Oxytocin modulates proliferation and stress responses of human skin cells: implications for atopic dermatitis

Verena Deing¹, Dennis Roggenkamp¹, Jochen Kühnl¹, Alisa Gruschka¹, Franz Stäb¹, Horst Wenck¹, Alexander Bürkle² and Gitta Neufang¹

¹Beiersdorf AG, Research Skin Care, Hamburg, Germany; ²Molecular Toxicology, University of Konstanz, Konstanz, Germany

Correspondence: Gitta Neufang, PhD, Beiersdorf AG, Research Skin Care, Unnastraße 48, 20245 Hamburg, Germany, Tel.: +49 40 4909 6676, Fax: +49 40 4909 186676, e mail: gitta.neufang@beiersdorf.com

Abstract: The neuropeptide hormone oxytocin (OXT) mediates a wide spectrum of tissue specific actions, ranging from cell growth, cell differentiation, sodium excretion to stress responses, reproduction and complex social behaviour. Recently, OXT expression was detected in keratinocytes, but expression of its receptor and function are still unexplored in human skin. Here, we showed that both OXT and its receptor are expressed in primary human dermal fibroblasts and keratinocytes. OXT induced dose dependent calcium fluxes in both cell types demonstrating that the OXT receptor (OXTR) is functionally expressed. We also showed that OXT decreases proliferation of dermal fibroblasts and keratinocytes in a dose dependent manner. In order to further investigate OXT mediated functions in skin cells, we performed OXTR knockdown experiments. OXTR knockdown in dermal fibroblasts and keratinocytes led to elevated

levels of reactive oxygen species and reduced levels of glutathione (GSH). Moreover, OXTR depleted keratinocytes exhibited an increased release of the pro inflammatory cytokines IL6, CCL5 and CXCL10. Our data indicate that the OXT system modulates key processes which are dysregulated in atopic dermatitis (AD) such as proliferation, inflammation and oxidative stress responses. Furthermore, we detected a downregulation of the OXT system in peri lesional and lesional atopic skin. Taken together, these data suggest that the OXT system is a novel neuroendocrine mediator in human skin homeostasis and clinically relevant to stressed skin conditions like AD.

Key words: atopic dermatitis – inflammation – oxidative stress – oxytocin system – proliferation

Introduction

The nonapeptide hormone oxytocin (OXT) plays a key role in certain kinds of behavioural regulation, such as social recognition, attachment and anxiety (1,2), as well as in physiological functions, including milk ejection during lactation and uterine contraction during labour (3). OXT is mainly produced in the paraventricular and supraoptic nuclei of the hypothalamus and released from hypothalamic nerve terminals of the posterior pituitary into the blood stream (4,5). Apart from this, OXT is also synthesized in uterus, ovary, testis and myocardial tissue (3).

Oxytocin acts through its receptor, which belongs to the rhodopsin type class I G protein coupled receptor superfamily (6). The OXT receptor (OXTR) is differentially expressed in various tissues correlating with the pattern of sex steroids (3), and its expression is regulated by cytokines (7,8). Differential pharmacological profiles of the OXTR in the brain and peripheral tissues are associated with the existence of OXTR subtypes (9,10). Furthermore, the OXTR displays promiscuous coupling to G_q, G_s and G_i protein isoforms forming heterotrimeric complexes, depending on the localization of the receptor within the plasma membrane. The variety of OXTR subtypes coupling with different G protein isoforms results in the activation of multiple signalling pathways, and consequently, of diverse physiological functions of OXT in different cell types (11,12). In fact, OXT is able to promote, to inhibit or to have no effect on proliferation of various cell types (13,14). However, the proliferative effects are altered by cholesterol depletion of membranes or changes in OXTR caveolae interaction (15), resulting in contrary observations (16,17). Inhibitory effects of OXT are mediated by activation of a cAMP protein

kinase A pathway, whereas mitogenic effects are associated with an increase of intracellular calcium and tyrosine phosphorylation (14). Further functions of OXT are the modulation of neuroendocrine stress responses and inflammatory processes (18,19). OXT dampens the hypothalamo pituitary adrenal (HPA) axis by lowering corticosterone/cortisol levels in response to acute exposure to stressors (18,20). Human skin takes part in the local as well as the systemic neuroendocrine network (21). For instance, all key mediators of the HPA axis including the neuropeptides CRH, POMC derived ACTH, α MSH, β endorphin and cortisol/corticosterone are synthesized by skin cells (21–23). In term myometrium, CRH receptor function is inhibited by OXT via activation of protein kinase C (24). In view of possible cross talks, CRH receptors are also expressed by dermal fibroblasts and keratinocytes (22,25,26). However, with respect to inflammation, subcutaneously given OXT has been demonstrated to prevent sepsis induced depletion of glutathione (GSH) contents in colonic and uterine tissues of rats (27). Furthermore, wound healing, which involves a well organized inflammatory phase, has been shown to be facilitated by OXT administration (28,29). Moreover, in a rat model of dried latex induced paw oedema, OXT treatment showed anti inflammatory and antinociceptive activity, thus pointing to a role of OXT in skin inflammation (30). Inflammation is typically accompanied by oxidative stress. In an *in vitro* model, OXT decreased not only IL6 secretion, but also NADPH dependent superoxide production in vascular cells and THP 1 macrophages suggesting that OXT attenuates vascular oxidative stress (31).

In humans, chronically inflamed skin is, amongst others, evident in atopic dermatitis (AD). The skin of patients suffering

from AD shows lichenification, erythema and pruritic lesions. AD is a multifactorial skin disease: genetic predisposition, bacterial infections, skin barrier disruption, immunological alterations and psychological stress have all been discussed as causes and aggravation factors (32,33). Epidermal hyperplasia is a common feature of AD and attributed to T cell derived cytokines such as tumor necrosis factor α and interferon γ which are potent inducers of epidermal growth factors and their receptor (34–36). Additionally, these cytokines initiate inflammatory cascades and have been shown to induce an abnormal chemokine production profile in keratinocytes of AD patients (37).

A recent study showed that in human skin, OXT is also produced by epidermal keratinocytes (38). However, its role in cutaneous homeostasis remains unknown. The aim of the present study was the characterization of the OXT system in human skin and the analysis of its functional activity and relevance in skin physiology. We showed that dermal fibroblasts and keratinocytes express OXT and its receptor. Additionally, we revealed the role of OXT in the modulation of cutaneous proliferation, inflammation and oxidative stress responses with possible implications for AD.

Methods

In vivo studies

All *in vivo* studies were conducted according to the Declaration of Helsinki and approved by the ethics committees of the Medical Associations at Hamburg, Kiel and Freiburg, Germany. All study participants gave written informed consent. AD patients displayed a local SCORAD of 5.8 ± 0.3 on test arm area. Ages of healthy ($n = 32$) and AD ($n = 24$) participants were equivalent (healthy: 36.6 ± 2.1 , atopic: 35.0 ± 1.7 years). Punch biopsies were taken under local anaesthesia from peri lesional/lesional areas of the forearm of atopic patients and healthy volunteers. Suction blisters were prepared as described by Kiistala (39). In an additional study, suction blister fluids were taken from the inner upper arms of six healthy women and six healthy men aged 20–40 at t_0 and t_1 . At t_1 , participants caressed themselves with a soft brush every 30 min for 5 min during 3–4 h around the investigated area (tactile stimulation) leaving the other arm unstimulated (control).

Isolation and culture of primary human dermal fibroblasts and keratinocytes

Skin cells were isolated from biopsies and cultured as described by Roggenkamp et al. (40), and used at passages 1–5. As determined by ELISA, basal OXT concentrations in culture medium for fibroblasts and keratinocytes were 66 pM and 2.7 nM, respectively. Physiological plasma OXT levels range from 5 to 300 pM (41–43). In knockdown experiments, no additional OXT was applied. OXT doses used in the experiments to determine Ca^{2+} fluxes and proliferation have been previously elaborated (44).

Immunofluorescence analyses

Cells grown on glass chamber slides (Thermo Fischer Scientific, Waltham, MA, USA) were fixed with 4% paraformaldehyde (Sigma Aldrich, Munich, Germany) in PBS and permeabilized with 0.1% Triton X 100 (Sigma Aldrich) in PBS. Cryosections of human skin were fixed with ice cold acetone. Cells and sections were blocked with 1% BSA in PBS and incubated with antibodies directed against human OXT (fibroblasts, keratinocytes: 1:200, LS C37953; LifeSpan BioSciences (Seattle, WA, USA), skin: 1:500, MAB5296; Millipore, Billerica, MA, USA) and OXTR (1:200, SYC592; Eurogentec, Seraing, Belgium). For staining of nuclei and visualization, cells and sections were incubated with Alexa 488 conjugated

secondary antibodies (1:1000; Invitrogen, Darmstadt, Germany) mixed with DAPI (1:2000, 5 mg/ml stock; Sigma Aldrich). Microscopy was performed with an Axiovert S100 (Zeiss, Jena, Germany).

Real time semi quantitative PCR

Total RNA from fibroblasts, keratinocytes and suction blister derived epidermis was isolated using RNeasy Kit (Qiagen, Hilden, Germany). Concentration and purity of RNA were assessed utilizing a NanoDrop 1000 (Peqlab, Erlangen, Germany). RNA was subjected to cDNA synthesis with the high capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). Expression levels were detected by semiquantitative real time polymerase chain reaction (RT-PCR) using gene expression assays mixed with TaqMan Master Mix performed in a 7900HT Fast RT-PCR System (all from Applied Biosystems). The gene expression assays Hs00792417 g1 (OXT) and Hs00168573 m1 (OXTR) were utilized. Expression was quantified by the comparative $\Delta\Delta C_t$ method using 18S expression as endogenous control.

Detection of oxytocin and cytokine protein levels

OXT concentrations in suction blister fluids were measured using an OXT ELISA (Enzo Life Sciences, Lörrach, Germany) and normalized to total protein contents determined by BCA quantification (Interchim, Montluçon, France). Cytokine concentrations were measured using a 27 Plex (BioRad, Munich, Germany).

Oxytocin receptor knockdown

One day after seeding cells at a density of 2×10^5 /well into six well plates, they were transfected with 20 nM (fibroblasts) or 50 nM (keratinocytes) of scrambled (No.1027281; Qiagen) or OXTR specific (AM16708, ID1766; Invitrogen) siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

Measurement of Ca^{2+} currents

Intracellular calcium concentrations were measured by flow cytometry as described previously (45). Briefly, cells were incubated with PBS containing 3 $\mu\text{g}/\text{ml}$ Fluo 4 AM, 10 $\mu\text{g}/\text{ml}$ Fura Red, 0.02% Pluronic Detergent F 127 and 4 mM Probenecid and 10% FCS (all from Invitrogen). Ca^{2+} currents were induced by OXT (Tocris, Bristol, UK) diluted in 10% FCS in PBS and recorded using the flow cytometer FACSCanto™ (BD Bioscience, Heidelberg, Germany). Inhibition of OXT induced Ca^{2+} currents was performed by pre addition of the OXTR specific antagonist L371,257 (Tocris). Data were analysed using FlowJo 7.2.1 (BD Bioscience). Ca^{2+} currents were normalized to cell number. Imaging of Ca^{2+} fluxes was carried out using the confocal microscope SP5 (Leica, Bensheim, Germany).

Proliferation assay

Fibroblasts and keratinocytes were seeded in 96 well plates. One day after plating, cells were treated daily with OXT alone or in combination with the OXTR specific antagonist L371,257. At day 4 and 7 after initial treatment, cells were fixed and stained with 6.2 μM propidium iodide (46) (Invitrogen) and 17% ethanol in PBS. Proliferation was quantified by cell counting using an Axiovert 200M (Zeiss).

UV irradiation

For UV exposure (47), cells grown in six well plates were irradiated in 1 ml PBS/well. Fibroblasts were irradiated with 5 J/cm² UVA light using a Psorisan 900/5164 H1 (Dr. Hönle Medizintechnik, Kaufering, Germany). Keratinocytes were irradiated with solar simulated light (SSR) using an Oriel 1600W (Newport Corporation, Stratford, CT, USA) at a dose of 100 mJ/cm² UVB. UV doses were determined with an IL1700 Research Radiometer (International Light, Newburyport, MA, USA). The UV spectra of the light

sources used are provided as supplemental figures (Figs S1 and S2). Directly after irradiation, cells were subjected to reactive oxygen species (ROS) and GSH determination assays.

Determination of reactive oxygen species

Cells were incubated with 150 ng/ml 2',7'-dichlorodihydrofluorescein diacetate (Invitrogen) in PBS according to the manufacturer's instructions. Fluorescence intensities were measured at 535 nm using a Safire reader (Tecan, Crailsheim, Germany) and normalized to cell number.

Detection of glutathione

Cells were incubated with 20 μ M ThiolTracker™ Violet dye (Invitrogen) in PBS according to the manufacturer's instructions. This dye was used to estimate the cellular level of reduced GSH, because it represents the majority of intracellular free thiols in the cell. Fluorescence intensities were measured at 526 nm using a Safire reader (Tecan) and normalized to cell number.

Statistics

Data were analysed with Prism 5 (GraphPad Software, San Diego, CA, USA) and are presented as the mean $\pm$ SEM. Normality was checked by the Shapiro Wilk test. If the hypothesis of normality was rejected, groups were compared using the Mann Whitney U test (unpaired data) or the Wilcoxon signed rank test (paired data). Otherwise, Student's *t* test (paired/unpaired data) was used for group comparison. *P* values < 0.05 were considered statistically significant.

Results

The oxytocin system is functionally expressed in primary human skin cells

Oxytocin expression in keratinocytes has recently been reported (38), but expression of the OXT receptor (OXTR) and its role in human skin have not been investigated so far. Immunofluorescence analyses revealed that both OXT and the OXTR are expressed in dermal fibroblasts and keratinocytes (Fig. 1a–d). Furthermore, OXT is localized throughout the epidermis (Fig. 1e). In contrast, the OXTR is mainly detected in the basal layer of the epidermis (Fig. 1f). RT-PCR analyses confirmed OXT and OXTR expression in skin cells (Fig. 1g,h). The highest mRNA expression levels of OXT and the OXTR were detected in fibroblasts compared to keratinocytes or epidermis tissue. OXT expression in keratinocytes was eightfold higher than in the epidermis. This was presumably due to a higher expression of OXT in proliferating keratinocytes (38), which were predominant in the *in vitro* monoculture, compared to differentiated keratinocytes, predominant in the epidermis *in vivo/ex vivo*. Hypothalamus mRNA served as positive control for OXT/OXTR expression.

To examine OXT protein levels in human skin *in vivo*, we analysed suction blister fluids, taken from both arms of 12 healthy volunteers. With respect to gender, OXT levels were equivalent (males: 6.5 ± 0.6 pg OXT/mg protein, females: 6.4 ± 1 pg OXT/mg protein) (Fig. 1i). As higher OXT concentrations are found in the saliva upon tactile stimulation (48), we investigated whether OXT is also released locally by tactile stimulation from skin tissue. To this end, suction blister fluids were taken either after repeated tactile stimulation or from the corresponding, unstimulated arm. Significantly higher OXT concentrations were observed in suction blister fluids taken from stimulated arms (6.8 ± 0.7 pg OXT/mg protein) compared to unstimulated arms (5.9 ± 0.4 pg OXT/mg protein) (Fig. 1j), indicating a local OXT release from tactile stimulated skin.

OXTRs predominantly couple to G_q proteins and enhance inositol 3 phosphate signalling, leading to increased intracellular Ca^{2+}

Figure 1. The oxytocin system is expressed in primary human skin cells. (a–f) Immunofluorescence localization of OXT (green) and the OXTR (green) was performed on dermal fibroblasts (FIB), keratinocytes (KER) and the epidermis of a skin section. Blue DAPI staining depicts nuclei. Scale bar: 20 μ m. (g, h) Transcript levels for OXT and the OXTR in fibroblasts (*n* = 81), keratinocytes (*n* = 28), suction blister derived epidermis (EPI) (*n* = 12) and hypothalamus (HYP) (*n* = 1) were assessed by RT-PCR. (i, j) OXT concentrations in human skin suction blister fluids (SBF) taken from both arms of male (*n* = 6) and female (*n* = 6) volunteers. (j) OXT concentrations in SBF derived from tactile stimulated and control areas. Wilcoxon signed rank test, **P* < 0.05; Mann Whitney U test, ****P* < 0.001.

fluxes (3). In order to test, whether OXTR activation leads to increased Ca^{2+} fluxes in dermal fibroblasts and keratinocytes, we performed flow cytometry analyses. In both cell types, OXT led to a significant increase in Ca^{2+} currents in a dose dependent manner, indicating that the OXTR is functional (Fig. 2a,c). Induction with 1 μ M OXT resulted in a twofold increase in Ca^{2+} currents compared to baseline. This effect was significantly inhibited by OXTR knockdown or by the OXTR antagonist L371,257. Visualization of OXT induced Ca^{2+} fluxes via fluorescence analyses confirmed OXTR signalling in dermal fibroblasts and keratinocytes (Fig. 2b,d).

Figure 2. Oxytocin induces Ca²⁺ currents in skin cells and decreases their proliferation rate. (a, c) OXT induced intracellular Ca²⁺ fluxes relative to baseline were measured in dermal fibroblasts (FIB) (*n* = 6) and keratinocytes (KER) (*n* = 6). OXTR knockdown in fibroblasts (siOXTR) and the treatment of keratinocytes with the OXTR inhibitor L371,257 (L375) served as negative controls. (b, d) Representative images of OXT induced Ca²⁺ fluxes (green). (e, f) Proliferation of OXT treated fibroblasts (*n* = 6) and keratinocytes (*n* = 6) relative to untreated control at day 7 (e) and day 4 (f). The OXTR inhibitor L371 served as negative control. Asterisks show significance of OXT induced effects versus untreated or negative control. Paired *t* test, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Oxytocin decreases proliferation of dermal fibroblasts and keratinocytes

As OXT either promotes or inhibits cell growth of different cell types (13,14), we investigated whether OXT modulates proliferation of skin cells.

Oxytocin treatment of fibroblasts and keratinocytes significantly decreased their proliferation rate (Fig. 2e,f). At day 7 of daily treatment with 1 nM OXT, proliferation of fibroblasts was significantly reduced ($14 \pm 2\%$) (Fig. 2e). This growth inhibiting effect of OXT was dose dependently more pronounced using higher concentrations (1 μM OXT: $37 \pm 6\%$ decrease). Cotreatment with the OXTR antagonist L371,257 (1 μM) counteracted the antiproliferative effects of 1, 10 and 100 nM OXT. In comparison to fibroblasts, keratinocytes responded to OXT treatment with a less pronounced but significant decrease in cell proliferation (10 nM OXT: $7 \pm 3\%$, 10 μM OXT: $22 \pm 4\%$) (Fig. 2f). Cotreatment with the OXTR antagonist L371,257 (10 μM) significantly antagonized the antiproliferative effect of OXT.

Oxytocin receptor knockdown induces oxidative stress, reduces glutathione levels and leads to enhanced pro inflammatory cytokine release of skin cells

To elucidate OXTR mediated functions in the skin, we performed transient OXTR knockdown in dermal fibroblasts and keratinocytes.

Next, we investigated whether OXTR knockdown in skin cells affects their oxidative stress status. Determination of ROS levels revealed significantly increased intracellular ROS concentrations in OXTR depleted fibroblasts ($22 \pm 7\%$) and keratinocytes ($30 \pm 9\%$) (Fig. 3a). As expected, both cell types responded to UV irradiation with increased ROS formation (fibroblasts: $60 \pm 19\%$, keratinocytes: $25 \pm 7\%$). OXTR knockdown significantly further aggravated the ROS status of irradiated skin cells (fibroblasts: $68 \pm 30\%$, keratinocytes: $26 \pm 11\%$ increase).

Additionally, we determined intracellular GSH concentrations to elucidate whether higher ROS levels correlate with lower GSH levels in OXTR depleted dermal fibroblasts and keratinocytes. In OXTR knockdown fibroblasts, GSH levels decreased significantly ($38 \pm 5\%$), whereas only a slight decrease was observed in OXTR knockdown keratinocytes (Fig. 3b). Irradiation of fibroblasts with 5 J/cm² UVA light diminished GSH concentrations significantly ($40 \pm 4\%$). Irradiation of keratinocytes with 100 mJ/cm² SSR light led to a marginal lowering of GSH level ($4 \pm 6\%$). Nevertheless, irradiated OXTR knockdown cells were more susceptible to GSH depletion than control cells. They exhibited a significant further decrease of GSH contents by approximately 13%.

OXT modulates inflammatory processes (19,31,49). In order to investigate whether the OXT system might be involved in the inflammatory stress response of the skin, we screened 27 cytokines concerning their regulation in OXTR knockdown skin cells. Out of these cytokines, the release of IL 6, CCL5 and CXCL10 was upregulated in keratinocytes in response to OXTR knockdown (Fig. 3c). The enhanced release was significant for all three cytokines and the strongest effect was observed for CXCL10 (IL6: $164 \pm 65\%$, CCL5: $226 \pm 90\%$, CXCL10: $1297 \pm 632\%$). In contrast, secretion of these cytokines was not modulated in OXTR depleted fibroblasts.

The oxytocin system is downregulated in atopic skin

Our data revealed modulation of cytokine release, oxidative stress status and proliferation by the OXT system in skin cells (Figs 2e,f and 3). As these parameters are aberrant in AD (50,51), we explored whether the OXT system might be dysregulated in atopic skin. Thus, we analysed mRNA expression of OXT and its receptor in dermal fibroblasts and keratinocytes derived from biopsies of healthy volunteers and atopic skin patients. RT PCR analyses

Figure 3. Oxytocin receptor knockdown leads to elevated concentrations of reactive oxygen species (ROS), reduced glutathione (GSH) levels and an increased release of pro inflammatory cytokines. (a–c) Primary human dermal fibroblasts (FIB) and keratinocytes (KER) were transfected with either scrambled (siControl) or OXTR specific (siOXTR) siRNA. (a) ROS levels in fibroblasts ($n = 6$) and keratinocytes ($n = 12$) were determined directly after sham irradiation and irradiation with 5 J/cm^2 UVA or 100 mJ/cm^2 solar simulated light (SSR). (b) GSH levels in fibroblasts ($n = 6$) and keratinocytes ($n = 6$) were determined directly after sham irradiation and irradiation with 5 J/cm^2 UVA or 100 mJ/cm^2 SSR. (c) Cytokine concentrations in culture supernatants of OXTR knockdown fibroblasts ($n = 4$) and keratinocytes ($n = 11$) relative to siControl were determined. Supernatants were collected 24 h after addition of fresh media at day 4 post transfection. Paired t test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

revealed significantly reduced OXT expression in atopic lesional fibroblasts ($2.8 \pm 2.6\%$) and in atopic peri lesional keratinocytes ($0.9 \pm 0.1\%$) compared to healthy controls (Fig. 4a). Atopic peri lesional fibroblasts and atopic lesional keratinocytes also showed reduced OXT expression. Additionally, OXTR expression was significantly decreased in both, atopic peri lesional and lesional fibroblasts ($620 \pm 52\%$, $560 \pm 65\%$, respectively), compared to healthy fibroblasts (Fig. 4b). Atopic keratinocytes also showed a decreased OXTR expression (Fig. 4b). Accordingly, a similar trend to reduced OXTR expression levels was detected in epidermis

Figure 4. Expression of oxytocin and oxytocin receptor is reduced in atopic skin. (a, b) Transcript levels for OXT and its receptor in healthy (h), peri lesional (pl) and lesional (l) atopic (a) dermal fibroblasts (FIB), keratinocytes (KER) and suction blister derived epidermis (EPI) were assessed by RT PCR. (c) OXT concentrations in suction blister fluids (SBF) of peri lesional atopic and healthy skin were detected. Unpaired t test, Mann–Whitney U test, $*P < 0.05$, $**P < 0.01$.

derived from atopic peri lesional suction blisters (Fig. 4b). To evaluate OXT levels in atopic versus healthy skin *in vivo*, we measured OXT concentrations in suction blister fluids. Suction blister fluids derived from peri lesional atopic skin exhibited a slight decrease of OXT concentration compared to the healthy control (Fig. 4c). These data suggest that atopic skin displays a deficit in OXT signalling.

Discussion

The neurohypophyseal hormone OXT exerts a wide spectrum of central and peripheral effects, including facilitation of reproduction at several levels, establishment of complex social behaviour, neuroendocrine modulation and immunological processes (3,31). Apart from studies, investigating the impact of subcutaneously injected OXT on different inflammation models (29,30), the role of the OXT system in skin has not been described so far. Recently, Denda

et al. (38) demonstrated that OXT is expressed in human skin keratinocytes and released in response to calcium influx via P2X receptors.

In this study, we showed that OXT is expressed not only in keratinocytes, but also in human skin derived dermal fibroblasts. Moreover, we were able to detect mRNA transcripts of the OXTR in dermal fibroblasts and keratinocytes. A previous study reported OXTR expression in human foreskin fibroblasts (52). Our immunohistochemical stainings of OXT and its receptor in human skin showed an expression of OXT in all epidermal layers and a preferential expression of the OXTR in the basal layers. Denda et al. (38) also found OXT immunoreactivity throughout the epidermis.

In a study with 12 healthy volunteers, we showed that tactile stimulation of the skin increases OXT concentrations in suction blister fluids. In contrast to former studies reporting on elevated systemic OXT levels after partner hugs in humans (53) or repeated massage like stroking in rats (54), our observation demonstrates local cutaneous OXT release *in vivo*. In anaesthetized rats, plasma OXT levels rise in response to touch or vagal and sciatic nerve activation proposed to originate from peripheral nerves in the skin (55). With regard to our findings of cutaneous OXT expression, the release might also originate from skin cells such as dermal fibroblasts and keratinocytes.

Stimulation of dermal fibroblasts and keratinocytes with OXT induced an increase in intracellular Ca^{2+} demonstrating the functionality of the OXTR in these skin cells. The specificity of this response was confirmed by either blocking Ca^{2+} signalling via OXTR knockdown or application of the OXTR antagonist L371,257. In smooth muscle cells, activation of the OXTR also induces G_q mediated increases in Ca^{2+} leading to contraction (56).

Depending on cell type, the activation of the OXTR is either associated with stimulation (14,57) or inhibition (11,44) of proliferation. We found a suppressive effect of OXT on the proliferation of dermal fibroblasts and keratinocytes. The regulation of proliferation by OXT is complex. It depends on expression levels and coupling of G_q and G_i protein isoforms, OXT concentration and OXTR recruitment to caveolae and cholesterol content in the cell membrane (15,44,58). The regulatory mechanisms underlying the modulation of proliferation by OXT signalling in dermal fibroblasts and keratinocytes need to be elucidated in future studies.

By inhibiting OXTR signalling, our data revealed an impact of OXT on the modulation of oxidative stress, intracellular GSH levels and cytokine release in dermal fibroblasts and keratinocytes. OXTR knockdown skin cells showed increased susceptibility to oxidative stress. This might indicate a cytoprotective role of OXT with regard to oxidative stress, as the formation of ROS leads to damage to nucleic acids, proteins and membrane lipids (59). OXT has anti oxidative activity by scavenging radicals and preventing the oxidation of lipoproteins (60). Furthermore, OXT improves the anti oxidative state of colonic tissue in a colitis model in rat (49) and balances ROS with cardioprotective efficacy in ischaemic rat heart (61). ROS formation is closely related to a decrease in GSH levels (62), thereby reducing the cellular anti oxidative defense (63,64). Consistent with higher ROS levels in OXTR knockdown dermal fibroblasts and keratinocytes, these cells also displayed decreased intracellular GSH concentrations. Sepsis induced pelvic inflammation is also associated with increased ROS and decreased GSH levels. OXT administration prevents the depletion of tissue GSH in this inflammatory condition (27). The treatment of pregnant rats with the OXTR

antagonist atosiban elevates oxidative stress in the hearts of the new borns (65). In keratinocytes, the effects of OXTR knockdown on ROS formation and GSH levels were less pronounced than in dermal fibroblasts, indicating a cell type specific susceptibility of skin cells to oxidative stress and its modulation by OXT. In accordance with this finding, Marionnet et al. (66) reported a higher sensitivity to oxidative stress of dermal fibroblasts in comparison with keratinocytes in UV irradiated skin models.

In contrast to dermal fibroblasts, keratinocytes released larger quantities of the cytokines IL6, CCL5 and CXCL10 in response to OXTR knockdown, thus confirming a cell type differential response to impaired OXT signalling. OXT has been shown to decrease the release of interleukins and other inflammatory mediators in various tissues and on a systemic level (19,31,49). In myometrial cells, OXT treatment reduces CCL5, CXCL5 and CCL20 expression (67). Administration of OXT in humans reduces endotoxin induced increases in plasma cytokines amongst others IL6 and CXCL10 (68).

The impact of OXT on the modulation of oxidative and inflammatory stress responses in skin cells suggests a role in chronic inflammatory skin conditions such as AD. Actually, OXT and its receptor were downregulated in dermal fibroblasts and keratinocytes derived from atopic skin. In a translational approach, we confirmed the aberrant expression of the OXT system in atopic skin as suction blister derived epidermis and fluids from AD patients displayed decreased OXTR and OXT levels.

The dynamic modulation of OXT and OXTR expression has been demonstrated in various studies: The OXT system is highly regulated during gestation, parturition and lactation, thus enabling circulating OXT to target a particular organ depending on the precisely regulated tissue specific expression of the OXTR (69,70). Furthermore, the OXTR is regulated by oestrogen, foetal bovine serum and lysophospholipids *in vitro* (71,72). The OXTR promoter also displays several potential interleukin response elements (73) explaining the negative regulation of OXTR gene expression by IL6, IL1 β and interferon (7,8). Sepsis, which is associated with massive production of inflammatory mediators and neuroendocrine alterations, decreases OXT gene expression in the brain (74). As the skin of AD patients is chronically inflamed, displaying a plethora of up regulated cytokines (75–77), elevated levels of inflammatory mediators might account for the decreased OXT and OXTR gene expression in atopic skin. As a consequence, deficits in OXT signalling possibly enhance oxidative stress and inflammation in atopic skin resulting in a vicious circle.

Altogether, we identified the OXT system as a novel neuroendocrine mediator of skin cell proliferation and stress responses *in vitro*. We speculate that the OXT system is also involved in skin neuroendocrine networks. Cutaneous CRH/POMC system activity is, amongst others, determined by cytokine release and is proposed to form a function analogous to the HPA axis (23,25,78). As OXT plays a role in modulating the HPA axis (79–81) and CRH signalling in term 'myometrium' (24), it is tempting to suggest that OXT might also interact with the skin's local neuroendocrine mediators, such as CRH. Moreover, the OXT promoter can be stimulated by oestrogen receptors and thyroid hormone receptors (82,83), which are also produced by skin cells (21,84–86). In line with the finding that the skin shares numerous mediators with the CNS (22), OXT turns out to be a new candidate of a brain and skin derived neuropeptide. Additionally, as we were able to dem

onstrate OXTR expression by skin cells, either skin cell derived or plasma derived OXT might signal in an autocrine as well as in a paracrine way in the skin. This would link into the hypothesized bidirectional brain skin connection (22,87).

In summary, the OXT system was characterized in human skin demonstrating its relevance in skin physiology. Dermal fibroblasts and keratinocytes responded to OXT via Ca^{2+} influx, thus proving functionality of the OXTR in skin cells. OXTR knockdown via siRNA or inhibition by a specific antagonist revealed an impact of OXT on the modulation of proliferation, ROS formation, GSH content and cytokine release in dermal fibroblasts and keratinocytes. The effects of OXT signalling on skin cell physiology point

to a role of OXT in cutaneous inflammation. Indeed, we found a reduced expression of the OXT system in AD skin indicating its clinical relevance to chronic inflammatory skin diseases.

Author contribution

V. D., J. K., F. S. and G. N. designed the research. V. D. and A. G. performed the research. V. D. and G. N. analysed the data. F. S. and H. W. contributed essential reagents and tools. V. D. and D. R. wrote the paper. A. B. and G. N. critically revised the manuscript. All authors have approved the submitted and final version of the manuscript and confirm that the submitted work complies with the ethical policies of *Experimental Dermatology*.

Conflict of interests

The authors have declared no conflicting interests.

References

- Kirsch P, Esslinger C, Chen Q *et al.* *J Neurosci* 2005; **25**: 11489–11493.
- Ferguson J N, Young L J, Hearn E F *et al.* *Nat Genet* 2000; **25**: 284–288.
- Gimpl G, Fahrenholz F. *Physiol Rev* 2001; **81**: 629–683.
- Gutkowska J, Jankowski M. *Pharmaceuticals* 2009; **2**: 168–183.
- Brownstein M J, Russell J T, Gainer H. *Science* 1980; **207**: 373–378.
- Vrachnis N, Malamas F M, Sifakis S *et al.* *Int J Endocrinol* 2011; **2011**: 350546.
- Ivell R, Bathgate R A, Walther N *et al.* *Adv Exp Med Biol* 1998; **449**: 297–306.
- Schmid B, Wong S, Mitchell B F. *Endocrinology* 2001; **142**: 1380–1385.
- Peter J, Burbach H, Adan R A *et al.* *Cell Mol Neurobiol* 1995; **15**: 573–595.
- Verbalis J G. *Front Neuroendocrinol* 1999; **20**: 146–156.
- Rimoldi V, Reversi A, Taverna E *et al.* *Oncogene* 2003; **22**: 6054–6060.
- Zhou X B, Lutz S, Steffens F *et al.* *Mol Endocrinol* 2007; **21**: 740–752.
- Cassoni P, Marrocco T, Bussolati B *et al.* *Mol Cancer Res* 2006; **4**: 351–359.
- Cassoni P, Sapino A, Marrocco T *et al.* *J Neuroendocrinol* 2004; **16**: 362–364.
- Reversi A, Cassoni P, Chini B. *J Mammary Gland Biol Neoplasia* 2005; **10**: 221–229.
- Cassoni P, Sapino A, Stella A *et al.* *Adv Exp Med Biol* 1998; **449**: 245–246.
- Bakos J, Strbak V, Ratulovska N *et al.* *Cell Mol Neurobiol* 2012; **32**: 891–896.
- Amico J A, Mantella R C, Vollmer R R *et al.* *J Neuroendocrinol* 2004; **16**: 319–324.
- Ivell R, Russell J A. *Rev Reprod* 1996; **1**: 13–18.
- Neumann I D. *Prog Brain Res* 2002; **139**: 147–162.
- Slominski A, Wortsman J. *Endocr Rev* 2000; **21**: 457–487.
- Slominski A T, Zmijewski M A, Skobowiat C *et al.* *Adv Anat Embryol Cell Biol* 2012; **212**: v, vii, 1–115.
- Slominski A, Wortsman J, Luger T *et al.* *Physiol Rev* 2000; **80**: 979–1020.
- Grammatopoulos D K, Hillhouse E W. *Endocrinology* 1999; **140**: 585–594.
- Slominski A, Pisarchik A, Tobin D J *et al.* *Endocrinology* 2004; **145**: 941–950.
- Slominski A, Ermak G, Hwang J *et al.* *FEBS Lett* 1995; **374**: 113–116.
- Iseri S O, Sener G, Saglam B *et al.* *J Surg Res* 2005; **126**: 73–81.
- Vitalo A, Frichione J, Casali M *et al.* *PLoS ONE* 2009; **4**: e5523.
- Iseri S O, Gedik I E, Erzik C *et al.* *Burns* 2008; **34**: 361–369.
- Padhy B M, Kumar V L. *Mediators Inflamm* 2005; **2005**: 360–365.
- Szeto A, Nation D A, Mendez A J *et al.* *Am J Physiol Endocrinol Metab* 2008; **295**: E1495–E1501.
- De Benedetto A, Agnihotri R, McGirt L Y *et al.* *J Invest Dermatol* 2009; **129**: 14–30.
- Proksch E, Folster Holst R, Jensen J M. *J Dermatol Sci* 2006; **43**: 159–169.
- Valyi Nagy I, Jensen P J, Albelda S M *et al.* *J Invest Dermatol* 1992; **99**: 350–356.
- Matsuura H, Sakaue M, Subbaramaiah K *et al.* *J Biol Chem* 1999; **274**: 29138–29148.
- Mascia F, Mariani V, Girolomoni G *et al.* *Am J Pathol* 2003; **163**: 303–312.
- Giustizieri M L, Mascia F, Frezzolini A *et al.* *J Allergy Clin Immunol* 2001; **107**: 871–877.
- Denda S, Takei K, Kumamoto J *et al.* *Exp Dermatol* 2012; **21**: 535–537.
- Kiistala U. *J Invest Dermatol* 1968; **50**: 129–137.
- Roggkamp D, Falkner S, Stab F *et al.* *J Invest Dermatol* 2012; **132**: 1892–1900.
- Amico J A, Seif S M, Robinson A G. *J Clin Endocrinol Metab* 1981; **52**: 988–993.
- Paredes J, Szeto A, Levine J E *et al.* *Psychoneuroendocrinology* 2006; **31**: 1062–1075.
- Eriksson M, Uvnas Moberg K. *Neuroendocrinology* 1990; **51**: 237–240.
- Guzzi F, Zanchetta D, Cassoni P *et al.* *Oncogene* 2002; **21**: 1658–1667.
- Novak E J, Rabinovitch P S. *Cytometry* 1994; **17**: 135–141.
- Dengler W A, Schulte J, Berger D P *et al.* *Anti cancer Drugs* 1995; **6**: 522–532.
- Izykowska I, Cegielski M, Gebarowska E *et al.* *In Vivo* 2009; **23**: 739–745.
- Holt Lunstad J, Birmingham W A, Light K C. *Psychosom Med* 2008; **70**: 976–985.
- Iseri S O, Sener G, Saglam B *et al.* *Peptides* 2005; **26**: 483–491.
- Tsuboi H, Kouda K, Takeuchi H *et al.* *Br J Dermatol* 1998; **138**: 1033–1035.
- Tsukahara H, Shibata R, Ohshima Y *et al.* *Life Sci* 2003; **72**: 2509–2516.
- Kinsey C G, Bussolati G, Bosco M *et al.* *J Cell Mol Med* 2007; **11**: 96–110.
- Light K C, Grewen K M, Amico J A. *Biol Psychol* 2005; **69**: 5–21.
- Lund I, Ge Y, Yu L C *et al.* *Eur J Neurosci* 2002; **16**: 330–338.
- Stock S, Uvnas Moberg K. *Acta Physiol Scand* 1988; **132**: 29–34.
- Yazawa H, Hirasawa A, Horie K *et al.* *Br J Pharmacol* 1996; **117**: 799–804.
- Tom N, Assinder S J. *Int J Biochem Cell Biol* 2010; **42**: 202–205.
- Busnelli M, Sauliere A, Manning M *et al.* *J Biol Chem* 2012; **287**: 3617–3629.
- Girard P M, Pozzebon M, Delacote F *et al.* *DNA Repair (Amst)* 2008; **7**: 1500–1516.
- Moosmann B, Behl C. *Mol Pharmacol* 2002; **61**: 260–268.
- Faghihi M, Alizadeh A M, Khor V *et al.* *Peptides* 2012; **37**: 314–319.
- Moysan A, Marquis I, Gaboriau F *et al.* *J Invest Dermatol* 1993; **100**: 692–698.
- Lu S C. *Mol Aspects Med* 2009; **30**: 42–59.
- Valko M, Leibfritz D, Moncol J *et al.* *Int J Biochem Cell Biol* 2007; **39**: 44–84.
- Simsek Y, Celik O, Karaer A *et al.* *Arch Gynecol Obstet* 2012; **285**: 655–661.
- Marionnet C, Pierrard C, Lejeune F *et al.* *PLoS ONE* 2010; **5**: e12059.
- Hua R, Pease J E, Sooranna S R *et al.* *Endocrinology* 2012; **153**: 481–491.
- Clodi M, Vila G, Geyeregger R *et al.* *Am J Physiol Endocrinol Metab* 2008; **295**: E686–E691.
- Soloff M S, Alexandrova M, Fernstrom M J. *Science* 1979; **204**: 1313–1315.
- Breton C, Di Scala Guenot D, Zingg H H. *J Mol Endocrinol* 2001; **27**: 175–189.
- Bale T L, Dorsa D M. *Endocrinology* 1997; **138**: 1151–1158.
- Jeng Y J, Soloff S L, Anderson G D *et al.* *Endocrinology* 2003; **144**: 61–68.
- Inoue T, Kimura T, Azuma C *et al.* *J Biol Chem* 1994; **269**: 32451–32456.
- Oliveira Pelegrin G R, Aguilera F A, Basso P J *et al.* *Peptides* 2010; **31**: 1847–1852.
- Gittler J K, Shemer A, Suarez Farinas M *et al.* *J Allergy Clin Immunol* 2012; **130**: 1344–1354.
- Raap U, Weissmantel S, Gehring M *et al.* *Pediatr Allergy Immunol* 2012; **23**: 285–288.
- Nomura I, Goleva E, Howell M D *et al.* *J Immunol* 2003; **171**: 3262–3269.
- Slominski A, Wortsman J, Tuckey R C *et al.* *Mol Cell Endocrinol* 2007; **265–266**: 143–149.
- Windle R J, Shanks N, Lightman S L *et al.* *Endocrinology* 1997; **138**: 2829–2834.
- Neumann I D, Torner L, Wigger A. *Neuroscience* 2000; **95**: 567–575.
- Neumann I D, Wigger A, Torner L *et al.* *J Neuroendocrinol* 2000; **12**: 235–243.
- Adan R A, Cox J J, Beischlag T V *et al.* *Mol Endocrinol* 1993; **7**: 47–57.
- Richard S, Zingg H H. *J Biol Chem* 1990; **265**: 6098–6103.
- Torma H, Rollman O, Vahlquist A. *Acta Derm Venereol* 1993; **73**: 102–107.
- Hasselquist M B, Goldberg N, Schroeter A *et al.* *J Clin Endocrinol Metab* 1980; **50**: 76–82.
- Punnonen R, Lovgren T, Kouvonen I. *J Endocrinol Invest* 1980; **3**: 217–221.
- Arck P C, Slominski A, Theoharides T C *et al.* *J Invest Dermatol* 2006; **126**: 1697–1704.