L-arginine uptake by cationic amino acid transporter 2 is essential for colonic epithelial cell restitution


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INFLAMMATORY BOWEL DISEASE (IBD) consists of the disease expression, and inhibition of arginase with epithelial cell restitution L-arginine uptake by cationic amino acid transporter 2 is essential for colonic colitis induced by L-arginine (L-Arg) is a semiessential amino acid that reduces murine colonic epithelial barrier is an important response during colitis. 302: G1061–G1073, 2012. First published February 23, 2012; doi:10.1152/ajpgi.00544.2011.

Restoration of the colonic epithelial barrier is an important response during colitis. L-arginine (L-Arg) is a semiessential amino acid that reduces murine colitis induced by Citrobacter rodentium. Cationic amino acid transporter (CAT) proteins increase L-Arg uptake into cells. L-Arg is utilized to produce nitric oxide (NO), by inducible NO synthase (iNOS), or L-ornithine (L-Orn) by arginase (Arg) enzymes. The latter is followed by generation of polyamines by ornithine decarboxylase (ODC) and L-proline (L-Pro) by ornithine aminotransferase (OAT). We show that L-Arg enhanced epithelial restitution in conditionally immortalized young adult mouse colon (YAMC) cells in a wound repair model, and in isolated mouse colonic epithelial cells (CECs), using a cell migration assay. Restoration was impaired by C. rodentium. Wounding induced CAT2, and inhibition of L-Arg uptake by the competitive inhibitor L-lysine (L-Lys) or by CAT2 shRNA, but not CAT1 shRNA, decreased restitution. Migration was impaired in CECs treated with L-Lys or from CAT2−/− mice. Wounding increased Arg1 expression, and inhibition of arginase with S-(2-boronoethyl)-L-cysteine (BEC) or Arg1 shRNA inhibited restitution in YAMC cells; cell migration in CECs was also impaired by BEC. Inhibition of ODC or iNOS did not alter restitution. L-Orn or L-Pro restored restitution in cells treated with BEC or Arg1 shRNA, whereas the polyamine putrescine had no benefit. Wounding increased OAT levels, OAT shRNA inhibited restitution, and L-Pro restored restitution in cells with OAT knockdown. Uptake of L-Arg, and its metabolism by Arg1 to L-Orn and conversion to L-Pro by OAT is essential for colonic epithelial wound repair.

arginine metabolism; epithelium; proline

INFLAMMATORY BOWEL DISEASE (IBD) consists of the disease types of ulcerative colitis and Crohn’s disease (5, 6). These chronic diseases are characterized by frequent exacerbations that can severely compromise the quality of life of affected individuals. It is estimated that there are 1.4 million people affected with IBD in the United States (5, 6). IBD has been the subject of intense investigation over the past few decades, and one consideration is that the response to injury, in particular, epithelial wound repair, is an important determinant of disease status (18). While the precise mechanisms underlying inflammation and dysregulated immune responses are still being actively investigated, various inflammatory mediators, including chemotactic peptides and proinflammatory cytokines, have been implicated in IBD (34). In particular, biologic agents targeting these mediators are available, but these therapies are only effective in inducing remission in approximately half of patients (30, 37), indicating that new treatment approaches are still necessary.

In addition to limited effectiveness, treatments for IBD are compromised by side effects and high cost. There is a strong need to develop therapies that lack such deleterious effects. Many patients with IBD are in search of new treatments, and some may take supplements; one such agent is L-arginine (L-Arg). L-Arg treatment has been aggressively marketed in the lay press and online as a beneficial agent for immunologic disorders, as well as general vitality. It is considered to be a semi-essential amino acid, as it can be depleted under conditions of severe stress (23), but its use in clinically ill patients is controversial. L-Arg supplementation has been shown to be effective in reducing endothelial dysfunction and improving insulin sensitivity in patients with type 2 diabetes (35), and there are ongoing studies of its use in a variety of cardiovascular conditions.

L-Arg uptake into cells is primarily dependent on the γ+ transport system (27). This includes the cationic amino acid transporter (CAT) family of proteins (16, 27, 38). CAT1 is constitutively expressed and involved in uptake of L-Arg for basic metabolism. CAT2 is recognized as an inducible form, which includes the alternatively spliced isoforms CAT2A, a low-affinity transporter primarily in liver, and CAT2B, the high-affinity L-Arg transporter known to be abundant in macrophages (12). The CAT2 protein is part of a larger family of solute carriers, and thus is also known as solute carrier 7A2 (12, 52). CAT3 and CAT4 also have been described (52). CAT3 is found in brain and thymus, and the function of CAT4 is unknown at this time.

Once transported into cells, L-Arg is utilized by nitric oxide (NO) synthase (NOS) and arginase (Arg) enzymes. Inducible NOS (iNOS; NOS2) is upregulated during inflammation and has been implicated as having both beneficial and deleterious effects in IBD and in animal models (17, 23, 50, 51). Arginase enzymes are the endogenous antagonists to iNOS functional activity because they compete for the same L-Arg substrate by metabolizing it to L-ornithine (L-Orn) and urea (36). There are two isoforms of Arg: Arg1 is abundant in liver and is important for the urea cycle, and Arg2 is abundant in kidney and localizes to mitochondria (31, 58). L-Orn is used by ornithine decarboxylase (ODC) to produce the polyamine putrescine, which is then converted into the polyamines spermidine and spermine by constitutively expressed spermidine and spermine syn-
thases, respectively. L-Orn can also be acted upon by ornithine aminotransferase (OAT) to produce L-proline (L-Pro) (33, 40). Specifically, L-Orn is converted into glutamic semialdehyde by OAT, which then is spontaneously converted into pyrroline-5-carboxylate, which is then converted into L-Pro by pyrroline-5-carboxylate reductase (3, 32, 33, 48).

We have previously reported that in the Citrobacter rodentium model of experimental colitis L-Arg supplementation is beneficial and inhibition of arginase is deleterious (23). Because alterations in the epithelial response to injury in terms of the ability of cells to migrate and repair wounds has been implicated in the pathogenesis of colitis (8), we now sought to determine whether L-Arg may be of benefit in epithelial repair in response to wounding. We show in the current report that the restitution of epithelial wounds is mediated by L-Arg in a concentration-dependent manner and that this repair is impaired by infection with C. rodentium. Moreover, we demonstrate that epithelial restitution and ex vivo migration of colonic epithelial cells (CECs) isolated from mice requires uptake of L-Arg by CAT2, followed by its utilization by Arg1 to generate L-Orn, and OAT to generate L-Pro. These studies provide a mechanism whereby L-Arg supplementation could represent a therapy for IBD by enhancing epithelial wound repair.

MATERIALS AND METHODS

Reagents. Reagents for cell culture and RNA extraction were from Invitrogen (Carlsbad, CA). Real-time PCR reagents were from Bio-Rad (Hercules, CA). The arginase inhibitor, S-(2-boronooethyl)-L-cysteine (BEC) was synthesized by J.-L. Boucher, as described (31). The ODC inhibitor, α-difluoromethylornithine (DFMO) was used as described (4, 11, 12). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Bacteria, cells, and culture conditions. A wild-type strain of C. rodentium (DBS100) was used as described (23, 50). C. rodentium was cultured on Luria agar plates and then transferred to Luria broth in a standing culture overnight. The bacterial concentration was calculated by optical density and confirmed by serial dilution and culture (50). Young adult mouse colon (YAMC) cells were derived from colonic crypts from the immortomouse, such that they are immortalized with SV40 large T-antigen with a temperature-sensitive interferon (IFN)-γ inducible promoter (54). YAMC cells were maintained under permissive growth conditions in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 50 μg/mL gentamicin, 100 U/mL penicillin, 100 μg/mL streptomycin, and 5 U/mL IFN-γ, in a humidified incubator with 5% CO2 at 33°C (50). Cells were used between passages 26–32 for experiments. For experiments assessing effects of C. rodentium on gene expression, cells were plated at confluency density in RPMI complete medium on 35-mm dishes at 33°C, followed by culture for 18 h under nonpermissive conditions at 37°C in RPMI containing 0.5% FBS, followed by washing and addition of medium without antibiotics; and C. rodentium or PBS vehicle was added.

Restitution assays and cell wounding. YAMC cells were plated at confluency density on 35-mm dishes coated with fibronectin and incubated for 3 h at 33°C, followed by culture for 18 h under nonpermissive conditions at 37°C in RPMI medium containing 0.5% FBS with antibiotics. Cells were then washed with PBS and kept in L-Arg-free medium for 2 h for arginine depletion. Medium was decanted and eight circular wounds of equal size (~1.5 mm2) were rapidly made in each monolayer using a drill press with a silicone drill bit (19, 21). Cellular debris was removed by washing twice with PBS, and culture medium was replaced with fresh L-Arg-free, serum-free medium, with varying amounts of L-Arg added, plus 0.4% BSA and no antibiotics. In some experiments, YAMC cells were first cocultured at 37°C with C. rodentium at a multiplicity of infection (MOI) of 200 for 4 h, washed vigorously three times with PBS, cultured in L-Arg-free, serum-free medium for 2 h, and then different concentrations of L-Arg were added along with 0.4% BSA. Wounds were photographed over time using a Nikon Eclipse TE2000-U inverted microscope and a Q-Imaging camera system and software (Surrey, BC, Canada) at ×40 magnification and measured using NIH ImageJ version 1.38x software (National Institutes of Health, Bethesda, MD) (19, 21). The percent closure over time for each wound was used as a measurement of restitution. To reduce potential variability of data, each series of experiments designed to address a specific question was conducted in sequential passages of YAMC cells.

Detection of apoptosis. YAMC cells under nonpermissive conditions were plated, cultured for 4 h in the presence or absence of C. rodentium at MOI 200, and washed. Apoptosis was measured at this time point and also after additional culture without C. rodentium for 18 h and 24 h. To quantify apoptosis, cells were stained with annexin V-FITC and 7-amino-actinomycin D (7-AAD; Oncogene Research Products, San Diego, CA). Cells were analyzed with a flow cytometer (FACS Calibur; BD Biosciences, San Diego, CA) using FlowJo software (Tree Star, Ashland, OR) as previously described (10).

Global protein translation. YAMC cells were plated, infected or not with C. rodentium, and depleted of L-Arg as described above. Global protein translation studies were initiated after 18 h of culture with different concentrations of L-Arg, by adding 10 μl of [35S]methionine (specific activity, >1,000 mCi/μmol) for 4 h. Cells were then washed three times with PBS, lysed with radioimmunoprecipitation assay (RIPA) buffer, and supernatants were collected. Cell lysates were resolved by 10% SDS-PAGE and phosphorimaged (Cy-clone; PerkinElmer, Boston, MA) as described (13).

mRNA analysis. For mRNA studies, 70 wounds were made in each monolayer in the 35-mm dishes. RNA was extracted from control, C. rodentium-activated, and wounded YAMC cells using Trizol reagent (50). Then 1 μg of RNA was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Each PCR was performed with 2 μl of cDNA using iQ SYBR Green Supermix (Bio-Rad). Primers for CAT1 (12–14), CAT2 (12–14), Arg1 (31), Arg2 (31), iNOS (50), and ODC (2, 14, 15) were used as previously described (31). The sequences for CAT1 primers were as follows: forward, 5'-ACCATGTCGCTCTGTCGCGGC-3'; and reverse, 5'-TGAAGTAC-GTCGCTGCTTCCACA-3'.

Western blot analysis. For protein studies, 70 wounds were made in each monolayer in the 35-mm dishes. Protein lysates were made from control, C. rodentium activated, and wounded YAMC cells using cell lysis buffer. Then 50 μg of protein lysates were used for each protein and immunoblotted as described (50). Polyclonal rabbit antibodies for CAT1 and CAT2 were provided by E. I. Closs (Johannes Gutenberg University, Mainz, Germany). Polyclonal rabbit antibodies for Arg1 (cat. no. Sc-20150; Santa Cruz Biotechnology, Santa Cruz, CA) and iNOS (cat. no. 61033-BD; Biosciences, San Jose, CA) were used. Polyclonal goat antibodies for Arg2 (Sc-18357) and ODC (Sc-21515) were used as previously described (12). All antibodies were used at 1:500 dilution, except iNOS, which was used at 1:1,000. Monoclonal mouse antibody for β-actin was used at 1:10,000 as described (50). All proteins were detected by chemiluminescence (50).

Animals. Male wild-type C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 6 wk of age. CAT2−/− mice on a congenic C57BL/6 background (38) were provided by Lesley Ellies (University of California, San Diego) and were bred in the animal facility at the Nashville Veterans Affairs Medical Center. Age-matched male wild-type and mutant mice were used for experiments at 7 wk of age. All procedures using mice were reviewed and approved by the Institutional Animal Care and Use Committee of the Vanderbilt University Medical Center and the Research and Development Committee of the Veterans Affairs Tennessee Valley Health-care System.
Isolation of CECs. CECs were isolated by a dissociation and dispersion method as described previously (51, 54). Briefly, mouse colons were removed, cut open longitudinally, cleaned, and cut in 2–3 mm pieces and then incubated in DTT and EDTA. Epithelial cells were detached by vigorous shaking and filtered with 70-μm nylon mesh. The purity of epithelial cells was determined by flow cytometry using E-cadherin antibody and was determined to be ~90% (51). Migration assay. Migration of YAMC and freshly isolated CECs from wild-type or CAT2−/− mice were measured using a migration kit from Chemicon International (Millipore, Billerica, MA; QCM Chemotaxis 8 μm 96-well Cell Migration Assay), according to the manufacturer’s instructions. Briefly, 2.5 × 104 cells were plated in 96-well migration chambers. Different concentrations of L-Arg were added to the medium in the feeder trays underlying the cell chambers, as well as in the migration chambers. Plates were incubated for 24 h at 37°C in a CO2 incubator. Cells and medium from the top side of the migration chambers were discarded and the migration chamber plates were placed on top of new 96-well feeder trays containing 150 μl of cell detachment solution, and incubated for 30 min at 37°C. CyQuant GR Dye was diluted with lysis buffer and added to each well of the feeder trays and incubated for 15 min at room temperature. Then 150 μl of the mixture from the feeder trays was transferred to new black 96-well plates and fluorescence was measured at 480/520 nm in a BioTek (Winooski, VT) Synergy 4 plate reader.

Lentiviral transduction. YAMC cells were plated at 3.2 × 104 cells per well in 96-well plates at 33°C for 16–20 h, and used at ~70% confluence at the time of transduction. The medium was removed, and 110 μl of medium containing hexadimethrine bromide was added to the cells to enhance transduction efficiency. Lentiviral particles containing CAT1, CAT2, ODC, Arg1, OAT, or control shRNAs (Sigma-Aldrich, St. Louis, MO) were added at an MOI of 0.5, 1, 2, or 5 in duplicate wells, followed by gentle swirling of the plates to mix, and incubated for an additional 20 h at 33°C in a humidified incubator in an atmosphere of 5% CO2. Next, the medium containing lentiviral particles was removed, cells were washed once with complete medium, and transduced cells were selected by using fresh medium with puromycin, which was replaced every 3–4 days, for 3 wk. The resistant colonies, indicating successful transduction, were then expanded, assessed for knockdown of CAT1, CAT2, ODC, Arg1, or OAT by real-time PCR, and used in restitition studies.

L-Arg uptake. YAMC cells were plated and depleted of L-Arg as described above. The uptake studies were initiated after 18 and 24 h of wounding by adding 10 μl of [14C]-L-Arg (specific activity, 346 mCi/mmol) for 5 min. Cells were then washed three times with PBS, lysed with RIPA buffer, and supernatants were collected. Cell lysates were mixed with scintillation fluid and the [14C] content was determined in a scintillation counter. Protein was measured in cell lysates by the bicinchoninic acid method as described previously (12). L-Arg transport values were expressed as pmol [14C]-L-Arg-min−1·mg protein−1 (12).

Statistical analysis. Quantitative data are shown as the means ± S.E. Statistical analyses were performed with Prism version 5.0c (GraphPad Software, San Diego, CA). When comparisons between multiple groups were made, ANOVA with the Student-Newman-Keuls post hoc multiple comparisons test was performed. When comparisons between only two groups were made, Student’s t-test was performed.

RESULTS

Restitution in CECs is L-Arg dependent. We used conditionally immortalized YAMC cells to assess the effect of L-Arg on epithelial restitution. YAMC cells were exposed to wounding using a drill press by which highly reproducible circular holes are placed in monolayers. We found that there was a concentration-dependent increase in restitution with L-Arg supplementation at each time point assessed from 6 to 24 h after wounding (Fig. 1, A and B). The largest effects of L-Arg were observed at 24 h after wounding with an increase in restitution from 38.4 ± 1.2% in cells cultured with 0 mM L-Arg to 64.3 ± 1.0% at 0.1 mM L-Arg, the physiologic circulating level in human and mouse serum (7, 12, 13), and 71.7 ± 1.3% in medium containing 1.6 mM L-Arg.

Because amino acid starvation is associated with impairment of protein synthesis (13), we assessed the effects of L-Arg on protein translation. As shown in Fig. 1C, global protein translation was low in the absence of L-Arg and was enhanced at 0.05 mM L-Arg and peaked at the physiologic level of 0.1 mM L-Arg, and was not further increased at higher concentrations of L-Arg. These findings suggest that protein translation is involved in the increase in restitution at 0.1 mM L-Arg, since wound repair has been linked to protein synthesis (55, 57). However, the further increase in restitution at higher concentrations of L-Arg > 0.1 mM (Fig. 1, A and B) was not associated with any further increase in protein translation (Fig. 1C).

C. rodentium inhibits epithelial restitution. Because the interaction of the enteric microflora and specific pathogenic bacteria have been directly implicated in the etiopathogenesis of IBD (45, 46), we tested in our murine cell model the effect of C. rodentium, an established colitis-inducing pathogen in mice (23). When cells were activated with C. rodentium, restitution was also enhanced in an L-Arg concentration-dependent manner, but at each level of L-Arg used, there was a significant reduction of restitution compared with uninfected cells (Fig. 2, A and B). It should be noted that this effect is not attributable to apoptosis, as we found no difference between control and C. rodentium-activated cells by annexin V/7-AAD staining and flow cytometry (levels were 1.1 ± 0.3% and 1.5 ± 0.6%; 1.0 ± 0.2% and 0.7 ± 0.2%; and 1.2 ± 0.3% and 0.6 ± 0.2% for control and C. rodentium-treated cells, at 4, 18, and 24 h postinoculation, respectively).

Alterations in expression of genes responsible for L-Arg metabolism in response to wounding and C. rodentium activation. L-Arg can be transported into cells by CAT1 or CAT2, and is metabolized by arginase and NOS enzymes. We focused on the inducible enzymes Arg1 and iNOS that we have previously shown to be upregulated in experimental colitis (23, 51). When YAMC cells were wounded and gene expression assessed after 18 h, there was no increase in CAT1 transcript or protein levels (Fig. 3A), but CAT2 mRNA and protein levels were increased (Fig. 3B). When genes involved in L-Arg metabolism were assessed, there was an increase in Arg1 (Fig. 3C) mRNA and protein levels, but no alteration in Arg2 mRNA or protein levels (Fig. 3D). Interestingly, a significant decrease in iNOS mRNA levels was observed (Fig. 3E) with wounding, but no iNOS protein could be detected in unwounded (or wounded) cells under the serum-free conditions of these experiments. mRNA levels of ODC, which is downstream of arginase, were increased by wounding (Fig. 3F), while ODC protein levels did not reveal any apparent increase.

When the effect of C. rodentium exposure was assessed prior to wounding, there was significant upregulation of mRNA levels of both CAT1 (Fig. 4A) and CAT2 (Fig. 4B). When assessed at the protein level, there was only a modest increase in CAT1, but a large increase in CAT2. In contrast to...
the effects of wounding, C. rodentium did not affect Arg1 transcript levels and also did not alter Arg1 protein levels (Fig. 4C), but increased Arg2 (Fig. 4D) and iNOS (Fig. 4E) at both the mRNA and protein levels, while ODC mRNA and protein levels (Fig. 4F) were unchanged. In summary, wounding increased expression of CAT2 and Arg1 at both the mRNA and protein level, while C. rodentium increased expression of CAT2, Arg2, and iNOS at both the mRNA and protein level and only modestly increased CAT1.

L-lysine, a competitive inhibitor of L-Arg uptake, reduces epithelial restitution. The amino acid L-lysine (L-Lys) is also transported by CAT1 and CAT2, and as such it functions as a
competitive inhibitor of L-Arg uptake (13, 16). Control and C. rodentium-activated YAMC cells were wounded and exposed to the physiologic concentration of 0.1 mM L-Arg in the absence and presence of L-Lys (Fig. 5, A and B). These data indicate that L-Arg uptake into CECs is an important step in enhancing epithelial wound repair in both uninfected and infected cells.

Inhibition of arginase impairs restitution, whereas inhibition of iNOS does not. Control and C. rodentium-activated YAMC cells were wounded and exposed to 0.1 mM L-Arg in the absence and presence of BEC (60 μM), a specific arginase inhibitor (31). Restitution was inhibited by 42.2 ± 5.7% by L-Lys treatment, with an additional 46.3 ± 5.5% reduction, compared with cells treated with C. rodentium alone (Fig. 5, A and B). These data indicate that L-Arg uptake into CECs is an important step in enhancing epithelial wound repair in both uninfected and infected cells.

Fig. 3. Wounding of YAMC epithelial cells alters expression of genes involved in L-Arg transport and metabolism. RNA was isolated, and protein lysates were prepared from wounded and unwounded control cells (70 wounds/35-mm dish) 18 h after wounding. Cells were cultured in L-Arg-free, serum-free medium containing 0.4% BSA, with 0.1 mM L-Arg added. mRNA expression and protein levels of the following genes were measured by real-time PCR and Western blot analysis, respectively: A: CAT1; B: CAT2; C: Arg1; D: Arg2; E: iNOS; and F: ODC. CAT, cationic amino acid transporter; Arg, arginase; iNOS, inducible nitric oxide synthase; ODC, ornithine decarboxylase; ns, not significant. *P < 0.05, **P < 0.01, ***P < 0.001 vs. unwounded (control) cells; n = 3 experiments for mRNA studies. For Western blots, data are representative of 2 experiments.

Fig. 4. C. rodentium alters expression of genes responsible for L-Arg transport and metabolism in YAMC epithelial cells. From uninfected control and C. rodentium-infected cells, RNA was isolated after 4 h and protein lysates were prepared after 24 h. Cells were cultured in RPMI medium containing 0.5% FBS. mRNA and protein expression of the following genes was measured by real-time PCR and Western blot analysis, respectively: A: CAT1; B: CAT2; C: Arg1; D: Arg2; E: iNOS; and F: ODC. **P < 0.01, ***P < 0.001 vs. uninfected cells; n = 3 experiments for mRNA studies. For Western blot analysis, data are representative of 2 experiments.
BEC (Fig. 5, C and D) in uninfected cells. In C. rodentium-treated cells there was still a reduction in restitution with BEC (Fig. 5, C and D). Consistent with our findings that wounding alone did not induce iNOS expression, the specific iNOS inhibitor 1400W had no effect on restitution in control cells (Fig. 5, E and F). However, in C. rodentium infection, where iNOS expression was substantially upregulated, the iNOS inhibitor enhanced restitution, restoring it to baseline levels, an increase of 52.1 ± 19.2% (Fig. 5, E and F). Taken together, these data indicate that a substantial amount of wound repair is dependent on activity of arginase, but not iNOS, in control cells, and that in the presence of C. rodentium, wound repair is inhibited by iNOS.

Migration of YAMC cells and isolated mouse CECs is dependent on L-Arg concentration, L-Arg uptake, and arginase activity. We sought to confirm our restitution data in freshly isolated CECs from mouse colon. However, when these cells were added to fibronectin-coated plates, they did not form monolayers of sufficient confluency to allow wounding assays to be performed. Therefore, we employed a cell migration assay to monitor motility, an essential component of wound repair. We developed this assay using YAMC cells, and found that these cells exhibited cell migration that occurred in an L-Arg concentration-dependent manner, with an increase at 0.1 mM L-Arg, and further increases at 0.4 and 1.6 mM L-Arg (Fig. 6A). Consistent with the wound repair data in Fig. 5, cell...
migration was impaired by inhibition of either L-Arg uptake by L-Lys, or arginase by BEC (Fig. 6B). When isolated mouse CECs were tested, there was also an L-Arg-dependent increase in cell migration (Fig. 6C). Both L-Lys and BEC inhibited cell migration in the CECs (Fig. 6D). It should be noted that we verified that the percentage of migrating cells that were E-cadherin positive were similar to the percent positive in the CECs loaded above the chambers (data not shown).

Restitution in YAMC cells and cell migration in CECs is dependent on CAT2. Because we found that blocking L-Arg uptake impaired restitution and migration in our two different assays, and wounding specifically induced CAT2, we sought to determine whether this transporter was a regulator of wound repair. We initially performed transfection of YAMC cells with three different siRNAs targeted to CAT2 using lipofectamine, employing our published techniques (14, 22). However, we were unable to obtain more than 30% knockdown. Therefore, YAMC cells were stably transduced with scrambled shRNA and CAT2 shRNA using lentivirus. This strategy resulted in 96% knockdown of CAT2 in CAT2 lentiviral shRNA transduced YAMC cells (Fig. 7A). As expected, CAT2 knockdown had no significant affect at 0 mM L-Arg, but at 0.1, 0.4, and 1.6 mM L-Arg, it resulted in significant reductions in restitution of 48.2 ± 4.2%, 45.1 ± 4.4%, and 50.9 ± 4.8%, respectively (Fig. 7B), compared with scrambled shRNA. It is notable that the restitution in cells with knockdown of CAT2 was reduced to 76–84% of baseline level compared with cells starved of L-Arg.

We also examined the role of CAT1, to exclude the involvement of this \( \gamma^+ \) transporter. It should be noted that we tested four different shRNA clones using lentiviral transduction, but even at MOI of 5, the maximal knockdown of CAT1 mRNA expression was in the range of 50% (Fig. 7C). When we assessed the effect on wound repair (Fig. 7D), CAT1 shRNA did not impair restitution in YAMC cells, and in fact, caused a modest increase in restitution. These findings are consistent with the lack of induction of CAT1 expression by wounding previously identified in Fig. 3, and indicate that CAT2, and not CAT1 is the primary regulator of the availability of L-Arg for restitution in wounded CECs.

We then studied cell migration in isolated CECs from wild-type and CAT2\(^{-/-} \) mice. The L-Arg-mediated increase in migration in CECs from wild-type mice was lost in cells from CAT2\(^{-/-} \) mice, and the level of migration was significantly attenuated even at 0.4 and 1.6 mM L-Arg (Fig. 7E).

Epithelial restitution is dependent on Arg1, but not ODC. As with CAT2, when we attempted knockdown of Arg1 using multiple siRNAs by a lipid-based approach, we were unable to obtain adequate reduction in expression in YAMC cells. Therefore, we generated YAMC cells with stable knockdown of Arg1 using lentiviral transduction of shRNA (82% knockdown, Fig. 8A). When restitution was measured in these cells, there was a 53.5 ± 4.1% inhibition in cells with Arg1 knockdown (Fig. 8B), which was consistent with the effect of the arginase inhibitor BEC in Fig. 5. In accordance with the utilization of L-Arg substrate by Arg1, cells with knockdown of Arg1 exhibited no further reduction in restitution in cells that were maintained at 0 mM L-Arg (data not shown). Because of reports that polyamines mediate wound repair in intestinal epithelial cells (53), we determined whether the
benefit of L-Arg on restitution was dependent on polyamines. In cells with knockdown of ODC with lentiviral transduction of ODC shRNA (90% knockdown; Fig. 8A), there was no loss of restitution in cells treated with 0.1 mM L-Arg (Fig. 8B). In addition, in cells that were pretreated for 4 days with the ODC inhibitor, DFMO, there was no attenuation of the enhanced restitution with L-Arg (Fig. 8C). DFMO also had no effect at 0 mM L-Arg, and similarly, knockdown of ODC also had no effect in the absence of L-Arg (data not shown).

Recovery of epithelial restitution with L-Orn or L-Pro in cells with inhibition of arginase or knockdown of Arg1. We next sought to determine the mechanism whereby arginase activity enhances restitution. When cells exposed to the arginase inhibitor BEC were treated with either supplemental L-Orn or L-Pro in the presence of physiologic L-Arg (0.1 mM), there was a complete recovery of restitution (Fig. 8D). In contrast, supplementation of BEC-treated cells with the polyamine putrescine did not restore restitution, as the decrease in wound repair with BEC treatment remained significant (Fig. 8D). When L-Orn or L-Pro were added to cells not treated with BEC, there was no additive effect with L-Arg on restitution (Fig. 8D), indicating a common mechanism of action of these amino acids. In cells with reduction in restitution due to knockdown of Arg1, the impairment in wound repair was reversed by supplementation with either L-Orn or L-Pro (Fig. 8E). Similar to the findings in BEC-treated cells, treatment with putrescine did not ameliorate the impairment of restitution caused by Arg1 knockdown (Fig. 8E).

Increased L-Arg uptake with epithelial wounding. Because we had observed increased CAT2 expression with wounding, and a beneficial effect of L-Arg on restitution, we directly assessed L-Arg uptake in wounded YAMC cells. At 18 h, and to a greater degree at 24 h after wounding, there was increased transport of [14C]-L-Arg into cells compared with unwounded cells (Fig. 8F). As expected, the well-established L-Arg transport inhibitor L-Lys reduced transport of L-Arg into YAMC cells (Fig. 8F), consistent with its inhibition of restitution in Fig. 5. L-Orn, which is also known to compete with L-Arg for transport into cells, inhibited uptake of L-Arg more profoundly, when assessed at both 18 h and 24 h after wounding (Fig. 8F). Taken together with the data in Fig. 8D and Fig. 8E, these uptake data suggest that L-Orn can act to enhance restitution downstream of Arg1 by effectively replacing the effect of L-Arg.

Inhibition of restitution by knockdown of OAT, and recovery with L-Pro supplementation. Because L-Orn is a substrate for L-Pro synthesis via OAT, we assessed the expression of the latter and found that it was upregulated in wounded YAMC cells (Fig. 9A). We therefore conducted lentiviral knockdown of OAT with shRNA, and obtained 87% inhibition of expression (Fig. 9B). There was a 43.2 ± 2.2% inhibition of restitution with OAT shRNA (Fig. 9, C and D). When L-Orn was supplemented in cells with knockdown of OAT, there was no improvement in restitution, and, in fact, there was a significant further worsening of restitution (Fig. 9, C and D), indicating a combined effect of inhibition of L-Arg uptake as well as inability of this supplemental L-Orn to be utilized by OAT. In contrast, L-Pro supplementation led to recovery of restitution, in a concentration-dependent manner, in the cells transduced with OAT shRNA (Fig. 9, C and D), indicating that bypassing
the block of OAT via supplementation with the OAT product, L-Pro, can restore wound repair. The proposed pathways for uptake and metabolism of L-Arg pertaining to epithelial restitution are illustrated in Fig. 10.

**DISCUSSION**

Migration of epithelial cells is important in both physiologic colonic epithelial cell function and in resealing defects that occur under pathologic conditions (29, 49, 56). We previously reported that L-Arg improved *C. rodentium*-induced colitis in C57BL/6 mice (23). Here we report that in YAMC cells cultured under conditions mimicking primary cells, restitution of wounded monolayers, and migration in chambers is dependent on L-Arg and that in primary CECs isolated from mice, cell migration is also L-Arg dependent. Enhancement of wound repair in rat IEC-6 cells and piglet jejunal cells has been reported with L-Arg, but small intestinal cells were used (44), and the concentrations of L-Arg studied were from 2 mM to 50 mM. While this range is 20- to 500-fold greater than the circulating physiologic level of 0.1 mM that is present in the serum of mice and humans (25), the authors of that study did not test lower concentrations of L-Arg, which might also have been of benefit in their system. We now show an enhancement of restitution in CECs with L-Arg, with most of the increase in YAMC cells occurring by 0.1 mM. In isolated CECs we also detected a...
significant increase in migration, at L-Arg levels below the level used in the previous study.

Our studies uniquely demonstrate that colonic epithelial restitution is dependent on the transport of L-Arg into cells by CAT2. This was demonstrated by shRNA knockdown of CAT2 in YAMC cells and by using CECs from CAT2−/− mice, and studies of wound repair and cell migration, respectively. It should be noted that CAT1 deletion is lethal in mice, so we could not conduct such studies in CECs. Consistent with our restitution and migration data, we have found that with dextran sulfate sodium treatment of mice, a model of acute epithelial injury, L-Arg supplementation during the recovery phase enhances clinical improvement, CAT2−/− mice exhibit marked exacerbation of colitis, and beneficial effects of L-Arg treatment are lost in CAT2−/− mice (data not shown).

Uptake of L-Arg leads to enhanced substrate availability for iNOS, Arg1, and Arg2. We found that wounding increased expression of Arg1, but not Arg2. Inhibition of arginase activity with BEC impaired restitution. These data are in agreement with our previous report that administration of BEC to mice markedly exacerbated colitis in the C. rodentium model (23). Also, our data showing that Arg1 knockdown inhibits restitution supports the importance of Arg1 in epithelial wound repair. Arg1 deletion is lethal in mice, so we could not perform such studies in CECs. However, floxed Arg1 mice have been described (20) so that future studies could be conducted in mice with colonic epithelial specific knockout of Arg1. In addition, we have treated Arg2−/− mice with DSS and observed no exacerbation of colitis (data not shown), also indicating that the effects on restitution are specific to Arg1.

Previous studies of epithelial wound repair have not directly examined the role of bacterial infection. We used C. rodentium, since it is a colitis-inducing pathogen that induces pro-inflammatory responses in vitro and in vivo (50, 51). When YAMC cells were activated with C. rodentium, there was less restitution than in control cells, and the degree of restitution was less at each concentration of L-Arg tested. It should be noted that we did not find any difference in apoptosis between control and C. rodentium-activated cells. C. rodentium induced expression of both CAT1 and CAT2, while wounding only induced CAT2; the findings that inhibition of L-Arg uptake with L-Lys caused the same degree of inhibition of restitution in both C. rodentium-treated and uninfected cells suggests that the ability of C. rodentium to induce CAT1 did not overcome the effects of L-Lys.

Consistent with our previous reports (23, 50), C. rodentium induced substantial increases in iNOS expression in CECs, and our findings herein demonstrate that inhibition of iNOS abolished the defect in restitution with C. rodentium infection of cells. This implicates enhanced L-Arg utilization by the arginase pathway in the potentiation of the beneficial effects of L-Arg by iNOS inhibition. Consistent with the lack of iNOS induction by wounding alone, its inhibition had no effect on restitution in the absence of C. rodentium. These data contrast with previous reports that NO mediates restitution in vitro (9, 24, 28, 44), but these studies were conducted in small intestinal

Fig. 9. Epithelial cell wounding upregulates ornithine aminotransferase (OAT) mRNA expression, and knockdown of OAT inhibits restitution, which is reversed by L-Pro but not by L-Orn. A: RNA was isolated from wounded and unwounded YAMC cells at 0.1 mM L-Arg after 18 h as in Fig. 3, and OAT mRNA levels were measured by real-time PCR. **P < 0.01 vs. control; n = 3 experiments. B: YAMC cells transduced with Sc shRNA or OAT shRNA were analyzed for knockdown of OAT by real-time PCR. C: YAMC cells were transduced with Sc shRNA or OAT shRNA, and restitution was determined 24 h after wounding in the presence of 0.1 mM L-Arg ± L-Orn (40 mM) or L-Pro (100 μM or 500 μM). ***P < 0.001 vs. cells transduced with Sc shRNA and exposed to L-Arg alone; $P < 0.05 and §§§P < 0.001 vs. cells transduced with OAT shRNA and exposed to L-Arg alone; n = 24 wounds. D: representative photomicrographs of wounds at 24 h postwounding, from Sc shRNA and OAT shRNA transduced cells, in the presence of the amino acids indicated.
A new finding in our study was that L-Orn or L-Pro could overcome the block of restitution by arginase inhibition or Arg1 knockdown. Our observation that L-Orn or L-Pro did not further increase restitution when combined with L-Arg in the absence of arginase inhibition suggests that L-Arg, L-Orn, and L-Pro are functioning through a common pathway, namely the conversion of L-Arg to L-Pro (Fig. 10). Consistent with the importance of the L-Arg to L-Pro pathway, knockdown of OAT impaired restitution, which was reversed with L-Pro. Furthermore, L-Pro alone did not facilitate restitution in cells starved of L-Arg (data not shown), indicating that L-Arg is also needed for restitution. We found that global protein translation in CECs requires physiologic L-Arg levels, similar to what we have reported in macrophages (13). Therefore, we speculate that L-Pro alone was not sufficient to facilitate restitution because L-Arg is needed for global protein translation (Fig. 10). Notably, inhibition of protein synthesis by cyclohexamide inhibits restitution in wounded colon carcinoma cell lines (55, 57). Despite its ability to inhibit L-Arg uptake, L-Orn restored restitution in cells with arginase inhibition or knockdown of Arg1. These data indicate that L-Orn can replace L-Arg in the enhancement of restitution, and that there was sufficient intracellular L-Arg to allow for protein translation.

L-Pro is an important precursor in collagen synthesis and has been demonstrated to be involved in wound healing and cell migration in fibroblasts (1), and in retinal pigment epithelial cells (59). Our findings have directly implicated OAT in L-Arg-mediated wound repair. One potential salutary role for OAT in the generation of L-Pro is that this can be converted by prolyl hydroxylase to L-hydroxyproline, which is required for collagen synthesis (26, 41) that has been implicated in wound repair (1). L-Pro restored cell migration in retinal pigment epithelial cells exposed to cis-hydroxyproline, an analog of L-Pro known to inhibit collagen synthesis (59). Similarly, mice supplemented with 0.5% L-Orn in the Chow exhibited enhanced wound breaking strength in skin wounds, increased collagen deposition at wounding sites, and increased L-Pro in wound fluids (47). Based on these data and our studies showing that OAT knockdown impairs wound repair, it would be very interesting to assess the role of OAT in colitis. Intriguingly, when we analyzed levels of OAT in murine DSS colitis and in human ulcerative colitis, we found a significant reduction in expression compared with normal tissues (unpublished data). Finally, an important implication from our data is that combined supplementation of amino acids should be investigated in experimental colitis and considered in human disease to provide both enhanced protein translation and cell migration needed for epithelial wound repair.

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Fig. 10. Summary diagram showing pathways involved in colonic epithelial restitution, including uptake of L-Arg and its utilization by metabolic enzymes. We have demonstrated that L-Arg is essential for restitution and cell migration and that the effect of L-Arg on restitution and migration is dependent on its transport into epithelial cells via CAT2. Hexagonal dots represent inhibition. L-Lys and L-Orn are competitive inhibitors of L-Arg uptake that use the same transporter. Once inside the cell, L-Arg can be utilized by iNOS or arginase enzymes. iNOS has no beneficial effect on restitution, as we showed the iNOS inhibitor 1400W (1) had no effect in wounded cells and actually improved restitution in C. rodentium-infected cells. In contrast, arginase, which converts L-Arg to L-Orn, enhances restitution of wounded cells, as inhibition of arginase with BEC or Arg1 knockdown (2) reduced restitution in our studies. Blockade of restitution with inhibition or knockdown of arginase was reversed by L-Orn supplementation in our experiments. L-Orn can be utilized by ODC or OAT. When acted upon by ODC, L-Orn is converted to polyamines, but inhibition of ODC with DFMO or knockdown of ODC (3) had no effect on restitution. OAT converts L-Orn to L-Pro. We have also demonstrated that knockdown of OAT inhibited restitution (4) and supplementation with L-Pro, but not L-Orn, restored restitution in these cells. L-Arg is also essential for global protein translation, and we speculate that a basal level of L-Arg is important for restitution (shown as a dotted line), as restoration of restitution by L-Orn or L-Pro in cells with blockade of arginase also requires physiologic levels of L-Arg. The conclusions represent findings in the current study using YAMC cells, with the role of L-Arg and CAT2 in cell migration confirmed in CECs from CAT2<sup>−/−</sup> vs. WT mice.

In previous reports, wound repair of small intestinal IEC-6 cells has been attributed to polyamines (43, 53). However, in our studies in YAMC cells, inhibition or knockdown of ODC had no effect on wound repair, and adding back the polyamine putrescine had no benefit in cells with inhibition of arginase or knockdown of Arg1. These dissimilarities in the role of polyamines may be due to differences in the cell types, as we used colon epithelial cells with a phenotype consistent with primary cells, whereas IEC-6 cells are small intestinal crypt-like cells (42). Also, we used wounds created with a drill press, while linear wounds made with a razor blade were used in the IEC-6 cells (43, 53).
REFERENCES

7. Beaumier L, Castillo L, Ajami AM, Young VR.
26. Murthy S, Cooper HS, Yoshitake H, Meyer C, Meyer CJ, Murthy NS. Combination therapy of pentoxifylline and TNF-alpha monoclonal antibody in...


