DOI: 10.1111/j.1600-0625.2012.01507.x
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Letter to the Editor

Oxytocin is expressed in epidermal keratinocytes and released upon stimulation with adenosine 5′-[γ-thio]triphosphate *in vitro*

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Abstract: Oxytocin is a neuropeptide produced primarily in the hypothalamus and is best known for its roles in parturition and lactation. It also influences behaviour, memory and mental state. Recent studies have suggested a variety of roles for oxytocin in peripheral tissues, including skin. Here we show that oxytocin is expressed in human skin. Immunohistochemical studies showed that oxytocin and its carrier protein, neurophysin I, are predominantly localized in epidermis. RT-PCR confirmed the expression of oxytocin in both skin and cultured epidermal keratinocytes. We also show that oxytocin is released from keratinocytes after application of adenosine 5′-[γ-thio]triphosphate (ATPγS, a stable analogue of ATP) in a dose-dependent manner. The ATPγS-induced oxytocin release was inhibited by removal of extracellular calcium, or by the P2X receptor antagonist 2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP). These results suggest that oxytocin is produced in human epidermal keratinocytes and is released in response to calcium influx via P2X receptors.

Abbreviations: ATPγS, adenosine 5′-[γ-thio]triphosphate; OT, oxytocin; RT, reverse transcription; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate.

Key words: ATP – keratinocyte – oxytocin – P2X receptor

Accepted for publication 5 April 2012

Background

Various similarities have been found between brain and epidermis, as might be expected, because both of them have a common embryonic origin (1). For example, epidermal keratinocytes synthesize a variety of neuropeptides (2, 3) and neurotransmitters (4–6).

Oxytocin (OT) is produced in the supraoptic and paraventricular nuclei of hypothalamus and is secreted from the posterior pituitary into blood (7). It contracts smooth muscle during parturition and lactation (8). It is also involved in behaviour, memory and social bonding (9, 10). Systemic OT infusion reduced the repetitive behaviour of patients with autism and Asperger's syndrome (11). Moreover, tactile stimuli increased plasma OT level and influenced emotional state (12, 13). Thus, OT might be a mediator between tactile sensation and emotional state.

Recent studies suggest that OT has important roles in skin. OT inhibited latex-induced inflammatory hyperalgesia (14), and improved skin damage after hot water treatment (15).

Questions addressed

Is OT produced by epidermal keratinocytes and secreted from the epidermis in response to external stimuli?

Experimental design

We examined the presence of OT in human skin and epidermal keratinocytes by means of immunohistochemistry and RT-PCR. We also measured release of OT from cultured human keratinocytes. Methods are described in detail in the supplementary information.

Results

Immunoreactivity to OT antibody was observed throughout the epidermis of human skin (Fig. 1a). The specificity of the staining was confirmed by blocking experiments with OT peptide (Fig. S1). Prepro-OT mRNA contains regions encoding OT and neurophysin I (16, Fig. S2). As OT is synthesized together with its carrier protein neurophysin I, and they exist as a complex in secretory vesicles in neuronal cytoplasm, we next tried to detect neurophysin I protein in epidermis. Immunoreactivity to neurophysin I antibody was also observed throughout the epidermis (Fig. 1c). Immunoreactivity to OT and neurophysin I antibodies was also observed in both proliferative and differentiating keratinocytes (Fig. 1e–h). Western blotting analysis showed a single 14 kDa band of neurophysin I protein in human epidermis, as well as in pituitary gland (Fig. 1i). Conventional RT-PCR showed that prepro-OT mRNA was present in human skin and keratinocytes (Fig. 1j). The OT expression in keratinocytes was not significantly changed after the induction of differentiation by high Ca^{2+} (Fig. S3). To confirm that OT peptide is present in human epidermis and keratinocytes, the OT content of protein extracts from the tissues and cells was measured by EIA (Table S1). The OT content of human epidermis (160 ± 27 pg/g wet weight) was similar to that of sheep testis (60–130 pg/g by radioimmunoassay) (17). These data suggest that both epidermis and cultured keratinocytes synthesize oxytocin peptide.

As ATP is an important signalling molecule for epidermis and keratinocytes (4, 18) and induces cytokine release (19), we hypothesized that ATP also stimulates keratinocytes to release OT. To test this hypothesis, we first compared the OT-releasing effects of ATP and ATPγS (adenosine 5′-[γ-thio]triphosphate tetralithium salt; a stable analogue of ATP) (20) and found that ATPγS is more potent than ATP, and the effect of ATP showed considerable variation (data not shown). As the variability seemed likely to be a consequence of hydrolysis of ATP in the keratinocyte medium, we

Experimental Dermatology

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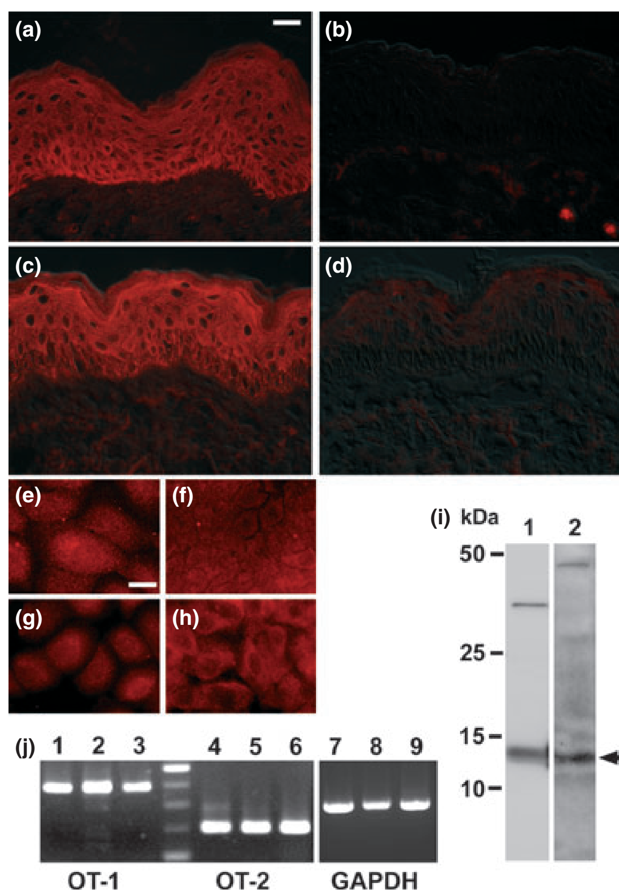

Figure 1. Expression of oxytocin (OT) and neurophysin I in skin and epidermal keratinocytes. (a–h) Immunohistochemical study of human skin (a–d) and cultured keratinocytes, both proliferative (e, g) and differentiating (f, h). (a, e, f) Anti-OT antibody; (c, g, h) anti-neurophysin I; (b, d) without primary antibodies, negative control for (a, c), respectively. Scale bar = 20 μm. (i) Western blot analysis with neurophysin I antibody. Lane 1, bovine pituitary extract; lane 2, human epidermis. (j) Detection of OT expression by RT-PCR. Lanes 1, 4, 7, brain (positive control for OT); lanes 2, 5, 8, skin; lanes 3, 6, 9, differentiating keratinocytes (2 days after induction). The PCR product sizes obtained with primer sets OT-1 and OT-2 were 390 and 201 bp, respectively.

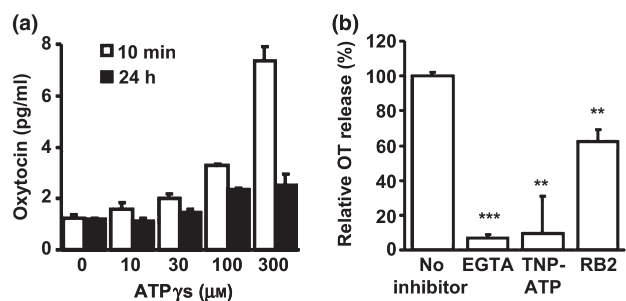

Figure 2. oxytocin (OT) release from cultured keratinocytes. (a) Dose dependence of the effect of ATPγS. Differentiating keratinocytes (2 days after induction) were incubated in the presence of the indicated concentration of ATPγS. After 10 min, the medium was collected and replaced with fresh medium containing ATPγS, and then incubation was continued at 37°C for 24 h. OT in the collected media was measured by EIA, and the data were expressed as concentration in the medium. (b) Effect of inhibitors. Differentiating keratinocytes were treated with the indicated reagents for 5 min before and during adenosine 5′-[γ-thio]triphosphate (ATPγS) application (300 μM) for 10 min. Then, the OT content in the medium was measured and expressed as% of that with ATPγS alone (No inhibitor). EGTA, 2 mM: 2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP), 100 nM; RB2, Reactive Blue 2, 50 μM. Asterisks denote statistical significance as compared to No inhibitor (n = 3). $**P < 0.01$, $***P < 0.001$.

used ATPγS instead of ATP for further analysis. As shown in Fig. 2a, OT was released from keratinocytes in the presence of ATPγS, in a dose-dependent manner. The OT release was detected within 10 min after ATPγS application. As the OT content in the medium did not increase during the subsequent 24 h, ATPγS-induced OT release may represent transient secretion from the intracellular pool, rather than *de novo* synthesis of the peptide.

Removal of Ca^{2+} from the medium with EGTA and application of the P2X receptor antagonist 2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP) significantly blocked the OT release induced by ATPγS (Fig. 2b). The P2Y and P2X2 receptor antagonist Reactive Blue 2 (21, 22) also inhibited the release.

Discussion and conclusions

In mouse hypothalamus, the intracellular Ca^{2+} concentration is amplified via ryanodine receptors after electrical signalling and calcium influx, and the increase of intracellular Ca^{2+} concentration induces OT secretion; for normal social behaviour, the sensitivity of the ryanodine receptors must be increased by cyclic ADP-ribose, the product of CD38 enzymatic activity (23). As epidermal keratinocytes express ryanodine receptors (24), the endogenous mechanism of OT secretion from epidermal keratinocytes might be similar to that of hypothalamus.

OT release by ATPγS was blocked by the removal of Ca^{2+}, and this supports the idea that the influx of calcium ion might be the trigger for OT release. Moreover, application of TNP-ATP also blocked the OT release. Previous reports demonstrated that multiple P2X receptors are expressed in keratinocytes (25, 26). Thus, OT generated in epidermal keratinocytes might be released in response to calcium influx induced by activation of these P2X receptors.

Oxytocin inhibits the ATP-activated current in dorsal root ganglion neurons (27). Further, ATP induces pain mediated by P2X3 receptor on dorsal root ganglion (28). ATP was released from epidermal keratinocytes upon barrier insult or air exposure (4, 29) and it might induce pain via P2X3 in peripheral nerve fibres. As we showed in the present study that OT is released from keratinocytes in response to a stable analogue of ATP, it is plausible that OT is released from epidermal keratinocytes in response to ATP release after barrier function impairment and plays a role in regulating skin nociception.

Tactile stimuli might induce ATP secretion from epidermis (30), leading to OT release from epidermal keratinocytes. As brain-derived OT and epidermis-derived OT in the blood cannot be distinguished, it will be necessary to produce keratinocyte-specific OT knockout mice to further evaluate these ideas. Further investigation of the roles of epidermis-derived OT may lead to new treatment methodologies for both skin and mental disorders.

Author contributions

SD and MD designed the study. SD, KT and JK performed the research. MG and MT contributed the essential reagents or tools. SD analysed the data. SD and MD wrote the paper.

Conflict of interests

The authors have declared no conflicting interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Peptide-blocking of anti-OT antibody staining.

Figure S2. Structure of prepro-OT mRNA.

Figure S3. Quantitative PCR analysis of OT mRNA level in keratinocytes.

Table S1. Oxytocin content.

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DOI: 10.1111/j.1600-0625.2012.01511.x
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Letter to the Editor

Pheomelanin in the skin of *Hymenochirus boettgeri* (Amphibia: Anura: Pipidae)

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Abstract: Pheomelanin is supposed to be the first type of melanin found in vertebrates, in contrast to the main type – eumelanin. Our study aimed at detecting pheomelanin in the skin of *Hymenochirus boettgerii*. We employed electron paramagnetic resonance (EPR) spectroscopy, and transmission electron microscopy (TEM), supplemented with standard histology and immunochemistry. We identified pheomelanin in the dorsal skin of adult frogs (not only in the dermis, but also in the epidermis) and in the dorsal tadpole. Our work identifies *Hymenochirus boettgerii* as a model in the basic study on the mechanism, evolution and role of melanogenesis in animals, including human.

Key words: amphibians – melanocytes – melanogenesis – pigmentation – UV.

Accepted for publication 11 April 2012

Background

Vertebrates synthesize two types of melanin in the skin: black eumelanin and yellow–red pheomelanin, in various proportions (1,2). Both are the end-products of complex reactions of oxidation and polymerization of L-tyrosine with (pheomelanin) or without contribution of L-cysteine (eumelanin). Eumelanins are polymorphous nitrogenous biopolymers of dihydroxyindole (DHI) and DHI-carboxylic acid (DHICA), while pheomelanins have the backbone of benzothiazine units. Both contain free radical paramagnetic centres embodied deeply in their structure, but because of the difference in the character of their monomers, the centres reveal different character, namely of semiquinone (eumelanin) or semiquinonimine (pheomelanin) (3). The latter contain nitrogen atom in their structure, affecting their electron paramagnetic resonance (EPR) properties, placing EPR among the most important research technique in melanin study (1–5).

Despite production of only two types of melanins, colouration of vertebrate skin is rich and diverse. In the most ancestral tetra-

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