SUPPLEMENTARY MATERIALS

Lysophosphatidic acid stimulates the intestinal brush border Na^+/H^+ exchanger 3 and fluid absorption via LPA_5 and NHERF2

by

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Supplementary Materials and Methods

Real-time RT-PCR:

Mouse tissue was rinsed with PBS and epithelial cells were collected by lightly scrapping the surface with a glass cover-slip [1]. Total RNA was isolated from colonic tissues using TRIzol (Invitrogen) and cDNA was subsequently synthesized using the First Strand Synthesis kit (Invitrogen). Real-time RT-PCR was performed as previously described [1] using iQ SYBR Green Supermix (Bio-Rad) on the iCycler (Bio-Rad). The primers sets were designed to cross an exon-intron junction to avoid any contamination from the genomic DNA. Primers used for qRT-PCR are:

LPA_1: 5'-ACACCAGCCTGACAGCTTCT-3' and 5'-CTGTAGAGGGGTGCCATGTT-3';

LPA_2: 5'-TCACTGGTCAATGCAGTGGT-3' and 5'-AAGGGTGGAGTCCATCAGTG-3';

LPA_3: 5'-AGGGCTCCCATGAAGCTAAT-3' and 5'-TTCATGACGGAGTTGAGCAG-3';

LPA_4: 5'- TGCATCAGTGTGGATCGTTT-3' and 5'-GAAGCCTTCAAAGCAAGTGG-3';

LPA_5: 5'- GCTCCAGTGCCCTGACTATC-3' and 5'- GGGAAGTGACAGGGTGAAGA-3';

18S: 5'- GCAATTATTCCCCATGAACG -3' and 5'- GGCCTCACTAAACCATCCAA -3'.

In vitro overlay assay:

Overlay assay was performed as previously described by us [2]. Briefly, His-tagged recombinant proteins were resolved on 10% SDS-PAGE gels, blotted, blocked in blot buffer (10 mM Hepes, pH 7.4, 50 mM NaCl, 2% dry milk, 0.1% Tween 20) for 30 min at room temperature, and overlaid with 50 nM GST-LPA_5 fusion protein or GST control protein in blot buffer for 1 h at room temperature. The membranes were washed three times in blot buffer, and incubated with HRP-conjugated anti-GST antibody to detect GST fusion protein bound to His-fusion proteins.

Na^+-dependent Intracellular pH Recovery:

The Na^+-dependent changes in intracellular pH (pH_i) by NHE3 was determined with the use of the ratio-fluorometric, pH-sensitive dye 2',7'-bis-(2-carboxyethyl)-5-carboxyfluorescein acetoxymethyl ester (BCECF-AM) as previously described [3]. Briefly, cells were seeded on coverslips, grown to 70-80% confluence for PS120 or 5 days post-

confluence for Caco2 cells, and then serum starved overnight. Cells were washed in Na^+ buffer (mM: 30 NaCl, 20 HEPES, 5 KCl, 1 tetramethylammonium-PO_4 (TMA-PO_4), 2 $CaCl_2$, 1 $MgSO_4$, and 18 glucose) and then were dye-loaded by incubating for 20 min with 6.5 μM BCECF-AM in the same solution. The coverslips were mounted on a perfusion chamber mounted on an inverted microscope, and were superfused with NH_4^+ buffer (mM: 40 NH_4Cl, 90 NaCl, 20 HEPES, 5 KCl, 1 TMA-PO_4, 2 $CaCl_2$, 1 $MgSO_4$, and 18 glucose) and subsequently with TMA^+ buffer (mM: 130 TMA-Cl, 20 HEPES, 5 KCl, 1 TMA-PO_4, 2 $CaCl_2$, 1 $MgSO_4$, and 18 glucose) containing 1 μM LPA or 1% BSA in PBS as a control for 2-5 min. Na^+ buffer was then reintroduced to drive Na^+-dependent pH recovery. Calibrations of the fluorescence signal and data analysis were performed as previously described [3]. S3224 and HOE694 were kindly provided by Drs. Lang and Punter at Aventis Pharma.

Surface Biotinylation:

Surface biotinylation of NHE3 was performed as previously described [3]. Briefly, cells grown in 10-cm petri dishes were treated with LPA or carrier for 5 min, followed by rinsing twice in PBS and 10 min incubation in borate buffer (mM:154 NaCl, 7.2 KCl, 1.8 $CaCl_2$, and 10 H_3BO_3, pH 9.0). Cells were then incubated for 40 min with 0.5 mg/ml NHS-SS-biotin (Pierce, Rockford, IL) in borate buffer. Unbound NHS-SS-biotin was quenched with Tris buffer (mM: 20 Tris-HCl, pH 7.4, 120 mM NaCl). Cells were then rinsed with PBS, scraped, lysed in the lysis buffer described above, and sonicated for 2 × 15 s. The lysate was agitated for 30 min and spun at 14,000 × *g* for 15 min to remove the insoluble cell debris. An aliquot was retained as the total fraction representing the total cellular NHE3. Protein concentration was determined and 1 mg of lysate was then incubated with streptavidin-agarose beads (Pierce) for 2 h. The strepavidin-agarose beads were washed 3 times in lysis buffer and twice in PBS. All the above procedures were performed at 4 °C or on ice. The proteins representing surface NHE3 were then eluted by boiling the beads at 95 °C for 10 min. Dilutions of the total and surface NHE3 were resolved by SDS-PAGE, and immunoblotted with anti-VSVG antibody or anti-HA monoclonal antibody. Densitometric analysis was performed using Scion Image software (NIH).

Immunofluorescence and Confocal studies:

In vivo NHE3 Trafficking studies were performed as follows. The last 3 cm of ileum was cannulated in anesthetized mice and perfused with 50 μM LPA or saline for 20 min. The mice were sacrificed, the ileum excised and processed for immunohistochemical staining of NHE3 and the actin in the apical microvilli and terminal web as previously described [4], with the following modification: An anti-NHE3 was from Alpha Diagnostic. After the secondary antibody incubation and washes, the tissue was incubated with Alexa Fluor 633 phalloidin (Invitrogen) for 30 min (5U/ml in PBS with 1%BSA, 0.2%TritonX100) at room temperature, followed by 6 washes of 1 minute each, then staining of the nuclei and covering using slow fade with DAPI (Invitrogen). Cover slides were imaged on a confocal microscope (Leica DM IRB with a TCS SP2 AOBS scan head equipped with a 405nm laser for excitation of blue dyes). Sections from *Nherf2*$^{-/-}$ and WT Ileum were imaged with identical confocal settings. For (semi-)quantification of NHE3 trafficking from the terminal web to the tip of the microvilli, images were analyzed by determining total background-subtracted fluorescent signal using Leica software as illustrated in supplementary Figure S2.

***Statistical analysis*:**

Results are presented as the means $\pm$ standard error (SE). Unpaired Student's *t*-test was performed to test significance between groups. Results were considered significant at $p < 0.05$. Statistic analysis was performed using Origin 8 (OriginLab Corp., Northampton, MA).

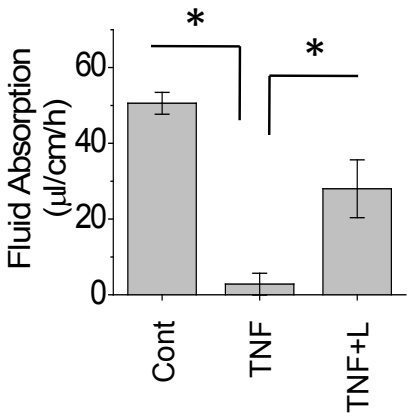
References for Supplementary

1. Lin, S, Wang, D, Iyer, S, et al. The absence of LPA_2 attenuates tumor formation in an experimental model of colitis-associated cancer. Gastroenterology 2009;136:1711-1720.
2. Zhang, H, Wang, D, Sun, H, et al. MAGI-3 regulates LPA-induced activation of Erk and RhoA. Cell Signalling 2007;19:261-268.

3. Wang, D, Sun, H, Lang, F, et al. Activation of NHE3 by dexamethasone requires phosphorylation of NHE3 at Ser663 by SGK1. Am J Physiol 2005;289:C802-810.
4. Cinar, A, Chen, MM, Riederer, B, et al. Nhe3 inhibition by cAMP and Ca2+ is abolished in PDZ-domain protein PDZK1-deficient murin enterocytes. J Physiol 2007;581:1235-1246.

SUPPLEMENTARY FIGURES

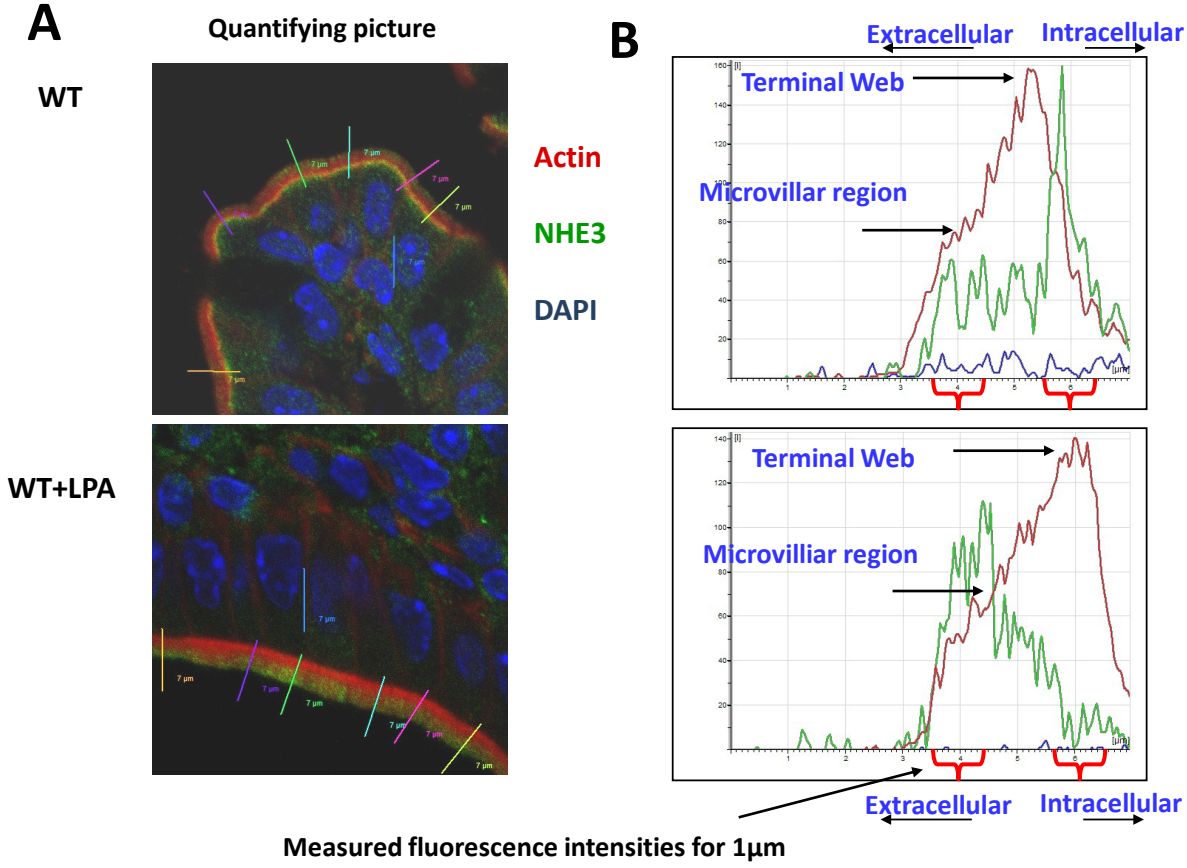
Supplementary Figure S1.



The effect of LPA on TNF-induced diarrhea

Injecting mice with 5 μg TNF-α resutled in a marked reduction of fluid absorption. Inclusion of 20 μM LPA in the perfusion buffer significantly augmented the amount of fluid absorbed in the intestine of TNF-treated mouse. n = 3. *, $p < 0.05$.

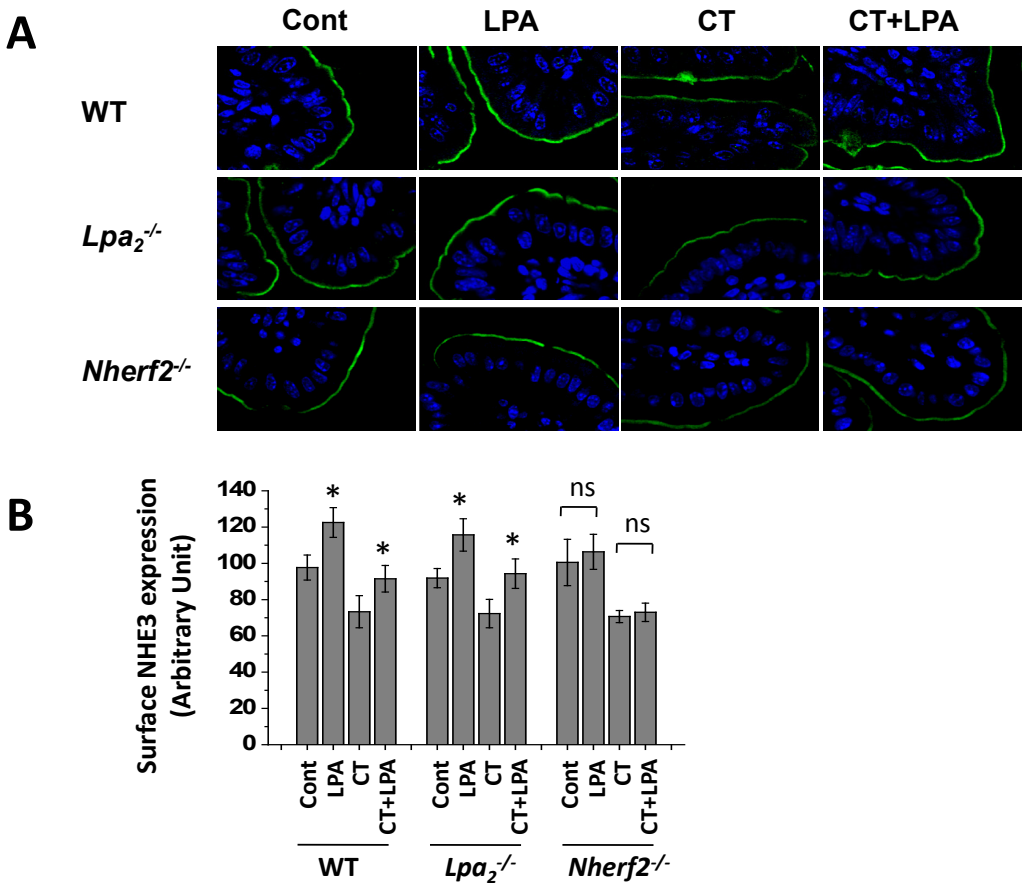
Supplementary Figure S2



Quantification of NHE3 in the terminal web and microvilli of ileum

(A) Confocal images were taken where the BBM had been cut exactly perpendicular, as assessed by measuring the length of the F-Actin/NHE3 stained BBM. A 7µm length line was drawn perpendicular to the membrane over the microvilli and terminal web region. A total of six lines were drawn per calculation, and one line was drawn intracellularly to assess background fluorescence. **(B)** The fluorescence intensity curves along the lines were obtained at three wavelengths corresponding to: Green=NHE3, Red= Actin and Blue= DAPI. The red fluorescence intensity peak indicated the terminal web region. Then the green fluorescence intensity was averaged for two 1 µm long parts of the curve (see brackets) with a fixed distance between them, corresponding to regions within the upper third of the microvilli, and the area of the terminal web. The upper panel is an example for the NHE3 distribution in the distal ileum under basal conditions, with the green NHE3-fluorescence intensity peak in the inner border of the terminal web region. The lower panel is an example for a LPA-treated ileum, with the majority of NHE3 found in the microvilli.

Supplementary Figure S3



Immunohistochemical staining of NHE3 in mouse intestine

(A) The intestine of WT, *Lpa*$_2^{-/-}$, and *Nherf2*$^{-/-}$ mice were perfused with LPA with or without CT as described in the text and materials and Methods. After 3 h of perfusion the intestine was excised and ileal segments were fixed in formalin and imbedded in paraffin blocks as previously described (53). Sections were deparaffinized and antigen unmasking was performed by microwave treatment. Non-specific blocking was obtained by incubation in PBS with 10% normal goat serum for 30 minutes at 37^{o}C. Sections were then incubated with an anti-NHE3 antibody for 1 h at room temperature. Sections were washed with PBS, incubated with Alexa Fluor 488 (Invitrogen) for 30 min and Topo-3 (Invitrogen) for 5 min at room temperture. The sections were washed 3 times 10 min each with PBS and visualized by confocal microscopy using a Zeiss LSM510 laser confocal microscope. Green: NHE3, Blue: Topo-3. **(B)** Quantification was performed by the green fluorescence signal at the brush border membrane at randomly selected regions as indicated in Suppl. Figure 2. At least 6 regions per visual field and 3 pairs of mice were used for the quantification. *, $p < 0.05$. ns = not significant.