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CONCISE COMMUNICATION

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Inducible nitric oxide synthase is required for epidermal permeability barrier homeostasis in mice

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Abstract

Nitric oxide (NO) regulates a variety of epidermal functions, including epidermal proliferation, differentiation and cutaneous wound healing. However, whether nitric oxide (NO) and its synthetic enzymes regulate epidermal permeability barrier homeostasis is not clear. In the present study, we employed inducible nitric oxide synthase (iNOS) KO mice to explore the role of iNOS in epidermal permeability barrier homeostasis. Our results showed that iNOS mice displayed a comparable levels of basal transepidermal water loss rates, stratum corneum hydration and skin surface pH to their wild-type mice, but epidermal permeability barrier recovery was significantly delayed both 2 and 4 hours after acute barrier disruption by tape stripping. In parallel, expression levels of mRNA for epidermal differentiation-related proteins and lipid synthetic enzymes were lower in iNOS KO mice versus wild-type controls. Topical applications of two structurally unrelated NO donors to iNOS KO mice improved permeability barrier recovery kinetics and upregulated expression levels of mRNA for epidermal differentiation-related proteins and lipid synthetic enzymes. Together, these results indicate that iNOS and its product regulate epidermal permeability barrier homeostasis in mice.

KEYWORDS
barrier function, epidermal differentiation, INOS, keratinocytes, nitric oxide, permeability

1 | BACKGROUND

Nitric oxide (NO) is a small free radical molecule, synthesized by three distinct nitric oxide synthases (NOS), that is endothelial, neural and inducible NOS. Although endothelial and neural nitric oxide synthases are mainly expressed in endothelial cells and neurons, respectively, keratinocytes also express these two enzymes.[1,2] In contrast, inducible NOS (iNOS) is expressed in a broad variety of cell types, including keratinocytes.[1,3] A number of stimuli, including cytokines (interferon and IL-10), wounding and UV irradiation, can increase iNOS expression and activity, as well as NO production.[4-6] NO regulates a wide spectrum of function in multiple organs and tissues, including the skin. Previous studies have showed that either deficiency in or inhibition of iNOS delays cutaneous wound healing.[7,8] Conversely, topical applications of nitric oxide accelerate cutaneous wound healing.[9] However, deficiency in either endothelial or neural

Erle Dang and George Man contributed equally to this work.

NOS accelerates epidermal permeability barrier recovery following acute barrier disruption in mice,[2] while one study showed that knockout of iNOS did not alter epidermal permeability barrier homeostasis in mice.[2] Because NO can stimulate keratinocyte differentiation and proliferation,[10,11] which both are linked to epidermal permeability barrier function, iNOS deficiency could alter epidermal permeability barrier function.

2 | QUESTIONS ADDRESSED

Because in the previous study, epidermal permeability barrier function of iNOS-deficient mice was assessed on the ears, which can respond differently to stimuli,[12,13] we assessed here whether iNOS deficiency alters epidermal permeability barrier homeostasis on the flank of the iNOS-deficient mice.

3 | EXPERIMENTAL DESIGN

All animal procedures were approved by the Animal Studies Subcommittee (IACUC) of the San Francisco Veterans Administration Medical Center and performed in accordance with their guidelines. 6- to 8-week iNOS knockout mice (B6;129P2-*Nos2*^{tm1Lau}/J), generated from cross between C57BL/6J-Aw−J and 129/J were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). For topical treatment, both flanks of iNOS knockout mice were treated topically with 60 μL of either 2 mmol/L S-nitroso-N-acetylpenicillamine (SNAP) or 2 mmol/L NOC-18 (both from Santa Cruz Biotechnology, Dallas, TX, USA) twice daily for three days, while mice treated with vehicle (propylene glycol: ethanol: water = 2:1:1, v/v) alone served as controls. Eighteen hours after the last NO treatment, basal epidermal permeability barrier function was assessed by measuring transepidermal water loss rates (TEWL), using TM300 connected to MPA5 (C&K, Cologne, Germany).[14] For barrier recovery in mice, TEWL was measured 0, 2 and 4 hours after tape stripping for 3 times (10-fold increase in TEWL measured with a Meeco electrolytic moisture analyser or over twofold increase measured with TM300. see figure legend of Figure 1), and per cent barrier recovery was calculated as described earlier.[14]

3.1 | Measurement of mRNA expression

Quantitative polymerase chain reaction (PCR) technique was used to measure expression level of mRNA. Total RNA was isolated from the epidermis, using TRI Reagent (Sigma). First-strand cDNA was synthesized from 1ug of total RNA with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The real-time PCR contained 20 ng of reversed transcribed total RNA, 450 nmol/L forward and reverse primers, and 10 μL of 2 × LightCycler 480 SYBR Green I Master in a final volume

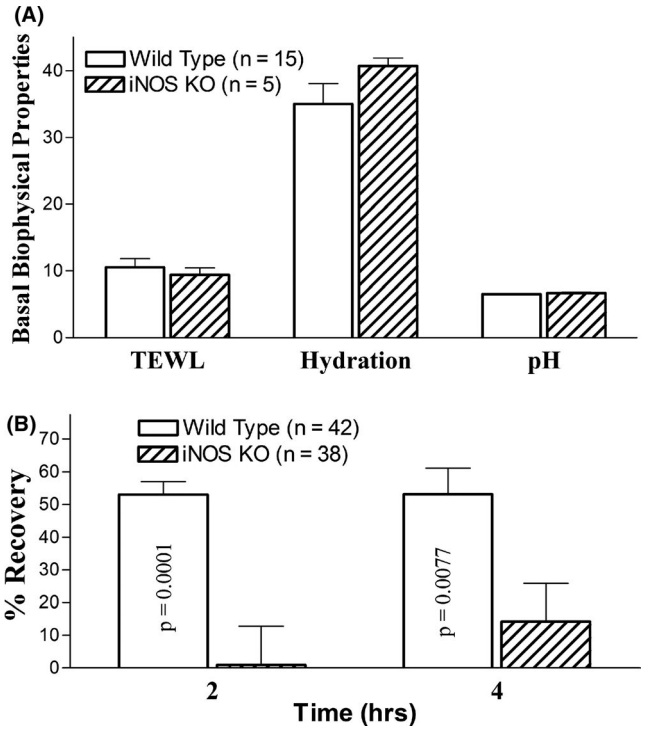

FIGURE 1 Deficiency in iNOS Delays Permeability Barrier Recovery in Mice. Basal epidermal permeability barrier function, skin surface pH and stratum corneum (SC) hydration were assessed with a MPA5 (CK electronic GmbH, Cologne, Germany) connected to TM 300, pH905 and Corneometer 825. Two readings were taken from each mouse for basal TEWL, hydration, as well as pH. For barrier recovery, TEWL was measured at 0, 2 and 4 h after tape stripping (detailed in the Methods section). A, Basal levels of TEWL, stratum corneum hydration and skin surface pH; (B) Barrier recovery. TEWL rates in Figure 1B were measured with a Meeco electrolytic moisture analyser (Meeco, Warrington, PA, USA). Numbers and significances are indicated in the figures

of 20 μL in 96-well plates using Mx3000P™ Real-Time PCR System (Stratagene, La Jolla, CA). Quantification was performed by the comparative C_T method with GAPDH used for normalization. The primers for lipid synthetic enzymes such as 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCoA), serine palmitoyl transferase 1 (SPT1), fatty acid synthase (FAS), filaggrin, involucrin and loricrin are listed in Table S1. Relative expression of the mRNAs compared to wild-type control mRNA was calculated. Data are expressed as percentage of control (as 100%).[14]

3.2 | Statistics

Data are expressed as the mean + SEM. GraphPad Prism 5 software (San Diego, CA, USA) was used for all statistical analyses. Unpaired two-tailed Student's *t* test was used to determine the statistical significances when two groups were compared.

4 | RESULTS

4.1 | Disruption of epidermal permeability barrier increases expression levels of epidermal iNOS mRNA in wild-type mice

Disruption of epidermal permeability barrier increases expression levels of mRNA for proteins required for permeability barrier homeostasis within 2 hours after barrier disruption.[15,16] And intraperitoneal injection of endotoxin significantly increased expression levels of iNOS mRNA in the kidneys within 1 hour[17] and iNOS protein in the lung within 3 hours.[18] Therefore, we first determined whether barrier disruption also increases expression levels of epidermal iNOS mRNA in wild-type mice. Indeed, disruption of epidermal permeability barrier significantly increased expression levels of epidermal iNOS mRNA as early at 1 hour after barrier disruption (231.15 ± 45.32 vs 100 ± 22.43, $P < .05$ for barrier disrupted vs. intact normal epidermis), followed by a slight decline at 2 hours (175.8 ± 29.1 vs 100 ± 54.5, $P < .05$). These results indicate that barrier disruption increases expression of iNOS mRNA in the epidermis of mice.

4.2 | Deficiency in iNOS delays epidermal permeability barrier recovery

We next assessed whether iNOS deficiency compromises epidermal permeability barrier homeostasis. The gross appearance of the skin did not noticeably differ between iNOS knockout and wild-type controls. Likewise, basal TEWL, stratum corneum (SC) hydration and skin surface pH were comparable between iNOS knockout and wild-type mice (Figure 1A). Moreover, the changes in TEWL rates after 3 tapes were also comparable between wild-type and iNOS knockout mice, assessed with a Meeco electrolytic moisture analyser (2.24 ± 0.23 vs. 2.27 ± 0.24 mg/cm^2/h). However, iNOS-deficient mice displayed a marked delay in permeability barrier recovery (Figure 1B). These results demonstrate that iNOS deficiency compromises epidermal permeability barrier homeostasis in mice.

4.3 | Expression levels of mRNA for epidermal differentiation marker-related proteins and lipid synthetic enzymes decline in the epidermis of iNOS knockout mice

Because both keratinocyte differentiation and epidermal lipid production are crucial for the maintenance of the epidermal permeability barrier,[19] we next assessed expression levels of mRNA for keratinocyte differentiation marker-related proteins and epidermal lipid synthetic enzymes in iNOS knockout vs. wild-type mice. As shown in Figure 2A, iNOS knockout mice exhibited significantly lower expression levels of mRNA for filaggrin and involucrin, while expression levels of loricrin mRNA were higher, but did not achieve statistical

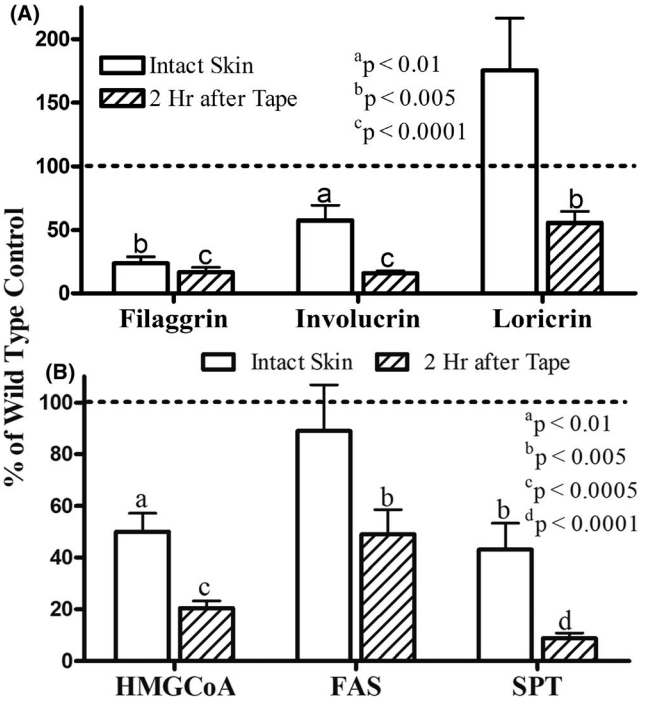

FIGURE 2 Mice with iNOS Deficiency Exhibit Lower Expression Levels of mRNA for Epidermal Differentiation Marker-Related Proteins and Lipid Synthetic Enzymes. Total epidermal RNA was isolated from both intact skin and 2 h after barrier disruption (detailed in the Methods section). Levels of mRNA expression were measured by qPCR using SYBR Green Master Mix. Relative expression of the mRNAs compared to GAPDH was calculated. Data are normalized to wild-type controls (setting wild-type controls as 100% indicated by the dotted line on figures). $N = 18$ for basal levels, and $N = 20$ for 2 h after barrier disruption. Significances are indicated in the figures

significance, in comparison with wild-type controls. Two hours after acute barrier disruption, expression levels of mRNA for all three differentiation marker-related proteins were lower in iNOS knockout than in wild-type controls, consistent with previous finding that low NO decreased keratinocyte differentiation.[10] Similarly, expression levels of mRNA for the rate-limiting enzymes for cholesterol and ceramides were also lower both under basal condition (except for fatty acid synthase, FAS) and 2 hours after acute barrier disruption in iNOS knockout than wild-type controls (Figure 2B). These results indicate that iNOS-deficient mice display lower expression levels of mRNA for epidermal differentiation marker-related proteins and lipid synthetic enzymes.

4.4 | Topical nitric oxide donors improve epidermal permeability barrier homeostasis in iNOS knockout mice

We determined next whether topical applications of the product of iNOS, NO, can correct the barrier abnormality in iNOS knockout mice. We first treated iNOS mice with a common NO donor, SNAP, twice daily for 3 days. As expected, topical SNAP accelerated

barrier recovery following acute barrier disruption (Figure 3A), while basal levels of TEWL remained comparable between iNOS knockout and wild-type mice (6.51 ± 0.41 in iNOS knockout mice; 5.35 ± 0.61 in wild-type mice, $P = .1259$). To confirm the impact of topical NO on barrier function, we treated iNOS knockout mice with another structurally unrelated NO donor, NOC-18. Although the results did not achieve a statistical significance ($P = .0637$), a substantial acceleration in barrier recovery rates was observed in NOC-18-treated mice compared with vehicle-treated mice (Figure 3B). NO donor-induced improvements in epidermal permeability barrier homeostasis were paralleled by a remarkable upregulation of expression levels of mRNA for epidermal differentiation and lipid synthetic enzymes, to the levels comparable to wild-type controls (Figure 3C), while expression levels of these mRNAs in vehicle-treated iNOS knockout mice remained lower than in wild-type controls (Figure 3C). Taken together, these results show that topical NO enhances epidermal permeability barrier function in iNOS-deficient mice, indicating a requirement for NO in epidermal permeability barrier homeostasis.

5 | DISCUSSION

Previous studies have demonstrated that deficiency in either endothelial or neural NOS accelerates epidermal permeability barrier recovery on the mouse ear.[2,20] Likewise, inhibition of neural NOS accelerates barrier recovery on the mouse flank.[20] In contrast, barrier repair kinetics were not altered either in iNOS-deficient mice or in hairless mice treated with iNOS inhibitor.[2,20] However, we show here instead that iNOS deficiency impedes permeability barrier homeostasis. The discrepant results between the present and prior studies can be attributed to the different methods employed in the studies. Tape stripping induces the production and release of pro-inflammatory cytokines, as well as NO.[20-24] Although certain cytokines and NO can benefit epidermal function barrier,[25,26] excessive inflammation and/or NO content can compromise epidermal function and induce inflammation.[27,28] Thus, reduction in NO content may benefit epidermal function in inflamed skin. In prior study on iNOS knockout mice, ears were used to assess barrier recovery. Because inflammatory reactions to the same stimuli on the ear are

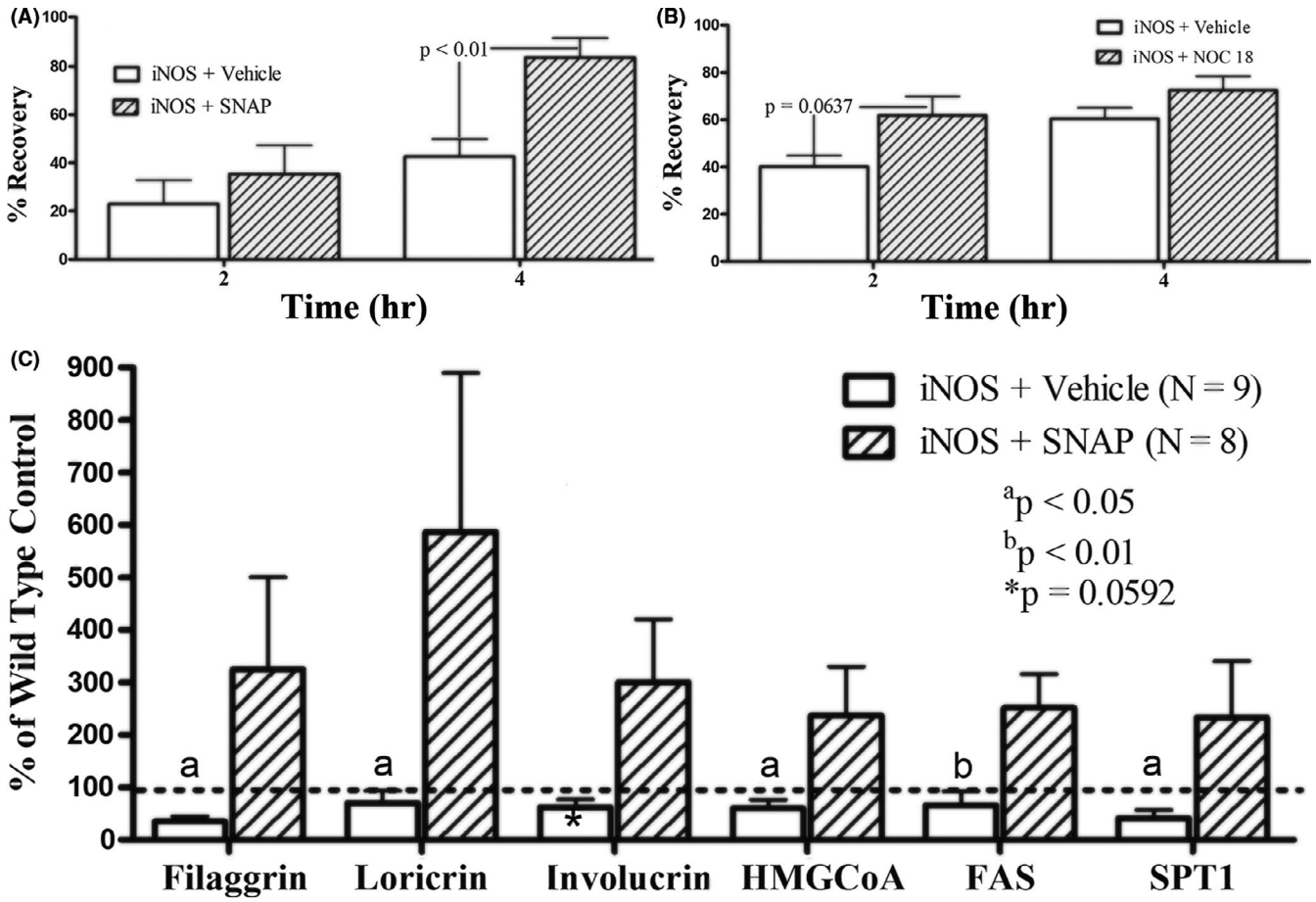

FIGURE 3 Topical NO Donors Improve Epidermal Permeability Barrier Homeostasis in iNOS Knockout Mice. Both epidermal permeability barrier and expression levels of mRNA were assessed following 3-d treatment with topical SNAP (detailed in the Materials and Methods section). Figure 3A and B depicts barrier recovery rates 2 and 4 h after acute barrier disruption. $N = 20$ for all; Figure 3C shows expression levels of mRNA for epidermal differentiation marker-related proteins and lipid synthetic enzymes in vehicle-treated vs NO donor-treated iNOS knockout mice. Data are normalized to wild-type controls (setting wild-type controls as 100% indicated by the dotted line on figures). Student's *t* test was used to determine the significances between vehicle- and NO donor-treated mice. Significances and numbers of mice are indicated in the figures

more severe than that on the flank of normal mice,[13] tape stripping-induced inflammation and NO release in the ear of NOS-deficient mice could be less severe than in normal mice. Hence, following tape stripping, the ears of NOS-deficient mice can display either accelerated or unaltered barrier recovery in comparison with the wild-type controls. However, further studies will be required to assess these speculations.

The underlying mechanisms by which iNOS deficiency impedes barrier recovery could be attributable, at least in part, to reductions in expression levels of mRNA for epidermal differentiation and lipid production, which both are crucial for maintenance of the epidermal permeability barrier function.[19] Although the exact signalling pathway that links NO to epidermal differentiation and lipid production is not clear, evidence suggests a involvement of peroxisome proliferator-activated receptor gamma (PPARγ). First, PPARγ activators stimulate keratinocyte differentiation and lipid production, resulting in an acceleration in permeability barrier recovery.[29,30] Second, iNOS inhibitor, such as L-N^G-nitroarginine methyl ester, inhibits PPARγ activity, while NO donors increase PPARγ activity via enhancing PPARγ binding to DNA and transactivation of its responsive reporter.[31,32] Therefore, reduced PPARγ activity can contribute to the reductions in expression of mRNA for keratinocyte differentiation and lipid production in iNOS-deficient mice.

6 | CONCLUSIONS

Deficiency in iNOS compromises epidermal permeability barrier homeostasis, possibly resulting from decreased PPARγ activity, consequently leading to reductions in epidermal differentiation and lipid production. However, further studies are needed to further determine the link between iNOS and epidermal function.

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CONFLICT OF INTEREST

All authors have no conflicts of interest.

AUTHOR CONTRIBUTIONS

ED, GM, JZ and DL performed experiments. ED and MQM analysed the data. PME, TMM and MQM interpreted the data. MQM originated the concept, designed experiments and drafted the manuscript. PME and TMM critically reviewed the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Table S1. Primer sequences

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