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Trefoil Factor 2 Requires Na/H Exchanger 2 Activity to Enhance Mouse Gastric Epithelial Repair*[S]

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Background: Trefoil factors promote tissue repair, but *in vivo* mechanisms are unknown.
Results: Trefoil factors fail to stimulate repair of individual gastric lesions when NHE2 is inactivated.
Conclusion: Trefoil factors require activity of a specific Na/H exchanger isoform to stimulate gastric epithelial repair.
Significance: NHE2 is newly identified as a target for enhancing gastric repair and as a downstream effector protein of trefoil factors.

Trefoil factor (TFF) peptides are pivotal for gastric restitution after surface epithelial damage, but TFF cellular targets that promote cell migration are poorly understood. Conversely, Na/H exchangers (NHE) are often implicated in cellular migration but have a controversial role in gastric restitution. Using intravital microscopy to create microscopic lesions in the mouse gastric surface epithelium and directly measure epithelial restitution, we evaluated whether TFFs and NHE isoforms share a common pathway to promote epithelial repair. Blocking Na/H exchange (luminal 10 μM 5-(*N*-ethyl-*N*-isopropyl) amiloride or 25 μM HOE694) slows restitution 72–83% in wild-type or NHE1$^{-/-}$ mice. In contrast, HOE694 has no effect on the intrinsically defective gastric restitution in NHE2$^{-/-}$ mice or TFF2$^{-/-}$ mice. In TFF2$^{-/-}$ mice, NHE2 protein is reduced 23%, NHE2 remains localized to apical membranes of surface epithelium, and NHE1 protein amount or localization is unchanged. The action of topical rat TFF3 to accelerate restitution in TFF2$^{-/-}$ mice was inhibited by AMD3100 (CXCR4 receptor antagonist). Furthermore, rat TFF3 did not rescue restitution when NHE2 was inhibited [TFF2$^{-/-}$ mice +HOE694, or NHE2$^{-/-}$ mice]. HOE694 had no effect on pH at the juxtamucosal surface before or after damage. We conclude that functional NHE2, but not NHE1, is essential for mouse gastric epithelial restitution and that TFFs activate epithelial repair via NHE2.

The integrity of the gastric epithelium is essential to protect the body (and the stomach tissue) from the noxious contents of the stomach lumen (including acid, proteases, and the pathogens ingested with food). When epithelial integrity is breached by acute damage to the surface epithelium, adjacent healthy cells rapidly migrate in to restore continuity of the cell layer (1). When this process of epithelial restitution fails, tissue damage can expand and lead to ulceration. These severe consequences have led to a prolonged effort to define the mechanisms regulating restitution and repair so that mucosal defenses could be bolstered clinically.

One of the major endogenous regulators of repair is the trefoil factor (TFF)[3] protein family, protease-resistant peptides that are released from gastric mucous cells to promote healing (2). TFFs have been extensively studied *in vivo* and *in vitro* using various species and in model systems from cell culture to genetically manipulated animals (3). All of the currently available results indicate TFFs, especially the endogenous gastric TFF2, accelerate gastric epithelial restitution (4). The downstream effectors of TFFs have been elusive, although it has recently been shown that TFF2 activates calcium-dependent signaling and ERK1/2 phosphorylation via the CXCR4 receptor, providing the first validation of a TFF receptor (5). As discussed in a recent review, the expanding information about signaling events stimulated by TFFs is broadening understanding of how restituting cells loosen contacts with neighboring cells, enhance their resistance to apoptosis, and initiate migration across a wound (3). The TFF effector proteins that are required to stimulate migration remain the most poorly defined.

In numerous cell types, the plasma membrane Na/H exchange proteins are necessary for wound repair and/or cellular migration. Most commonly, the ubiquitous NHE1 isoform supports these functions through transport activity (affecting both the intracellular and extracellular pH milieu), and the dynamic regulation of protein scaffolds that can affect signal transduction and cytoskeleton activity (6–13). The gastric epithelium expresses at least four NHE isoform Na/H exchangers; apical NHE2 (most abundant in surface/neck mucous cells), apical NHE3 (in rat but not rabbit parietal cells), basolateral NHE1 (all cells), and basolateral NHE4 (parietal, chief cells) (14–17). It has been suggested that Na/H exchange function can be important for gastric repair, although no work has yet been directed to evaluating the role of specific NHE isoforms in

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[3] The abbreviations used are: TFF, trefoil factor; EIPA, 5-(*N*-ethyl-*N*-isopropyl) amiloride; NHE, Na/H exchanger; HOE 694, Hoechst 694.

gastric repair. In isolated guinea pig fundus, NHE1 has been implicated in repair because serosal (but not luminal) amiloride blocks restitution of the gastric surface epithelium (18, 19). Results suggest that endogenous or exogenous EGF can promote healing via serosal Na/H exchange (18). Curiously, NHE1 KO mice have modest changes in gastric gland morphology, but no evidence of altered surface epithelium has been reported, as might be expected if the tissue was unable to repair in this mutant mouse (20). In contrast, NHE2 KO mice show a progressive gastritis starting at the tenth day of life that leads to gastric atrophy (21, 22), and cultured rat gastric epithelial cells (RGM1) were tentatively suggested to utilize NHE2 to repair acid-induced damage (23). Even the fundamental role of Na/H exchange in gastric repair remains controversial, as Na/H exchange inhibition had no effect on restitution of isolated bullfrog gastric mucosa in the presence of HCO_3/CO_2 (24). Using an intravital microscopy technique that allows creation of microscopic lesions (with two-photon laser photodamage) and measurement of repair in real-time, we use a combination of genetic and pharmacologic approaches to test whether TFFs and Na/H exchange share roles in the repair of the mouse gastric surface epithelium.

EXPERIMENTAL PROCEDURES

Animal Husbandry—Experiments used C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME), in-house bred TFF2 KO mice (backcrossed onto a C57BL/6J background until >90% of genomic microsatellite markers were from C57BL/6J) (25), in-house bred NHE1 (*Slc9A1*) KO mice (129/SvJ and Black Swiss mixed background) and in-house bred NHE2 (*Slc9A2*) KO mice (FVBN background). We are very grateful to Drs. Gary Shull and Vikram Prasad for supplying animals from the NHE1 KO colony. Pups were genotyped by genomic PCR as described (20, 21, 26) and used for experimentation at 2–4 months of age. For experiments examining TFF2 or NHE null −/− genotypes, wild-type controls were composed of +/− and/or +/+ genotypes from the same colony.

Animal Surgery—The surgical preparation of animals has been described previously (27, 28). Briefly, mice were anesthetized, and then the gastric mucosal surface was surgically exposed. The mouse was placed prone on the heated stage of an inverted confocal/two-photon microscope (Zeiss LSM 510 NLO), with a portion of the exposed mucosa protruded into a perfusion chamber. The mucosal surface was superfused (0.2 ml/min) with a pH 3 solution containing 150 mM NaCl, 1 mM homopipes (Research Organics, Cleveland, OH) and a fluorescent pH sensor (10 μM Cl-NERF, Invitrogen). In some experiments, 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA, Sigma) or Hoescht 694 (HOE694, from Dr. H. J. Lang, Sanofi-Aventis, Frankfurt, Germany) were added directly to perfusate. When required, the CXCR4 antagonist AMD3100 (Sigma) was added into luminal perfusate (10 μM) as well as given subcutaneously (5 mg/kg in saline, 1 h prior to experiments). In some experiments, the rat trefoil factor 3 peptide (rTFF3, 2 mg/ml in saline, solubilized on the day of the experiment) was topically applied to the exposed gastric surface at two to three times within 20 min (29). We are very grateful to Dr. Daniel Podolsky for supplying the rTFF3 peptide. Due to limited reagent, rTFF3 was not included in the perfusion solution. All experimental procedures were approved by the Animal Care and Use Committee of the University of Cincinnati.

Live Tissue Imaging and Inducing Focal Lesions in Gastric Surface Epithelium—Our methods for confocal/two-photon imaging of the exposed gastric mucosa and creation of microscopic lesions in the surface epithelium by two-photon photodamage have been reported previously (25, 30, 31). Briefly, the mucosal surface of the gastric corpus was observed using a Zeiss C-Apo ×40 objective, and data were collected in a time series. Multiple images were collected for each time point, reporting tissue autofluorescence (two-photon excitation 710 nm, emission 435–485 nm), confocal reflectance (reflecting 710 nm light to show cell/tissue structure), and confocal images of 550–600 nm Cl-NERF fluorescence in response to alternating 514 nm/458 nm excitation. To create focal lesions, a small rectangle region (200 μm^2) of gastric surface epithelium was repetitively scanned with 710 nm of light at 350 milliwatt average laser power for 100 iterations (5–10 s duration). The resultant photobleaching of endogenous fluorophores (putative NAD(P)H), led to a sequence of surface epithelial damage and repair that was recorded by time course imaging. The damage-repair cycle was measured independently several times per animal in different locations of the corpus. As described previously (25, 30, 31), post-acquisition image analysis (Metamorph software, Molecular Devices, Downington, PA) measured the lesioned area as defined by cellular loss of NAD(P)H autofluorescence in direct comparison with simultaneously recorded confocal reflectance images that confirmed location of cellular structures. The changing size of the damaged area over time was used to estimate rates of epithelial restitution, by fitting data to a single exponential decay curve as described and reporting the rate constant of repair as a measure of the restitution rate (25). Extracellular pH near the gastric surface was measured from Cl-NERF ratio images as described previously (25, 27, 28, 31).

Western Blots—We followed published procedures to separate corpus gastric mucosa from muscle layer, solubilize/homogenize tissue, separate proteins on 7.5% SDS-PAGE gel, and transfer to a PVDF membrane (Immobilon-P; Millipore Corp., Billerica, MA) (25). Membranes were incubated for 1 h with Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE) and then incubated with rabbit polyclonal anti-murine NHE1–3 or anti-TFF2 (1:1000 dilution) mixed with mouse monoclonal anti-murine GAPDH (1:10000 dilution). Membranes were then washed and incubated for 1 h in goat anti-rabbit secondary antibody (Alex Fluor 680, 1:1000 dilution; Invitrogen) and goat anti-mouse secondary antibody (IR800 dye, 1:10000, Rockland, Gilbertsville, PA). Fluorescent blots were imaged and quantified using an Odyssey infrared imaging system (Li-Cor Biosciences). Immunoreactive bands were quantified using background-corrected integrated pixel intensity, with results normalized *versus* GAPDH in the same gel lane. Genotype comparisons (between lanes) were always made from the same gel and exposure.

Immunofluorescence—Isolated mouse stomach was fixed with 4% paraformaldehyde for 2 h at 4 °C, rinsed with PBS, and transferred to 30% sucrose for 2–4 h at 4 °C. Ten μm sections

were prepared with a microtome cryostat at −20 °C. Antigen retrieval was performed for NHE antibodies by citrate buffer (pH 6.0) for 10 min. Sections were incubated with the primary antibodies for 30 min in room temperature, using 1:100 dilution of the antiserum to TFF2, NHE1, or NHE2. The polyclonal rabbit antisera against NHE1 and NHE2 have previously been shown to be specific when staining murine tissues (32). Secondary antiserum (1:200 dilution Alexa Fluor 488-labeled goat anti-rabbit IgG, Invitrogen) was incubated for 30 min at room temperature. The sections incubated with TFF2 antisera were mounted in medium with DAPI (Vector Laboratories, Burlingame, CA), whereas nuclear DNA staining was performed for sections incubated with NHE antisera by incubation with Hoechst 33342 (HOE 33342, Molecular Probes, Eugene, OR) at 1 μg/ml for 1 min. Sections were imaged with confocal microscopy.

Statistical Analysis—All compiled values are reported as mean ± S.E. from multiple experiments. The number of repetitions (*n*) is reported as the number of independent damage-repair cycles analyzed. All results were reproduced in at least three animals. Statistical significance was determined using an unpaired Student's *t* test, or for multiple comparisons by one-way analysis of variance with Dunnett's post hoc test. A p value of <0.05 was considered significant.

RESULTS

We previously established an *in vivo* model for creation of microscopic lesions in the gastric surface epithelium of anesthetized mice and reported that TFF peptides are important to allow efficient repair of the gastric epithelium (33). We now examine whether this response requires NHE activity.

Contribution of Na/H Exchanger Isoforms to TFF2-dependent Gastric Epithelial Repair—We first compared the effect of two structurally unrelated Na/H exchange inhibitors, EIPA (10 μM) and Hoescht 694 (HOE694, 25 μM), on gastric epithelial restitution in WT mice. Drug was added into the superfusate bathing the gastric mucosal surface for 20 min, and then laser photodamage was applied to the superficial epithelial cells. As shown qualitatively in the compiled time course experiments in Fig. 1*A*, and in the calculated rates of repair in Fig. 1*B*, both drugs produced a significant slowing of epithelial repair compared with control ($p < 0.05$). Given similar effects of both drugs and subsequent focus on effects of NHE1 and NHE2, two Na/H exchanger isoforms that are inhibited more selectively by HOE94 (34, 35), all further experiments used HOE694.

The NHE1 Na/H exchanger isoform is ubiquitously expressed and has been shown to promote cell migration in other cell types (6–13). We therefore measured gastric epithelial restitution rates in NHE1 KO mice. Surprisingly, the NHE1 null stomach had normal rates of lesion recovery compared with WT controls and still demonstrated a significant block of that repair rate by HOE694 (Fig. 1, *C* and *D*). In contrast, we also evaluated NHE2 KO mice, and found that NHE2 null stomachs demonstrated a significantly impaired gastric restitution (compared with WT controls) that was not decreased further in the presence of HOE694 (Figs. 1, *E* and *F*). Results strongly suggest that NHE2, but not NHE1, is the target of HOE694 inhibition and is required for gastric epithelial repair.

Similar experiments were performed in TFF2 KO mice. As we reported previously, disruption of the TFF2 gene impaired gastric repair rates (25). However, HOE694 had no further effect on epithelial repair rates in TFF2 null stomachs (Fig. 2). Raising the HOE694 concentration to 50 μM did not change outcomes (data not shown). Results suggest that TFF2 and NHE2 may share a common mechanism to facilitate epithelial restitution after injury.

Comparison of NHE Isoform Expression in WT and TFF2$^{-/-}$—Based on these results, it was possible that TFF2 null mice had ineffective gastric repair due to down-regulation of Na/H exchange proteins in the gastric mucosa. Therefore, experiments used Western blots to compare levels of NHE proteins in gastric mucosal homogenates from WT and TFF2 null mice, as shown in Fig. 3*A*. Results were quantified from multiple blots, and results are compiled in Fig. 3*B*. As shown, TFF2 null mucosa had a significant but modest 23% decrease in NHE2 protein compared with WT mucosa (using GAPDH to control for protein loading). No significant change in NHE1 or NHE3 was detected. The localization of NHE2 in the stomach body was also evaluated using immunofluorescence staining. As shown in Fig. 4, NHE1 was present in the basolateral membranes of all gastric epithelial cells (36), and NHE2 was present in the apical membrane of surface and neck epithelial cells. The specificity of the NHE2 antisera in gastric tissues was confirmed by negative staining of the gastric surface epithelium in NHE2 null mice (supplemental Fig. 1). No striking difference in NHE2 localization was noted when comparing WT with TFF2 null tissues, although there was a tendency for the NHE2 staining of the null stomach to be more tightly restricted to the surface cells compared with pit/neck cells.

We also tested whether the NHE2 null stomach had diminished TFF2 expression *versus* NHE2 WT stomach. As shown in supplemental Fig. 2, preliminary data showed sustained (maybe increased) expression of TFF2 in the NHE2 null gastric mucosa, with expanded immunolocalization of TFF2 to deeper tissue locations than in WT tissue. There was no basis to suggest that lack of TFF2 could explain inefficient gastric repair in NHE2 null mice.

Exogenous rTFF3 Requires Na/H Exchange Activity to Accelerate Gastric Restitution—It has been shown that either exogenous TFF3 or TFF2 can promote healing of gastric injury, likely due to receptor cross-talk between these related peptides (37). We have previously reported that exogenous rat TFF3 (rTFF3) can rescue the impaired gastric restitution rates of TFF2 null mice, so that the speed of repair becomes similar to WT animals (25). In contrast, the same rTFF3 peptide was not able to rescue the gastric restitution of TFF2 null mice that had been pre-treated with HOE694 for 20 min. This is shown qualitatively in the representative experiments of Fig. 5*A* and in the compiled measures of gastric repair rate in Fig. 5*B*. Results suggest that the trefoil peptide requires Na/H exchange activity to mediate gastric repair or that the HOE694 may non-specifically interfere with rTFF3 action. Further experiments using NHE2 null mice showed that the rTFF3 was also ineffective to accelerate repair in this genotype in the absence of HOE694 (representative experiments in Fig. 5*C* and compiled outcomes in Fig.

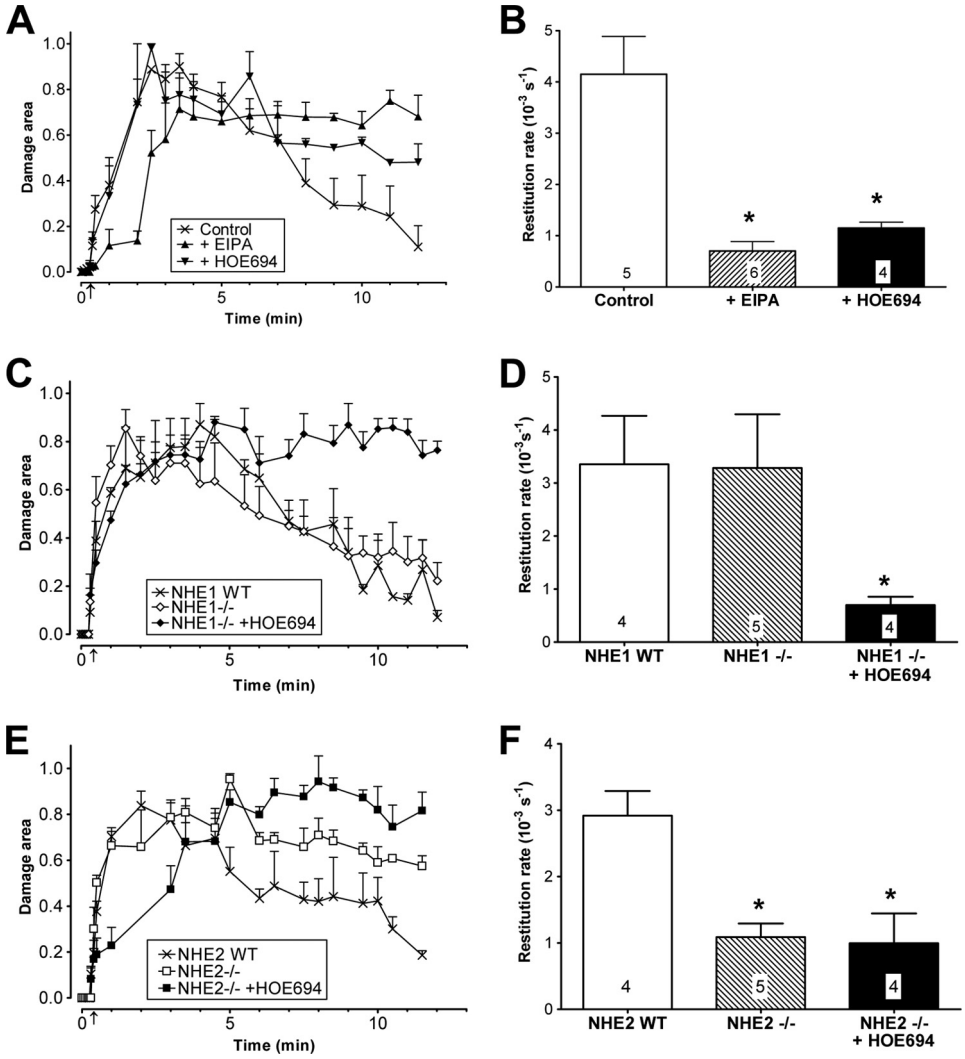

FIGURE 1. **Requirement for Na/H exchange activity during gastric epithelial restitution.** As indicated in the figure, experiments compared the effects of adding EIPA (10 μM) or HOE694 (25 μM) to perfusate bathing the exposed gastric mucosa of different mouse genotypes. Results evaluate epithelial repair when adding drugs to C57Bl/6 (*A* and *B*), NHE1 KO$^{-/-}$ mice (*C* and *D*), or NHE2 KO$^{-/-}$ mice (*E* and *F*). Time course graphs (*A*, *C*, and *E*) track the size of epithelial damage after inducing laser photodamage to the gastric mucosal surface (*arrow* on time axis indicates time of damage). Each time point is mean ± S.E. from three to six experiments. Bar graphs (*B*, *D*, and *F*) show compiled restitution rate constants calculated from the time course results as described under "Experimental Procedures." The number of experiments is indicated as a *number* in each *bar*. *, $p < 0.05$ *versus* WT or control.

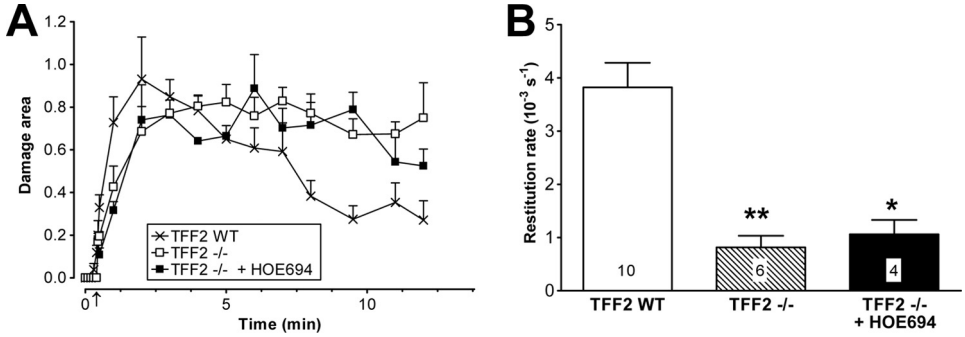

FIGURE 2. **Gastric restitution in TFF2 KO$^{-/-}$ gastric mucosa.** Experiments induced damage and measured repair as described in the legend to Fig. 1. *A*, compiled time course experiments in the indicated genotypes measuring the size of epithelial damage after inducing laser photodamage to the gastric mucosal surface (*arrow* on time axis). HOE694 (25 μM) was added to TFF2 KO$^{-/-}$ mice as indicated. Each time point is mean ± S.E. from 4–10 experiments. *B*, for each of the indicated genotypes and conditions, restitution rate constants were calculated from the time course results as described under "Experimental Procedures." The number of experiments is indicated as a *number* in each *bar*. *, $p < 0.05$ *versus* WT; **, $p < 0.01$ *versus* WT.

5*D*). Results strongly suggest that the trefoil peptides accelerate gastric repair by a mechanism that requires activity of the NHE2 Na/H exchanger.

Effect of CXCR4 Receptor Antagonist on Gastric Restitution— The CXCR4 antagonist AMD3100 has been shown to block TFF2 action in a cultured gastric cell line (5), so we asked whether this

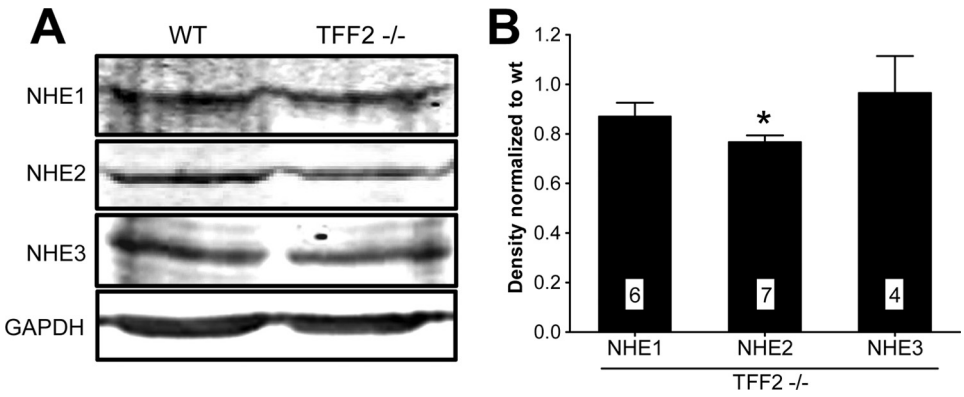

FIGURE 3. **Expression of NHE1, NHE2, and NHE3 isoforms in WT and TFF2 KO$^{-/-}$ gastric mucosa.** WT tissue was from animals in the TFF2 KO colony. *A*, representative Western blots applied to gastric mucosal homogenates obtained from WT and TFF2 KO$^{-/-}$ mice, using antibodies directed against NHE1, NHE2, NHE3, or GAPDH, as indicated. *B*, compiled densitometric analysis of Western blots as in *A*, with results normalized to GAPDH (loading control). Results for the indicated NHE isoform are presented as the ratio of normalized TFF2 KO$^{-/-}$ values divided by normalized WT values, mean ± S.E. The number of experiments is indicated as a *number* in each *bar*. *, $p < 0.05$ *versus* WT.

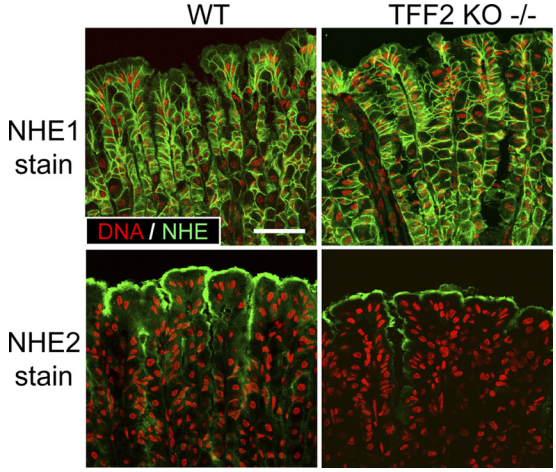

FIGURE 4. **Immunostaining of WT (*left panels*) or TFF2 KO$^{-/-}$ (*right panels*) gastric corpus.** WT tissue was from animals in the TFF2 knock-out colony. As described under "Experimental Procedures," tissue was prepared for immunofluorescence visualization using NHE1 (*top panels*) or NHE2 antisera (*bottom panels*). NHE staining is shown in *green*, and the cell nucleus counterstain (HOE33342) is shown in *red*. *Scale bar*, 50 μm.

compound affected the speed of gastric restitution *in vivo*. As shown in Fig. 6, the drug slowed restitution in TFF2 WT stomach and also slowed the rTFF3-stimulated recovery in TFF2 null stomach. Results suggest that trefoils act to promote gastric restitution by engaging the CXCR4 receptor.

Effect of Na/H Exchange Activity on Gastric Surface pH—Because the application of exogenous rTFF3 has been shown to increase surface pH in the juxtamucosal space near the gastric surface, and this environment that has been proposed to provide a favorable environment for gastric repair (25), we examined whether gastric Na/H exchange affected pH in this microenvironment. Using our established methods to image pH in this space *in vivo* (25, 27, 28, 31), we first compared the resting pH under conditions where Na/H exchange was either inhibited (EIPA or HOE694) or was genetically disrupted (NHE2 null mice). As shown in Fig. 7*A*, addition of NHE inhibitors had no effect on resting pH, but NHE2 null tissue had significantly raised surface pH compared with WT controls. After imposition of photodamage, surface pH significantly increased in the presence of NHE inhibitors or in the NHE2 null mouse stomach. Fig. 7*B* compiles the maximal pH excursion observed after damage, and no significant difference was noted among conditions (one-way analysis of variance). Results suggest that although Na/H exchange is required to promote efficient gastric repair, it is not because this transporter acutely regulates pH in the juxtamucosal environment following damage.

DISCUSSION

TFF peptides are well established *in vitro* as motogens that stimulate cell motility and *in vivo* as promoters of ulcer healing (38). Using a recently introduced model of microscopic damage allowing real-time monitoring of gastric repair, this gap in knowledge has been bridged to confirm that TFFs acts to promote the speed of epithelial restitution *in vivo* (25). The work in this article seeks to understand the mechanism of action of TFFs, building on the novel observation that NHE2 activity (in addition to TFFs) is a required component of gastric repair.

In numerous cell types, Na/H exchange is necessary for wound repair and/or cellular migration. The NHE1 isoform is most commonly reported in this role (6–13). In isolated guinea pig fundus, NHE1 has been implicated in repair since serosal (but not luminal) amiloride blocks gastric restitution (18, 19), although a role for Na/H exchange is more controversial in the presence of HCO_3/CO_2 (using isolated bullfrog gastric mucosa) (24). In contrast, our intravital approach shows that NHE1 is not required for gastric restitution in mouse but does implicate NHE2 as an important component of gastric restitution. There are only a few reports of NHE2 affecting wound repair or cellular migration. Most notably, Blikslager *et al.* (39, 40) have shown compelling evidence that genetic elimination of NHE2 weakens recovery of mouse intestinal epithelium after ischemic damage, yet in pig, the pharmacologic inhibition of NHE2 activity strengthens ischemic recovery. The two species manifest qualitatively opposite responses in transepithelial ion permeability after addition of 25 μM HOE694, and the basis for these differences remains unresolved. In our work, overlapping pharmacologic and genetic approaches help minimize concerns about confounding adaptive responses that might occur in the NHE2 null mice or the off-target effects of inhibitors, and both approaches point to a role of NHE2 activity in promoting gastric repair.

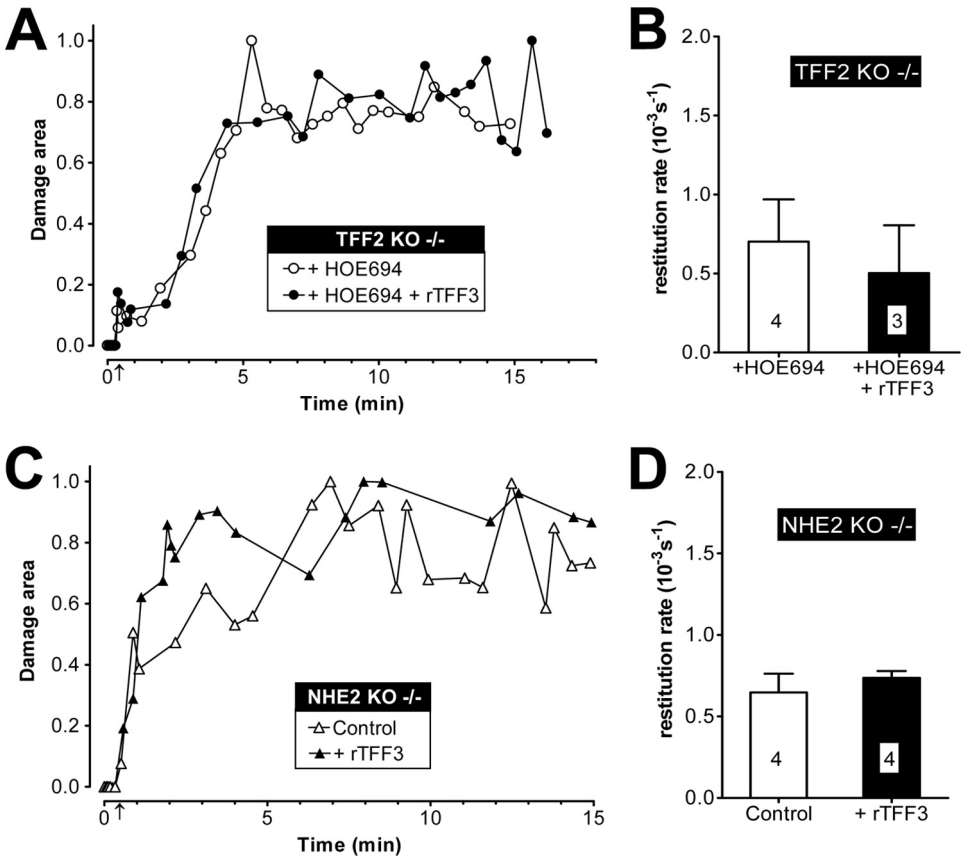

FIGURE 5. **Effect of topical addition of rat TFF3 (rTFF3) on gastric restitution when NHE2 is inactivated.** *A*, representative time course results from the same TFF2 KO$^{-/-}$ animal treated with 25 μM HOE694, comparing gastric repair (at two sites in the corpus) before and after topical addition of rTFF3 as described under "Experimental Procedures." Damage was imposed at the time indicated by the arrow on the *x* axis. *B*, results compiled from multiple experiments using TFF2 KO$^{-/-}$ mice treated with HOE694 comparing restitution rates in the presence *versus* absence of rTFF3 ($n = 3$ or 4 as indicated). *C*, representative time course results from the same NHE2 KO$^{-/-}$ animal comparing gastric repair (at two sites in the corpus) before and after topical addition of rTFF3. *D*, results compiled from multiple experiments using NHE2 KO$^{-/-}$ mice comparing restitution rates in the presence *versus* absence of rTFF3 ($n = 4$). Not all comparisons could be made in the same animal, as in *A* and *C*, so unpaired statistics were applied to the compiled outcomes to evaluate significance.

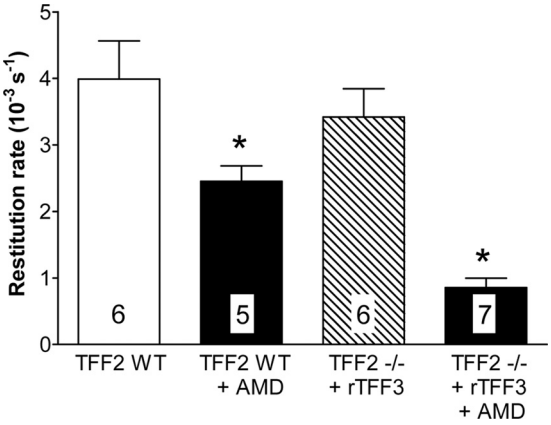

FIGURE 6. **Effect of AMD3100, a CXCR4 receptor antagonist, on gastric restitution.** For each of the indicated genotypes and conditions, restitution rate constants were calculated from the time course results as described under "Experimental Procedures." Experiments evaluated gastric repair in TFF2 KO$^{-/-}$ mice after topical addition of rTFF3, or in TFF2 WT mice. Experiments evaluated the effect of AMD3100 (*AMD*; injected sc and present in luminal fluid as described under "Experimental Procedures"). The number of experiments is indicated as a *number* in each *bar*. Statistical comparisons were only made to relevant genotype controls. *, $p < 0.05$.

We evaluated the hierarchal interdependence between the two effectors that are shown to be important for gastric repair: NHE2 and trefoil factor proteins. As HOE694 (shown to be specific *in vivo* for NHE2, Fig. 1) produces no additional inhibition of repair in TFF2 null mice (Fig. 2), initial results suggested a mutual dependence between the actions of TFF2 and NHE2. We then showed that the ability of exogenous rTFF3 to rescue repair of TFF2 null stomach is ineffective in the presence of the HOE694 Na/H exchange inhibitor (Fig. 5). In complementary experiments, the exogenous rTFF3 is also shown to be ineffective at restoring the defective repair in the stomachs of NHE2 KO mice (Fig. 5). We had no means to test the obverse hypothesis by asking whether selective activation of NHE2 could rescue the gastric repair of TFF2 null mice but did confirm that inhibition of NHE2 activity (HOE694) was sufficient to block repair in wild-type mice (Fig. 1) and that the faulty epithelial repair of NHE2 null mice was unlikely to be due to lack of TFF2 (supplemental Fig. 2). We conclude that the NHE2 Na/H exchanger acts as a downstream effector of trefoil factor peptides, mediating the action of TFF proteins to promote gastric epithelial restitution.

Experiments discriminated whether the TFF requirement for NHE2 was likely related to NHE2 transport activity, altered NHE2 protein amount, or altered NHE2 protein localization. Results show that the slowed repair of gastric surface epithelium in TFF2 null mice is not due to elimination of NHE2 protein in the gastric mucosa or relocalization of NHE2 protein

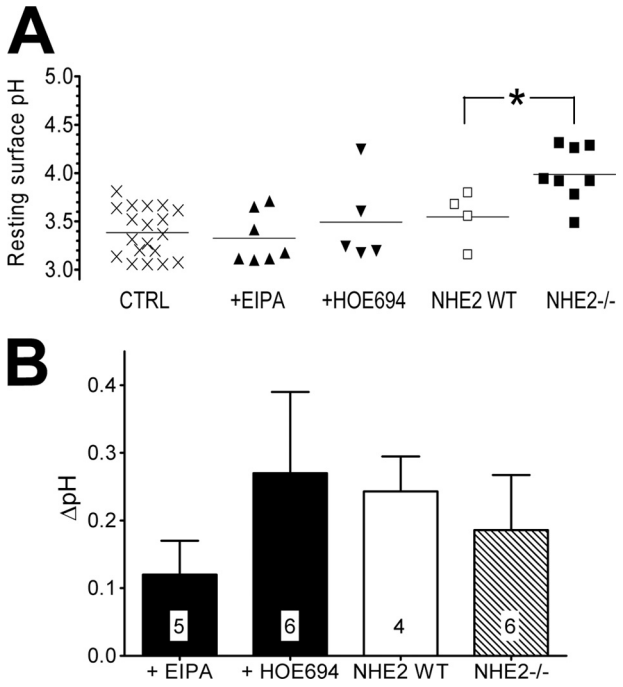

FIGURE 7. **Effect of inactivating NHE2 on gastric surface pH.** Juxtamucosal pH in the gastric corpus was measured using confocal imaging of the pH-sensitive CI-NERF dye, as described under "Experimental Procedures." Experiments compared outcomes in C57BL/6 in the absence of drug addition (*CTRL*) or after incubation with 10 μM EIPA (+EIPA) or 25 μM HOE694 (+HOE694). Experiments also measured outcomes in NHE2 KO$^{-/-}$ (NHE2$^{-/-}$) mice *versus* genotype controls from the NHE2 KO colony (NHE2 WT). *A*, each symbol is a measurement collected from a separate animal, reporting the resting surface pH prior to any damage. Statistical comparisons were only made to relevant genotype controls. *, $p < 0.05$. *B*, compiled values of maximal juxtamucosal (surface) pH increase (ΔpH) observed after damage. The number of experiments is indicated as a *number* in each *bar*.

away from the apical membrane of gastric surface cells (Figs. 3 and 4). We did note TFF2 null mice had a modest (~20%) reduction in NHE2 protein amount in the gastric mucosa in parallel with lowered NHE2 staining in gastric pit epithelium (Figs. 3 and 4) but believe this unlikely to impact on the surface cell repair we measured. It should be noted that although we did not observe NHE2 immunostaining in gastric glands, others have reported NHE2 mRNA and transport activity in parietal cells (14, 16). In our measures made at the gastric surface, a role for NHE2 transport activity is confirmed by the ability of HOE694 to block repair in response to exogenous trefoil peptide and is supported by the positive effect of HOE694 to block repair in wild-type mice. We conclude that either baseline or TFF-stimulated NHE2 transport activity is necessary for TFF-stimulated repair.

The activity of an apical Na/H exchanger in the gastric surface epithelium is difficult to predict. This apical membrane is exposed to an inward 1000-fold hydrogen ion gradient (pH 4 outside, pH 7 inside) that is larger than any reasonable prediction of the outward sodium gradient. In this condition, the ion gradient-dependent NHE transporters at the apical membrane will either inactivate or run in a reverse mode resulting in cellular hydrogen ion uptake and sodium ion extrusion. It is clear that the forward mode of heterologously expressed NHE2 Na/H exchange is fully inhibited when extracellular pH is below pH 6 (41). Elegant experiments have shown that the apical NHE3 present in rat parietal cells inactivates when acid secretion is ongoing (17) but that the basolateral NHE in guinea pig surface cells is active at luminal pH 1.5–5 (18). Although NHE2 activation by either damage or TFF remains unproven, in this scenario, it could produce an advantageous raising of surface pH to promote epithelial repair with cells exposed to a less stressful pH environment (as long as the resultant cellular acid load could be handled by buffers or basolateral transporters). The activation of NHE2 might logically be expected to be related to the surface pH change that occurs after damage, and so it was exciting to note that (*a*) TFF2 null mice have a diminished surface pH increase after damage that was restored by exogenous rTFF3 (25) and that (*b*) increases in extracellular pH will kinetically activate Na/H exchangers including NHE2 (41). However, we have previously shown that damage causes activation of the SLC26A9 Cl/HCO_3 exchanger to partially mediate an increase in surface pH (30), and we now show that an extracellular pH increase of similar magnitude occurs after damage even in the presence of NHE2 inhibition or NHE2 genetic deletion (Fig. 7). It seems paradoxical that the starting surface pH prior to damage was modestly elevated in NHE2 null mice but not after direct inhibition of NHE2 (+ HOE694) in wild-type mice (Fig. 7). This could be explained by the diminished parietal cells in the NHE2 null genetic model (21), but it is also possible that the NHE2 exchanger in migrating cells shifts to a new membrane localization after damage so that it has minimal effect on surface pH (42). Alternatively, NHE2 could be activated by the damage-induced cytosolic acidification, but an EIPA-sensitive acidification was only observed in cells that are fated to be exfoliated from the monolayer (25) and so would not be a viable mechanism in the healthy restituting cells that are the presumed target of TFF-mediated repair. It remains possible that NHE2 in restituting cells is activated in a manner that masks cytosolic or surface pH change via other compensatory mechanisms (18, 24, 30), but at present, we can only conclude that the apical NHE2 does not mediate its novel beneficial effect on gastric repair via changes in surface pH.

In conclusion, this is the first report defining NHE2 as required for gastric restitution and also the first report that any Na/H exchanger is a downstream target of trefoil factor peptides. Our work offers NHE2 as a new target to help decipher the pathways regulating gastric repair. We have limited information about the signaling pathways regulating NHE2 activity. However, our work with the CXCR4 receptor antagonist (AMD3100) suggests that exogenous rTFF3, as well as tissue repair in normal mice, engage the CXCR4 receptor to drive epithelial repair *in vivo*. TFF2 has recently been shown to activate calcium-dependent signaling and ERK1/2 phosphorylation via the CXCR4 receptor (5), and others have previously noted that ERK1/2 activation is a shared feature between the actions of TFF2 and TFF3 (3). Still, NHE2 is activated by α2-adrenergic agonists in intestinal cells (likely mediated by decreased cAMP and phospholipase C mediated activation of PI3K and Akt), but NHE can be stimulated by cAMP in rabbit parietal cells (14, 43). Thus, although the present study shows that TFFs can rapidly stimulate gastric restitution by a mechanism that engages the CXCR4 receptor and requires activity of

the apical NHE2 Na/H exchanger, the gastric-specific pathways for both TFF signaling and NHE2 action remain to be resolved.

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