Urea Uptake Enhances Barrier Function and Antimicrobial Defense in Humans by Regulating Epidermal Gene Expression

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Urea is an endogenous metabolite, known to enhance stratum corneum hydration. Yet, topical urea anecdotally also improves permeability barrier function, and it appears to exhibit antimicrobial activity. Hence, we hypothesized that urea is not merely a passive metabolite, but a small-molecule regulator of epidermal structure and function. In 21 human volunteers, topical urea improved barrier function in parallel with enhanced antimicrobial peptide (AMP; LL-37 and β-defensin-2) expression. Urea stimulates the expression of, and is transported into, keratinocytes by two urea transporters (UTs), UT-A1 and UT-A2, and by aquaporins 3, 7, and 9. Inhibitors of these UTs block the downstream biological effects of urea, which include increased mRNA and protein levels of (i) transglutaminase-1, involucrin, loricrin, and filaggrin, (ii) epidermal lipid synthetic enzymes, and (iii) cathelicidin/LL-37 and β-defensin-2. Finally, we explored the potential clinical utility of urea, showing that topical urea applications normalized both barrier function and AMP expression in a murine model of atopic dermatitis. Together, these results show that urea is a small-molecule regulator of epidermal permeability barrier function and AMP expression after transporter uptake, followed by gene regulatory activity in normal epidermis, with potential therapeutic applications in diseased skin.

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INTRODUCTION

Human epidermis is the most external tissue of the body, serving at least three critical functions, i.e., provision of a barrier against (i) transcutaneous water loss, (ii) penetration of microbial pathogens, and (iii) ingress of potentially toxic xenobiotics (Marks, 2004). These major barrier functions localize to the outermost layer of the epidermis, i.e., the stratum corneum, which consists of extracellular lipid-enriched lipid lamellar bilayers enriched in ceramides, cholesterol, and free fatty acids, surrounding anucleate corneocytes, filled with filaggrin and its metabolites and keratin filaments (Loden, 2005). Individual corneocytes are surrounded by a unique cornified envelope composed of proteins (e.g., involucrin and loricrin), which are cross-linked into a rigid, mechanically resistant structure, by the calcium-dependent enzyme, transglutaminase 1 (TG-1; Iismaa *et al.*, 2009).

During epidermal differentiation, keratinocytes progress vertically from mitotically active basal cells into transcriptionally active spinous and granular cells to flattened, differentiated squames in the stratum corneum. This carefully orchestrated program allows continuous epidermal regeneration over a lifetime. Epidermal barrier function is regulated by several small molecules, including lipid and hormonal activators of nuclear hormone receptors, cytokines, and divalent cations, particularly calcium (Proksch *et al.*, 2008). Although topical urea has been used for decades to enhance hydration, as well as a keratolytic agent in psoriasis, ichthyosis, and dermatophytosis (Loden, 2005; Scheinfeld, 2010), anecdotal reports also suggest that urea improves barrier function after topical use, and that urea is beneficial for cutaneous infectious diseases, such as onychomycosis. Although it is widely assumed that urea acts solely by virtue of its moisturizing capacity, all of these disorders are also characterized by a perturbation of epidermal barrier function and/or altered antimicrobial defense. These observations together lead us to hypothesize that the beneficial effects of urea extend beyond its passive role as a moisturizer or

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Abbreviations: AD, atopic dermatitis; AMP, antimicrobial peptide; AQP, aquaporin; NHK, normal human keratinocytes; Ox, oxazolone; SPT, serine palmitoyl transferase; TEWL, transepidermal water loss; TG-1, transglutaminase 1; UT, urea transporter

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keratolytic agent, suggesting instead that urea could have additional regulatory activities within the nucleated layers of the epidermis, which could impact cutaneous barrier function and/or antimicrobial defense.

In previous studies, we have shown that normal human keratinocytes (NHK) possess an osmolyte strategy that maintains cell volume as a part of their response to exogenous perturbations, such as UV radiation (Warskulat *et al.*, 2004; Rockel *et al.*, 2007). Urea is a non-toxic, water-soluble carrier of excreted nitrogen, which can only be further metabolized by urease-positive, micro-organisms within the gut (Walser and Bodenlos, 1959). In many extracutaneous cell types, exogenous urea is taken up by specific urea transporters (UTs), UT-A and UT-B (Lucien *et al.*, 1998; Bagnasco *et al.*, 2001; Sands, 2002). The first gene encodes several, alternatively spliced isoforms, named UT-A1–UT-A6, which are expressed primarily in the renal tubules, except for UT-A5, which is expressed only in testis (Smith and Rousselet, 2001). The major renal UT-A isoforms, UT-A1, UT-A2, and UT-A3, act in concert to concentrate urea in the renal medulla, thereby negating the osmotic effects of urea in the urine. This action, together with that of vasopressin-regulated aquaporins (AQPs), allows water reabsorption across the medullary collecting ducts and excretion of hyperosmotic urine (Smith, 2009). In contrast, the UT-B gene is primarily expressed not only in erythrocytes, but also in endothelial cells of the kidney and brain (Stewart *et al.*, 2004). Whether one or more of the above mentioned UTs are expressed in NHK, the downstream metabolic consequences of such transport, as well as the potential clinical relevance of urea transport and uptake into epidermis are not known.

In this study, we first assessed whether topical urea enhances epidermal barrier function, and the potential biochemical basis for such improvement. We then analysed whether one or more functionally active UTs are expressed by human keratinocytes. We then determined whether genes that are involved in skin barrier formation are regulated by exogenous urea. Specifically, we studied the effects of exogenous urea on the expression of TG-1, involucrin, loricrin, and filaggrin, which have important roles in keratinocyte differentiation, genes encoding for epidermal lipid and antimicrobial peptide (AMP; i.e., LL-37 and β-defensin-2) production (Braff and Gallo, 2006). Once secreted within the extracellular spaces of the stratum corneum, these AMPs are well localized to inhibit invading pathogens. Moreover, at least one of these AMP, the carboxypeptide cleavage product of human cathelicidin LL-37 is also necessary for normal permeability barrier function (Aberg *et al.*, 2008), demonstrating the convergence of these two critical defensive functions (Elias, 2007).

RESULTS

Topical urea enhances human cutaneous permeability barrier function and AMP expression in normal human skin *in vivo*

Several anecdotal reports suggest that topical urea could improve epidermal permeability barrier function. To assess the impact of urea on cutaneous barrier function, we conducted a placebo-controlled, double-blinded study of 10 and 20% urea versus placebo applications in 21 healthy human volunteers. After 4 weeks of once-daily applications, epidermal barrier function was assessed as changes in transepidermal water loss (TEWL) on the arms and buttocks, followed by biopsies for real time PCR and immunohistochemistry from treated and untreated sites on the buttocks. Basal urea concentration is $\sim$150 nmol cm^{-2}, corresponding to $\sim$125 mM, which can be raised from 3.5- to 5-fold upon application of as little as 2% and 4% urea, respectively (Gabard and Chatelain, 2003). Although 10% urea did not significantly alter TEWL, 20% urea significantly improved skin barrier function, shown as a 31% decrease in TEWL levels (from 10.0 ± 0.9 to 6.9 ± 0.5 g h^{-1} m^{-2}; $P = 0.003$) in the 21 healthy volunteers (Figure 1a). Biopsies taken at the end of urea treatments showed that both 10 and 20% urea significantly enhanced expression of markers for epidermal antimicrobial defense. Improved barrier function in normal human volunteers treated with 20% urea was paralleled by increased mRNA levels for TG-1, involucrin, loricrin, and filaggrin (Figure 1b), as well as LL-37 and β-defensin-2 (Figure 1c). Nevertheless, although 10% urea treatment also significantly upregulated the expression of genes involved in epidermal differentiation and AMP production, it should be noted that TEWL levels did not significantly improve at this urea concentration. These studies indicate that topical applications of 20% urea improve cutaneous barrier function and expression of antimicrobial defense in normal human skin.

UT-A1 and A2, as well as AQPs 3, 7, and 9, function as UTs in keratinocytes

To begin to assess the basis for urea-induced barrier improvement, we first determined whether urea is taken up by NHK, and the responsible transporters. As exogenous urea has been shown to induce the expression of UTs in a variety of cell types (Smith and Rousselet, 2001; Stewart *et al.*, 2004), we first assessed basal and urea-induced expression for the four UTs that have been cloned to date (i.e., the human isoforms UT-A1, UT-A2, UT-A6, and UT-B (Fenton and Knepper, 2007)) in NHK under normosmotic (10 mM urea) and hyperosmotic conditions, such as 100 mM urea, 192 mM NaCl (Warskulat *et al.*, 2004), and 600 mM sorbitol. Expression levels of UT-A6 and UT-B could only be identified in the control cell lines HepG2 and caCo-2 (for details see Supplementary Figure S1c and d online). Both UT-A1 and UT-A2 are expressed in *NHK* (Figure 2a), and their expression increased by 2.9-fold and 2.1-fold under normosmotic conditions, respectively, comparable to changes in the physiological ranges that occur in human serum (1–10 mM; Wu, 2006). The extent of upregulation of UT-A1 and UT-A2 at 10 mM was similar to those observed in non-keratinocyte cell lines. Finally, the subsequent decline in UT-A1 and UT-A2 expression to background levels after treatment with 100 mM urea for 24 hours in NHK was not due to cell death because viability was not affected despite an increased osmolarity increased (i.e., to 400 mosmol l^{-1}; for details see Supplementary Figure S1a and b online).

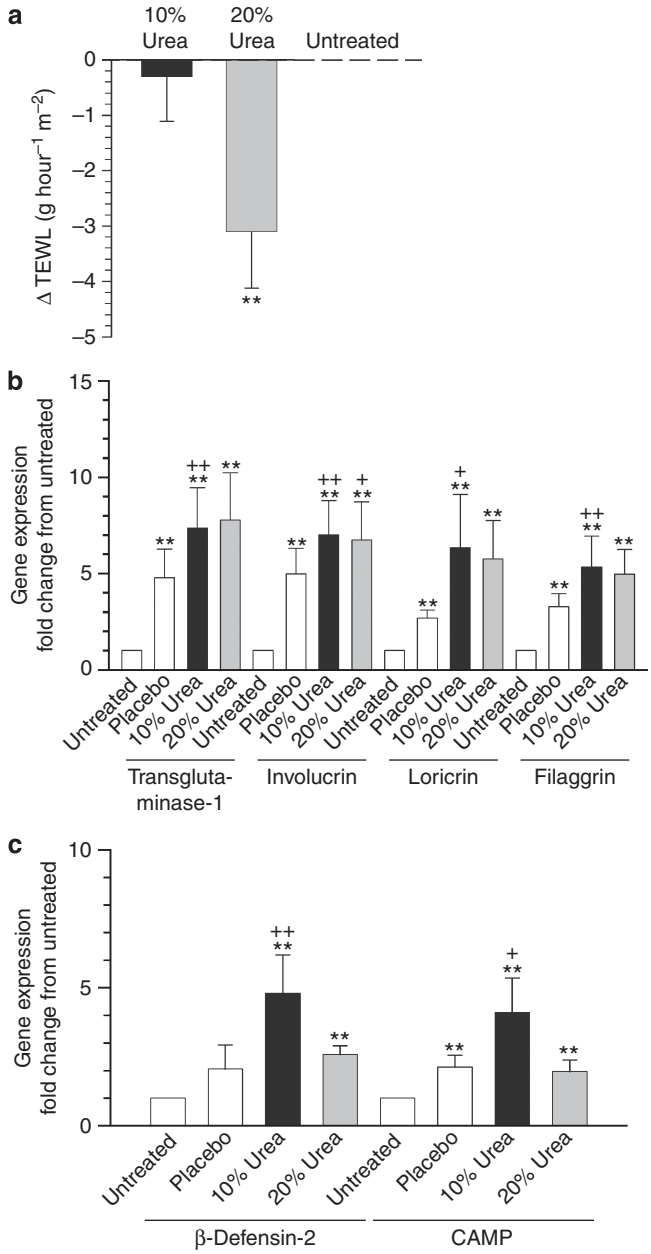

Figure 1. *In vivo* effect of urea treatments on barrier function of normal human volunteers. (**a**) Twenty-one normal volunteers were treated once daily for 4 weeks at three different areas of the left forearm (placebo), the right forearm (10% urea), and the right upper arm (20% urea). Skin barrier function was measured as transepidermal water loss (TEWL). Upregulation of (**b**) skin differentiation markers and (**c**) antimicrobial peptide was also assessed in biopsies taken from buttocks of the same 21 volunteers with normal skin after treatment once daily over a period of 4 weeks receiving either no treatment (untreated), or placebo with 0, 10, or 20% urea. Gene expression is normalized to 18S rRNA. All data represent mean ± SE. Statistical significance was tested by Wilcoxon's signed-rank test: (**a**) $^{**}P<0.01$ before versus after treatment; (**b**, **c**) $^{**}P<0.01$ versus untreated, $^{+}P<0.05$, $^{++}P<0.01$ versus placebo. CAMP, human cathelicidin gene.

One or both UTs is (are) functional, because NHK took up ^{14}C-labeled urea in a manner that could be significantly inhibited by coapplications of either phloretin or the pore-blocking analog, thiourea (Figure 2b), which also inhibited facilitated transport of urea in kidney, liver, and red blood cells (Chou and Knepper, 1989; Inoue *et al*, 2004; Zhao *et al*, 2007; Figure 2b). Since neither phloretin nor thiourea completely blocked urea transport, we next assessed whether AQPs, which are known to transport small molecules, such as glycerol (Rojek *et al.*, 2008), also transport urea. Both AQP-3 and -9 are expressed in the differentiating layers of human epidermal skin equivalents (Sugiyama *et al.*, 2001) and AQP-7 localizes to superficial epithelial cells of the gastrointestinal tract (Laforenza *et al.*, 2005). Yet, all 3 AQPs, including AQP-7, are expressed in NHK after stimulation with relatively low doses of exogenous urea (i.e., 1–10 mM; Figure 2c). By binding to cysteine residues (Cys^{11} in human AQP-3), mercury inhibits water and glycerol transport by mammalian AQPs (Kuwahara *et al*, 1997), and both nickel and copper cations inhibit glycerol permeability in human lung epithelial cells by interference with the extracellular amino acids Trp^{128}, Ser^{152}, and His^{241} (Zelenina *et al*, 2003, 2004). Accordingly, all three of these divalent cations also significantly inhibited ^{14}C-labeled urea uptake into NHK (Figure 2d), indicating that AQPs also contribute to the net uptake of urea by NHK. As urea transport in *Xenopus laevis* oocytes has been described for active cotransporters such as the low affinity Na^+-glucose cotransporter, the Na^+-iodide cotransporter, and the Na^+Cl-GABA cotransporter (Leung *et al.*, 2000) in the absence of their proper substrates, we also tested urea-inducible expression of these cotransporters after urea applications in NHK (Figure 2e). Leung and coworkers (2000) presented evidence that urea transport by Na^+-glucose cotransporter is not inhibited by urea analogs, such as thiourea. We then studied inhibition of urea transport of 34% by the UT-inhibitor, thiourea (Figure 2b), and 66% by the AQP inhibitor, $HgCl_2$ (Figure 2d). Finally, determination of copy numbers yielded the highest copy numbers for UT-A2 (62 ± 9 SE copies μl^{-1}), followed by AQP-3 (32 ± 4 SE copies μl^{-1}) and UT-A1 (19 ± 4 SE copies μl^{-1}), whereas copy numbers for Na^+-glucose cotransporter, Na^+-iodide cotransporter, and Na + Cl-GABA cotransporter were found to be as low as 7 ± 1.2 SE, 6 ± 1.4 SE, and 2 ± 0.4 SE, respectively, indicating only a minor relevance by these cotransporters (Figure 2f) (for further details see also Supplementary Figure S2a–c online).

Urea upregulates mRNA expression of differentiation markers, lipid synthetic enzymes, and AMP

We next assessed the basis for the benefits of urea for permeability barrier function and antimicrobial defense in normal epidermis. At the gene level, skin barrier function actively depends on two parallel processes, i.e., keratinocyte differentiation, which generates the corneocytes that contribute to barrier function by several mechanisms (Elias, 2007), and epidermal lipid synthesis, which generates the extracellular lamellar bilayers (Elias, 2005) as well as generation of tight junction proteins (Furuse *et al.*, 2002). Keratinocyte differentiation is orchestrated by regulation of a number of genes (Eckert and Welter, 1996), including involucrin, filaggrin, loricrin, and TG-1 (Chen *et al.*, 1995). While TG-1 mRNA levels did not

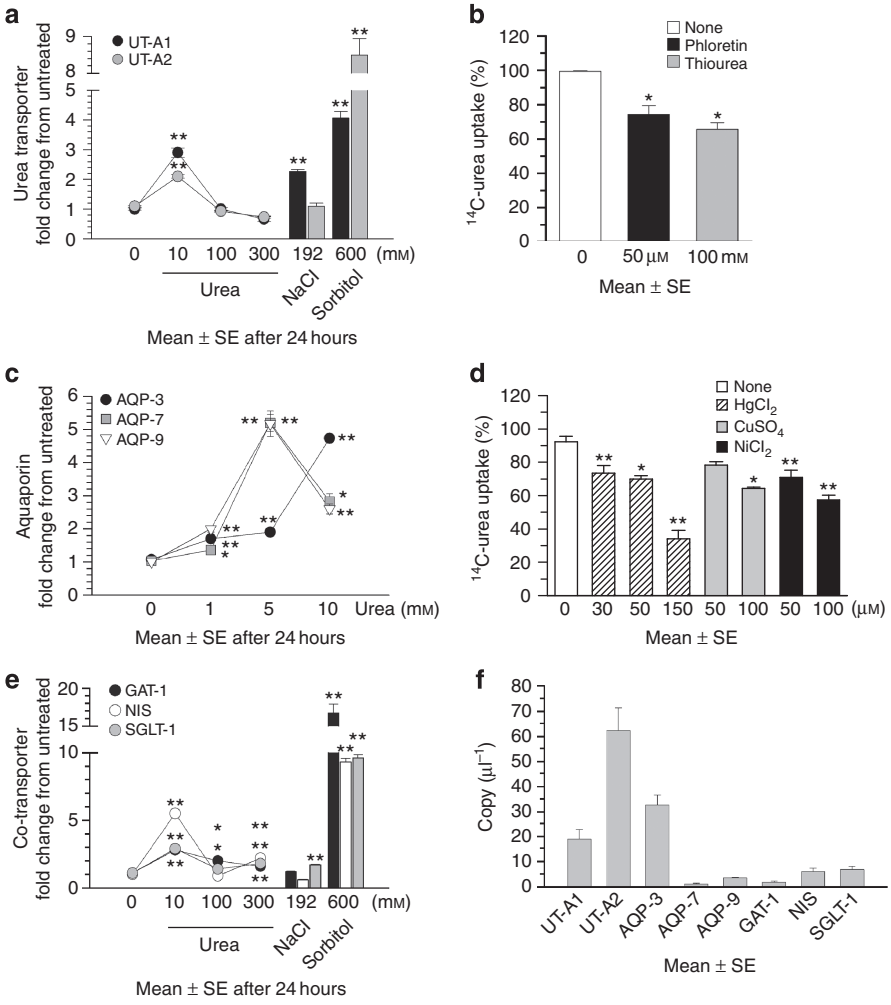

Figure 2. Expression and functionality of urea transporters (UTs) and aquaporins (AQPs) in human keratinocytes. Human keratinocytes express and upregulate mRNA encoding UTs (**a**) and AQPs (**c**). Under the various conditions indicated, functionality of transporters is shown by inhibition of uptake of ^{14}C-labeled urea in the presence of phloretin or thiourea (**b**) and/or $HgCl_2$, $CuSO_4$, or $NiCl_2$ (**d**). Expression of cotransporters such as Na^+-glucose cotransporter (SGLT-1), the Na^+-iodide cotransporter (NIS) and the Na^+Cl^-GABA cotransporter (GAT) in normal human keratinocytes (NHK) is presented (**e**) and copy numbers for all transporters assessed in untreated NHK are given in (**f**). Paired Student's *t*-test was used for statistical analyses: $*P<0.05$, $**P<0.01$ versus control.

change over the initial 24 hours of incubation, mRNA levels increased significantly after exposure of NHK to 5–50 mM urea for 48 hours, and at even lower urea doses after 72 hours (Figure 3d). Similar results were observed for both involucrin and loricrin at 48 hours, although filaggrin expression increased primarily by 72 hours of exposure (Figure 3b–d). Specifically, upregulation of involucrin mRNA (Figure 3b) reached a 1.8-fold ± 0.1 SE increase after stimulation with 5 mM urea for 48 hours and a 1.28 ± 9.3 SE increase in involucrin protein after 10 mM urea treatment for 24 hours (Figure 5a). These changes are comparable to changes in involucrin when keratinocytes are treated with 1.2 mM Ca^{2+} for 24 or 48 hours (Morizane *et al.*, 2010). By contrast, exogenous urea at 5–10 mM did not upregulate expression of the tight junction protein, claudin-1, at any of the time points (data not shown).

As noted above, formation of the permeability barrier requires not only the participation of corneocytes, but also epidermal lipid synthesis, as well as at least one AMP, i.e., the cathelicidin carbocyterminal peptide, LL-37, which is required not only for antimicrobial defense but also for permeability barrier function. Accordingly, gene expression of enzymes involved in lipid synthesis, such as serine palmitoyl transferase ((SPT)-1/SPT-2), 3-hydroxy-3-methyl-glutaryl-CoA reductase, and acidic sphingomyelinase increased after 48 hours of exposure to urea (Figure 3e). Moreover, urea stimulated mRNA expression of both β-defensin-2 and cathelicidin in NHK over a wide dose range, and at four time points assessed (Figure 3f and g). Although the most striking enhancement by urea on cathelicidin expression occurred even at low concentrations, β-defensin-2 levels increased mainly after exposure to ⩾20 mM urea for 72–96 hours.

We next confirmed these mRNA results at the protein and lipid level. Immunohistochemical analyses revealed that expression of TG-1, loricrin, involucrin, filaggrin, LL-37, and β-defensin-2, as well as lipid content increased after 10% urea application compared to untreated skin (Figure 4a–g).

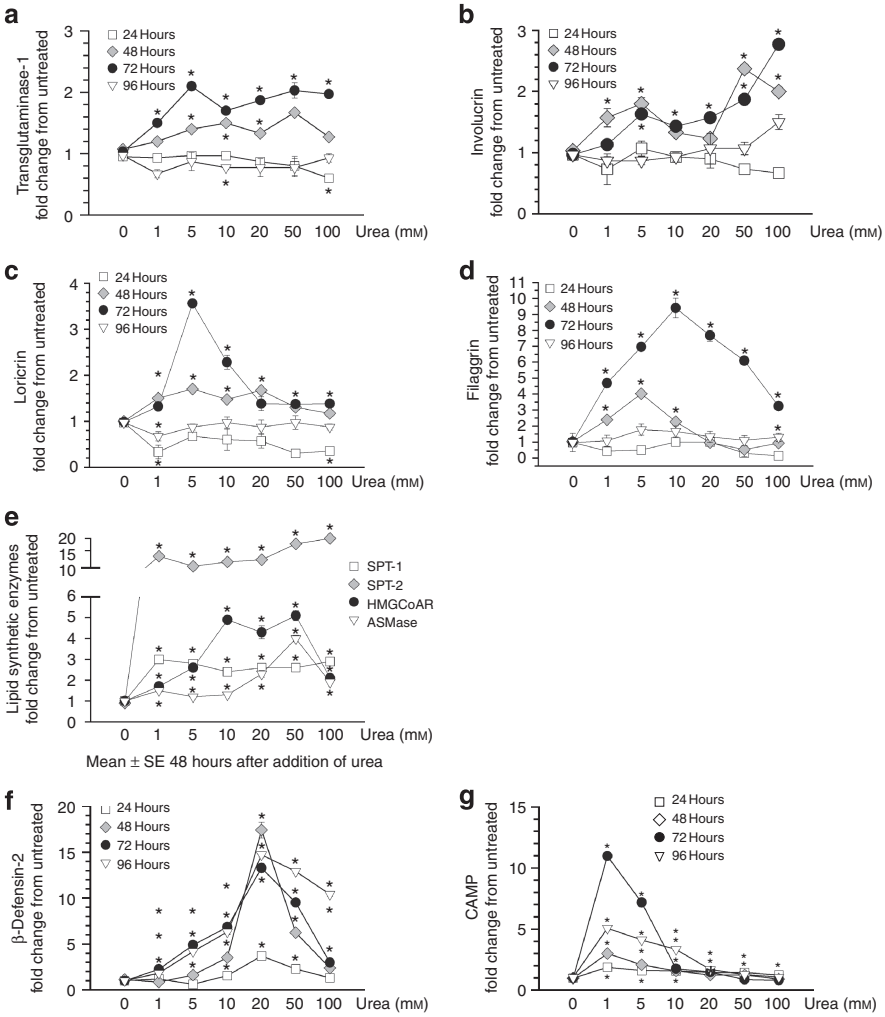

Figure 3. Upregulation of mRNA encoding keratinocyte differentiation markers and antimicrobial peptide (AMP) by urea *in vitro*. Expression of epidermal differentiation markers given as mean ± SE for the indicated time points: (**a**) transglutaminase-1, (**b**) involucrin, (**c**) loricrin, (**d**) filaggrin, (**e**) major epidermal lipid synthetic enzymes, and AMP (**f**) β-defensin-2 and (**g**) LL-37 has been assessed as indicated in normal human keratinocytes. Paired Student's *t*-test with $*P<0.05$ versus time-matched untreated sample. ASMase, acidic sphingomyelinase; CAMP, human cathelicidin gene; HMGCoAR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; SPT, serine palmitoyl transferase.

For volunteer no. 17, we observed a 7.2-fold increase of loricrin on the protein level after 10% urea treatment corresponding to a 9.1-fold increase on the mRNA level. Within the volunteers assessed ($n=3$), epidermal thickness did not change significantly (92.5 μm ± 1.5 SE for untreated skin, 91.5 μm ± 1.5 SE for 10% urea, $P=0.647$ paired Student's *t*-test). Together, these results indicate that exogenous urea not only stimulates urea transport, but also transcription of genes involved in keratinocyte differentiation, epidermal lipid synthesis, and AMP production, providing a mechanistic basis for improved barrier function and antimicrobial defense.

Urea-induced enhancement of epidermal barrier function and AMP expression requires urea uptake

We next assessed whether improved barrier function and AMP expression require prior urea uptake. As we used an inhibitor approach, we first determined the concentration ranges of inhibitors that were not toxic to NHK. Urea-induced upregulation of involucrin, filaggrin, and TG-1 expression, as demonstrated above is completely inhibited by phloretin or thiourea (Table 1). Also, a partial but significant inhibition of urea-induced gene expression was detected following application of inhibitors for AQPs such as $HgCl_2$ or $CuSO_4$ (Table 1). Western blotting of extracts from urea-treated NHK in the presence or absence of inhibitors also showed that urea-induced increase of involucrin protein expression was significantly inhibited by $HgCl_2$, although phloretin affected involucrin expression to a lesser extent due to its own inducing effects on this protein (Figure 5a and b). In addition, urea-induced expression of filaggrin protein was significantly diminished by coincubation with either $HgCl_2$ or phloretin (Figure 5c and d) (Simon *et al.*, 1996). Likewise, expression of the genes involved in lipid synthesis was largely blocked by coapplication of the urea transport inhibitors. Although urea-induced upregulation of SPT-1 was completely inhibited

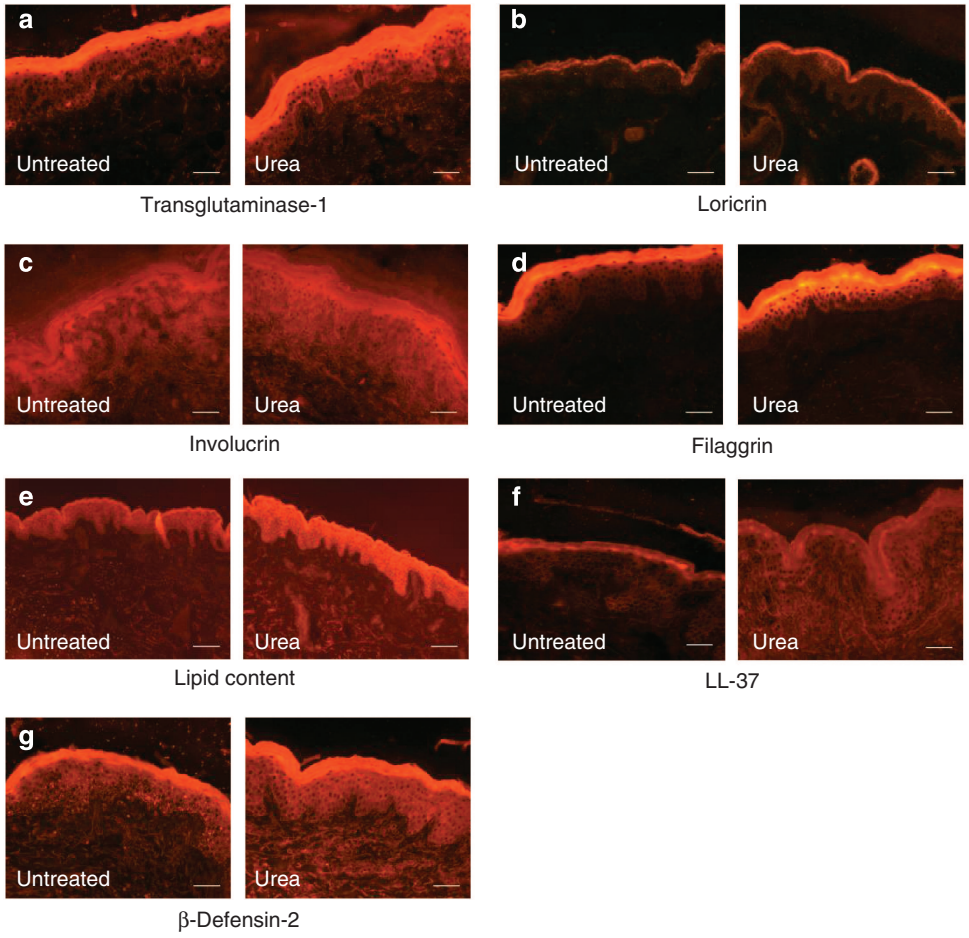

Figure 4. Increased skin differentiation, lipid content, and antimicrobial peptide expression after application of 10% urea to human skin. Immunocytochemistry shown in cryosections for (**a**) transglutaminase (TG)-1, (**b**) loricrin, (**c**) involucrin, (**d**) filaggrin, (**e**) total lipid, (**f**) LL-37, and (**g**) β-defensin 2 of three randomly chosen volunteers. Quantification with Image J resulted in increases of 3.1-fold for TG-1 (volunteer no. 17), 27.2-fold for loricrin (volunteer no. 17), 1.5-fold for filaggrin (volunteer no. 7), 1.7-fold for involucrin (volunteer no. 17), 1.5-fold for lipid content (volunteer no. 7), 2.7-fold for LL-37 (volunteer no.16), and 1.7-fold for β-defensin-2 (volunteer no. 16) after treatment with 10% urea as compared with the untreated skin area. Bar = 50 μm.

by the UT inhibitors above, SPT-2 and 3-hydroxy-3-methyl-glutaryl-CoA reductase upregulation were partially diminished (Table 1). Because the expression of LL-37 and β-defensin-2 occurs relatively late in differentiation, the effects of UT and AQP blockade on AMP expression were assessed 72 hours post exposure to 5 mM urea. In contrast to the complete inhibition of urea-induced stimulation of differentiation markers by phloretin and thiourea, the urea-induced stimulation of β-defensin-2 and LL-37 expression was partially diminished in NHK coincubated with phloretin, or thiourea (Table 1). In addition, incubation with AQP inhibitors, $HgCl_2$ or $CuSO_4$, blocked urea-induced upregulation of AMP to a similar extent (Table 1). Together, these results indicate that urea transport and uptake is necessary for the urea-induced stimulation of epidermal differentiation, lipid synthesis, and AMP expression.

Urea improves barrier function and upregulates AMP in a mouse atopic dermatitis (AD) model

We asked next whether these newly appreciated characteristics of urea predict potential clinical utility. For these studies, we assessed the effects of topical urea in an established murine model with AD (Man *et al.*, 2008; Hatano *et al.*, 2009, 2010), a disease that displays abnormalities in both permeability barrier function and antimicrobial defense. AD-like dermatosis is generated by repeated hapten (oxazolone (Ox)) challenges (Ox-AD model). We first assessed whether applications of urea concurrent with hapten challenges improved epidermal function in Ox-AD mice. In placebo-treated, Ox-AD mice, repeated Ox challenges induced the expected decline in AMP expression (Man *et al.*, 2008). But, cotreatment with 20% urea reverted both murine cathelin-related antimicrobial peptide and mouse β-defensin 3 (murine homologs of cathelicidin and human β-defensin-2, respectively) expression in Ox-AD mice (Figure 6a and b). Additionally, 10% urea significantly improved stratum corneum hydration (Figure 6c) and barrier function, assessed as TEWL levels (Figure 6d). These results suggest that topical applications of 10 or 20% urea improve both epidermal function and AMP expression in this AD model. Epidermal thickness gradually increased by urea

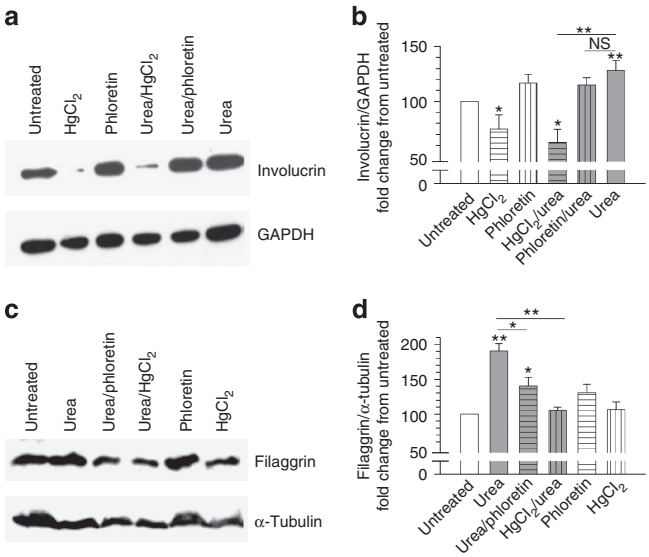

Figure 5. Inhibition of urea transport by phloretin (50 μM) or $HgCl_2$ (150 μM) decreases the expression of epidermal differentiation markers. Urea-induced (10 mM) expression of involucrin ($n=5$) (**a**) or filaggrin ($n=3$) (**c**) was assessed by western blotting of total cell extracts from normal human keratinocytes treated for 24 or 72 hours, respectively. Quantification (**b**, **d**) of data is given as mean ± SE. Paired Student's *t*-test was used for statistical analyses: $*P<0.05$, $**P<0.01$ versus untreated or as indicated. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NS, not significant.

treatment from 10 μm (only Ox treated) to 12 μm with 5% urea and to 25 μm with 10 and 20% urea.

DISCUSSION

In the present study, we describe the mechanistic basis for the unexpected, new properties of topical urea. Human epidermal keratinocytes appear to take up urea via specific transport mechanisms including UT-A1, UT-A2, and AQPs (i.e., 3, 7, and/or 9). Although these conclusions are based upon inhibitor studies, rather than a gene knock-down approach, we utilized non-toxic doses of these agents, and demonstrated comparable effects with several chemically unrelated molecules. Also, urea uptake is followed by gene regulatory events, including increased expression of the UTs mentioned above, and more importantly, of a number of genes that are critical for cutaneous skin barrier function and antimicrobial defense. To our knowledge this is previously unreported that urea, which has previously been relegated to solely relatively minor role in the skin as an endogenous moisturizer, probably has much more than a merely passive role in the maintenance of epidermal homeostasis. In fact, urea appears to be a highly active small-molecule regulator of genes that impact keratinocyte differentiation, lipid synthesis, and AMP production, together leading to improved permeability barrier function and probably to antimicrobial defense as well.

Although the signaling pathways involved in urea-induced changes in gene expression are currently not known, urea-induced gene regulation does not result from osmotic stress, because they occur under normal osmotic conditions, and require the presence of specific transporters. The time course of urea-induced gene expression points to an indirect mechanism, because significant upregulation of some of the genes, i.e., loricrin, filaggrin, LL-37, and β-defensin 2, can only be observed 48–72 hours after urea exposure. One explanation for the delayed effects of urea might be that urea activates the non-energy-dependent, sodium-proton exchanger (NHE1), which could contribute to optimal barrier function by controlling not only intracellular pH (Mauro *et al.* 1995) but also acidification of extracellular domains of the lower stratum corneum. Acidification in turn activates pH-sensitive enzymes that both form the epidermal permeability barrier and facility desquamation of cells, mainly in the outer layers of the stratum corneum. Additionally, urea-induced non-enzymatic carbamylation might also contribute to delayed gene expression, although the effect of carbamylation on protein modification, assessed for hemoglobin, only results in a 1.7-fold increase of protein modification, when urea concentrations were raised from 4.8 to 30 mM (Wynckel *et al.*, 2000). Besides this more indirect effect, urea can activate transcription, translation of, and trans-activation by the immediate early gene transcription factor Egr-1, probably via serum-responsive elements together with adjacent Ets motifs in physiological doses with regards to the renal medulla (Cohen *et al.*, 1996). Although further studies with transporter-knockout animals might be useful, it is possible that deletion of one transporter could alter the expression of other transporters, as shown for UT-B deletions in erythrocytes, which upregulate UT-A2, AQP-2, and AQP-3, in a compensatory fashion (Klein *et al.*, 2004). Similarly, UT-B has been shown to transport water, when AQP-1 is knocked out in erythrocytes (Yang and Verkman, 2002). Further studies will be required to clarify the signalling mechanisms that account for the stimulation of the multiple proteins and enzymes shown here.

Our results may explain the many anecdotal observations that patients with perturbed barrier function and increased susceptibility towards skin infections benefit from topical urea application (Stüttgen, 1992; Bissonnette *et al.*, 2010). Accordingly, we showed that urea can prevent development of abnormal barrier function and reverse the decrease in AMP expression in a murine AD model. Also, stimulation of AMP expression could explain in part the clinical observation that patients with infectious skin diseases, such as onychomycosis, benefit from urea treatment by improving barrier function, since tinea infections abrogate the barrier (Bonifaz and Ibarra, 2000; Baran and Tosti, 2002). Urea also could be useful for the treatment of other skin conditions that display both altered permeability and antimicrobial defense, such as AD. Accordingly, acute and chronic lesions of AD patients are characterized by a decreased expression of β-defensin-2 and LL-37, which probably explains their propensity to be colonized by *Staphylococcus aureus* and viral pathogens (Kim *et al.*, 2007). UVB radiation was recently described to induce expression of human β-defensin-2 (Glaser *et al.*, 2009) and the precursor of LL-37, hCAP18 (Mallbris *et al.*, 2005), in human skin *in vivo*. Accordingly, suberythemal UVB also enhances barrier function and murine cathelin-related

Table 1. Effect of inhibitors for UTs (thiourea 100 mM and phloretin 50 μM) and AQPs ($HgCl_2$ 30 μM, and $CuSO_4$ 50 μM) on urea-induced expression of markers involved in epidermal skin barrier formation at time points indicated

			Gene Expression in %				
				Inhibition of UT		Inhibition of AQP	
Marker	Time (h)	Concentration of urea (mM)	Urea	+Thiourea	+Phloretin	+$HgCl_2$	+$CuSO_4$
Transglut-1	48	5	100 ± 8	0 ± 0*	0 ± 5*	17 ± 2*	29 ± 4*
Involurin	48	5	100 ± 2	0 ± 8**	0 ± 15**	45 ± 6**	22 ± 5**
Filaggrin	48	5	100 ± 5	0 ± 11*	0 ± 11*	42 ± 6**	17 ± 4**
Loricrin	48	5	100 ± 0.6	0 ± 0*	0 ± 8**	39 ± 4**	17 ± 6**
Transglut-1	48	50	100 ± 3	0 ± 17*	0 ± 14*	ND	ND
Involucrin	48	50	100 ± 3	0 ± 5**	0 ± 5**	ND	ND
Filaggrin	48	50	100 ± 9	5 ± 5*	0 ± 10*	ND	ND
Loricrin	48	50	100 ± 3	0 ± 8**	0 ± 0**	ND	ND
Transglut-1	48	100	100 ± 14	0 ± 0*	0 ± 0*	ND	ND
Involucrin	48	100	100 ± 12	0 ± 0*	0 ± 9*	ND	ND
Filaggrin	48	100	100 ± 14	0 ± 12*	0 ± 25*	ND	ND
Loricrin	48	100	100 ± 10	0 ± 14**	0 ± 0*	ND	ND
SPT-1	48	5	100 ± 2	0 ± 6**	0 ± 6*	00 ± 6**	67 ± 8 NS
SPT-2	48	5	100 ± 2	31 ± 2**	0 ± 3**	9 ± 7**	48 ± 2**
HMGCoAR	48	5	100 ± 3	62 ± 2*	40 ± 2*	28 ± 6*	51 ± 6*
BD2	72	5	100 ± 0.9	46 ± 14**	54 ± 7**	49 ± 4*	39 ± 4**
CAMP	72	5	100 ± 0.9	51 ± 0.9**	28 ± 17**	60 ± 0**	40 ± 5*
BD2	72	10	100 ± 6	ND	ND	52 ± 0 NS	55 ± 4 NS
CAMP	72	10	100 ± 2	ND	ND	32 ± 0**	51 ± 2**

Abbreviations: AQP, aquaporin; BD2, β-defensin 2; CAMP, human cathelicidin gene; HMGCoAR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; ND, not detected; NS, not significant; SPT, serine palmitoyl transferase; Transglut-1, transglutaminase-1; UT, urea transporters.
Given is the gene expression based on 18S rRNA. Gene expression under urea treatment for the indicated markers was set 100%, and the effect of inhibitors is given as the remaining gene expression.
Paired Student's *t*-test versus urea treatment. *$P<0.05$, **$P<0.01$.

antimicrobial peptide production in mice (Hong *et al.*, 2008; Rodriguez-Martin *et al.*, 2011). Barrier function was also increased after topical application of calcipotriol in healthy human skin (Weber *et al.*, 2005), and in lesional psoriatic skin, vitamin D analogs increase LL-37, but decrease β-defensin-2 expression (Peric *et al.*, 2009).

The present studies add urea to the list of potential inducers of antimicrobial defense in human skin. Since urea is a widely used molecule, with a very low toxicity profile, these observations should prompt further interest in the use of urea and urea-related molecules as potential stimulators of skin antimicrobial defense mechanisms.

In addition to antimicrobial defense, the skin of AD patients also exhibits defective permeability barrier function. Accordingly, not only involucrin and loricrin mRNA and protein expression but also lipids such as ceramides are greatly diminished both in affected and in unaffected skin of AD patients (Kim *et al.*, 2008), and the current observation that urea can increase the expression of these differentiation genes and the lipid synthetic enzymes, e.g., SPT, provides another rational for the beneficial effects of urea treatment in this skin disease. Interestingly, among all differentiation markers studied, filaggrin expression showed the strongest upregulation after urea treatment. Accordingly filaggrin expression, which is reduced in a substantial proportion of AD patients (Weidinger *et al.*, 2006), might be improved by topical applications of urea to the skin of heterozygous null allele carriers (Sandilands *et al.*, 2006; Sergeant *et al.*, 2009).

MATERIALS AND METHODS

Volunteers

Approval had been obtained from the Ethics Committee of the Heinrich-Heine University. The double-blind, placebo-controlled study was conducted according to the ethical rules stated in the Declaration of Helsinki Principles and the ICH GCP guideline was observed insofar as applicable. Twenty-one healthy human volunteers (seven female and fourteen male) were enrolled after written informed consent. Their age ranged from 21 to 59 years with a mean

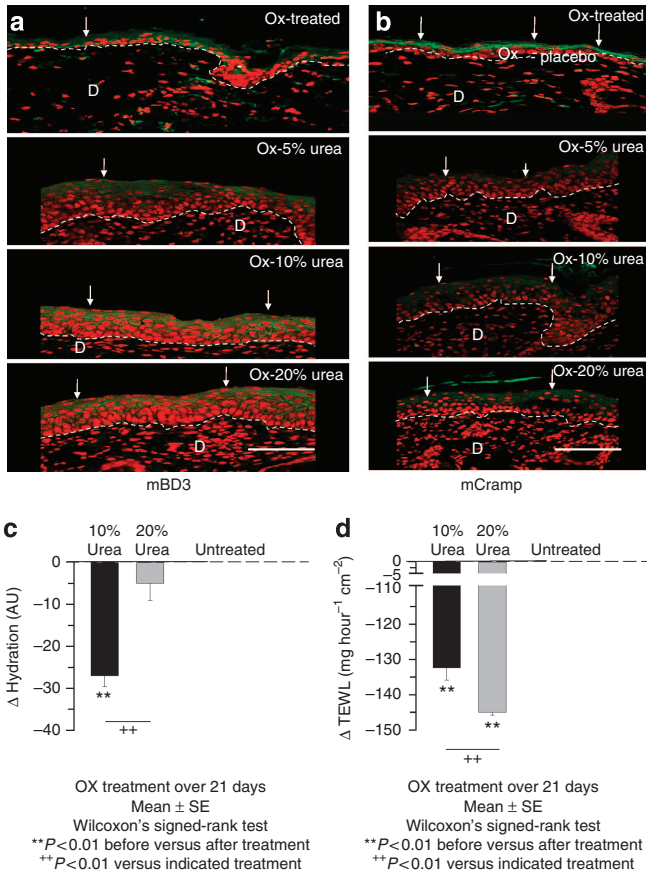

Figure 6. Urea increases the expression of antimicrobial peptide, increases skin hydration, and improves transepidermal water loss (TEWL) in a mouse atopic dermatitis model. After 10 oxazolone (Ox) challenges over a period of 3 weeks and coapplication of urea treatment twice daily, immunocytochemistry was performed in paraffin sections (5 μm) for (**a**) murine cathelin-related antimicrobial peptide (mCramp) and (**b**) mouse β-defensin 3 (mBD3) as compared with a sample with 10 OX challenges alone (untreated with regard to urea or placebo). Bar = 40 μm. (**c**) Skin hydration was measured by corneometry and (**d**) skin barrier function as TEWL in groups of eight animals each. The dotted line represents the dermo-epidermal junction. D, dermis.

age of 40 ± 2.7 SE years, and all individuals were non-smokers and had no history of any skin disease.

Topical treatment

The volunteers were requested not to use any topical preparations for at least 2 weeks before the study. Urea was used in concentrations of either 0, 10, or 20% in a placebo, prepared and provided by ISDIN (Barcelona, Spain), containing isopropyl myristate, paraffin, glyceryl stearate, PEG-40 stearate, sorbitol, cetearyl ethylhexanoate, cetyl alcohol, dimethicone, Persea Gratissima, stearic acid, sodium carbomer, acrylates, allantoin, methylparaben, propylparaben, lactic acid, parfum, and 2-bromo-2-nitropropane-1,3 diol in water. For skin physiological assessments, volunteers were treated once daily for 4 weeks at three different 4 cm × 4 cm areas on the left inner forearm (placebo), the right inner forearm (10% urea), and the right upper arm (20% urea). In parallel, three different 4 cm × 4 cm large skin areas on the buttocks were treated identically to obtain 4 mm punch biopsies (Grether-Beck *et al.*, 2008a), which were cut in halves for either RNA isolation or immunohistochemical staining.

Assessment of permeability barrier function

TEWL levels were measured before and after applications of the treatments with an evaporimeter (Tewameter TM300, Courage and Khazaka Electronic GmbH, Cologne, Germany), according to the guidelines provided by the European Society of Contact Dermatitis (Pinnagoda *et al.*, 1990). The inner forearms and upper arms have been chosen as treatment locations, because intra- and inter-individual variation coefficients are reasonably small. The results were expressed as $\Delta TEWL = TEWL_{aftertreatment} - TEWL_{beforetreatment}$ in $mg\,h^{-1}\,m^{-2} \pm SE$.

Cells and cell culture

Primary human epidermal keratinocytes (NHK) were prepared from neonatal foreskin and maintained in culture under serum-free conditions as described previously (Grether-Beck *et al.*, 2008a). For induction of differentiation, NHK were seeded and grown up to confluence. Urea (diaminomethanal, carbamide) was obtained from ISDIN (Barcelona, Spain). See the Methods section and Supplementary Figure S1(a) and (b) online in the Supplementary material online at http:/www.nature.com/jid for further details on cell viability and osmolarity.

RNA isolation and PCR

Total RNA was extracted from cell culture dishes or from frozen biopsies and gene expression measured by a two-step reverse transcription real time PCR as described (Grether-Beck *et al.*, 2008a, b). The primer sequences are available in supplements (Supplementary Table S1 online). For comparison of relative expression in real time PCR untreated control cells/biopsies and treated cells/biopsies the $2^{-\Delta\Delta C(t)}$ method was used (Livak and Schmittgen, 2001).

Expression of UTs, AQPs, and cotransporters

The mRNA expression of UTs was assessed using quantitative real time PCR. See the Methods section and Supplementary Figures S1(c) and (d) online in Supplementary material online at http:/www.nature.com/jid for further details on validation of primers in control cell lines and determination of copy numbers.

Measurement of [^{14}C]-urea transport

To test their function in NHK, expression of the transporters was induced 16 hours before initiation of radiolabeling by 10 mM urea in the absence of bovine pituitary extract. For radiolabeling, 74 kBq (2 μCi) ^{14}C urea (Hartmann Analytic GmbH, Braunschweig) was added and the cells were incubated for another 4 minutes as described (Klein *et al.*, 1999). Classical inhibitors (all from Sigma-Aldrich, Steinheim, Germany) of urea transport (Inoue *et al.*, 2004), e.g., phloretin (50 μM), and thiourea (100 mM), were added together with ^{14}C urea, whereas mercury ($HgCl_2$, 30, 50, and 150 μM (Kuwahara *et al.*, 1997)), nickel ($NiCl_2$, 50 and 100 μM (Zelenina *et al.*, 2003)), and copper ($CuSO_4$, 50 and 100 μM (Zelenina *et al.*, 2004)) to inhibit AQP were preincubated for 24 hours. The urea influx was stopped simultaneously by three rapid washes with ice-cold solution containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$,

and 0.05 mM phloretin using a centrifuge. Supernatants and dried cell pellets were harvested separately. Cell pellets were solubilized in 0.5 ml of 25 mM NaOH with 0.5% deoxycholate, 100 µl from each well was used to measure the protein content with the BCA assay (Pierce, Rockford, IL), and 500 µl was used to count the cells by a standard scintillation procedure (Beckman Coulter LS 6000IC, Beckman Coulter GmbH, Krefeld, Germany) using CytoScint ES (ICN, Irvine, CA) as scintillation fluid. Transport was calculated as Bq per mg protein (nmol urea per mg protein) and set as 100% for urea-stimulated cells.

Western blotting

Confluent NHK were stimulated with urea and/or the inhibitor and cultivated for 24 or 72 hours. The cells were lysed in a buffer composed of 60 mM Tris-HCl (pH 7.8), 150 mM NaCl, 5 mM EDTA, 10% glycerol, and 60 mM n-octyl-β-D glucopyranoside. In all, 10 µg of the lysates for involucrin and 50 µl lysate in Laemmli final sample buffer for filaggrin were resolved by SDS-Page and immunoblotted on nitrocellulose membrane (Trans-blot; Bio-Rad, Munich, Germany). Antibodies were obtained from Santa Cruz (Santa Cruz, CA): rabbit polyclonal antibody against human involucrin (H-120, 1:250) and a rabbit polyclonal antibody against human filaggrin (H-300). GAPDH (Millipore, Temecula, CA) or α-tubulin (Abcam, Cambridge, UK) served as loading control. The blots were visualized with the SuperSignal West Pico detection system (Pierce, Rockford, IL), and were scanned and quantified using spot denso function from AlphaEase FluorChem 8900, Version 3.2.3 (Alpha Innotech, San Leandro, CA).

Immunohistochemical staining

Acetone-fixed frozen sections (6 µm) were stained with the following primary antibodies overnight at 4°C: from Abcam (Cambridge, UK) polyclonal rabbit anti human filaggrin antibody (1:1,000), polyclonal mouse anti loricrin antibody (1:500), monoclonal mouse anti involucrin antibody (1:200), and monoclonal mouse anti-TG-1 antibody (1:20); from Santa Cruz (Santa Cruz, CA) polyclonal rabbit anti human LL-37 antibody (1:500) and polyclonal rabbit anti human β-defensin 2 antibody (1:500). After rinsing in phosphate-buffered saline, the sections were visualized using a Vectastain ABC-AP kit (Axxora, Lörrach, Germany). Total lipid staining using Nile Red from Sigma-Aldrich (Steinheim, Germany) was performed in frozen sections as described, (Gunathilake *et al.*, 2009) and examined with an Olympus BX60 fluorescence microscope (Olympus Imaging Europe GmbH, Hamburg, Germany). The entire field of each section was examined at ×400 magnification. For quantification of fluorescence intensity, ImageJ software (1.41; US National Institutes of Health, Bethesda, MD, http://imagej.nih.gov/ij/, 1997–2011) was used. In detail, the images were saved as tif files, the ratio of stained areas versus background areas was calculated for each file, setting this ratio to 1 for untreated skin samples. Epidermal thickness was measured using ImageJ software. For each treatment (urea or untreated), ten different sites on three different skin sections were measured and the mean epidermal thickness was calculated.

Mouse AD model

Female hairless mice (hour/hour) aged 6–8 weeks were purchased from Charles River Laboratories (Wilmington, MA) and were fed a standard mouse diet (Ralston-Purina, St Louis, MO) and water *ad libitum*. All animal procedures were approved by the Animal Studies Subcommittee of the San Francisco Veterans Administration Medical Center and performed in accordance with their guidelines.

Animals were sensitized with a single topical application of 1% Ox (50 µl) to the flank. One week after sensitization, hairless mice were treated topically with 60 µl of 0.1% Ox on both flanks once every other day for an additional period of 3 weeks (totally, 10 challenges over 20–21 days) (Man *et al.*, 2008). These mice with an AD-like dermatosis were divided into four groups (eight animals each) at the beginning of challenges, and were treated with placebo containing 0%, 5, 10, or 20% urea. The placebo (Cetaphil, Galderma, Düsseldorf, Germany) cream contains water, petrolatum, glyceryl polymethacrylate, dicaprylyl ether, glycerol, dimethicone, glyceryl stearate, cetyl alcohol, *Prunus amygdalus dulcis* (sweet almond) oil, PEG-30 glyceryl stearate, tocopheryl acetate, benzyl alcohol, phenoxyethanol, sodium hydroxide, acrylates/C10-30 alkyl acrylate crosspolymer, dimethiconol, disodium EDTA, and propylene glycol. Treatments were applied twice daily at an interval of 6 hours, and at least 1 hour after prior Ox treatments. Both before challenges and at the end of the treatment periods, TEWL was measured with an electrolytic water analyzer (Meeco, Warrington, PA) and stratum corneum hydration was assessed as capacitance, with a Corneometer CM820 (Courage & Khazaka, Cologne, Germany).

Full-thickness skin specimens were fixed in 10% formaldehyde and embedded in paraffin. Sections (5 µm) were cut and immunostained for mouse β-defensin 3 and murine cathelin-related antimicrobial peptide (primary antibodies from Santa Cruz (Santa Cruz, CA)). After deparaffinization, sections were rehydrated sequentially with 100%, 90%, and 70% ethanol and incubated for 15 minutes in Tris-buffered saline to inactivate endogenous peroxidases. Samples then were treated for 10 minutes with a blocking serum solution and incubated overnight at 4°C with the primary antibody (1:100 dilution). After several washes in Tris-buffered saline, they were incubated for 40 minutes with a secondary antibody, FITC-conjugated goat anti-rabbit (Alpha Diagnostics, San Antonio, TX) at room temperature. Slides were counterstained with propidium iodide for nuclear visualization, and examined on a Leica TCS-SP laser confocal microscope (Leica Microsystems, Buffalo Grove, IL). The thickness of epidermis in mice was measured in three confocal pictures for each therapy and the ox-treated animals (three different measures each) using NIS-Elements 3.0 (Nikon Instruments, Melville, NY).

Statistical analyses

NHK cell culture experiments were performed in three independent experiments with cells derived from three different donors. Data shown in each figure are from experiments performed in one representative individual in triplicate, unless otherwise indicated. Data are presented as mean ± SE. Student's *t*-test or Wilcoxon signed rank test was used for comparison between two groups, $P<0.05$ was considered significant.

CONFLICT OF INTEREST

PME and JK are members of the Scientific Advisory Board of ISDIN, Barcelona, Spain. CT is an employee of this company.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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