ORIGINAL ARTICLE

Topical tranexamic acid improves the permeability barrier in rosacea

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A B S T R A C T

Objective: To evaluate the influence of tranexamic acid on epidermal permeability barrier function in rosacea and its potential mechanisms.
Methods: A randomized, vehicle controlled, split-face study was performed on 30 rosacea patients. This study involved 2 weeks of 3% tranexamic acid solution treatment and vehicle control treatment. Skin physiological parameters, including skin surface pH, stratum corneum hydration, and transepidermal water loss, were measured. The expression of protease-activated receptor 2 (PAR-2) in rosacea and normal skin samples was assessed with immunohistochemical staining. The expression of PAR-2 in HaCaT keratinocytes was determined using reverse transcription polymerase chain reaction after stimulation with tranexamic acid. Changes of intracellular calcium induced by PAR-2 activation were measured using Fluo-4 NW calcium assay.
Results: Individuals with rosacea expressed a higher baseline level of PAR-2 compared with normal skin. Tranexamic acid improved the permeability barrier function in rosacea patients and inhibited calcium mobilization in keratinocytes induced by PAR-2 activation. The PAR-2 expression was not altered by tranexamic acid stimulation.
Conclusion: Topical tranexamic acid could improve the epidermal permeability barrier function and clinical signs of rosacea, likely resulting from inhibition of PAR-2 activation and consequent calcium influx. Thus, tranexamic acid could serve as an adjuvant therapy for rosacea.

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Introduction

Rosacea is a common inflammatory facial disorder with compromised epidermal permeability barrier function. The common manifestations of rosacea include recurrent erythema, telangiectasia, inflammatory papules and pustules on the mid face, or rhinophyma in severe cases. The current therapies for rosacea have certain limitations, and prevention of relapse requires a long-term maintenance therapy.[1] The treatments are often short of targets, largely due to its unknown pathogenesis.[2,3] Recent studies have suggested that protease-activated receptor 2 (PAR-2) could be involved in the pathogenesis of rosacea. PAR-2 is a G-protein-coupled 7-transmembrane domain receptor, which mediates inflammation in various tissues upon activation by serine proteases (SPs) such as kallikrein.[4,5] Consistently, the activities of SPs are increased in rosacea. SPs degrade the epidermal antimicrobial peptide to its active form, cathelicidin LL-37. The latter can mediate vascular action and inflammation, which are features of rosacea.[6–8]

Compromised epidermal permeability barrier function is another feature of rosacea.[9] While rosacea exhibits a higher level of transepidermal water loss, improvement of permeability barrier function alleviates rosacea.[10] Whether the defective permeability barrier is the cause or the resultant of rosacea is not clear, it is likely linked to the increased SP activity. Previous studies showed that the increased SP activity is associated with certain dermatoses, such as atopic dermatitis, accompanied by defective permeability barrier.[11] Conversely, inhibition of SP activity by either lowering stratum corneum pH or topical SP inhibitors, improves epidermal permeability barrier homeostasis in barrier-disrupted skin. As an SP inhibitor, tranexamic acid has been proved to accelerate the restoration of the damaged skin barrier. In this study, we chose tranexamic acid as a candidate treatment for rosacea, since it can act as an inhibitor of SP and accelerate the recovery of the damaged skin barrier resulting from tape stripping, acetone, sodium dodecyl

sulfate, and other physical or chemical inducers.[12,13] Here, we hypothesize that in rosacea, activation of PAR-2 by SP results in inflammation and defective permeability barrier.

In the present study, we determined whether inhibition of SP activity by tranexamic acid improves rosacea *in vivo* and affects calcium mobilization induced by PAR-2 activation *in vitro*, which may further prove the importance of barrier abnormality in the etiology of rosacea, and indicate a new direction for rosacea treatment.

Methods

Participants

The study was conducted following the principles outlined in the Declaration of Helsinki. The protocol was read and approved by the Ethics Committee of Peking University First Hospital, Beijing, China. Written informed consent was obtained from all patients prior to the study. Thirty patients aged 18—65 years were recruited according to the diagnostic criteria of rosacea.[1] The exclusion criteria included people with facial acne, steroid-dependent dermatitis, or other skin or systemic diseases that might influence skin assessment; using antirosacea drugs (including antibiotics), steroids, or vasodilating agents topically during the past 2 weeks or orally during the past 1 month; allergic to the test ingredients; during gestation or lactation. A randomized, vehicle controlled, split-face study was performed on 30 rosacea patients. One side of each patient's face was topically treated with 5% tranexamic acid solution twice daily for 2 weeks while the vehicle-treated side served as a control. During the study, no other topical or systemic agents were allowed. This study was conducted in March—May 2011, in Beijing, China.

Physiological assessment

After facial cleansing at each visit, the patients were kept in a test room with a controlled temperature (20—22°C) and humidity (40—60%) for 30 minutes before physiological measurements. Corneometer CM825 (COURAGE+KHAZAKA electronic GmbH, Köln, Germany) was used to measure the water content in the stratum corneum. Tewameter TM300 and pH meter pH900 (both from COURAGE+KHAZAKA electronic GmbH, Köln, Germany) were used to measure transepidermal water loss and pH value, respectively. Chromameter CM2600d (Konica Minolta, Inc., Tokyo, Japan) was used for the measurement of Chroma, an indicator for the severity of erythema[14]; VISIA Image System (Canfield Scientific, Inc. Fairfield, NJ, USA) was used for image analysis.

Cell culture and reagents

Immortalized human keratinocyte cell line HaCaT were purchased from the China Center for Type Culture Collection (Wuhan, China) and cultured 1% penicillin-streptomycin at 37° in Dulbecco's Modified Eagle's medium (Gibco, Langley, OK, USA) supplemented with 10% fetal bovine serum and C in a humidified CO_2 incubator (95% air, 5% CO_2). For stimulation, cells were cultured in 12-well plates (Corning, Inc., Corning, NY, USA). At 60—70% confluence, culture medium was replaced with serum-free medium, followed by incubation with tranexamic acid (200 μg/mL) for an additional 24 hours. Then the keratinocytes were harvested for analysis.

MTT assay

Three-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) assay was used to determine the viability of HaCaT cells and the appropriate concentration of tranexamic acid to treat cells.[15] Tranexamic acid of 400 μg/mL, 200 μg/mL, 100 μg/mL, and 50 μg/mL diluted in *phosphate-buffered saline* (PBS) were used to treat cells for 24 hours.[16] After incubation with MTT for 4 hours, the optical density of each well was read at 540 nm using a microplate reader.

Histology and immunohistochemistry

Four μm thin-sections of formalin-fixed, paraffin-embedded skin samples were stained using immunohistochemistry. Briefly, fixed sections were deparaffinized, rehydrated and washed in PBS (2 × 5 minutes). For antigen retrieval, the sections were put into 10mM citrate buffer (pH 6.0) and boiled for 15 minutes. After cooling at room temperature, sections were treated with 3% H_2O_2 for 15 minutes, and incubated with primary antibodies (rabbit polyclonal antihuman, Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:200) overnight at 4°C. Secondary antibodies were polyperoxidase-antimouse/rabbit immunoglobulin G. Staining color was developed using 3,3′-diaminobenzidine for 1—3 minutes. The reaction was stopped with distilled water while being observed under a microscope. Harris hematoxylin was used for counterstaining. Slides were examined at 200 × and 400 × magnification. Images were evaluated using the Leica microscope with imaging software (Leica Application Suite, Leica Microsystems Limited, Switzerland).

RNA isolation, cDNA synthesis, and quantitative real-time polymerase chain reaction

After stimulation for 24 hours, keratinocytes were washed with PBS, and RNA was isolated using RNeasy 96 Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For cDNA synthesis RNA was reverse-transcribed with iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) and real-time polymerase chain reaction (PCR) was carried out with ViiA 7 Real-time PCR system (Applied Biosystem Life Technology, USA) according to the manufacturer's instructions. The relative expression of the target genes were calculated by comparing with the housekeeping gene GAPDH using a formula described previously.[17] The primers used are PAR-2 : forward 5′-ACATGGCAACAACTGGGTCT-3′ ; reverse, 5′-CGAT CACCCAGTACCTCTGC-3′. GAPDH: forward 5′-TGACGTGCCGCCTG GAGAAA-3′; R5′-AGTGTAGCCCAAGATGCCCTTCAG-3′. All real-time PCR experiments were performed at least in triplicate and the specificity of the reactions was confirmed by sequencing the PCR products.

Fluo-4 NW calcium assay to detect the in-cell calcium signaling of PAR-2 activation in HaCat

HaCaT cells were cultured on the chamber slice (Lab-Tek Nunc, Thermo Scientific, USA) and used at 50% confluent. After incubation with basal medium or tranexamic acid for 24 hours, the medium was removed and washed with Ca^{2+} free assay buffer. To each well 100 μL of the dye loading solution was added. The plates were incubated at 37°C for 30 minutes, then at room temperature for an additional 30 minutes. As a PAR-2 agonist 100nM trypsin was used; fluorescence images were measured and recorded using a laser confocal microscope (Leica TCS SP5 microsystem, Germany) for excitation at 488 nm.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA, USA). Calculations are based on the means of independent experiments (each individual

experiment with 3–4 replicates) Comparison between the control and the treated side was performed using Student t test and Mann–Whitney test, while comparison between prior-treatment and post-treatment was performed by paired t test and Wilcoxon signed ranks test. A p value < 0.05 was considered statistically significant.

Results

Influence of tranexamic acid on skin barrier function in rosacea patients

The mean age of the rosacea patients was 39.34 ± 11.43 years. Forty percent (12/30) of these patients were male and 60.0% (18/30) were female. As shown in Figure 1, the baseline stratum corneum functions on the control side were similar to the treated side. After 2 weeks of treatment, all skin biophysical parameters on the treated side were significantly improved compared to baseline, while the vehicle treatment induced no changes in stratum corneum functions. In parallel with the improvement of stratum corneum functions, clinical sign and symptoms were also improved dramatically after treatments with topical tranexamic acid (Figure 2 and Table 1). These results demonstrate that topical tranexamic acid improves both stratum corneum functions, and clinical signs and symptoms of rosacea.

Rosacea patients express high level of PAR-2

Since activation of PAR-2 is involved in the pathogenesis of rosacea,[18] we next assessed the expression of PAR-2 in rosacea patients with immunohistochemical staining. As seen in Figures 3B and 3D, PAR-2 was weakly expressed in the nuclei and cytoplasm of the normal epidermis whereas a more intense staining of PAR-2 was shown in both nuclei and cytoplasm in rosacea patients (Figures 3A and 3C). These results indicate that the epidermal PAR-2 expression is elevated in rosacea patients.

Tranexamic acid inhibits calcium mobilization induced by PAR-2 activation

Previous studies have shown that activation of PAR2 by protease stimulates Ca^{2+}.[19] We then determined whether tranexamic acid inhibits Ca^{2+} mobilization induced by PAR2 activation, providing a potential mechanism whereby tranexamic acid improves rosacea. The optimal concentration of tranexamic acid in keratinocyte cultures was determined using MTT assay. Our results showed that 200 ug/L caused no changes in cell viability. Therefore, 200 μg/L of tranexamic acid was used in all our studies *in vitro*. As seen in Figure 4, the addition of trypsin, a potent activator of PAR2, HaCaT cells induced an immediate increase in the intensity of fluorescence (blue line in Figure 4A and top panel in Figure 4B), indicating the stimulation of Ca^{2+} mobilization. The maximum signal (975.88 ± 120.27) was obtained 40 seconds after the addition of trypsin. However, following the treatment of cells with 200 μg/L tranexamic acid for 24 hours , trypsin-induced peak signal of fluorescence was much lower (464.06 ± 72.35, red line in Figure 4A) than without tranexamic acid. Moreover, the appearance of peak signal was delayed by 120 seconds (Figure 4A, $p < 0.05$). To determine whether the tranexamic acid-induced inhibition of Ca^{2+} mobilization is due to inhibition of PAR-2 production, the expression levels of PAR-2 mRNA were measured in HaCaT cells after tranexamic acid treatment. As shown in Figure 5, incubation of HaCaT cells with tranexamic acid did not affect the expression levels of PAR-2 mRNA. The mRNA level of PAR-2 in all the groups was comparable, indicating tranexamic acid did not influence PAR-2 expression. Together, these results indicate that tranexamic acid inhibits PAR-2 activity, resulting in inhibition of Ca^{2+} mobilization.

Discussion

Rosacea is one of the most common inflammatory skin disorders. The pathogenesis of rosacea is unknown. However, evidence suggests that compromised epidermal permeability barrier could play
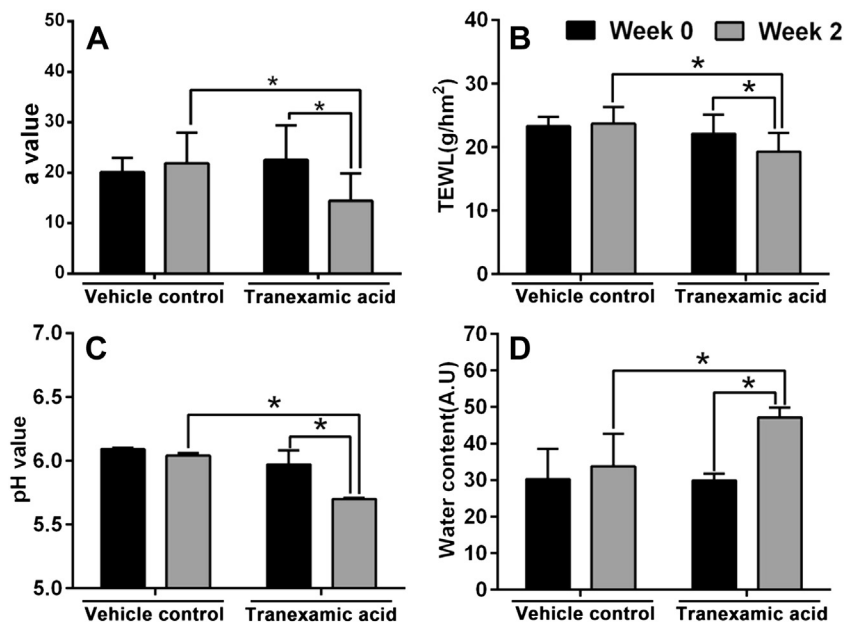

Figure 1 Changes in physiological parameters. (A) Stratum corneum biophysical properties were measured as described in the materials and methods section. Chroma; on the treated side, Chroma a value decreased significantly after 2-week treatment ($p < 0.05$), indicating an obvious improvement of erythema severity. (B) While on the treated side, after 2-week treatment, transepidermal water loss; (C) and skin surface pH value decreased significantly ($p < 0.05$). (D) Stratum corneum hydration increased, indicating the impaired barrier function of rosacea was improved significantly ($p < 0.05$). The statistical significances are shown in the figures (* $p < 0.05$).
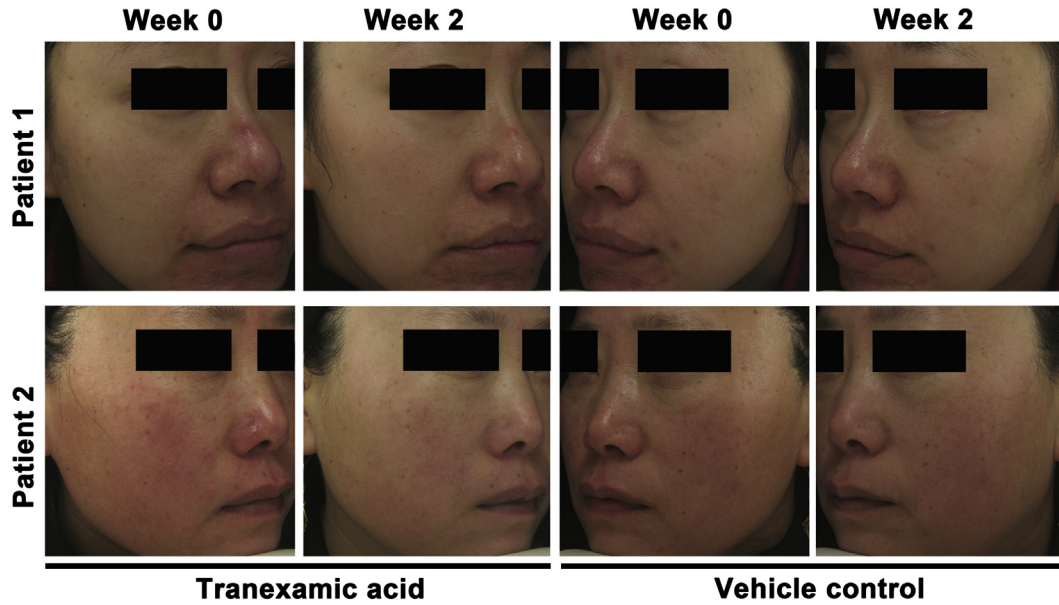
Figure 2 Clinical pictures of two female patients before and after treatment. After a 2-week treatment, tranexamic acid improved the clinical manifestations of rosacea with fewer inflammatory lesions. The right side was tranexamic acid-treated while the left side was vehicle-treated.

Table 1 Lesion assessment of rosacea patients before and after treatment. Lesion count including papules and pustules were assessed by two experienced dermatologists and the same investigator independently. The investigators' assessment also revealed that the lesion counts decreased more significantly on the treated side than on the control side ($p < 0.05$).

Control side			Treated side			p2
Before	After	p1	Before	After	p1	
6.5 (0, 40)	2.5 (0, 25)	0.000	6.5 (2, 40)	1 (0, 16)	0.000	0.043

Data are represented as median (minimum, maximum value).
p1 = comparison of the score between Week 0 and Week 2 (Wilcoxon signed ranks test).
p2 = comparison of the score variation between control and treated side (Mann-Whitney test).

a vital role in the development of rosacea. Rosacea displays a defective permeability barrier.[20] Previous studies have demonstrated that disruption of epidermal permeability barrier stimulates cytokine expression, inflammatory cell infiltrate, LL-37 expression, as well as expression of vascular growth factor, all of which are features of rosacea.[21] In addition, rosacea exhibits a higher level of SP activity, which activates PAR2. PAR-2 signaling influx of calcium ions in *stratum granulosum* keratinocytes could inhibit *lamellar body* secretion, which is one of the mechanisms that PAR-2 is involved in the regulation of permeability barrier homeostasis.[11] Activation of PAR-2 decreases E-cadherin adhesion,[22] whereas loss of E-cadherin function causes a defective epidermal permeability barrier.[23,24] Inhibition of SP by tranexamic
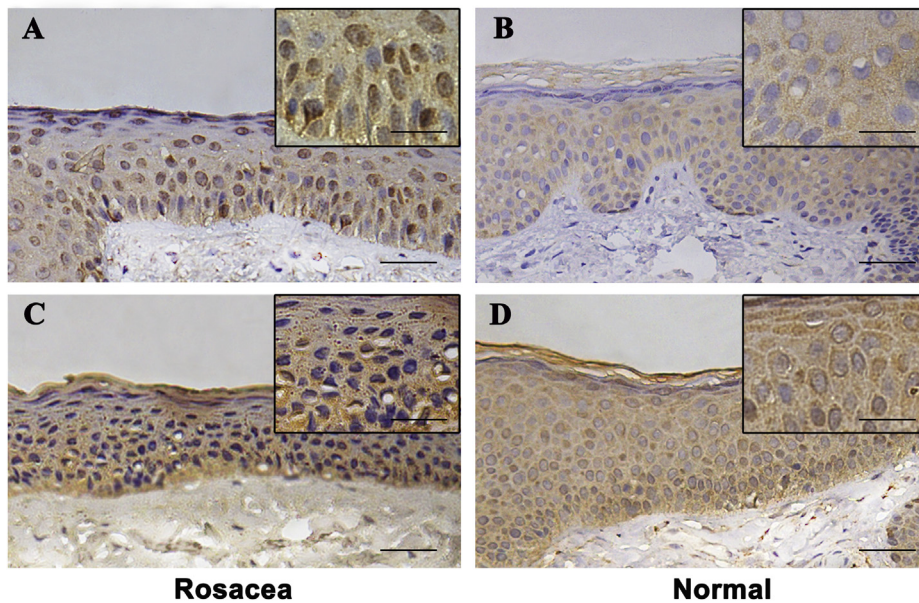

Figure 3 Immunohistochemical findings of PAR-2 in rosacea and normal skin. PAR-2 staining in rosacea skin from two different patients (A, C × 200, × 400), PAR-2 staining in normal skin from two different donors (B, D × 200, × 400). Magnification bar = 100 μm for 200 ×, and = 40 μm for 400 ×.
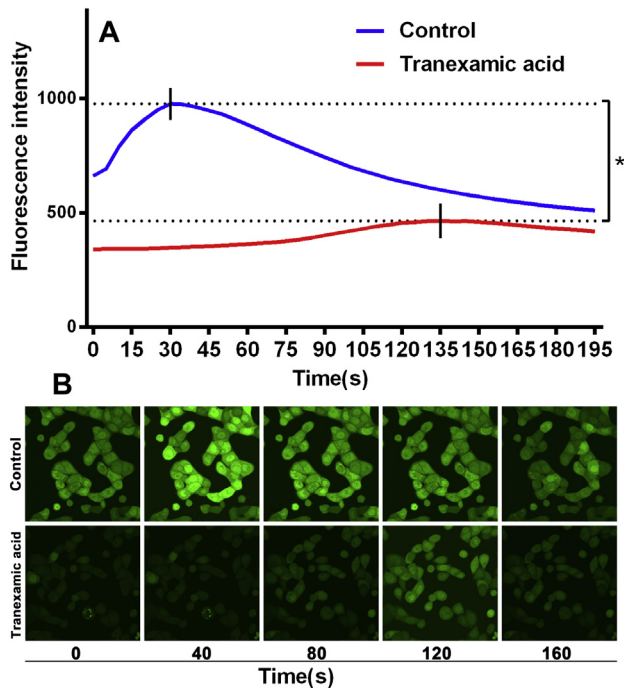
Figure 4 Ca^{2+} mobilization responses to trypsin with or without tranexamic acid treatment in HaCaT cells. HaCaT cells were pretreated with 200 μg/L tranexamic acid and triggered by 100nM trypsin. The maximum signal was decreased and the peak time was delayed in tranexamic acid group compared with the trypsin control group. (A) Time response curves; (B) fluorescence intensity.

acid [4-(aminomethyl)cyclohexane carboxylic acid,t-AMCHA], accelerates barrier recovery and inhibits epidermal hyperplasia induced by repeated barrier disruption.[25]

Logically, administration of tranexamic acid would benefit rosacea. Indeed, we demonstrate here that topical tranexamic acid improves rosacea. The mechanisms by which tranexamic acid benefits rosacea could be due to the improvement of permeability barrier function, likely resulting from the inhibition of SP. The assumption is based on findings from present studies and previous studies from others.[26] First of all, both the expression levels of PAR-2, as shown in present studies, and SP activity increases in rosacea.[7] Secondly, inhibition of either SP or PAR-2 improves permeability barrier homeostasis.[6,27] As with PAR-2 inhibition, the present study shows that pretreatment of keratinocytes with tranexamic acid

inhibits protease stimulated calcium mobilization. Thus, the beneficial effects of tranexamic acid on rosacea can be attributed to inhibition of SP activity.

However, as a plasmin inhibitor, tranexamic acid suppresses plasmin-induced angiogenesis, and also inhibits neovascularization induced by basic fibroblast growth factor (bFGF). It has been reported that topical tranexamic acid could reduce erythema and vessel numbers in the melasma lesion. We found in our study that the erythema of rosacea was also reduced by tranexamic acid, which might be caused by the antiangiogenic action of tranexamic acid.[28]

The treatment of rosacea has been a big challenge for both patients and clinicians, mainly due to the lack of approaches targeting pathogenic pathway. Although the pathogenesis of rosacea is unknown, a line of evidence suggests that the development of rosacea is driven by a defective permeability barrier. In addition to the induction of pre-inflammatory cytokine releases, barrier disruption increases the epidermal SP activity[6] and LL-37 expression.[29] Epidermal proteases, including kallikrein-related peptidases 5 and 7 (KLK5, KLK7), degrade LL-37 to different active fragments of LL-37, including FA-29.[7] The active forms of LL-37 cause erythema and vasodilatation, as well as cytokine release.[30,31] Moreover, LL-37 upregulates vascular endothelial growth factor expression.[32] However, activation of PAR-2 by SP also undermined the permeability barrier. The latter could exacerbate rosacea. The clinical evidence that improvement of permeability barrier alone benefits rosacea[33] also supports the pathogenic role of the permeability barrier in rosacea. The putative pathogenic role of the permeability barrier in rosacea and the mechanisms by which tranexamic acid benefits rosacea are illustrated in Figure 6.
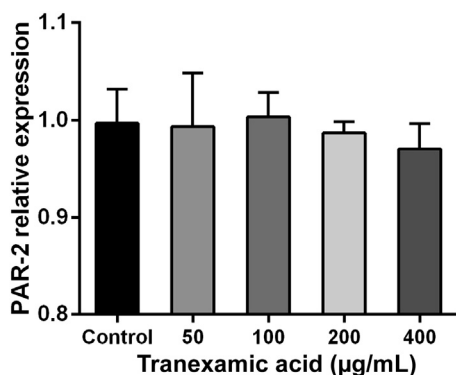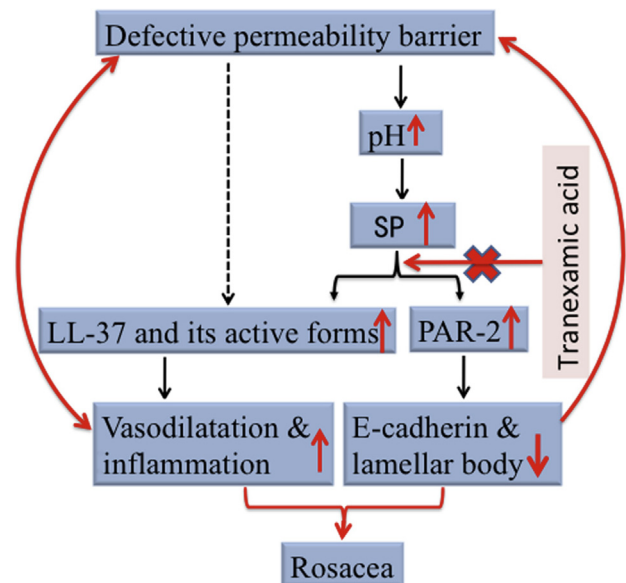

Figure 5 Effect of tranexamic acid on the mRNA expression of PAR-2 in HaCaT cells. Real time RT-PCR of PAR-2 in HaCaT cells after 24-hour tranexamic acid treatment. Each data point represents the mean (± SEM) result from three independent experiments. No statistical significance was found.

Figure 6 The possible pathogenic role of the permeability barrier in rosacea and the mechanisms by which tranexamic acid benefits rosacea. The impaired permeability barrier function can increase serine protease (SP) activity, resulting from the elevation of pH. Activation of SP activates SP-PAR2 and increases the production of active forms of LL-37, which in turn induces inflammatory cell infiltrate and angiogenesis. Activation of SP-PAR2 pathway can cause further damage in the permeability barrier via the inhibition of lamellar body secretion and E-cadherin adhesion. In addition, defective permeability barrier alone could increase LL-37 expression and induce inflammation. However, inflammation can worsen permeability barrier. Ultimately, inflammation and defective permeability together lead to the development of rosacea. Tranexamic acid benefits rosacea via inhibition of SP activity, leading the reduction of inflammation and improvement of permeability barrier function.

In summary, the present study demonstrates that topical applications of tranexamic acid benefit rosacea, likely due to the improvement of the permeability barrier function. Epidermal permeability barrier could be involved in the pathogenesis of rosacea. Approaches targeting the pathogenic pathway could be an optional therapy for rosacea.

Acknowledgments

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