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Aquaporin-3 mediates hydrogen peroxide-dependent responses to environmental stress in colonic epithelia

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The colonic epithelium provides an essential barrier against the environment that is critical for protecting the body and controlling inflammation. In response to injury or gut microbes, colonic epithelial cells produce extracellular hydrogen peroxide (H_2O_2), which acts as a potent signaling molecule affecting barrier function and host defense. In humans, impaired regulation of H_2O_2 in the intestine has been associated with early-onset inflammatory bowel disease and colon cancer. Here, we show that signal transduction by H_2O_2 depends on entry into the cell by transit through aquaporin-3 (AQP3), a plasma membrane H_2O_2-conducting channel. In response to injury, AQP3-depleted colonic epithelial cells showed defective lamellipodia, focal adhesions, and repair after wounding, along with impaired H_2O_2 responses after exposure to the intestinal pathogen *Citrobacter rodentium*. Correspondingly, AQP3$^{-/-}$ mice showed impaired healing of superficial wounds in the colon and impaired mucosal innate immune responses against *C. rodentium* infection, manifested by reduced crypt hyperplasia, reduced epithelial expression of IL-6 and TNF-α, and impaired bacterial clearance. These results elucidate the signaling mechanism of extracellular H_2O_2 in the colonic epithelium and implicate AQP3 in innate immunity at mucosal surfaces.

aquaporin-3 | hydrogen peroxide | colon | epithelium | microbe

In the mammalian colon, a single-cell-thick continuous layer of epithelial cells (ECs) forms the physical barrier that constitutes the interface between the body and the gut lumen, a compartment heavily laden with environmental microbes and other foreign and potentially toxic substances. Major gastrointestinal diseases, including infections and inflammatory bowel disease (IBD), are associated with dysfunction of the epithelial barrier (1). In both health and disease, mechanisms are in place to restore barrier function after injury and to defend against microbial colonization and invasion.

One of the pathways underlying barrier restitution and innate host defense against gut microbes depends on the generation of extracellular hydrogen peroxide (H_2O_2) by the epithelial monolayer. Exactly how extracellular H_2O_2 signals are transduced to cellular responses in the host epithelium remains incompletely characterized, especially at mucosal surfaces, where commensal microbes can produce H_2O_2 in the absence of epithelial injury or pathogenic infection.

H_2O_2 is a stable reactive oxygen species (ROS) molecule that acts as an extracellular and intracellular signal that mediates pleiotropic effects in tissues, including recruitment of immune cells to damaged areas and cell migration (2–4). In the intestine, the generation of extracellular H_2O_2 at the mucosal or serosal surface is carried out by NADPH oxidase (NOX) enzymes, such as NOX1 and dual oxidase 2 (DUOX2) (5, 6). NOX1 is highly expressed in the mammalian colon, and the NOX enzymes are important in the defense against microbial pathogens and for regenerative homeostasis (7, 8). In addition, NOX enzymes are thought to play a role in the pathogenesis of IBD, with inactivating mutations recently described in children with early-onset IBD and strong DUOX2 up-regulation found in inflamed pediatric ileal biopsy specimens (9, 10).

Aquaporin-3 (AQP3), a member of the aquaporin water channel family, is a water-, glycerol-, and H_2O_2-transporting protein implicated in various cellular functions (11, 12). AQP3-mediated H_2O_2 transport has been reported to modulate epidermal growth factor signaling in ECs (13) and TNF-α signaling in skin keratinocytes (14, 15). In addition, AQP3-mediated H_2O_2 transport has been shown to be important for T-cell and breast cancer cell migration (16, 17), although how AQP3 contributes to these changes in cellular function remains undefined. In the colon, AQP3 is expressed in ECs, with changes in expression found in response to inflammation, and our previous studies showed that mice lacking AQP3 experienced impaired recovery after chemical-induced colitis (18, 19).

In the present study, we tested whether AQP3 mediates the entry of extracellular H_2O_2 into ECs lining the colon, and, if this explains the H_2O_2-dependent mucosal responses to epithelial injury and microbial infection.

Results

AQP3 Renders the Colonic Epithelium Responsive to Extracellular H_2O_2. H_2O_2 transport was measured by live cell imaging using a ratiometric H_2O_2-sensitive fluorescent protein (HyPer) stably transfected in Caco-2 colon ECs depleted of AQP3 (shRNA; AQP3-KD) or in control Caco-2 cells expressing AQP3 (scrambled shRNA; AQP3-SC). HyPer fluorescence ratios reflect changes in cytosolic H_2O_2 concentration and represent an indirect measure of extracellular H_2O_2 entry into cells. AQP3 is localized to the plasma membrane in Caco-2 cells, and analysis of AQP3 protein expression demonstrated efficient knockdown (Fig. S1*A*). When exposed to medium containing H_2O_2, cytosolic increases in H_2O_2 were significantly faster in cells expressing AQP3 compared with cells depleted of AQP3 or in which AQP3 transport was acutely

Significance

The extracellular production of hydrogen peroxide (H_2O_2) by membrane or secreted oxidases has been linked to epithelial wound repair, defense against infection, and inflammation. Here, we elucidate the pathway that explains how extracellular H_2O_2 transduces a signal into the cell to induce these critical cell functions. Our study shows the central component of this pathway in the mammalian colon is the H_2O_2-conducting aquaglyceroporin AQP3 and implicates this channel in innate immune responses at mucosal surfaces.

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The authors declare no conflict of interest.

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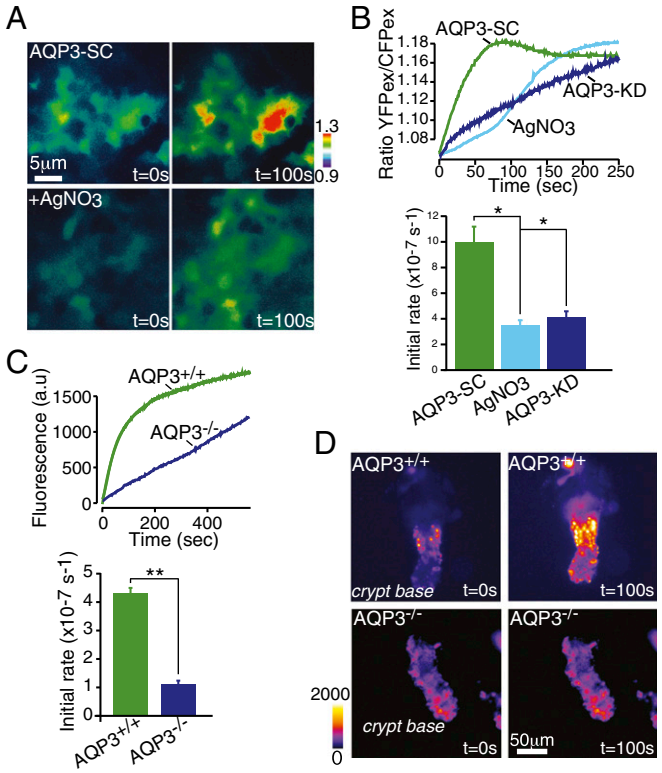

Fig. 1. AQP3 transports extracellular H_2O_2 into colonic epithelia. (*A*) Caco-2 cells expressing H_2O_2-sensitive YFP (HyPer) show increased intracellular H_2O_2 (ratio fluorescence) on exposure to 100 μM H_2O_2, which is inhibited by $AgNO_3$ (5 μM), supporting AQP3-facilitated H_2O_2 transport. (*B*) Representative traces of increases in the HyPer ratio after the addition of 100 μM H_2O_2 in Caco-2 cells transfected with control shRNA (AQP-SC) or after treatment with $AgNO_3$ (5 μM) or in AQP3-KD cells (*Upper*), and mean initial rates of increase in cellular H_2O_2 (*Lower*); $n = 6$. (*C*) Representative traces of increases in CM-H_2-DCFDA fluorescence after addition of 100 μM H_2O_2 in $AQP3^{+/+}$ and $AQP3^{-/-}$ intestinal crypts (*Upper*) and mean initial rates of increase in cellular H_2O_2 (*Lower*); $n = 4$. (*D*) Representative images showing increased H_2O_2 entry in intestinal crypts from $AQP3^{+/+}$ and $AQP3^{-/-}$ mice measured using CM-H_2-DCFDA after the addition of extracellular 100 μM H_2O_2 pulse.

inhibited by $AgNO_3$ (Fig. 1 *A* and *B*). Similarly, cells lacking endogenous AQP3 (FRT cells) showed greatly increased rates of H_2O_2 elevation after transfection with AQP3 (Fig. S1*B*).

Because antioxidant cytosolic proteins, such as glutathione peroxidases (Gpx) and peroxiredoxins (Prx), rapidly remove free cellular H_2O_2, the rate-limiting step for the cytosolic increases in H_2O_2 in our model systems is determined primarily by H_2O_2 membrane permeability. Thus, increased H_2O_2 membrane permeability in the presence of AQP3 should cause increases in cytosolic H_2O_2 at much lower concentrations of extracellular H_2O_2. Dose–response studies have shown this prediction to be correct. AQP3-containing Caco-2 cells responded to extracellular H_2O_2 at 100-fold lower concentrations compared with Caco-2 cells depleted of AQP3 (Fig. S1*C*). Importantly, uptake was observed in the concentration range critical for cellular H_2O_2 signaling (1–100 μM) (3, 13, 21), and the expression of antioxidant proteins was not different between AQP3-KD and AQP3-SC cells (Fig. S1 *D* and *E*). At much higher and toxic concentrations of H_2O_2 (>1 mM), cellular entry was not rate-limited by AQP3, but was governed by the intrinsic membrane permeability to H_2O_2. Thus, the presence or absence of membrane AQP3 allows colonic ECs to discriminate extracellular H_2O_2 signals.

To test this idea in primary ECs in situ, we isolated colonic crypts from $AQP3^{-/-}$ and $AQP3^{+/+}$ mice. ECs in WT ($AQP3^{+/+}$) mice express AQP3 on both apical and basolateral membranes (Fig. S1*F*). Colonic crypts from $AQP3^{-/-}$ mice and exposed to exogenous H_2O_2 showed a much slower increase in cytoplasmic H_2O_2 compared with crypts from $AQP3^{+/+}$ mice, consistent with AQP3-facilitated H_2O_2 entry in $AQP3^{+/+}$ mice (Fig. 1 *C* and *D*).

Epithelial Repair and Wound Healing Depends on H_2O_2 Transport via AQP3. To test whether AQP3-dependent H_2O_2 transport underlies signal transduction in the intestine, we investigated cell migration and barrier repair in response to injury, an important EC function modulated by extracellular H_2O_2. Studies on monolayers of WT Caco-2 cells after an initial scratch wounding showed that the addition of H_2O_2 increased cell migration and wound closure (Fig. 2 *A* and *B*), consistent with previous reports (20). Wound closure was slowed in AQP3-KD cells, as well as in control AQP3-SC cells after inhibition of AQP3 channel function by $AgNO_3$ or Auphen (21, 22). Wound closure was similarly slowed in control (AQP3-SC) Caco-2 cells after the addition of diphenyleneiodonium (DPI), which inhibits the NOX enzymes normally involved in production of extracellular H_2O_2 after cell injury, and also by catalase, which enzymatically depletes extracellular H_2O_2 (Fig. 2*C*). For each of these inhibitors, the H_2O_2 specificity of their effect on wound healing was confirmed by showing their lack of effect on AQP3-dependent water transport (Fig. S1*C*). Finally, imaging of intracellular H_2O_2 at the wound edge of scratch-injured Caco-2 monolayers showed reduced H_2O_2 accumulation in AQP3-KD cells, consistent with the interpretation that wound healing is facilitated by H_2O_2 transport via AQP3 (Fig. 2 *D* and *E* and Fig. S2*A*).

Closure of epithelial wounds after injury begins with migration of the wound-associated ECs from either side of the wound (23), which requires the production of numerous actin-driven lamellopodial projections. If AQP3 underlies wound signal transduction by H_2O_2, then the induction of lamellopdia also should be AQP3-dependent. To test this possibility, we imaged lamellopodia by actin staining in wound-associated Caco-2 cells depleted or not in AQP3 and in corresponding cells expressing eGFP-actin (Life-Act). Depletion of AQP3 or inhibition of H_2O_2 signaling by incubation with catalase or DPI decreased the numbers and sizes of actin-containing lamellopodia (Fig. 3*A* and Fig. S2 *B* and *C*). For lamellipodial assembly and cell migration, wound-associated ECs require active turnover of focal adhesion complexes controlled by phosphorylation of key focal adhesion proteins, such as paxillin and focal adhesion kinase. We tested for this by examining the induction of focal adhesion complexes. Both proteins were found to be depleted in wound-associated ECs lacking AQP3 (AQP3-KD cells) (Fig. 3*B* and Fig. S2*B*). In addition, the ratio of phosphorylated to total paxillin was reduced in AQP3-KD cells, suggesting reduced active focal adhesions at the wound edge (Fig. 3*B*). The assembly of lamellopodia at the wound edge was accompanied by the expression and colocalization of AQP3 and NOX1 at these sites in AQP3-expressing cells (Fig. 3*C*), suggesting active regulation of cell migration by spatially coordinated H_2O_2 production (NOX1) and membrane H_2O_2 permeability (AQP3).

To test this pathway in vivo, we created endoscopic wounds in the colons of $AQP3^{-/-}$ and $AQP3^{+/+}$ mice and assessed wound healing by endoscopic visualization as described previously (24). $AQP3^{-/-}$ mice had distinctly impaired superficial wound healing, as assessed by analysis of the healed wound area at 6 d after injury (Fig. 3 *D* and *E* and Fig. S3*A*). ECs at the edge of the induced wounds showed a small increase in phosphorylated paxillin on day 1 postinjury in $AQP3^{+/+}$ mice compared with $AQP3^{-/-}$ mice (Fig. S3 *B* and *C*). Thus, AQP3-facilliated H_2O_2 entry underlies the native NOX/H_2O_2-dependent epithelial response to injury and barrier repair.

Host Defense Against *Citrobacter rodentium* Infection Depends on H_2O_2 Transport via AQP3. Because colonic ECs form the primary barrier against microbes and microbial products, we next tested

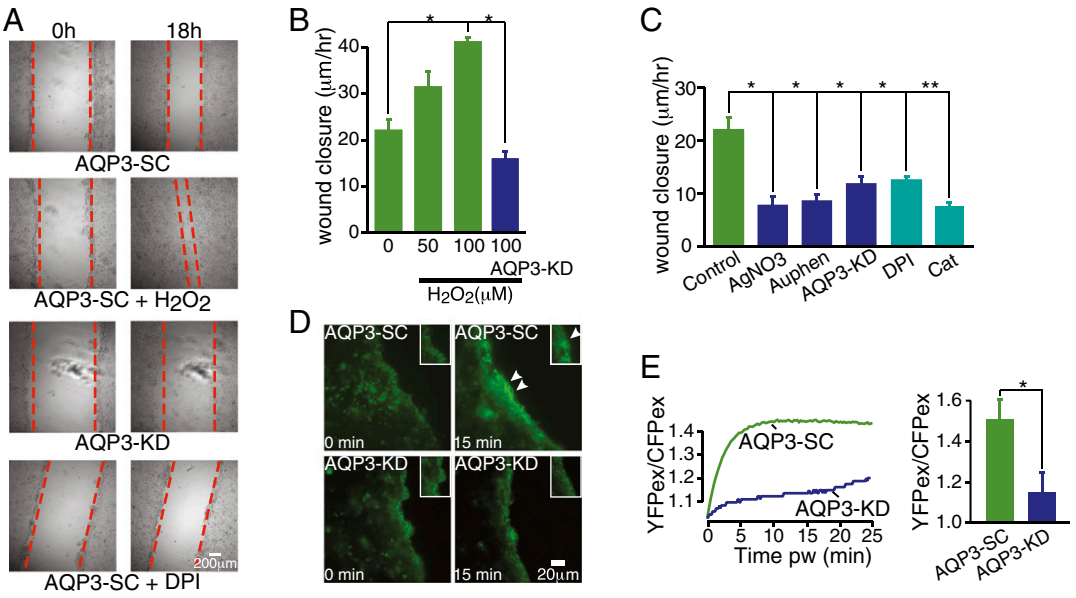

Fig. 2. H_2O_2 entry through AQP3 in ECs facilitates wound repair. (*A*) Representative images of colonic epithelial monolayers at 0 h and 18 h after scratch wounding with the addition of H_2O_2 (100 μM), or in AQP3 knockdown cells after the addition of DPI (20 μM). (*B*) Mean rates of wound closure in epithelial monolayers after addition of H_2O_2, $n = 4$. (*C*) Mean rates of wound closure in AQP3-SC cells in the absence of added H_2O_2 after the addition of $AgNO_3$ (5 μM), Auphen (10 μM), and in AQP3 knockdown cells (AQP3-KD) or AQP3-SC cells in the presence of catalase (200 U) or DPI (20 μM). $n = 4$. (*D*) Representative images of CM-H_2-DCFDA fluorescence showing H_2O_2 at the wound edge after scratch wounding in AQP3-SC and AQP3-KD cells. (*E*) Representative traces of the HyPer ratio change with time (*Left*) and mean ratio (*Right*) at the wound edge at 15 min after wounding. $n = 4$.

whether AQP3-dependent H_2O_2 transport may modulate signal transduction in host defense. *N*-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLF) is a uniformly expressed microbial peptide that acts as potent chemotactic peptide by binding formyl peptide receptors of immune and nonimmune cell types. In the intestine, activation of formyl peptide receptors of surface ECs

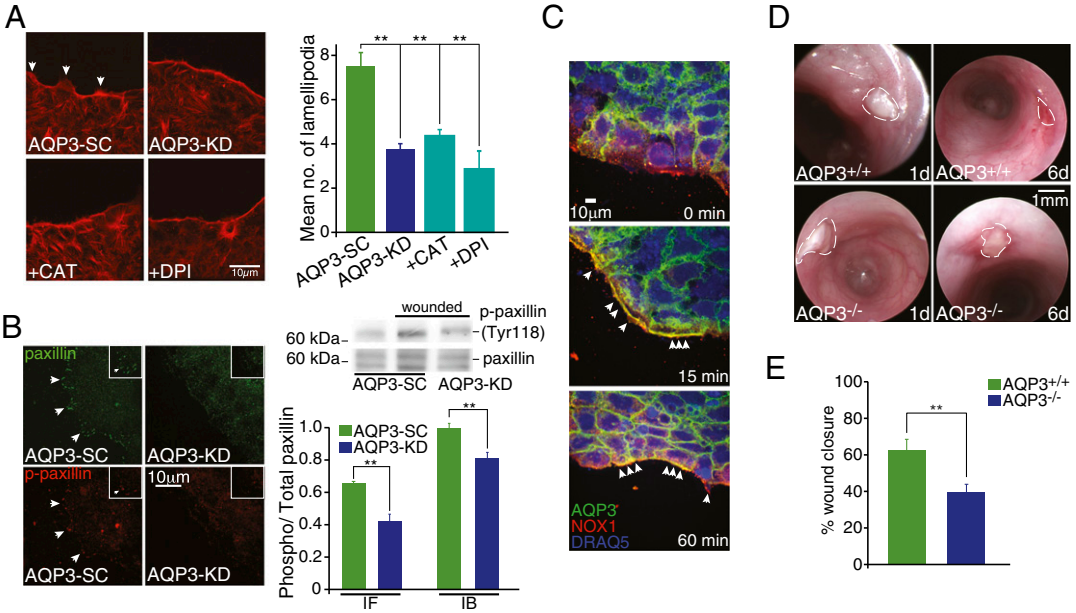

Fig. 3. AQP3-mediated H_2O_2 entry induces lamellipodia formation at the wound edge. (*A*) Representative images of actin showing lamellipodia in wound-associated colonic ECs at 1 h after wounding in AQP3-SC cells and after the addition of catalase (200 U) or DPI (20 μM) and in AQP3-KD cells (*Left*), and mean number of lamellipodia measured from imaging (*Right*). $n = 4$. (*B*) Representative images of total paxillin (green) and phosphorylated paxillin (red) in wound-associated colonic ECs after wounding (1 h) in AQP3-SC cells and in AQP3-KD cells (*Left*); immunoblots of total and phosphorylated paxillin in wounded AQP3-SC cells and in AQP3-KD cells (*Right*, *Upper*); and mean ratio of total/phosphorylated paxillin measured from imaging (IF; $n = 4$) and immunoblot (IB; $n = 3$) (*Right*, *Lower*). (*C*) Representative images showing AQP3 (green), NOX1 (red), and DRAQ5 (blue) in AQP3-SC wound-associated colonic ECs after wounding. Arrows indicate colocalization of AQP3 and NOX1 and/or lamellipodia. (*D*) Endoscopic images of mouse distal colon after superficial biopsy wounds in $AQP3^{+/+}$ (*Upper*) and $AQP3^{-/-}$ (*Lower*) mice at 1 d and 6 d after wounding. Dotted lines indicate the wound area. (*E*) Summary of wound healing in $AQP3^{+/+}$ and $AQP3^{-/-}$ mice expressed as percentage of wound closure at 6 d postwounding. $n = 6$ mice, three to five wounds per mouse.

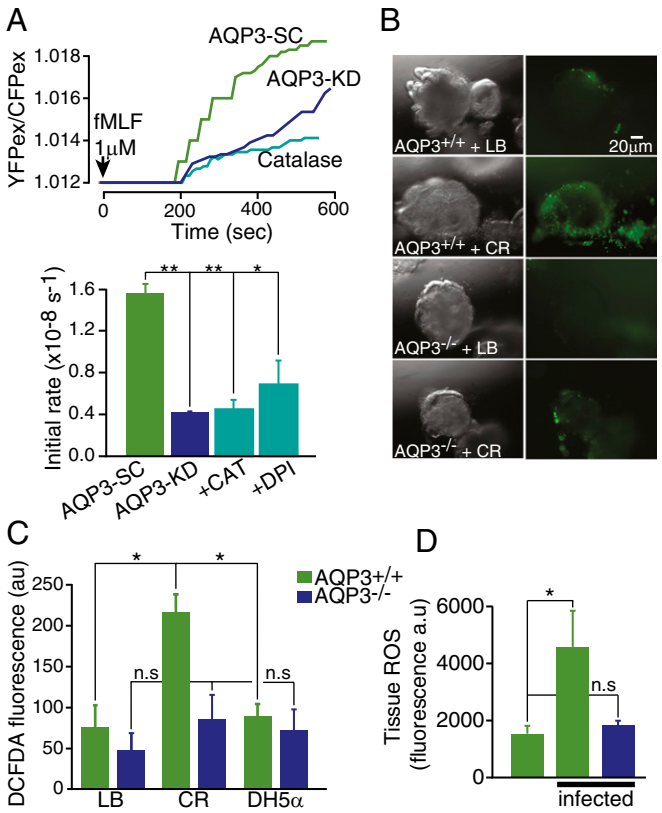

Fig. 4. fMLF and *C. rodentium* induce epithelial H_2O_2 entry via AQP3. (*A*) Representative traces of HyPer ratio change in Caco-2 colonic ECs after the addition of fMLF (1 μM) (*Upper*), and summary of the mean initial rate of H_2O_2 increase (*Lower*). $n = 5$. (*B*) Images of mouse distal colonoids from $AQP3^{+/+}$ and $AQP3^{-/-}$ mice after microinjection into the colonoid lumen of media (LB) or *C. rodentium* (CR, 1×10^7 cfu) showing CM-H_2-DCFDA fluorescence and brightfield. (*C*) Summary of mean CM-H_2-DCFDA fluorescence in $AQP3^{+/+}$ and $AQP3^{-/-}$ colonoids microinjected with LB, CR, or DH5α. $n = 3$. (*D*) Summary of accumulation of colonic epithelial ROS species in colon from $AQP3^{+/+}$ and $AQP3^{-/-}$ mice measured by ROS_{550} fluorescence at 3 d post-infection with CR. $n = 3$.

leads to activation of NOX1 and generation of extracellular H_2O_2, which mediates the epithelial response to wound healing. In alignment with this and our earlier results (Figs. 1–3), the addition of fMLF to colon ECs produced a time-dependent increase in cellular H_2O_2 that was attenuated in AQP3-KD monolayers and blocked in WT Caco-2 cells by depletion of extracellular H_2O_2 using catalase and inhibition of NOX1 using DPI (Fig. 4*A*).

To test whether a colonic pathogen can induce such a response, and whether this action is AQP3-dependent, colonoids generated from $AQP3^{+/+}$ and $AQP3^{-/-}$ mice were exposed to the noninvasive, attaching, and effacing pathogen *C. rodentium*, the murine equivalent of enteropathogenic *Escherichia coli* in humans. *C. rodentium* or nonpathogenic *E. coli* (DH5α) were microinjected into the lumen of the colonoids (Fig. 4*B* and Fig. S4*A*). Luminal incubation with *C. rodentium*, but not with growth medium alone or *E. coli* DH5α, induced a robust increase in cellular H_2O_2 that was significantly attenuated in $AQP3^{-/-}$ colonoids (Fig. 4 *B* and *C* and Fig. S4*B*). Similarly, colonic tissue from $AQP3^{-/-}$ mice infected with *C. rodentium* showed reduced accumulation of ROS in surface colonic ECs compared with infected $AQP3^{+/+}$ mice (Fig. 4*D*).

In vivo, colonic infection by *C. rodentium* induces a robust early mucosal inflammatory response required for subsequent bacterial clearance and recovery. Induction of this response depends on direct interaction of the bacteria with the epithelium and ROS signaling (25, 26). To test whether AQP3 mediates this recovery, we studied *C. rodentium* infection in $AQP3^{+/+}$ and $AQP3^{-/-}$ mice. $AQP3^{+/+}$ mice infected with *C. rodentium* showed an early loss in body weight at 3–6 d postinfection with subsequent crypt hyperplasia, indicative of the early inflammatory response. Body weight then recovered, as described previously (27) and in contrast to $AQP3^{-/-}$ mice (Fig. 5 *A* and *B*). $AQP3^{-/-}$ mice showed no initial loss of weight or crypt hyperplasia at early stages of infection, and no weight gain later (days 18–25), consistent with the lack of an initial inflammatory response and persistent *C. rodentium* infection. This was further evidenced by the failure of $AQP3^{-/-}$ mice to show increased expression of the inflammatory cytokines IL-6 and TNF-α at early stages of infection, as well as the failure to clear the bacteria from the colon at later stages (Fig. 5 *C* and *D*). At later stages of infection (day 25), $AQP3^{+/+}$ mice exhibited reduced crypt hyperplasia in conjunction with resolution of the infection. In contrast, $AQP3^{-/-}$ mouse crypts showed a delayed increase in crypt length, possibly a result of persistent bacterial colonization (Fig. S5*B*). Importantly, in both $AQP3^{+/+}$ and $AQP3^{-/-}$ mice, Nox1 expression was up-regulated in surface ECs after infection (Fig. 5*E* and Fig. S5*C*). Furthermore, $AQP3^{+/+}$ mice given *N*-acetyl cysteine (NAC) in drinking water, a well-tolerated antioxidant that removes free H_2O_2, phenocopied $AQP3^{-/-}$ mice at early stages of *C. rodentium* infection (Fig. 5 *F* and *G*) and showed impaired bacterial clearance and cytokine expression at later stages of infection (Fig. S5 *A*, *B*, and *D*). Thus, AQP3 is required for H_2O_2 signaling in the colon and for the epithelial response to infection by *C. rodentium*.

Discussion

The innate immune function of ECs lining the colon is thought to be critical for the maintenance of epithelial transport and barrier functions in the face of a complex and potentially hostile luminal environment (1). Epithelial generation of H_2O_2 has been shown to be an important signal for wound repair and recruitment of inflammatory cells after injury. NOX and DUOX complexes at the intestinal epithelial plasma membrane generate H_2O_2 or superoxide (O^-), which is rapidly converted to H_2O_2 by extracellular superoxide dismutase. In the distal colon, this appears to occur primarily via NOX1 based on its high regional expression (6). Studies in *Drosophila, Caenorhabditis elegans*, and zebrafish intestine have shown that epithelial production of H_2O_2 is part of the host response to gut infection and is involved in microbial clearance and responses to gut symbionts and pathogens (28–30). In mammals, a similar role has been postulated based on studies of pathogen infection in stomach and airway ECs (31, 32).

In the present study, we have shown that AQP3 mediates H_2O_2 signal transduction in colonic epithelia. The increased membrane permeability to H_2O_2 in AQP3-containing colonic ECs allows them to respond to external H_2O_2 at concentrations (1–100 μM) relevant for cellular signaling processes, such as migration and pathogen recognition. This was demonstrated both in vitro and in vivo by studying wound repair after injury and host defense against *C. rodentium* infection.

In the case of wound repair, various signaling proteins may be involved in translating the rapid H_2O_2 uptake facilitated by AQP3 into the signals required for the observed epithelial cytoskeletal and cell migratory responses. Previous studies have identified various candidate ROS-sensitive intracellular proteins, including the src family kinases and several tyrosine phosphatases, including SHP2 and PTP-PEST (4, 15, 26). These proteins contain free cysteines that can alter function and are affected by changes in cellular H_2O_2 levels. Given that the specific proteins involved are often cell type-specific, further studies are needed in primary EC systems to fully understand the signaling pathways and effectors of AQP3-facilliated H_2O_2 cellular entry.

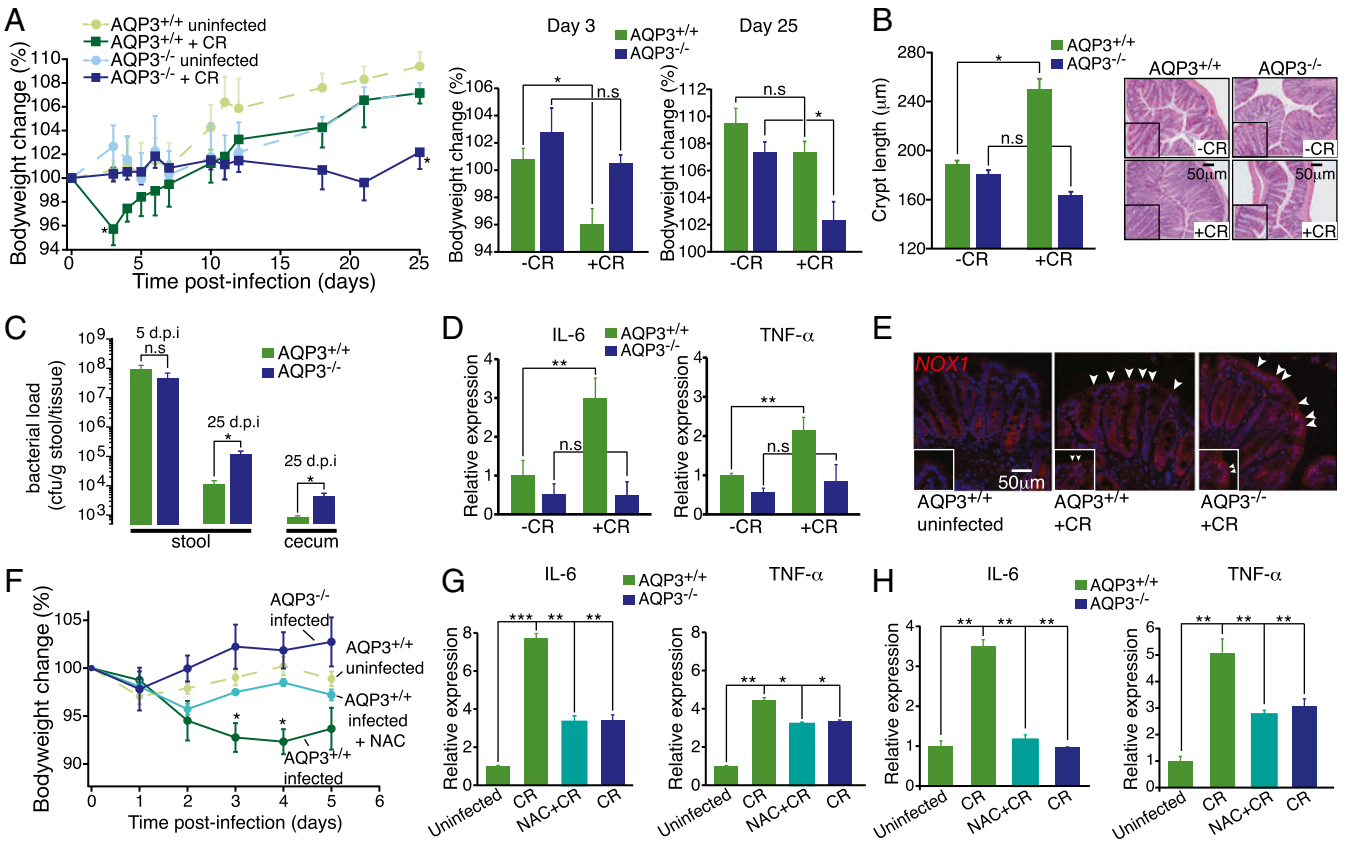

Fig. 5. AQP3-dependent mucosal response to *C. rodentium infection* in vivo. (*A*) Time course of body weight change after *C. rodentium* (CR) infection in AQP3$^{+/+}$ and AQP3$^{-/-}$ mice, and mean bodyweight change at days 3 and 25 postinfection. $n = 6$. (*B*) Crypt length (μm) in AQP3$^{+/+}$ and AQP3$^{-/-}$ mice at 12 d postinfection and representative H&E images of crypt hyperplasia in AQP3$^{+/+}$, but not AQP3$^{-/-}$, distal colon at 12 d postinfection. $n = 6$. (*C*) CR bacterial load in stool at 5 and 25 d postinfection and cecum tissue at 25 d postinfection. $n = 6$. (*D*) Relative expression of IL-6 and TNF-α mRNA in isolated ECs at 5 d postinfection. $n = 6$. (*E*) Images of NOX1 (red) expression in distal colon at 5 d postinfection. Arrows indicate increased expression in surface ECs after infection. (*F*) Time course of mean body weight change after CR infection in AQP3$^{+/+}$ mice, AQP3$^{+/+}$ mice administered NAC, and AQP3$^{-/-}$ mice. $n = 5$. (*G*) Relative expression of IL-6 and TNF-α mRNA in isolated ECs from AQP3$^{+/+}$ mice, AQP3$^{+/+}$ mice administered NAC, and AQP3$^{-/-}$ mice at 5 d postinfection. $n = 5$. (*H*) Relative expression of IL-6 and TNF-α mRNA at 25 d postinfection. $n = 4$.

Recent studies also have pointed to an important role for ROS signaling in relation to pathogen infection and microbial recognition, as well as in the mucosal inflammation seen in chronic inflammatory bowel diseases, which is associated with alterations in gut microbes (9, 28, 31, 33, 34). In the case of *C. rodentium* infection, ROS signaling has been shown to be required for a robust TH_{17} response and bacterial clearance (26). The results reported here suggest that this response is likely dependent on AQP3-mediated H_2O_2 signal transduction. In the skin epidermis, another bacterially colonized mucosal surface, AQP3-dependent H_2O_2 responses have been shown to play a role in the pathogenesis of psoriasis and in T-cell migration (14, 15). Further studies will help characterize how colonic epithelial cytokine secretion is regulated by AQP3-dependent H_2O_2 signals, and how this may modulate other epithelial innate immune pathways, such as Toll-like receptor signaling and activation of the Nlrp6 inflammasome (35, 36).

A recent study identified several mutations in epithelial NOXs in children with very-early-onset IBD. In these patients, defective production of H_2O_2 by ECs was linked to impaired initial host resistance to pathogenic infections (9), as we found here for AQP3$^{-/-}$ mice. Although AQP3 has not been identified as a monogenic cause of IBD, patients with ulcerative colitis have significantly lower expression of epithelial AQP3 during both inflammation and clinical remission (17). Conversely, AQP3 expression is highly increased in many colon carcinomas and is correlated with metastatic spread (37). As shown here, AQP3-dependent H_2O_2 cellular entry increases colonic epithelial migration, and it is plausible that AQP3-facilliated H_2O_2 transport may play a role in the development of colonic cancers and metastases.

Given that AQP3 is expressed in mucosal surface cells lining the colon, airway, genitourinary tract, and skin, we speculate that AQP3-faciliated H_2O_2 entry represents a general mechanism for mediating EC responses to the external environment. Environmental changes in commensal microbial composition or pathogenic infection generate a variety of signals that mucosal ECs are poised to recognize, including changes in redox status and the production of stable ROS species, such as H_2O_2, in the extracellular milieu. The mechanisms by which the host ECs discriminate between pathogenic "danger" H_2O_2 signals and homeostatic commensal H_2O_2 signals remains unknown, however. Accumulation of H_2O_2 at colonic EC surfaces can occur in a cell-autonomous manner through activation of NOX1 (5, 6), in a paracrine manner after release from gut commensal bacteria (34), or as a product of secreted enzymes, such as xanthine oxidase (38). In this context, and perhaps dependent on the source of H_2O_2 (cell-autonomous or paracrine) and/or its localization (luminal or subepithelial), AQP3-facilliated H_2O_2 transport in the epithelium may be intrinsic to host responses to the environment, including the acute and chronic adaptive response to gut commensal or pathogenic microbes.

Materials and Methods

The materials and experimental procedures used in this study are described in detail in *SI Materials and Methods*. Primer sequences for qRT-PCR are shown in Table S1.

Mice. AQP3$^{+/+}$ and AQP3$^{-/-}$ mice in a CD1 genetic background were generated as described previously. Animals were maintained in a specific pathogen-free animal facility at Boston Children's Hospital. Experiments were conducted after approval from Animal Care Systems and in accordance with regulations of the Institutional Animal Care and Use Committee.

H_2O_2 Fluorescence Ratio Imaging. Stably transfected cells containing the H_2O_2-sensitive probe HyPer (39) on glass coverslips were placed in a microperfusion chamber (Warner Instruments) with HBSS with glucose at 37 °C and imaged.

In Vivo Colonoscopy. AQP3$^{+/+}$ and AQP3$^{-/-}$ mice (n = 3–5) were anesthetized by i.p. injection of a ketamine (100 mg/kg)/xylazine (10 mg/kg) solution. The distal colon was imaged using a high-resolution colonoscopy system (Karl Storz). To create superficial wounds, endoscopic biopsies (three to six per colon) using biopsy forceps were made in the distal colon and followed at 1 and 6 d postwounding by repeat endoscopy and image capture.

Colonoid Microinjection. Epithelial colonoids were generated and plated in Matrigel on glass-bottom Petri dishes. At 4–5 d after plating, colonoids were microinjected with a pulled glass pipette containing injection solutions (*C. rodentium* 1×10^7 cfu, LB, *E. coli* DH5α) attached to a Nanoject (World Precision Instruments). Colonoids (25–40 per condition) were injected with 2 nL of solution. Some colonoids were injected with FluoSpheres (Thermo Fisher Scientific) to mark injections (Fig. S4*B*). Injected colonoids were incubated at 37 °C for 6 h.

Statistics. Quantitative data are expressed as mean ± SEM for each treatment group. Statistical comparisons were performed using the two-tailed Student's t test or ANOVA with Tukey's multiple-comparison post hoc test. P values < 0.05 were considered significant (*P < 0.05; **P < 0.01; ***P < 0.001).

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