Organic osmolytes increase expression of specific tight junction proteins in skin and alter barrier function in keratinocytes*

C. El-Chami 1,2 A.R. Foster 1 C. Johnson 2 R.P. Clausen 3 P. Cornwell 4 I.S. Haslam 1,5 M.C. Steward 6 R.E.B. Watson 1,7 H.S. Young 1,8 and C.A. O’Neill 1

1Centre for Dermatology Research, Division of Musculoskeletal and Dermatological Sciences, School of Biological Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Oxford Road, Manchester M13 9PT, UK
2School of Electrical and Electronic Engineering, Faculty of Science and Engineering, University of Manchester, Oxford Road, Manchester M13 9PT, UK
3Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
4TRI Princeton, 601 Prospect Avenue, Princeton, NJ 08540, USA
5Department of Biological Sciences, School of Applied Sciences, University of Huddersfield, Queensgate, Huddersfield HD1 3DH, UK
6Division of Diabetes, Endocrinology and Gastroenterology, School of Medical Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Oxford Road, Manchester M13 9PT, UK
7NIHR Manchester Biomedical Research Centre, Manchester University NHS Foundation Trust, Manchester Academic Health Science Centre, Manchester, UK
8Department of Dermatology, Salford Royal NHS Foundation Trust, Manchester, UK

Linked Comment: Benedetto. Br J Dermatol 2021; 184:388–389.

Summary

Background The epidermal barrier is important for water conservation, failure of which is evident in dry-skin conditions. Barrier function is fulfilled by the stratum corneum, tight junctions (TJs, which control extracellular water) and keratinocyte mechanisms, such as organic osmolyte transport, which regulate intracellular water homeostasis. Organic osmolyte transport by keratinocytes is largely unexplored and nothing is known regarding how cellular and extracellular mechanisms of water regulation may interact.

Objectives We aimed to characterize osmolyte transporters in skin and keratinocytes, and, using transporter inhibitors, to investigate whether osmolytes can modify TJs. Such modification would suggest a possible link between intracellular and extracellular mechanisms of water conservation may interact.

Methods Immunostaining and quantitative polymerase chain reaction of organic osmolyte-treated organ-cultured skin were used to identify changes to organic osmolyte transporters, and TJ protein and gene expression. TJ functional assays were performed on organic osmolyte-treated primary human keratinocytes in vitro.

Results Immunostaining demonstrated the expression of transporters for betaine, taurine and myo-inositol in transporter-specific patterns. Treatment of human skin with either betaine or taurine increased the expression of claudin-1, claudin-4 and occludin. Osmolyte transporter inhibition abolished this response. Betaine and taurine increased TJ function in primary human keratinocytes in vitro.

Conclusions Treatment of skin with organic osmolytes modulates TJ structure and function, which could contribute to the epidermal barrier. This emphasizes a role for organic osmolytes beyond the maintenance of intracellular osmolarity. This could be harnessed to enhance topical therapies for diseases characterized by skin barrier dysfunction.

*Plain language summary available online
DOI 10.1111/bjd.19162
At the interface between the body and the outside world, skin faces major challenges in terms of water conservation, as there is a constant driving force for water to leave the skin. The epidermis contains two barriers to water loss: the hydrophobic stratum corneum and tight junction (TJ) complexes in the granular layer. Although considered unimportant for some years, TJs have been shown to play a direct role in the barrier to water loss from the epidermis.

Both the stratum corneum and TJ barrier function can be compromised in response to stressors such as ultraviolet (UV) radiation and wounding, and their functions also decline with age. Skin barrier function is also disrupted in inflammatory skin diseases like rosacea, psoriasis and atopic dermatitis (AD). AD is the most common chronic skin disease worldwide and affects about 20% of children and 5% of adults. In AD, defects in skin barrier functioning are considered the initial step in development of the disease.

Transepidermal water loss (TEWL) is a noninvasive measurement used to evaluate skin barrier function, and patients with AD have increased TEWL, reflecting skin barrier dysfunction in the disease.

In situations where barrier function is compromised, the driving force for water to leave the skin increases, which increases the risk of skin dehydration. To counteract this many skincare and emollient products contain humectants. These are compounds with hygroscopic properties, which attract water and trap it in the stratum corneum.

Moisturizers perform critical roles in the management of dry-skin conditions such as AD by improving skin barrier function. Furthermore, recent studies have demonstrated that increased TEWL in infancy predicts the development of AD at 1 year and that early use of moisturizers may reduce rates of development of AD.

One such humectant commonly used in prescribed and over-the-counter emollient preparations is betaine. Betaine belongs to a class of naturally occurring molecules known as the organic osmolytes, which also include taurine, myo-inositol, sorbitol, sarcosine and others. Studies in osmotically challenged tissues such as the kidney have demonstrated that organic osmolytes are transported into cells via specific transporters when cells are in danger of shrinkage. Limited studies have demonstrated that keratinocytes also use organic osmolytes to prevent potential changes to cell volume when they are at risk of osmotic stress. However, the mechanisms involved in osmolyte transport within keratinocytes are poorly defined. To date only TAUT, the sodium- and chloride-dependent taurine transporter (TAUT), is known to be expressed in the epidermis.

There is also a dearth of functional studies, which for the most part, have been performed only in isolated keratinocytes. These studies suggest that insults such as osmotic stress and UV radiation result in the intracellular accumulation of osmolytes to preserve keratinocyte volume. Other studies have suggested that taurine may also have a role in protecting keratinocytes against UVB.

To date few studies have addressed the potential for organic osmolytes to have effects independently of cell volume regulation, such as might occur when these molecules are applied to the skin as topical therapy. A single study in rat organotypic cultures demonstrated that betaine could induce changes to gene expression in this system, suggesting a potential role in cell signalling for organic osmolytes. A recent study showed that organic osmolytes preserved TJ integrity and function in UVB-irradiated rat epidermal keratinocytes. Thus, application of organic osmolytes in topical preparations may have direct effects on the barrier independently of the humectant and cell-volume-preserving properties of these molecules. These barrier effects could make their addition to topical treatments for dry-skin conditions extremely valuable.

We reasoned that control of water might be an integrated process in skin wherein the mechanisms controlling intra- and extracellular water may be interlinked. In particular, we hypothesized that if osmolytes can act as signalling molecules,
they may exert some of their effects on TJ structure and function. Therefore, the aims of this study were as follows. Firstly, to demonstrate the protein expression and localization of betaine, taurine and myo-inositol transporters in human skin. Secondly, to investigate the effects of organic osmolytes on TJ protein expression and function in keratinocytes and organ-cultured ex vivo human skin.

Materials and methods

Ex vivo skin organ culture

Human skin was obtained from eight healthy adults undergoing liposculpture procedures. The study was approved by the North West Research Ethics Committee (reference 14/NW/0185). All patients gave written, informed consent. Three 4-mm biopsy punches per donor (Integra Millex; Fisher Scientific, Loughborough, UK) were cultured for 72 h in 12-well plates at 37 °C and 5% CO₂–95% air. The culture medium – William’s E supplemented with 1% (v/v) l-glutamine, 1% (v/v) penicillin-streptomycin, 0-02% (v/v) hydrocortisone and 0-1% (v/v) insulin – was changed daily. In some experiments the medium was supplemented with 35 mmol L⁻¹ betaine, taurine or myo-inositol, with or without inhibitors of their transporters (Appendix S1; see Supporting Information). After culture, biopsies were embedded in optimal cutting temperature compound, snap frozen in liquid nitrogen and stored at −80 °C.

Immunostaining

Frozen sections (Appendix S1; see Supporting Information) from all eight volunteers were air dried at room temperature then fixed with methanol-acetone mix (50:50 v/v) for 20 min at −20 °C for TJ proteins, sodium/myo-inositol cotransporter (SMIT); or 100% acetone for betaine–γ-aminobutyric acid transporter (BGT)-1 and TAUT. Sections were then permeabilized with 0-5% Triton X-100 at room temperature, then blocked using 1% (w/v) bovine serum albumin and 10% (v/v) normal goat serum solution for 1 h. Slides were then incubated overnight at 4 °C with the corresponding antibody (Table 1), diluted in block solution. Slides were washed with Tris-buffered saline and labelled with a goat antimouse IgG conjugated to Alexa Fluor 488 (Thermo Fisher Scientific, Altrincham, UK) for 1 h at room temperature then counterstained by incubation for 1 min with 4’,6-diamidino-2-phenylindole (Sigma-Aldrich, St Louis, MO, USA). Slides were mounted and fluorescence intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

RNA extraction and quantitative reverse-transcriptase polymerase chain reaction

Total RNA was extracted from human tissue using the RNeasy Plus Universal Kit (Qiagen, Manchester, UK) according to the manufacturer’s instructions. cDNA was synthesized from 1 μg of total RNA using the cloned AMV first-strand cDNA synthesis kit (Invitrogen, Paisley, UK) according to the manufacturer’s instructions. Quantitative polymerase chain reaction was performed using Taqman gene expression assays (Applied Biosystems, Warrington, UK) (Table 2). Reactions were performed using the StepOne Plus Real-Time PCR machine (Applied Biosystems, Paisley, UK) with Taqman Fast Universal PCR Master Mix (Thermo Fisher Scientific) for 40 cycles. Samples were assayed in triplicate and gene expression changes were calculated using the comparative CT method. Relative expression was determined against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

Primary keratinocyte cultures

Normal human epidermal keratinocytes (NHEKs) (Promocell, Heidelberg, Germany) were maintained in keratinocyte basal serum solution for 1 h. Slides were then incubated overnight at 4 °C with the corresponding antibody (Table 1), diluted in block solution. Slides were washed with Tris-buffered saline and labelled with a goat antimouse IgG conjugated to Alexa Fluor 488 (Thermo Fisher Scientific, Altrincham, UK) for 1 h at room temperature then counterstained by incubation for 1 min with 4’,6-diamidino-2-phenylindole (Sigma-Aldrich, St Louis, MO, USA). Slides were mounted and fluorescence intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

**Table 1** List of primary antibody dilutions used in immunostaining and Western blot

| Antibody                  | Dilution used | Catalogue no. | Supplier       |
|---------------------------|---------------|---------------|----------------|
| Rabbit anti-claudin-1     | 1:50 (IF), 1:1000 (WB) | 71-7800       | Invitrogen     |
| Mouse anti-claudin-4      | 1:50 (IF), 1:1000 (WB) | 32-9400       | Invitrogen     |
| Rabbit anti-claudin-7     | 1:50 (IF)     | 34-9100       | Invitrogen     |
| Rabbit anti-claudin-12    | 1:50 (IF)     | 38-8200       | Invitrogen     |
| Mouse antioccludin        | 1:25 (IF)     | 33-1500       | Invitrogen     |
| Rabbit antioccludin       | 1:500 (WB)    | 71-1500       | Invitrogen     |
| Rabbit anti-BGT-1         | 1:50 (IF)     | HPA034973     | Sigma-Aldrich  |
| Rabbit anti-TAUT          | 1:25 (IF)     | HPA015028     | Sigma-Aldrich  |
| Rabbit anti-SMIT          | 1:50 (IF)     | AB5518        | Millipore      |
| Rabbit anti-HMIT          | 1:25 (IF)     | BMP026        | MBL            |
| Mouse anti-β-actin        | 1:10 000 (WB) | A1978         | Sigma-Aldrich  |

BGT, betaine–γ-aminobutyric acid transporter; HMIT, H⁺/myo-inositol cotransporter; IF, immunofluorescence; SMIT, sodium/myo-inositol cotransporter; TAUT, sodium- and chloride-dependent taurine transporter; WB, Western blot.
Table 2 List of predesigned TaqMan gene expression assays used in quantitative reverse-transcriptase polymerase chain reaction

| Gene name   | Species | Catalogue no. | Supplier                      |
|-------------|---------|---------------|-------------------------------|
| CLDN1       | Human   | Hs00221623_m1 | Thermo Fisher Scientific      |
| CLDN4       | Human   | Hs00976831_s1 | Thermo Fisher Scientific      |
| CLDN7       | Human   | Hs00600772_m1 | Thermo Fisher Scientific      |
| CLDN12      | Human   | Hs00273258_s1 | Thermo Fisher Scientific      |
| OCLN        | Human   | Hs00170162_m1 | Thermo Fisher Scientific      |
| SLC6A12 (BGT-1) | Human | Hs00758246_m1 | Thermo Fisher Scientific      |
| SLC6A6 (TAUT) | Human | Hs00161778_m1 | Thermo Fisher Scientific      |
| SLC5A3 (SMIT) | Human | Hs00272857_s1 | Thermo Fisher Scientific      |
| SLC2A13 (HMIT) | Human | Hs00369423_m1 | Thermo Fisher Scientific      |
| GAPDH       | Human   | Hs02758991_g1 | Thermo Fisher Scientific      |

BGT, betaine-γ-aminobutyracid transporter; HMIT, H^+/-myo-inositol cotransporter; SMIT, sodium/myo-inositol cotransporter; TAUT, sodium- and chloride-dependent taurine transporter.

were analysed by two-way ANOVA followed by Bonferroni’s correction. If only two experimental groups were investigated, a paired Student’s t-test was employed. Differences were considered statistically significant at a P-value < 0.05.

Results

Organic osmolyte transporters are expressed in human skin and induced by the presence of osmolytes

In agreement with a single previous study, the expression of TAUT was observed throughout the human epidermis (Figure 1a). BGT-1 was also expressed in all layers of the epidermis but with higher intensity in the granular layer (Figure 1b). However, SMIT and HMIT were expressed only in basal layers (Figure 1c, d).

To understand the relationship between the expression of transporters and the presence of organic osmolytes, we treated human skin in organ culture with doses of betaine, taurine or myo-inositol up to 50 mmol L⁻¹, which is the known taurine concentration in human tissues. Following 72-h incubation, the expression of the corresponding transporter was measured.

When skin biopsies were cultured in the presence of 35 mmol L⁻¹ betaine (which proved to be optimal for inducing expression; Appendix S2 and Figure S1; see Supporting Information), BGT-1 protein expression significantly increased (Figure 2a, b, c; P < 0.001 at 25 μm and P = 0.0011 at 125 μm distance from the stratum corneum). The addition of 10 μmol L⁻¹ N-(1-benzyl-4-piperidinyl)-2,4-dichlorobenzamide (BPDBA), a specific inhibitor of BGT-1, prevented the betaine-induced increase in BGT-1 expression (Figure 2c, e), but BPDBA did not affect the expression of BGT-1 per se (Figure 2d, e). The change in BGT-1 protein expression was complemented with a similar change in gene expression (Figure 2f; P = 0.0038).

In the presence of 35 mmol L⁻¹ taurine, the expression of TAUT significantly increased over that observed in untreated skin (Figure 3a, b, c; P < 0.001). Addition of 0.1 μmol L⁻¹ cyclosporin A (CsA), a TAUT inhibitor, to the culture
medium caused a significant reduction in TAUT protein expression throughout the epidermis (P < 0.001) (Figure 3c). Moreover, CsA also inhibited the taurine-induced increase in TAUT protein expression (Figure 3d, e).

Taurine-induced increases in TAUT protein expression were complemented by an increase in gene expression (Figure 3f). The addition of CsA had no effect on expression of the TAUT gene (SLC6A6) but abolished the taurine-induced increase in SLC6A6 mRNA (Figure 3f).

SMIT and HMIT were expressed only in the basal layer of the epidermis, and the presence of 35 mmol L⁻¹ myo-inositol had no effect on their protein or gene expression levels (Figure S2; see Supporting Information).

**Organic osmolytes enhance tight junction protein expression in organ-cultured human skin**

To determine whether TJ protein expression was altered following treatment with organic osmolytes, we examined claudins 1, 4, 7 and 12, and occludin protein and gene expressions, in organ-cultured human skin following 72-h treatment with osmolytes.

The protein and gene expressions of claudins 7 and 12 were not affected by the presence of the organic osmolytes betaine or taurine (Figure S3; see Supporting Information). Treatment with myo-inositol had no effect on the protein or gene expression of the examined TJ components (Figure S4; see Supporting Information). By contrast, protein expression of claudin-1 (P < 0.001), claudin-4 (P = 0.0012) and occludin (P < 0.001) all increased significantly in the presence of betaine (Figure 4a, b), but mRNA expression did not (Figure 4c).

While the presence of BPDBA alone in the culture medium had no impact on claudin-1, claudin-4 and occludin gene and protein expression (Figure S5; see Supporting Information), BPDBA did abolish the betaine-induced increase in claudin-1, claudin-4 and occludin (Figure 4a, b).

The presence of 35 mmol L⁻¹ taurine caused an increase in claudin-1 (P < 0.001), claudin-4 (P = 0.0017) and occludin (P < 0.001) protein expression (Figure 5a, b), an effect that was abolished by the presence of the TAUT inhibitor CsA. CsA alone had no effects on the gene or protein expression of these TJ proteins (Figure S5). Claudin-1 (P = 0.0021), claudin-4 (P < 0.001) and occludin (P < 0.001) gene expression also increased following incubation with taurine. This increase was abolished by the presence of CsA (Figure 5c).

**Organic osmolytes improve tight junction function in human primary cultured keratinocytes**

To assess the possible effects of osmolytes on TJ function, NHEKs were utilized as a model. However, we first investigated the expression of osmolyte transporters in NHEKs. We showed expression of both TAUT and BGT-1 in response to differentiation and substrate availability (Figure S6; see Supporting Information). Thus, NHEKs were a good model in which to investigate any crosstalk between osmolytes and TJs.

NHEKs were exposed to 5 mmol L⁻¹ betaine, taurine and myo-inositol (the optimal tested concentration; Figure S7; see Supporting Information). This resulted in a significant increase
in TEER, an established marker of TJ function, at 48 h with betaine \( P < 0.001 \), taurine \( P < 0.001 \) and myo-inositol \( P < 0.001 \). At 72 h this effect was maintained only by betaine \( P < 0.001 \) and taurine \( P < 0.001 \) (Figure 6a). Addition of organic osmolytes to already developed TJs also resulted in a significant increase in TEER at day 5 both \( P < 0.001 \); at day 6 betaine \( P = 0.0022 \) and taurine \( P < 0.001 \) (Figure 6b).

Similarly, the paracellular flux of a 4-kDa FITC–dextran in NHEKs treated with organic osmolytes for 72 h was lower than that measured in untreated cells. This decrease in dextran permeability was statistically significant in cells treated with betaine \( P < 0.001 \) and taurine \( P < 0.001 \) (Figure 6e). This significant improvement in TJ function was abolished by incubation of the cells with BPDBA or CsA (Figure 6c, d, f, g).

Immunoblotting demonstrated changes in protein expression of claudin-1, claudin-4 and occludin 72 h after incubation with 5 mmol L\(^{-1}\) organic osmolytes (Figure 7). Claudin-1 (betaine \( P = 0.021 \), taurine \( P = 0.028 \), myo-inositol \( P = 0.0035 \)) and claudin-4 (betaine \( P = 0.026 \), taurine \( P = 0.038 \), myo-inositol \( P = 0.010 \)) protein significantly increased in response to treatment with organic osmolytes in a similar manner to that seen in treated skin biopsies. Treatment with organic osmolytes had no effect on the protein expression of occludin (Figure 7).

**Discussion**

A single previous study investigated the expression of osmolyte transporters in human skin. Janeke et al. showed that TAUT was expressed in human epidermis with high expression in the stratum granulosum and no expression in the stratum basale layer. The present study expands on the previous

---

**Figure 2** Betaine–γ-aminobutyric acid transporter (BGT)-1 expression is augmented by the presence of betaine in human organ-cultured skin. BGT-1 expression in ex vivo human skin cultured for 72 h (a) under control conditions and in the presence of (b) 35 mmol L\(^{-1}\) betaine, (c) 35 mmol L\(^{-1}\) betaine and 10 μmol L\(^{-1}\) N-(1-benzyl-4-piperidinyl)-2,4-dichlorobenzamide (BPDBA) and (d) 10 μmol L\(^{-1}\) BPDBA. (e) Quantification of signal intensity as a function of distance from the stratum corneum (SC), showing a betaine-induced increase in BGT-1 protein expression in ex vivo skin, which does not occur in the presence of betaine and BPDBA. (f) BGT-1 gene expression increased 72 h after incubation with betaine, while its expression did not change with betaine and BPDBA. Data are presented as the mean ± SEM, two-way ANOVA, \( n = 8 \). Bars = 50 μm.

© 2020 The Authors. British Journal of Dermatology published by John Wiley & Sons Ltd on behalf of British Association of Dermatologists

British Journal of Dermatology (2021) 184, pp482–494
work by demonstrating the presence of transporters for betaine and myo-inositol in human skin.

We show that TAUT is uniformly expressed throughout the living layers of the human epidermis, which differs from the expression pattern reported by Janeke et al. This may be due to differences in the antibodies used in the two studies. The present study used a specific antibody, whereas Janeke et al. used antiserum. Alternatively, the difference may be due to time in culture, or anatomical site differences in the expression pattern.

The present study is the first to show BGT-1 expression throughout the living layers of the epidermis, with higher expression towards the granular layer. The stratum granulosum forms the outermost keratinocyte layer, and following stratum corneum disruption will be the first viable layer exposed to the outside environment. BGT-1 might therefore exhibit higher expression in the stratum granulosum as a first line of defence to maintain cell volume when under osmotic stress.

SMIT localizes only at the basal layer of the epidermis. For the first time, we also demonstrate the expression in skin of HMIT, which, like SMIT, shows predominantly basal expression. SMIT is induced by osmotic stress and is involved in the intracellular accumulation of myo-inositol in mammals. The role of HMIT is less well understood; myo-inositol is a key regulator of osmolarity, and, given the importance of the basal layer stem cells, it is tempting to speculate that expression of myo-inositol transporters may be crucial in protecting stem cells from volume disruption. However, myo-inositol is also a precursor of phosphatidylinositol, an important signalling molecule. Thus, a signalling role for myo-inositol within the epidermis cannot be excluded.

Figure 3 Sodium- and chloride-dependent taurine transporter (TAUT) gene and protein expression is induced by taurine in organ-cultured human skin. TAUT expression in organ-cultured human skin incubated for 72 h (a) under control conditions and in the presence of (b) 35 mmol L\(^{-1}\) taurine, (c) 0.1 μmol L\(^{-1}\) ciclosporin A (CsA) and (d) 35 mmol L\(^{-1}\) taurine and 0.1 μmol L\(^{-1}\) CsA. (e) Fluorescence intensity quantification showing an increase in TAUT protein expression in the presence of taurine, whereas the presence of CsA negated this increase. (f) TAUT gene expression increased 72 h after incubation with taurine, while it was similar to that in control conditions in cultures treated with CsA and taurine. Data are presented as the mean ± SEM, two-way ANOVA, n = 8. Bars = 50 μm.
Figure 4 Betaine increases tight junction protein expression in human organ-cultured skin. (a) Claudin-1, claudin-4 and occludin immunofluorescence in ex vivo human skin cultured for 72 h under control conditions, in the presence of 35 mmol L⁻¹ betaine, 10 μmol L⁻¹ N-(1-benzyl-4-piperidinyl)-2,4-dichlorobenzamide (BPDBA), and both 10 μmol L⁻¹ BPDBA and 35 mmol L⁻¹ betaine. (b) Quantification of fluorescence intensity showed that the presence of BPDBA mitigated the increase in expression of all three tight junction proteins caused by betaine. (c) Claudin-1, claudin-4 and occludin gene expression was not affected by the presence of betaine. Data are presented as the mean ± SEM, two-way ANOVA, n = 7. Bars = 50 μm. SC, stratum corneum.
Figure 5 Taurine increases tight junction protein expression in human organ-cultured skin. (a) Claudin-1, claudin-4 and occludin immunofluorescence in ex vivo human skin cultured for 72 h under control conditions, in the presence of 35 mmol L\(^{-1}\) taurine and following the addition of 0.1 µmol L\(^{-1}\) ciclosporin A (CsA) and 35 mmol L\(^{-1}\) taurine. (b) Quantification of fluorescence intensity showed that the presence of CsA mitigated the increase in expression of all three tight junction proteins caused by betaine. (c) Claudin-1, claudin-4 and occludin gene expression was similarly affected by the presence of taurine. Data are presented as the mean ± SEM, two-way ANOVA, n = 7. Bars = 50 µm. SC, stratum corneum.
Figure 6 Osmolytes increase tight junction expression and function in human primary keratinocytes. (a) Transepithelial electrical resistance (TEER) values of cells grown on permeable supports, supplemented with 5 mmol L⁻¹ organic osmolytes. (b) Cells were supplemented with 5 mmol L⁻¹ betaine or taurine after 4 days in high-calcium medium. (e) 4-kDa fluorescein isothiocyanate–dextran permeability was significantly lower in cells supplemented with 5 mmol L⁻¹ betaine. (c, d, f, g) Inhibition of the betaine transporter and taurine transporter significantly abolished the effect of betaine and taurine, respectively. Data are represented as the mean ± SEM, two-way ANOVA; (a) n = 7, (b), n = 3, (e) n = 5, (c, d, f, g) n = 3. BPDBA, N-(1-benzyl-4-piperidinyl)-2,4-dichlorobenzamide; CsA, ciclosporin A; o.osm, organic osmolyte.

Figure 7 Immunoblotting of tight junction proteins in human primary keratinocytes. (a) Immunoblotting analysis showed that the expression of claudin-1 and claudin-4 was induced in differentiated normal human epidermal keratinocytes incubated with 5 mmol L⁻¹ organic osmolytes. (b) Densitometric quantification showing the ratio of tight junction protein to β-actin protein expression. Data are presented as the mean ± SEM, two-way ANOVA, n = 3.
A notable observation from this study is that the presence of betaine and taurine (but not myo-inositol) induced an upregulation of specific TJ proteins. Claudin-1 is ubiquitously expressed in epithelia and is thought to be part of the backbone structure of TJs in the skin. Claudin-4 has been implicated in formation of the barrier to water in keratinocytes. The exact function of occludin is unclear but it is thought to be part of the barrier to free movement of molecules through TJs. Thus, betaine and taurine specifically increase expression of proteins important for the barrier-forming properties of TJs. On the other hand, claudins 7 and 12 are more associated with pore-forming properties and are unaffected by betaine or taurine.

Quantitative reverse-transcriptase polymerase chain reaction analysis, performed at 72 h post-treatment with organic osmolytes, showed that only taurine caused an upregulation in claudin-1, claudin-4 and occludin mRNA levels. These data suggest that different osmolytes increase levels of specific TJ proteins via diverse mechanisms. Taurine induced the increase in specific TJ protein expression through increased mRNA synthesis and therefore, most probably, synthesis of new protein. Betaine probably mediates its effects via stabilizing the existing TJ protein pool. Betaine is well known for its ability to stabilize proteins and might act via reduction in protein turnover or increased mRNA lifetime.

Inhibition of betaine and taurine transporters negated the osmolyte-induced increase in TJ proteins. Crucially, transport inhibitors did not inhibit expression of TJ proteins per se. Indeed, BPDBA has been demonstrated to be a specific inhibitor of BGT-1, and CsA removes TATU from the membrane thus reducing its capacity for osmolyte transport. These data suggest that the increases in expression of specific TJ proteins were induced by the presence of the organic osmolytes within keratinocytes.

Betaine and taurine enhanced keratinocyte TJ function, and this was abolished by treatment of the cells with the respective transporter inhibitors. The putative ionic (measured by TEER) and molecular (measured by FITC–dextran) pathways are ‘tightened’ by application of betaine and taurine to keratinocytes. This is likely due to modulation of tightening TJ protein expression in keratinocytes, suggestive of the idea that betaine- and taurine-induced increases in specific TJ proteins result in change in function. It should be noted that NHEKS constitute a minimalist model used to measure TJ function and may not fully replicate the more complicated situation in the stratum granulosum, where TJs form in skin.

At present, methods to study TJ function in a stratified epithelium are limited. A dye penetration assay has been employed by some authors to study TJ loosening. Enhancement of TJ function is not easily measured using this assay. Therefore, instead, and in common with all other investigators in the TJ field, we used a monolayer of keratinocytes that have been calcium switched to promote differentiation, which is required for TJ assembly. Although it is a simplistic model, it is nevertheless encouraging that the changes in TJ protein expression noted in the skin explant model are replicated in keratinocytes. As methodology improves, future studies should be conducted in stratified models of skin to explore fully the effects of organic osmolytes on TJ function.

Our data have demonstrated previously unrecognized effects of organic osmolytes commonly used in topical formulations. Specifically, we show that osmolytes can upregulate their own transporter expression and enhance the structure and function of TJs. Both of these would improve epidermal barrier function, especially following exposure to environmental stressors or in skin conditions associated with barrier dysfunction such as AD. Betaine and taurine in particular have been added to topical preparations for years, but their added value in skincare products beyond their humectant properties has not been recognized. Some of these compounds can initiate gene expression leading to the upregulation of TJ proteins, which could enhance the overall barrier function even in healthy skin.

Further investigation improving our understanding of how each organic osmolyte is capable of protecting TJ structure and function could prove particularly important to our understanding of how the skin tolerates stress. Furthermore, identification of compounds that augment transporter expression could translate to the development of novel therapeutics relevant to skin diseases such as AD that involve barrier dysfunction.

References
1 Muizzuddin N, Ingrassia M, Marenus KD et al. Effect of seasonal and geographical differences on skin and effect of treatment with an osmoprotectant: sorbitol. J Cosmet Sci 2013; 64: 165–74.
2 Bommannan D, Potts RO, Guy RH. Examination of the effect of ethanol on human stratum corneum in vivo using infrared spectroscopy. J Control Release 1991; 16: 299–304.
3 Yoshida K, Yokouchi M, Nagao K et al. Functional tight junction barrier localizes in the second layer of the stratum granulosum of human epidermis. J Dermatol Sci 2013; 71: 89–99.
4 Furuse M, Hata M, Furuse K et al. Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. J Cell Biol 2002; 156: 1099–111.
5 Kirschner N, Houdek P, Fromm M et al. Tight junctions form a barrier in human epidermis. Eur J Cell Biol 2010; 89: 839–42.
6 Simpson CL, Patel DM, Green KJ. Deconstructing the skin: cytoarchitectural determinants of epidermal morphogenesis. Nat Rev Mol Cell Biol 2011; 12: 565–80.
7 Yuki T, Hachiya A, Kusaka A et al. Characterization of tight junctions and their disruption by UVB in human epidermis and cultured keratinocytes. J Invest Dermatol 2011; 131: 744–52.
8 Kirschner N, Brandner JM. Barriers and more: functions of tight junction proteins in the skin. Ann N Y Acad Sci 2012; 1257: 158–66.
9 Kirschner N, Rosenthal R, Furuse M et al. Contribution of tight junction proteins to ion, macromolecule, and water barrier in keratinocytes. J Invest Dermatol 2013; 133: 1161–9.
10 Ghadially R, Brown BE, Sequeira-Martin SM et al. The aged epidermal permeability barrier. Structural, functional, and lipid biochemical abnormalities in humans and a senescent murine model. J Clin Invest 1995; 95: 2281–90.
11 Tagami H, Kobayashi H, Zhen XS, Kikuchi K. Environmental effects on the functions of the stratum corneum. J Invest Dermatol Symp Proc 2001; 6: 87–94.
12 White-Chu EF, Reddy M. Dry skin in the elderly: complexities of a common problem. Clin Dermatol 2011; 29:37–42.
13 Fowler J. Understanding the role of natural moisturizing factor in skin hydration. Pract Dermatol 2012; 36–40.
14 Dirschka T, Tromnier H, Felder-Heins H. Epithelial barrier function and atopic diathesis in rosacea and perioral dermatitis. Br J Dermatol 2004; 150:1136–41.
15Engelbreiten K, Thyssen J. Skin barrier function and allergens. Curr Probl Dermatol 2016; 49:90–102.
16 Tsakok T, Woolf R, Smith CH et al. Atopic dermatitis: the skin barrier and beyond. Br J Dermatol 2019; 180:464–7.
17 Czarnecki T, Krueger JG, Guttman-Yassky E. Novel concepts of prevention and immunomodulation of atopic dermatitis through barrier barrier and manipulations with implications for the atopic march. J Allergy Clin Immunol 2017; 139:1723–34.
18 Irvine AD, McLean WHI, Leung DYM. Filaggrin mutations associated with skin and allergic diseases. N Engl J Med 2011; 365:1315–27.
19 Draelos ZD. Active agents in common skin care products. Plast Reconstr Surg 2010; 125:S19–24.
20 Nolan K, Marmur E. Moisturizers: reality and the skin benefits. Dermatol Ther 2012; 25:229–33.
21 Kelcheier M, Dunn-Galvin A, Hourihane JOB et al. Skin barrier dysfunction measured by transdermal water loss at 2 days and 2 months predates and predicts atopic dermatitis at 1 year. J Allergy Clin Immunol 2015; 135:930–5.
22 DuPont. Discover osmolytes. Available at: http://biosciences.dupont.com/solutions/personal-care/gencare-osms-ba (last accessed 14 May 2020).
23 Pericu P, Gherardi C, Lambers H. Discovering the invigorating osmolyte. Available at: http://biosciences.dupont.com/fileadmin/duPont_magazineEU_OSMS_MI.PDF (last accessed 14 May 2020).
24 Garcia-Perez A, Burg MB. Importance of organic osmolytes for osmoregulation by renal medullary cells. Hypertension 1990; 16:595–602.
25 Yancey PH. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. J Exp Biol 2005; 208:2819–30.
26 Khan SH, Ahmad N, Ahmad F, Kumar R. Naturally occurring organic osmolytes: from cell physiology to disease prevention. JIBMB Life 2010; 62:891–5.
27 El-Chami C, Haslam IS, Steward MC, O’Neill CA. Role of organic osmolytes in water homeostasis in skin. Exp Dermatol 2014; 23:534–7.
28 Lang F, Busch GL, Ritter M et al. Functional significance of cell volume regulatory mechanisms. Physiol Rev 1998; 78:247–306.
29 Lang F. Mechanisms and significance of cell volume regulation. J Am Coll Nutr 2007; 26 (5 Suppl.):613S–623S.
30 Wehner F, Olsen H, Tinel H et al. Cell volume regulation: osmolytes, osmolyte transport, and signal transduction. Rev Physiol Biochem Pharmacol 2003; 148:1–80.
31 El-Chami C, Haslam IS, Steward MC, O’Neill CA. Organic osmolytes preserve the function of the developing tight junction in ultraviolet B-irradiated rat epidermal keratinocytes. Sci Rep 2018; 8:5167.
32 Janeke G, Stiefen W, Carsten S et al. Role of tauinine accumulation in keratinocyte hydration. J Invest Dermatol 2003; 121:354–61.
33 Warskulat U, Reinen A, Grether-Beck S et al. The osmolyte strategy of normal human keratinocytes in maintaining cell homeostasis. J Invest Dermatol 2004; 123:516–21.
34 Warskulat U, Brookmann S, Reinen A, Haeussinger D. Ultraviolet B radiation induces cell shrinkage and increases osmolyte transporter mRNA expression and osmolyte uptake in HaCat keratinocytes. Biol Chem 2007; 388:1345–52.
35 Rockel N, Esser C, Grether-Beck S et al. The osmolyte tauinine protects against ultraviolet B radiation-induced immunosuppression. J Immunol 2007; 179:3604–12.
36 Rauhala L, Hamalainen L, Dunlop TW et al. The organic osmolyte betaine induces keratin 2 expression in rat epidermal keratinocytes – a genome-wide study in UVB irradiated organotypic 3D cultures. Toxicol In Vitro 2015; 30:462–75.
37 Artursson P. Epithelial transport of drugs in cell culture. I: a model for studying the passive diffusion of drugs over intestinal absorptive (Caco-2) cells. J Pharm Sci 1990; 79:476–82.
38 Jacobsen JG, Smith LH. Biochemistry and physiology of tauinine and tauinine derivatives. Physiol Rev 1968; 48:424–511.
39 Kragholm B, Kivist T, Madsen KK et al. Discovery of a subtype selective inhibitor of the human betaine/GABA transporter 1 (BGT-1) with a non-competitive pharmacological profile. Biochem Pharmacol 2013; 86:521–8.
40 Jørgensen L, Al-Khawaja A, Kickinger S et al. Structure–activity relationship, pharmacological characterization, and molecular modeling of noncompetitive inhibitors of the betaine-/γ-aminobutyric acid transporter 1 (BGT1). J Med Chem 2017; 60:8834–46.
41 Speake PF, Zipitis CS, Houston A, D’Souza S. Taurine transport into fetal cord blood cells: inhibition by cycloporsine A. J Soc Gynaeol Investog 2004; 11:472–7.
42 Powell DW. Barrier function of epithelia. Am J Physiol 1981; 241:G275–88.
43 Yamauchi A, Kwon HM, Uchida S et al. MyoInositol and betaine transporters regulated by toxicity are basolateral in Mdcck cells. Am J Physiol 1991; 261:F197–202.
44 Kwon HM, Yamauchi A, Uchida S et al. Cloning of the cDNA for a Na+/myo-inositol cotransporter, a hyperosmotic stress protein. J Biol Chem 1992; 267:6297–301.
45 Yamauchi A, Uchida S, Preston AS et al. Hypertonicity stimulates transcription of gene for Na+-myo-inositol cotransporter in Madck cells. Am J Physiol 1993; 264:F20–3.
46 Warskulat U, Weik C, Häussinger D. myo-Inositol is an osmolyte in rat liver macrophages (Kupffer cells) but not in RAW 264.7 mouse macrophages. Biochem J 1997; 326:289–95.
47 Andronic J, Shirakashi R, Pickel SU et al. Hypotonic activation of the myo-inositol transporter SLCSA3 in HEK293 cells probed by cell volumetry, confocal and super-resolution microscopy. PLOS ONE 2015; 10(5):e0119990.
48 Van Itallie CM, Anderson JM. Claudins and epithelial paracellular transport. Annu Rev Physiol 2006; 68:403–29.
49 Tunggal JA, Helfrich I, Schmitz A et al. E-cadherin is essential for vpi epidermal barrier function by regulating tight junctions. EMBO J 2005; 24:1146–56.
50 Al-Sadi R, Khatib K, Guo S et al. Occludin regulates macromolecule flux across the intestinal epithelial tight junction barrier. Am J Physiol Gastrointest Liver Physiol 2011; 300:G1054–64.
51 Buschmann MM, Shen L, Rajapakse H et al. Occludin OCEL-domain interactions are required for maintenance and regulation of the tight junction barrier to macromolecular flux. Mol Biol Cell 2013; 24:1056–68.
52 Alexandre MD, Lu Q, Chen YH. Overexpression of Claudin-7 decreases the paracellular Cl– conductance and increases the paracellular Na+ conductance in LLC-PK1 cells. J Cell Sci 2005; 118:2683–93.
53 Fujita H, Sugimoto K, Inatomi S et al. Tight junction proteins Claudin-2 and -12 are critical for vitamin D-dependent Ca2+ absorption from enterocytes. Mol Biol Cell 2008; 19:1912–21.
54 Guinn EJ, Pegram LM, Capp MW et al. Quantifying why urea is a protein denaturant, whereas glycine betaine is a protein stabilizer. Proc Natl Acad Sci U S A 2011; 108:16932–7.
Organic osmolytes and tight junctions in human skin, C. El Chami et al.

55 Rydeen AE, Brustad EM, Pielak GJ. Osmolytes and protein–protein interactions. J Am Chem Soc 2018; 140:7441–4.
56 Papageorgiou GC, Murata N. The unusually strong stabilizing effects of glycine betaine on the structure and function of the oxygen-evolving photosystem-II complex. Photosynth Res 1995; 44:243–52.
57 Tran TT, Qian X, Edwards C, Sarkar HK. Inhibition of taurine transport by cyclosporin A is due to altered surface abundance of the taurine transporter and is reversible. In: Taurine 7. Advances in Experimental Medicine and Biology (Azuma J, Schaffer SW, Ito T, eds). New York: Springer Science+Business Media, LLC, 2009; 503–11.
58 Ding L, Zhang Y, Tatum R et al. Detection of tight junction barrier function in vivo by biotin. Methods Mol Biol 2011; 762:91–100.
59 Kitajima Y, Inoue S, Yaoita H. Effects of pemphigus antibody on the regeneration of cell–cell contact in keratinocyte cultures grown in low to normal Ca ++ concentration. J Invest Dermatol 1987; 89:167–71.
60 Kitajima Y, Aoyama Y, Seishima M. Transmembrane signaling for adhesive regulation of desmosomes and hemidesmosomes, and for cell–cell detachment induced by pemphigus IgG in cultured keratinocytes: involvement of protein kinase C. J Invest Dermatol Symp Proc 1999; 4:137–44.
61 Morita K, Itoh M, Saitou M et al. Subcellular distribution of tight junction-associated proteins (occludin, ZO-1, ZO-2) in rodent skin. J Invest Dermatol 1998; 110:862–6.
62 Pummi K, Malminen M, Alho H et al. Epidermal tight junctions: ZO-1 and occludin are expressed in mature, developing, and affected skin and in vitro differentiating keratinocytes. J Invest Dermatol 2001; 117:1050–8.
63 Yoshida Y, Morita K, Mizoguchi A et al. Altered expression of occludin and tight junction formation in psoriasis. Arch Dermatol Res 2001; 293:239–44.
64 Brandner JM, Kief S, Grund C et al. Organization and formation of the tight junction system in human epidermis and cultured keratinocytes. Eur J Cell Biol 2002; 81:253–63.
65 Yuki T, Haratake A, Koishikawa H et al. Tight junction proteins in keratinocytes: localization and contribution to barrier function. Exp Dermatol 2007; 16:324–30.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Appendix S1 Supplementary methods.
Appendix S2 Supplementary results.
Figure S1 Dose-dependent effect of organic osmolytes on claudin-1 expression.
Figure S2 Sodium/myo-inositol cotransporter and H⁺/myo-inositol cotransporter expression in human skin.
Figure S3 Organic osmolytes had no effect on claudin-7 and claudin-12 expression.
Figure S4 myo-Inositol had no effect on tight junction protein expression.
Figure S5 Tight junction protein expression in normal human skin cultured for 72 h in the presence of organic osmolyte inhibitors.
Figure S6 Organic osmolyte transporter expression and localization in cultured primary human keratinocytes.
Figure S7 Dose-dependent effect of organic osmolytes on transepithelial electrical resistance.
Powerpoint S1 Journal Club Slide Set.
Video S1 Author video.