTIVES: The aims of this study were to confirm the properties of selective agonist peptide (Rubixyl) contained in the spinach towards opioid receptor delta. In fact, agonist properties of both spinach peptides (Rubiscolin-5 and Rubixyl) towards opioid receptor delta were demonstrated by Zang *et al.*, but their effects on the other opioid receptors were not studied [1]. We also studied the expression of opioid receptor delta in epidermis under normal and stress condition (inflammatory) and its role in epidermis homeostasis under stress condition *in vitro* and *in vivo*.

METHODS: Agonist properties studies were performed using functional agonist cellular model containing human opioid receptors.

Opioid receptor delta expression and epidermis homeostasis were studied on human reconstructed epidermis under normal and stress conditions (inflammatory stress) using gene expression (RT-qPCR) and protein expression analysis (immunohistological analysis).

Skin repair properties of opioid receptor delta agonist were based on the following parameters TEWL (trans epidermal water loss, hydration and wrinkle depth at periocular and perilabial area) on human volunteers having either intrinsic ageing (more than 40 years old and non-smoker group) and both intrinsic ageing and extrinsic ageing (more than 40 years old and smoker group).

RESULTS: We have demonstrated that the Rubixyl peptide is a specific agonist of opioid receptor delta. We have demonstrated that opioid receptor delta expression is modulated under inflammatory condition. The agonist Rubixyl was able to block the depletion of opioid receptor delta seen under inflammatory condition in reconstructed human epidermis.

Inflammatory conditions lead to the unbalanced gene and protein expressions of markers involved in epidermis integrity and barrier function properties. The treatment of human reconstructed epidermis with the agonist Rubixyl leads to the normalization of unbalanced gene and protein expressions. *In vivo* study has confirmed the efficiency of the agonist Rubixyl to repair damaged skin by decreasing TEWL, increasing hydration and decreasing wrinkle depth at the periocular and perilabial area.

CONCLUSION: In this research, we have demonstrated *in vitro* (on inflamed reconstructed human epidermis, RHE) and *in vivo* (on human aged volunteers) that activation by natural agonist peptide of opioid receptor delta reduces the skin inflammation thus leading to improvement in epidermis differentiation and skin barrier properties.

Résumé

OBJECTIFS: Les objectifs de notre étude sont de confirmer pour le peptide Rubixyl son caractère d'agoniste spécifique au récepteur delta opioïde. La propriété d'agoniste du peptide Rubixyl a été démontrée par Zang *et al.* pour le récepteur delta uniquement [1]. Nous avons également étudié l'expression du récepteur delta dans l'épiderme humain reconstruit sous condition normale et stressée (inflammation induite) *in vitro* et sur des volontaires sains.

MÉTHODES: L'étude des propriétés agonistes a été réalisée à l'aide de modèles cellulaires fonctionnels exprimant les récepteurs opioïdes humains.

L'étude de l'expression du récepteur delta opioïde ainsi que celle de l'homéostasie épidermique ont été réalisées à partir d'épidermes humains reconstruits sous condition normale et stressée (inflammation induite) à l'aide de la technologie RT-qPCR (analyse de l'expression génique) et de l'immunohistologie (analyse de l'expression protéique).

L'étude des propriétés de réparation cutanée a été basée sur les paramètres suivants: TEWL, hydratation et profondeur des rides en région péri-buccale et péri-oculaire. Les volontaires sains sélectionnés avaient soit un vieillissement intrinsèque (plus de 40 ans et non fumeurs) soit un vieillissement extrinsèque (plus de 40 ans et fumeurs).

RÉSULTATS: Nous avons démontré que le peptide Rubixyl est un agoniste spécifique au récepteur delta opioïde, que l'expression du récepteur delta opioïde est modulée en condition inflammatoire. Le peptide Rubixyl est capable de bloquer la déplétion du récepteur delta opioïde en condition inflammatoire. Le stress inflammatoire conduit au dérèglement de l'expression des gènes et des protéines impliqués dans le maintien de l'intégrité épidermique et de la fonction barrière de l'épiderme. Le traitement des épidermes reconstruits avec le peptide Rubixyl permet la régulation de l'expression des gènes et des protéines dérégulés. L'étude sur volontaires sains a confirmé l'efficacité du peptide Rubixyl à réparer la peau endommagée en permettant une diminution du TEWL, une augmentation de l'hydratation et une diminution des rides en région péri-buccale et péri-oculaire.

CONCLUSION: Dans ces travaux, nous avons démontré *in vitro* sur un modèle d'épiderme reconstruit inflammatoire et *in vivo* (sur des volontaires âgés) que l'activation par un agoniste naturel du récepteur delta opioïde contribue à la diminution de l'inflammation de la peau ce qui permet une amélioration de la différenciation épidermique et de la fonction barrière.

Introduction

During the last 15 years, several experimental evidence have suggested skin physiology is regulated through the interaction of the

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nervous, immune, cutaneous, endocrine (NICE) systems [2]. In order to respond to the nervous system, the skin expresses several receptors, including opioids receptors [3]. These receptors are G protein-coupled receptors (GPCR) mediating the effects of opioid ligands either of endogenous or exogenous origin. Natural endogenous known ligands for opioid receptor delta, Mu-opioid receptors and kappa-opioid receptors are enkephalins, beta-endorphins and dynorphins, respectively [4]. Natural exogenous agonist peptides with opioid activity were found in pepsin hydrolysates of wheat gluten (Gliadorphin), milk casein (Caseomorphin) or spinach (Rubiscolin-5 and Rubiscolin-6 or Rubixyl) [1, 5].

Recent studies suggest that opioid receptors are not only implicated in pain control, but also in the maintenance of skin cells homeostasis [6, 7]. These studies have revealed an important role in human epidermis of opioid receptor delta in the control of cell differentiation, migration and cytokine expressions [6]. Based on the studies from Bigliardi *et al.* [6, 7] (showing modulation of cytokine expression and significant atrophy of the epidermis of knock-out mice for opioid receptor delta), we hypothesized that opioid receptor delta is a key biological targets for repair of damaged skin and for the control of skin ageing. These both kinds of skins are known to have a micro-inflammation environment with poor skin barrier properties and a thin epidermis.

In our studies, we have assessed the specific agonist properties of spinach peptides (Rubiscolin-5 and Rubixyl) towards opioid receptor delta. Then, to confirm the key role of opioid receptor delta in maintenance of epidermis homeostasis, we have performed expression studies of proteins and genes on inflammatory human reconstructed epidermis. These studies were focused on specific differentiation epidermis and barrier function markers.

To demonstrate the interest of the peptide in anti-ageing strategies and precisely anti-inflammaging strategies, we assessed the Rubixyl peptide on aged human volunteers' skin (including smoker volunteers with a higher level of stressed skins). The parameters studied were wrinkle's depth, skin smoothness and TEWL (transepidermal water loss).

Materials and methods

Peptides

The peptides used in this study were Rubiscolin-5 and Rubiscolin-6 (also called Rubixyl) with the respective following sequences YPLDL (5 amino acids) and YPLDLF (6 amino acids). These peptides are found in a large subunit of spinach D-ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco). Peptides with purity higher than 95% were obtained from Senn Chemicals (Dielsdorf, Switzerland). Both peptides were used at the following concentrations for agonists assay: 1.10^{-7} , 3.10^{-7} , 1.10^{-6} , 3.10^{-6} and 1.10^{-5} M.

Opioid receptors agonist assays

Agonist assays were performed by Cerep Company (Poitiers, France).

Agonist properties to OPRD2 (opioid receptor delta 2) of Rubiscolin-5 and Rubixyl peptides were assessed in cellular functional assays. OPRD agonist assay was performed with NG-10815 rat cells expressing endogenously delta-2 receptor using the CellKey[®] System detection method (Molecular Devices, LLC) [8]. The CellKey[®] measures the impedance of the monolayer which depends on the cells' shape and correlates with cytoskeleton reorganization

after agonist stimulation. Agonist properties to OPRD2 of Rubiscolin-5 and Rubixyl peptides were determined from concentration–response curve. Concentration–response curves were generated using mean triplicate values using Hill equation curve fitting. The results are expressed as a per cent of control agonist response [(measured response/control agonist response) × 100] obtained in the presence of the test compounds. A compound was considered agonist if it induced at least 25% agonist-like effect at the concentration tested. Agonist properties to OPRM (opioid receptor MU) [9] and OPRK (opioid receptor kappa) [10] of Rubixyl peptide at 4 μM were assessed in cellular assays (CHO recombinant cells from rat or human). The experiment was conducted in duplicate. DPDPE (at 0.3 or 1 μM, reference, H-2905, Bachem, Switzerland), DAMGO (at 0.3 μM, reference H2535, Bachem, Switzerland) and U50488 (at 1 μM, reference D8040, Sigma, France) were used, respectively, as control agonists for OPRD2, OPRM and OPRK.

The results are expressed as a percentage of control agonist response [(measured response/control response)*100]. The agonist response for OPRD2, OPRM and OPRK was determined following adenylyl cyclase activity [10].

Cell and tissue cultures

All cell and tissue studies (culture, immunohistochemistry and gene expression analysis) were performed by Bioalternatives (Gençay, France).

Cell cultures: NG-10815 rat cells were seeded onto 96-well plate coated with fibronectin at 2×10^5 cells/well in Hank's balanced salt solution (HBSS Gibco[®], life technologies TM, Saint-Aubin, France) and 20 mM HEPES (life technologies TM, France) with 0.1% bovine serum albumin (Sigma-Aldrich[®], Saint Quentin Fallavier, France) and allowed to equilibrate for min at 37°C before the start of the experiment. Solutions containing different agonist concentrations were added simultaneously to all wells using an integrated fluidics system. All experimental conditions were performed in triplicate.

Reconstructed human epidermises (RHE) preparation

RHE was prepared by Bioalternatives (Gençay, France) as previously described [11].

Normal human epidermal keratinocytes (NHEK) were isolated from surgical samples of healthy foreskin from male children admitted for circumcision as previously described [12]. Foreskin tissues were obtained from the departments of paediatric surgery (University-Hospital of Poitiers). NHEK were cultured in keratinocyte-SFM (serum-free medium) with 0.25 ng mL⁻¹ epidermal growth factor, 25 μg mL⁻¹ pituitary extract and 25 μg mL⁻¹ gentamicin at 37°C in 5% CO₂. Briefly, suspension of cultured keratinocytes was cultivated on polycarbonate culture inserts (Millipore, Molsheim, France), then transferred at the air–liquid interface (day 0: DO) in Epilife[®] medium supplemented with 1.5 mM calcium chloride (life technologies TM, France) and 50 μg mL⁻¹ ascorbic acid (life technologies TM, France). The medium was refreshed every 2 or 3 days. For the experiments, RHE of 10 days old (D10) were used.

Reconstructed human epidermises (RHE) cultures for gene expression analysis

The RHE were cultured in assay medium containing or not the Rubixyl peptide (YPLDLF) and pre-incubated for 24 h. Epidermises were then stimulated or not (non-stimulated control) with a cytokine mix (IL-17+ OSM + TNF-α) at 3 ng mL⁻¹, and the epidermises were incubated for 24 h. The cytokine mix is known to induce a strong

inflammatory profile and a psoriatic phenotype in keratinocytes [13]. All experimental conditions were performed in duplicate.

Reconstructed human epidermises (RHE) cultures for protein expression analysis

For normal conditions (non-inflammatory): RHE were incubated in assay medium containing Rubixyl peptide (YPLDLF) or the reference JAK inhibitor I at 10 μM (Reference 420099-500UG, Millipore, Molsheim, France) or not (control non-treated) for 72 h with a renewal treatment after 24 h.

For inflammatory conditions: RHE were pre-cultured for 6 h in assay medium (Bioalternatives, Gençay, France) containing or not (control), Rubixyl peptide or the reference (JAK inhibitor I at 10 μM). RHE were then treated for 48 h with a cytokine mix (for convenience, the cytokine mix is named CTK) containing IL-17, Oncostatin M and TNF-alpha at 3 ng mL^{-1} (all from R&D Systems[®], Minneapolis, MN, U.S.A.).

At the end of incubation for basal and stimulated conditions, the supernatants were collected and the RHE were washed in phosphate-buffered saline solution (PBS) for *in situ* immunolabelling. All experimental conditions were performed in triplicate.

Normal human skin explants were healthy foreskins obtained from the departments of paediatric surgery (University-Hospital of Poitiers) with approval of the relevant ethical committee.

Immunohistochemistry, image analysis and data management

To investigate opioid receptor delta expression and expression of corneodesmosin, involucrin, keratin 10 and occludin at the protein level, immunohistochemistry was performed on RHE treated by cytokines (inflammatory conditions) with or without the peptide or not (normal conditions).

For OPRD1 protein expression analysis

RHE were washed with PBS and formaldehyde-fixed and embedded in paraffin for immunohistochemistry analysis. The transversal sections were carried out using a microtome (5 μm thickness). Labelling was performed after removing paraffin and retaining antigenic sites using a Target retrieval solution pH 6 (Dako[®], Les Ulis, France S1700). After a short incubation with 3% hydrogen peroxide (Sigma-Aldrich[®], Saint Quentin Fallavier, France) and a wash, an incubation with primary antibody (anti-OPRD1 at 1/250, reference LS-A222, Clinisciences, LSBio LifeSpan Biosciences, Seattle, WA, U.S.A.) for 60 min followed. Then, labelling was revealed by secondary biotin-labelled antibody and peroxidase-conjugated streptavidine addition (Vector VECTASTAIN Universal Quick Kit, R.T.U., reference PK-7800, Vector Laboratories, Burlingame CA, USA), together with the peroxidase substrate (Dako AEC Substrate-chromogen, reference K3464). Nuclei were counter-stained with a haematoxylin solution. Sections were washed and mounted in aqueous medium and then visualized using a NIKON E400 microscope. Images were captured with a NIKON DS-Ri1 and processed with NIS-ELEMENTS 3.10 software. The validity and specificity of the antibody used were confirmed by immunostaining of brain sections known to express OPRD1. A negative control was performed without the primary antibody. Validation data of anti-OPRD1 antibody demonstrating its specificity were also obtained from the manufacturer.

For corneodesmosin, involucrin, keratin 10 and occludin protein expression analysis:

RHE were snap frozen in liquid isopentane/N2 and store at -80°C . The transversal sections were carried out using a micro-

tome (5 μm thickness). The sections were fixed with an acetone/methanol mix and dried. RHE sections were stained.

After PBS milk 5% saturation, the section were incubated with the specific primary antibody solutions (anti-corneodesmosin (Tebu, H00001041-M01 at 1/100), anti-involucrin (Biomedical technologies Inc., BT-601 at 1/2), anti-keratin 10 (Santa-Cruz, Dallas, Texas 75220 USA, SC-23877 at 1/100) and anti-occludin (Life technologies, Saint-Aubin, France 33-1500 at 1/50). After washes, the binding sites were revealed using appropriate secondary coupled antibody (Alexa fluor 488 Goat anti-Mouse, Invitrogen, Saint-Aubin, France A11001 and Alexa fluor 488 Goat anti-Rabbit, Invitrogen A11008 at 1/400) and the nuclei were stained with propidium iodide solution. The sections were washed and then observed under epifluorescence microscopy (epifluorescence using a NIKON E400 microscope. Images were captured with a NIKON DS-Ri1 and processed with NIS-ELEMENTS 3.10 software). Fluorescence intensity for each marker was quantified by image analysis (IMAGEJ software), and a mean fluorescence intensity was calculated per sample ($N = 3$ replicates). Statistical analysis of the data was then performed using 't-test' of Student for paired data.

RT-qPCR

RT-qPCR was performed to investigate the transcriptional effect of the peptide (Rubixyl) on gene expression of the following selected markers: OPRD1-related genes, keratinocyte differentiation markers, gap junction, adherent and tight junction markers), on inflammatory HRE model.

The OPRD1-related genes assessed were opioid receptor delta 1 (OPRD1), adrenergic, beta-2-receptor surface (ADRB2), cAMP responsive element binding protein 1 (CREB1), GNAS complex locus (GNAS), mitogen-activated protein kinase 1 (MAPK1), mitogen-activated protein kinase 14 (MAPK14), prostaglandin endoperoxide synthase 2 (PTGS2), signal transducer and activator of transcription 3 (STAT3), baculoviral IAP repeat-containing 5 (BIRC5), ornithine decarboxylase 1 (ODC1), caspase 3 apoptosis-related cysteine peptidase (CASP3), solute carrier family 1 (glial high-affinity glutamate transporter) member 3 (SLC1A3), epidermal growth factor receptor (EGFR). The keratinocyte differentiation markers were calmodulin-like 5 (CALML5), filaggrin (FLG), keratin 1 (KRT1), keratin 10 (KRT10), keratin 19 (KRT19), loricrin (LOR), involucrin (IVL), peptidyl arginine deiminase, type I (PADI1), small proline-rich protein 1A (SPRR1A), small proline-rich protein 2A (SPRR2A), small proline-rich protein 1B (SPRR1B), transglutaminase 1 (TGM1), cornulin (CRNN) and stratifin (SFN). GAP junction markers assessed were gap junction protein, alpha 1, gap junction protein, alpha 5, GJA5, gap junction protein, beta 2 (GJB2), gap junction protein, beta 3 (GJB3), gap junction protein, beta 4 (GJB4), gap junction protein, beta 5 (GJB5), gap junction protein, beta 6 (GJB6), gap junction protein, gamma 1 (GJC1), gap junction protein, delta 3 (GJD3) and Pannexin 1 (PANX1). Tight junction markers assessed were claudin 1 (CLDN1), claudin 2 (CLDN2), occludin (OCLN), F11 receptor (F11R), tight junction protein 1 (TJP1), tight junction-associated protein 1 (TJAP1). Adherent junctions markers assessed were cadherin 1 (CDH1), dystonin (DST), corneodesmosin (CDSN), desmoglein 1 (DSG1), desmoplakin (DSP) and epiplakin 1 (EPPK1).

Extracted mRNA was analysed on a customized PCR array containing 64 target genes including two housekeeping genes, ribosomal protein S28 (RPS28) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). After culture, RHE were washed with PBS (Life Technologies[™]) and immediately dry-frozen at -80°C . Tripli-

cates were pooled, and total RNA from each sample was extracted using 'TriPure Isolation Reagent' kit (Roche Applied Science). The amount and quality of RNA were evaluated using a lab-on-a-chip Bioanalyzer (Agilent technologies). Potential contaminant traces of genomic DNA were removed using the DNA-free™ system (Ambion by Life Technologies™). The reverse transcription of mRNA was conducted in the presence of oligo(dT) and Superscript™ II reverse-transcriptase (Life Technologies™). Quantification of cDNA was performed using NanoVue Plus™ (GE Healthcare) and adjustment of cDNA at 10 ng μL^{-1} . The PCRs (polymerase chain reactions) were performed using the LightCycler® system (Roche Molecular System Inc.). The incorporation of fluorescence in amplified DNA was continuously measured during the PCR cycles. This resulted in a 'fluorescence intensity' versus 'PCR cycle' plot allowing the evaluation of a relative expression (RE) value for each marker.

Clinical study on human volunteers

In vivo tests were carried out at Farcoderm (San Martino Siccomarino, Italy). A double-blind and placebo-controlled clinical evaluation was carried out with 20 women volunteers (age between 40 and 65 years old) showing clinical signs (periorcular and perilabial wrinkles) of skin ageing due to physiological ageing and smoking habits. Fifty per cent of the volunteers were smokers. All of the subjects participating in the study gave their informed consent signed at the beginning of the study.

The effect of the OPRD agonist peptide (Rubixyl) at 3 ppm on normal aged skin and smoker aged skin (inflammatory skin) was evaluated at days 15, 30 and 60, after daily product use (twice a day). The volunteers applied twice a day either a placebo cream on one side of their face or a cream containing the Rubixyl peptide. Cream compositions include aqua, octyldodecyl neopentanoate, octyldecanol, myristyl myristate, acrylates/C10-alkyl acrylate crosspolymer, sodium hydroxide, phenoxyethanol, methyl paraben, ethylparaben, butylparaben, propylparaben, isobutylparaben and ± 3 ppm Rubixyl peptide.

Dermatologists assessed the following face skin parameters: TEWL (transepidermal water loss), hydration, smoothness and wrinkles' depths.

TEWL was measured by means of the internationally recognized Tewameter® method. The instrument used was a Tewameter 300® (Courage + Khazaka, electronic GmbH). The following equation which represents the Diffusion law (discovered by Adolf Fick in 1855) is the basis for the measurement: $dm/dt = -DA dc/dx$.

The diffusion flow dm/dt indicates the mass per cm which is transported in a specific period of time. It is proportional to the area A and the change of concentration per distance (dc/dx). D is the diffusion coefficient of water vapour in the air. This law is only

valid within a homogenous diffusion zone, which is approximately formed by a hollow cylinder. The resulting density gradient is measured indirectly by two pairs of sensors (temperature and relative humidity) and is analysed by a microprocessor. The measuring head of the probe is a narrow hollow cylinder (10 mm diameter and 20 mm height), to minimize influences of air turbulence inside the probe. Measurement of TEWL was used for evaluation of the skin barrier function. Wrinkle's depth was performed using Primo's 3D (GF Messtechnik, GmbH) and by self-assessment of subjects (only after 60 days).

Skin hydration was measured using the corneometer method. The instrument used was Corneometer (Courage + Khazaka, electronic GmbH). This measurement is based on the dielectric constant of water. The probe shows changes of capacitance according to the moisture content of the measuring object. An electric scatter field penetrates the very first layer of the skin and determines the dielectricity. Measurement of skin hydration was used for evaluation of the skin repair function.

Primos 3D is a non-contact *in vivo* skin measurement device based on structured light projection. In conjunction with a comprehensive 3-D measurement and evaluation software, the sensor allows to evaluate skin surface properties (i.e. wrinkle depth, volume and roughness). In this study, the wrinkles' depth is calculated. Statistical analysis of the data was then performed using 't-test' of Student for paired data.

The statistic significances are reported as follows: n.s. not significant $P > 0.05$ and * significant $P < 0.05$.

Results and discussion

Our experiments on the rat cell culture model (NG-10815 cells) have demonstrated that both Rubiscolin-5 (YPLDL) and Rubixyl (YPLDLF) are agonist for OPRD and precisely for delta 2 opioid receptor (OPRD2). The agonist effects of YPLDL and YPLDLF are concentration dependent (Fig. 1).

Our results confirmed those published by Yang *et al.* [1]. They have demonstrated the agonist properties of both peptides (YPLDL and YPLDLF) for OPRD2 but also for OPDR1.

To confirm the specificity of Rubixyl towards OPRD only, we have investigated its agonist properties at 4 μM in OPRM and OPRK agonist assay models. In Fig. 2, the results showed for OPRM and OPRK an agonist response rate lower than 25%, whereas for OPRD2, an agonist response more than 40%. We can conclude that Rubixyl is a specific agonist for OPRD2 and is not agonist for OPRM and OPRK.

Until now, the published data have shown only an expression of OPRD in skin epidermis with no indication on its specific location

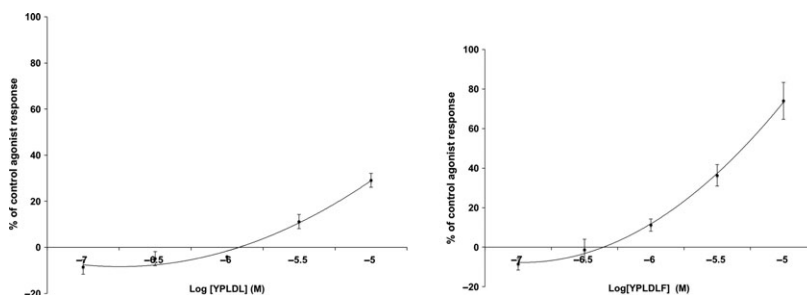


Figure 1 Agonist effect of Rubiscolin-5 (YPLDL) and Rubixyl (YPLDLF) for OPRD2, ($N = 3$ replicates for each data point).

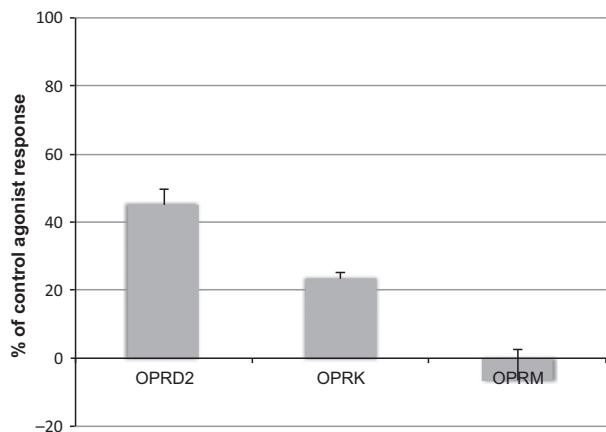


Figure 2 Assessment of agonist properties of Rubixyl for OPRD2, OPRK and OPRM ($N = 2$ replicates for each opioid receptor).

[6]. We have shown for the first time, on normal human skin explants (Fig. 3A, black arrow) and on normal RHE model (Fig. 3B, black arrow) that OPRD1 is expressed constitutively in the granular layer cell membrane.

In inflammatory RHE, a significant decreased of OPRD1 expression is seen (Fig. 4A, brown staining showed by black arrow). This decrease of OPRD expression under inflammatory conditions was also mentioned by other authors in the retina [14, 15]. The decrease of OPRD at the cell surface is probably due to their internalization as

mentioned in literature data [16, 17]. Nevertheless, this phenomenon is reversed by addition of increasing concentrations of agonist Rubixyl peptide in the culture media (Fig. 4B, data shown only for Rubixyl at 3 ppm). Pradhan *et al.* [16] have shown that some agonists for OPRD are able to block their internalization into the cell. We have demonstrated in inflammatory RHE that Rubixyl peptide has the same effect as the JAK inhibitor I on OPRD1 protein expression (Fig. 4C). OPRD1 protein is visible in both conditions (Fig. 4B and C, brown staining showed by black arrow), whereas in inflammatory RHE, non-treated, OPRD1 protein visualization is limited (Fig. 4A).

In mammals, the JAK/STAT [18] pathway is the principal stress signalling mechanism for cytokines in inflammatory condition. Many cytokines (such as TNF-alpha, IL-17, IL-1, IL-6 and IL-8) function by binding to cytokine receptors and lead to the secretion of chemokines which interact with chemokine receptors. These receptors in turn rely on the Janus kinase (JAK) family of enzymes for signal transduction. JAK activation induces cell proliferation, differentiation, migration, apoptosis and inflammation. Hence, molecules (such as JAK inhibitor I) that inhibit Janus kinase activity block cytokines signalling thus leading to block inflammation.

These results might be explained by several works [19–21] showing a functional interaction between chemokine and opioid receptors. By the way, Pello *et al.* [19] have suggested a model showing that chemokine and OPRD form heterodimeric complexes that are dynamically regulated by their ligands. In their model, knockdown of chemokine and OPRD signalling by heterodimerization might have repercussions on inflammatory process.

Additionally, it has been demonstrated that during inflammation, activation of proinflammatory chemokine receptors downre-

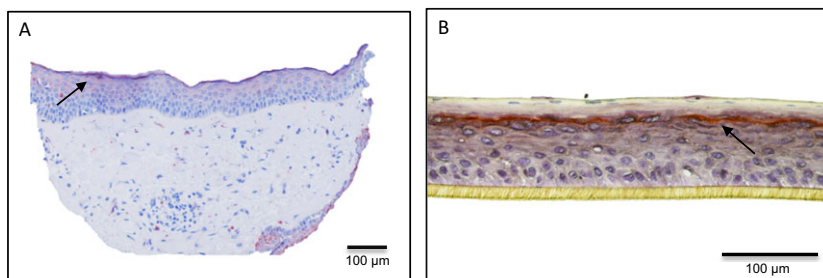


Figure 3 OPRD1 protein expression in normal human skin explants and in normal reconstructed human epidermis (RHE) (Immunohistochemistry).

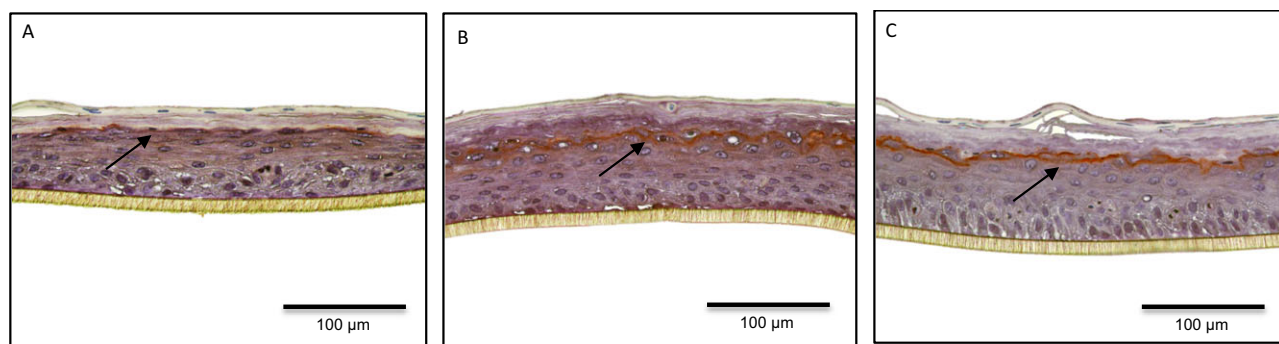


Figure 4 OPRD1 protein expressions in inflammatory RHE model non-treated (A) and treated with agonist Rubixyl peptide at 3 ppm (B) and treated with JAK inhibitor I (C). (Immunohistochemistry).

Table 1 Comparison of gene expression regulation under JAK inhibitor I or Rubixyl to normal reconstruct human epidermis (N = 3 replicates)

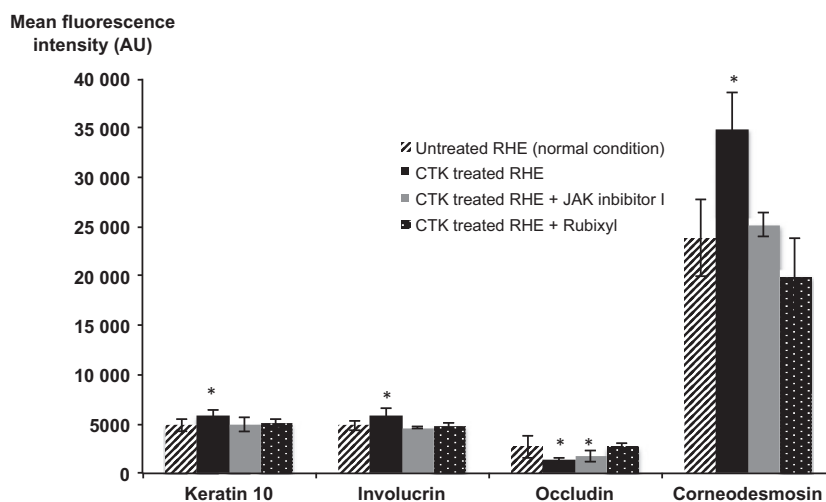
Markers	CTK treated RHE/Normal RHE	CTK treated RHE + JAK inhibitor I/ Normal RHE	CTK treated RHE + Rubixyl/ Normal RHE
FLG	0.22	3.3	1.19
KRT1	0.06	9.63	2.31
KRT10	0.05	9.14	1.49
IVL	0.17	3.88	2.29
SPPR2A	5.16	0.35	1.55
OCLN	0.83	1.19	2.37
CDSN	0.44	1.91	1.82
DST	0.21	2.32	0.61
STAT3	1.87	0.28	1.49

gulates the analgesic functions of opioid receptors (μ - and δ -receptors) [20, 21]. Opioid receptors in presence of their agonists are inversely able to desensitize chemokine responses [19]. This phenomenon is called heterologous desensitization of opioid receptors [21]. We can suggest that Rubixyl peptide acts as a desensitizer agent of cytokine receptors thus leading to block the inflammatory signalling pathway. We have shown in inflammatory RHE study model that the peptide has the same effect as JAK inhibitor I (a blocking agent of the inflammatory cascade through JAK/STAT3). Literature data [15] confirmed the anti-inflammatory effect of opioid receptor agonists in inflammatory tissues (retina and synovial). The authors mentioned that the mechanism implicated was the inhibition of the inflammation process. Another study has shown that stimulation of opioid receptor delta by an agonist reduces the production of pro-inflammatory mediators [22]. Based on all these data, it appears reasonable to propose that Rubixyl peptide has effective anti-inflammatory properties.

To further characterize the effect of the peptide on this inflammatory RHE model, we analysed gene and protein expressions of

selected epidermis markers involved in keratinocyte differentiation, function barrier and gene related to OPRD1. Table 1 showed only the expression profile of genes regulated by the peptide and/or the JAK inhibitor I. In the inflammation state, several keratinocyte differentiation markers and genes coding for barrier function maintenance (gap junction proteins, tight and adherent junctions) were deregulated. Most of the upregulated or downregulated genes in the inflamed state could be brought back to normal expression levels (value close to 1) by JAK inhibitor I or by the peptide (Table 1). Nevertheless, we noticed that the gene regulation performed by the peptide leads to a level closed or similar to normal RHE gene expression, whereas the regulation performed by JAK inhibitor I counteracts the inflammatory effect induced by cytokines, but the regulation does not lead to a normal RHE gene expression. With JAK inhibitor I, the genes were either hyper-upregulated or hyper-downregulated. The gene expression level of STAT 3 is not affected by the Rubixyl peptide in comparison with JAK inhibitor I (strong inhibitory effect confirmed, Fig. 5C). We can conclude that the signalling pathway involving the peptide is not via the JAK/STAT3 as seen with JAK inhibitor I.

At the protein expression level, the following proteins were assessed: calmodulin-like 5 (CALM5), claudin 1 (CLDN1), claudin 2 (CLDN2), corneodesmosin (CDSN), epiplakin (EPPK1), gap junction protein delta 3 (GJD3), involucrin (IVL), KRT1 (KRT1), keratin 10 (KRT10) and occludin (OCLN). In inflammatory RHE (CTK treated), at the protein expression level, there are less markers deregulated than at the gene expression level. Nevertheless, some important epidermis proteins for maintenance of skin barrier function (corneodesmosin: adherent junction protein and occludin: tight junction protein) and for epidermis differentiation (keratin-10 and involucrin) were also deregulated. In this inflammatory model, keratin 10, involucrin and corneodesmosin were upregulated, whereas occluding protein is downregulated in comparison with untreated RHE (Fig. 5). The peptide was able to normalize the expression of all the deregulated proteins (close to protein expression level of non-inflammatory model), whereas JAK inhibitor I is able to control only expression of KRT10, IVL and CDSN (Fig. 5). At the protein expression level, there are no differences between JAK inhibitor I and Rubixyl peptide. The normalization of occludin (OCLN) protein is only efficient in presence of peptide. JAK inhibitor

**Figure 5** Mean fluorescence intensity calculated for keratin 10, involucrin, occludin and corneodesmosin based on image analysis quantification. The stars represent the significant difference observed in comparison with untreated RHE (* $P < 0.01$, Student's t -test).

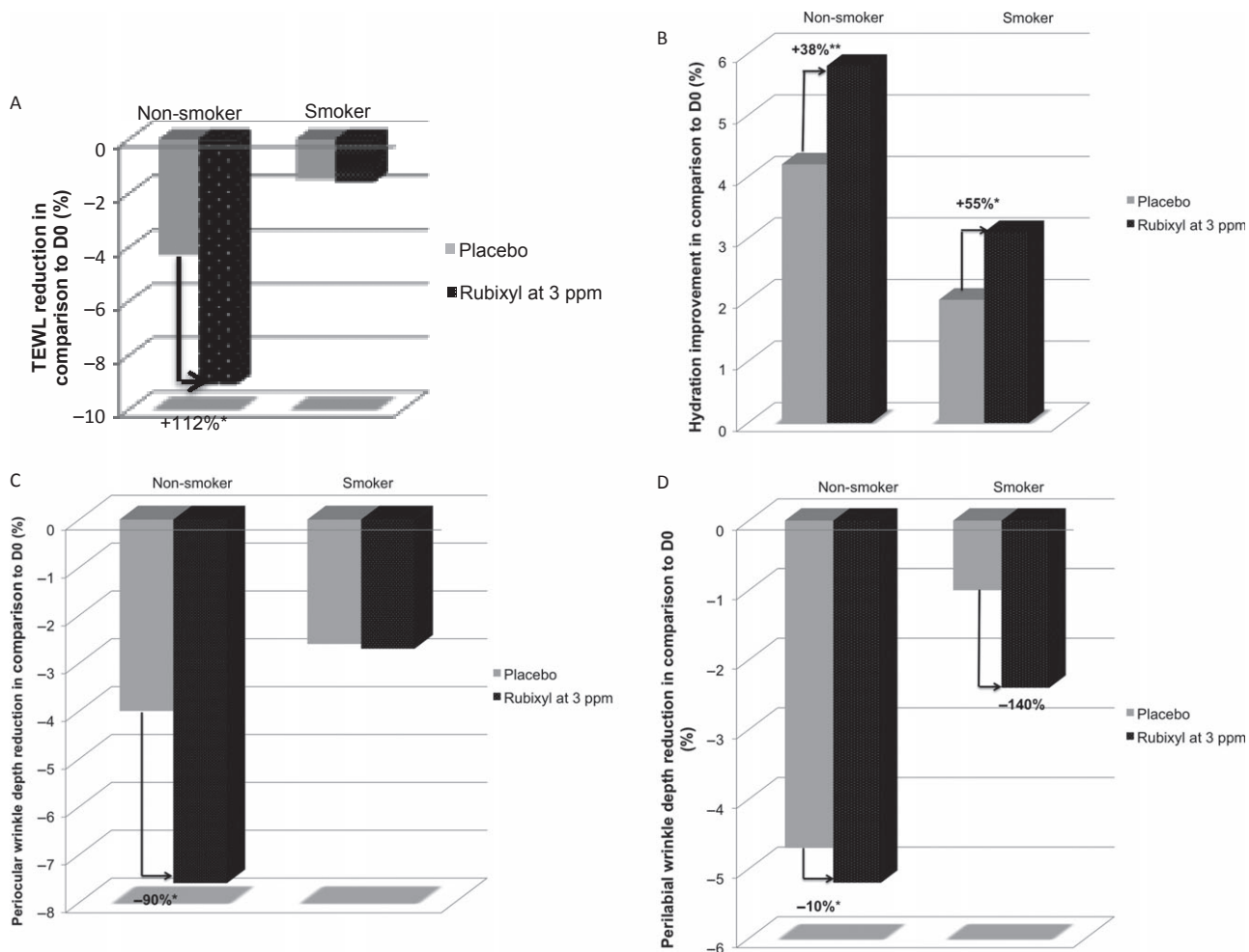


Figure 6 TEWL (A), hydration (B), periocular (C) and perilabial (D) wrinkle depth variations after 60 days of Rubixyl (at 3 ppm) or placebo treatments. The variations are expressed in percentage in comparison with D0 and in comparison with placebo. $N = 10$ volunteers in each group (smoker and non-smoker), stars represent values statistically significant with $*P < 0.05$, Student's t -test, $P < 0.01$.

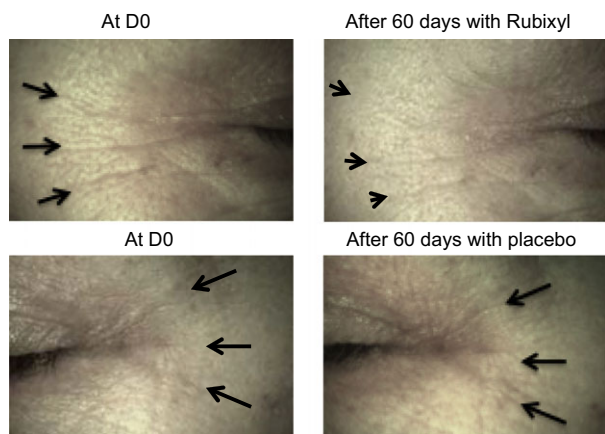


Figure 7 Wrinkle depth at D0 and after 60 days of Rubixyl (at 3 ppm) or placebo treatments (macrophotographies, 56-year-old woman non-smoker, periocular area).

I slightly increases the expression of occludin, but it is still far from the normal level. These data confirm the observation seen at the gene expression level for OCLN. Transcription of OCLN was modulated only by the peptide and not by the JAK inhibitor I.

To confirm the interest of the biological properties of Rubixyl to counteract aged skin micro-inflammation 'consequences', a double-blinded clinical study has been performed on human aged volunteers. The normal chronological ageing process is associated with a gradual increased level of micro-inflammation (production and circulation of pro-inflammatory cytokines such as IL-6, IL-8, TNF- α) in the skin referred to as 'inflammageing' [23–25]. The 'inflammageing' is also due to accumulation of the effect of external aggressions (UV irradiation [26] and smoking [27]). In stressed conditions [26, 28, 29] (such as in sun-exposed prematurely aged human skin induced by UV irradiation and smoker skins), cytokine (especially IL-1 β) and metalloproteinase levels are elevated. Cytokine IL-1 β inhibits type I collagen production and upregulates matrix metalloproteinases, which degrade collagen fibrils and elastic fibres. These fibres degradation leads in part to wrinkle apparition.

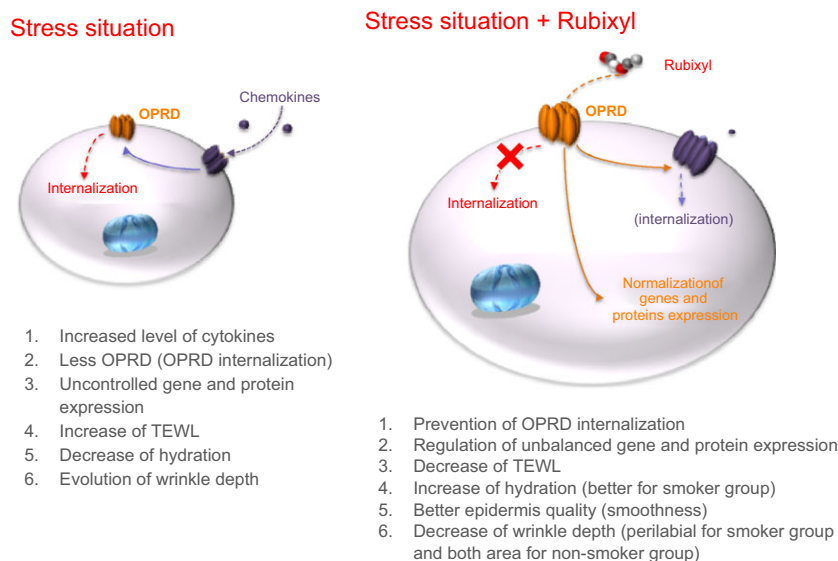


Figure 8 Mechanism of action of Rubixyl peptide in 'stress situation'.

The study has demonstrated that applying twice a day and during 60 days, on periorcular area a cream containing the peptide reduced TEWL and increase hydration (Fig. 6A,B).

TEWL is directly correlated with the state of skin barrier function [30]. Usually, an increased TEWL value means that the barrier function of the skin is altered. TEWL tends to decrease in normal aged skin [31], whereas in inflammatory stress condition (tobacco smoking, UV irradiation, irritation, oxidative stress, psoriatic skin), TEWL increases [32]. Fifty per cent of volunteers included in our clinical study were smokers with skin barrier function altered [33–35]. The significant decrease of TEWL seen with the peptide demonstrates that skin barrier function is repaired in stressed skin. This repair is seen very quickly only after 15 days (data not shown) and confirmed by increase of skin hydration after 60 days of treatment. The peptide by its anti-inflammatory properties decreased the skin inflammation thus leading to the limitation of water loss from the epidermis and better skin hydration. The clinical study on smoker and non-smoker skins has shown the improvement of the wrinkle depth at periorcular and perilabial areas (Figs 6C–D and 7) when treated by Rubixyl during 60 days. The results with placebo have shown no improvement in wrinkle depth after 60 days of treatment, whereas the peptide revealed a cumulative biological effect upon time. At D60, the decreases observed in wrinkle depth at periorcular and perilabial areas are statistically significant in comparison with D0 for non-smoker group. At D60, the decrease observed in wrinkle depth at perilabial area is close to significance for smoker group (Fig. 6D).

Conclusion

In this research, we have demonstrated *in vitro* (on inflamed reconstructed human epidermis RHE) and *in vivo* (on human aged volunteers) that activation by a natural agonist peptide of opioid receptor delta reduced skin inflammation thus leading to improvement of better epidermis differentiation and skin barrier properties.

We have shown in inflammatory RHE model that during inflammation, several keratinocyte differentiation markers and barrier

function markers (gap junction proteins, tight and adherent junctions) were deregulated (either upregulated or downregulated) at the gene and protein level. We have demonstrated that most of the upregulated or downregulated genes and proteins in the inflamed state could be brought back to normal expression levels by JAK inhibitor I or by the peptide. On human aged volunteer stressed skin, we have demonstrated the efficient effects of a cream containing the Rubixyl peptide. All parameters assessed were significantly improved after 60 days of treatment in comparison with placebo: reduction of wrinkle depth by 58%, increase of smoothness by 30% and decrease of TEWL by 82%. The significant decrease of TEWL confirms that skin barrier deficiency is repaired. This repair is seen very quickly only after 15 days. The Rubixyl peptide by its anti-inflammatory properties decreased the skin inflammation thus leading to the limitation of water loss from the epidermis.

A mechanism of action for the Rubixyl peptide in anti-ageing strategies proposed is the inhibition of inflammation induced by cytokines (via the desensitization of cytokine receptors), followed by the restoration of epidermis differentiation and barrier function markers. The restoration of epidermis differentiation and barrier function markers is a consequence of the equalization to a normal level of the markers upregulated or downregulated in inflammatory conditions by Rubixyl peptide (Fig. 8). Based on these studies performed on inflammatory RHE and on aged human volunteer smokers or not (with micro-inflamed skins), we could confirm the high potent anti-inflammatory properties of this new delta opioid agonist peptide (Rubixyl). Our studies highlight the interest of OPRD agonist peptides in anti-inflammatory strategies and more globally highlight the interest of OPRD as new biological targets in anti-ageing strategy for skincare research.

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