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ORIGINAL ARTICLE

Helicobacter pylori arginase mutant colonizes arginase Ⅱ knockout mice

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Abstract

AIM: To investigate the role of host and bacterial arginases in the colonization of mice by *Helicobacter pylori* (*H. pylori*).

METHODS: *H. pylori* produces a very powerful urease that hydrolyzes urea to carbon dioxide and ammonium, which neutralizes acid. Urease is absolutely essential to *H. pylori* pathogenesis; therefore, the urea substrate must be in ample supply for urease to work efficiently. The urea substrate is most likely provided by arginase activity, which hydrolyzes L-arginine to L-ornithine and urea. Previous work has demonstrated that *H. pylori* arginase is surprisingly not required for colonization of wild-type mice. Hence, another *in vivo* source of the critical urea substrate must exist. We hypothesized that the urea source was provided by host arginase Ⅱ, since this enzyme is expressed in the stomach, and *H. pylori* has previously been shown to induce the expression of murine gastric arginase Ⅱ. To test this hypothesis, wild-type and arginase (*rocF*) mutant *H. pylori* strain SS1 were inoculated into arginase Ⅱ knockout mice.

RESULTS: Surprisingly, both the wild-type and *rocF* mutant bacteria still colonized arginase Ⅱ knockout mice. Moreover, feeding arginase Ⅱ knockout mice the host arginase inhibitor S-(2-boronoethyl)-L-cysteine (BEC), while inhibiting > 50% of the host arginase Ⅰ activity in several tissues, did not block the ability of the *rocF* mutant *H. pylori* to colonize. In contrast, BEC poorly inhibited *H. pylori* arginase activity.

CONCLUSION: The *in vivo* source for the essential urea utilized by *H. pylori* urease is neither bacterial arginase nor host arginase Ⅱ; instead, either residual host arginase Ⅰ or agmatinase is probably responsible.

Key words: Arginase; *Helicobacter pylori*; S-(2-boronoethyl)-L-cysteine; Urease; Mice

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INTRODUCTION

Helicobacter pylori (*H. pylori*) causes gastritis[1-3], peptic ulcers[3], and is a significant risk factor for gastric adenocarcinoma[4]. Although the mechanisms behind this spectrum of diseases are not well understood, the very powerful bacterial urease is clearly central to pathogenesis[5-9]. Urease remains a key enzyme for diagnosing *H. pylori* infection[10].

Urease hydrolyzes urea to carbon dioxide (carbonic acid) and ammonium; the latter of which serves to protect the bacterium by neutralizing the acidic microenvironment[11]. Urease is the most abundant protein in *H. pylori*, and accounts for up to 10% of the total cellular protein[5]. It is assumed that urea, the substrate for urease, must be provided abundantly *in vivo* from either the bacterium or the host in order for urease to function efficiently (Figure 1). Indeed, the *in vivo* concentration of urea in the stomach is approximately 5 mmol/L[12,13], which is well above the K_m for urease, and yet the *in vivo* source of the urea for *H. pylori* urease has remained unknown. It originally had been hypothesized that the urea comes from the bacterial arginase, RocF. Arginase catalyzes the hydrolysis of arginine to ornithine and urea (Figure 1). The surprising finding that arginase (*rocF*) mutants of *H. pylori* can still colonize mice suggests that another *in vivo* source of urea must exist[14]. The urea may come from direct release from gastric epithelial cells or other cells in gastric pits lining the stomach. Alternatively, the urea may diffuse into the gastric juice from the bloodstream. In either case, host arginases would be responsible.

We hypothesized that the host arginases are responsible for the urea that is needed by the *H. pylori* urease. There are two known host arginases: arginase Ⅰ and arginase Ⅱ. Arginase Ⅰ is the cytoplasmic enzyme that is expressed heavily in the liver and at lower levels in a few other tissues, whereas arginase Ⅱ is the mitochondrion-associated enzyme that is expressed in many tissues and cells, including the stomach, kidneys and macrophages[15]. Further support for a host-derived stomach source of arginase also comes from the findings that: (1) the arginase Ⅱ gene is expressed in the stomach in mice and humans[16]; and (2) arginase immunohistochemistry staining is markedly elevated in human gastric cancer[17,18]. Both arginase Ⅰ and arginase Ⅱ knockout mice have become available[19,20]. Arginase Ⅰ knockout mice have severe health problems and die between neonatal days 10 and 14[19], which makes it nearly impossible to use this model with *H. pylori*. In contrast, arginase Ⅱ knockout mice have no reported overt health problems[20], although they appear to have reduced fecundity (McGee, unpublished observations).

Arginase Ⅱ is expressed in the human and mouse stomach[16], therefore, we reasoned that the arginase Ⅱ knockout mouse would allow us to determine the relative contributions of host *versus* bacterial arginase to colonization *in vivo*, through the production of urea. The *rocF* arginase mutant of *H. pylori* was included in this study in case the bacterial arginase became an important source of urea in the context of the arginase Ⅱ knockout mice. Also, recent evidence has indicated that *H. pylori* induces murine and human gastric arginase Ⅱ[16], which supports the logical choice of arginase Ⅱ knockout mice as the ideal model for these experiments.

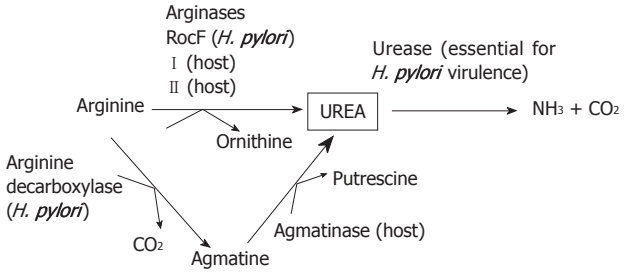

Figure 1 Overview of possible urea sources for *Helicobacter pylori* urease. *Helicobacter pylori* (*H. pylori*) urease is essential for colonization of mice, which indicates that the substrate for the enzyme, urea, is also essential. This study examines the three possible arginase-mediated sources for the urea (underlined): arginase from *H. pylori* (RocF), arginase Ⅰ (host) and arginase Ⅱ (host). Another possible urea source is host agmatinase. *H. pylori* does not have an agmatinase, but does have an enzyme, arginine decarboxylase, that can synthesize agmatine. Arginine is an essential amino acid for *H. pylori* and serves as a substrate for both arginase and arginine decarboxylase.

We infected wild-type or *rocF* mutant *H. pylori* into homozygous arginase Ⅱ knockout mice to decipher whether bacterial or host arginase Ⅱ was important for *H. pylori* colonization. To our surprise, the *rocF* mutant *H. pylori* colonized the arginase Ⅱ knockout mice similar to wild-type mice, which suggests that the *in vivo* source of urea for *H. pylori* urease hydrolysis is from another pathway. Partial inhibition of host arginase Ⅰ by the potent arginase inhibitor S-(2-boronoethyl)-L-cysteine (BEC)[21] still permitted colonization of the *rocF* mutant at or near wild-type levels in the arginase Ⅱ knockout mice.

MATERIALS AND METHODS

Bacterial strains, growth conditions, primers, and plasmids

H. pylori strains SS1[22] and the isogenic *rocF* mutant[14] were routinely grown on *Campylobacter* agar (Becton Dickinson, Sparks, MD, USA) with 10% (v/v) sheep defibrinated blood (CBA; blood from Quad Five, Ryegate, MT, USA) for 2 d, using the CampyPak Plus system (Becton Dickinson), or in a humidified microaerobic atmosphere (5% O_2, 10% CO_2, and 85% N_2). Kanamycin (10-15 μg/mL) was added as needed.

For mouse inoculation, wild-type SS1 and the isogenic *rocF* mutant were passaged an equal number of times from frozen stocks onto CBA. Strains were then grown overnight in a T 25 cm^2 tissue culture flask without aeration in 5 mL Mueller-Hinton broth that contained 1% heat-inactivated fetal bovine serum (FBS). Strains were then diluted 1:40 in 40 mL F-12 plus FBS (2%) in a T 75 cm^2 tissue culture flask and allowed to grow for an additional 16-18 h at 37℃ under microaerobic conditions. All strains grew equally well under these conditions and were inspected by light microscopy for motility and purity. Bacteria were harvested by centrifugation, washed with 1 × PBS (Invitrogen, Carlsbad, CA, USA), resuspended in 1 mL

PBS, and used for inoculation of animals.

Arginase activity by detection of ornithine

Arginase activity to detect ornithine production was determined as described previously[23] using a heat-activation step in the presence of 5 mmol/L cobalt chloride (*H. pylori*) or manganese chloride (mouse/rat) for 30 min at 50-55℃, followed by 1 h incubation at 37℃ in arginase buffer [15 mmol/L MES (pH 6.0) plus 10 mmol/L L-arginine for *H. pylori*; 15 mmol/L Tris (pH 9.0) plus 10 mmol/L L-arginine for mouse or rat]. Absorbance at 515 nm was determined.

Purification of H. pylori arginase

Enzymatically active *H. pylori* RocF was purified as a six-histidine-tagged fusion protein (His_6-RocF) as described previously[23].

Construction of pGEN222-rocF

Plasmid pGEN222, kindly provided by Dr. Jim Galen (University of Maryland, Center for Vaccine Development)[24], was digested with *Sal* I and *Bam*H I and the 1.1 kb *rocF* gene with its own promoter was excised from pBS-*rocF*[14] using the same enzymes. The ligated construct was transformed into *Escherichia coli* (*E. coli*) DH5α and confirmed by digestions and sequencing.

Rat arginase I

A plasmid carrying the rat arginase I gene, pARGr-2[25], kindly provided by Dr. Sid Morris (University of Pittsburgh), was transformed into *E. coli* DH5α, and plasmid extraction and restriction analysis showed that the construct was correct.

Mouse genotyping

Mouse genotyping was similar to that described by Shi *et al*[20], with modifications described below. Chromosomal DNA was isolated from mouse ear (approximately 2 mm diameter) or tail snips using a lysis buffer (10 mmol/L Tris, pH 7.8, 75 mmol/L NaCl, 25 mmol/L EDTA pH 8.0, 1% SDS, 500 μg/mL Proteinase K) followed by phenol-chloroform extraction, ethanol precipitation, and elution in 10 mmol/L Tris/1 mmol/L EDTA, pH 8.0. This template was used in PCR with 200 pmol of each primer, 0.25 mmol/L dNTPs, and *Taq* DNA polymerase under the following conditions: 94℃, 5 min for one cycle; 94℃ for 1 min, 53℃ for 1 min, 72℃ for 1 min for 35 cycles; and 72℃ for 5 min. Primer sequences were as follows: DM52: 5'-TCCTTTCTCCTGTCTAATTC-3'; DM53: 5'-CTAGCATCTAATTGACTGCC-3'; DM54: 5'-CCATGATGGATACTTTCTC-3'. Expected product sizes were: DM 52/53 = 500 bp (wild-type mice); DM 52/54 = 870 bp (arginase knockout mice). Heterozygotes have both products.

Mouse inoculation experiments

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal experiments were approved by the Institutional Animal Care and Use Committee at LSU Health Sciences Center-Shreveport (protocol # 06-020). Euthanasia was done by carbon dioxide asphyxiation. Arginase Ⅱ knockout mice were initially bred from a homozygous knockout female and a heterozygous male generously provided by Dr. W O'Brien (Baylor College of Medicine, Houston, TX, USA). Offspring were genotyped and homozygous knockouts were retained for further breeding. Heterozygotes were euthanized from the colony. Male and female homozygous knockouts were used. The wild-type mice were C57BL/6 (Charles River Laboratories; ⩾ 4 wk old), because homozygous wild-type littermates were euthanized from the breeding colony about 6 mo after the knockout line was established. Following the experiments, the breeding colony and all excess mice were euthanized due to insufficient resources.

Animals were inoculated orally (25-0 μL approximately 10^8-10^9 viable CFU/mL) with bacteria suspended in PBS (pH 7.4). Control animals received PBS. At various time points post-infection, animals were euthanized by cervical dislocation under anesthesia. Stomachs were removed, dissected longitudinally along the greater curvature, and the chyme removed. The rest of the stomach was usually dissected into antrum (distal region), body (middle region) and fundus/cardia (proximal region) portions, weighed, and homogenized (Ultra-Turrax T25, IKA Works, Inc; 10-15 s at a setting of 3) in 1.0 mL sterile PBS. There was insufficient stomach material to measure urea content. Stomach homogenates and dilutions thereof (serial 10-fold in 96-well microtiter dish) in PBS were plated for viable counts in duplicate on CBA plates that contained six antimicrobials to suppress normal flora as described previously[26]. *H. pylori* is resistant to these antimicrobials. Plates were incubated at 37℃ for 5 d in a microaerobic atmosphere. Data are presented as CFU/g stomach or tissue section. Other organs were removed, weighed and homogenized in PBS, and assayed immediately for arginase activity.

BEC experiments

BEC is a potent inhibitor of host arginases and was chemically synthesized according to published procedures[21]. BEC was given to animals (50 mmol/L) in the drinking water *ad libitum*, or directly given orally twice daily to mice (20 μL; approximately 50-100 μmol/L final concentration). BEC was also used *in vitro* for biochemical experiments.

Statistical analysis

Arginase and animal data were analyzed by unpaired, two-tailed *t* test, Welch corrected. $P < 0.05$ was considered significant. InStat software was used (GraphPad Software, San Diego, CA, USA).

RESULTS

Arginase I knockout mice breeding and genotyping

We received a heterozygous male and a homozygous female from the established arginase Ⅱ knockout mouse breeding colony[20], therefore, we internally bred for the ho-

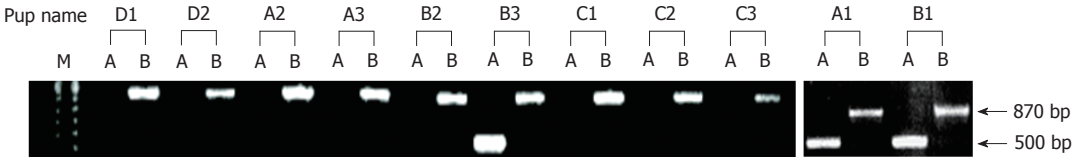

Figure 2 Genotyping of mice to determine whether they are heterozygous or homozygous arginase Ⅱ knockouts. Pups obtained from breeding heterozygous males to arginase Ⅱ knockout females were genotyped by PCR analysis. Wild-type band = 500 bp ("A" lanes); mutant band = 870 bp ("B" lanes). Eight of the 11 pups were homozygous knockouts. Pups A1, B1, and B3 were heterozygous because they contained wild-type and mutant copies of arginase Ⅱ. M: 100 bp marker (Bio-Rad).

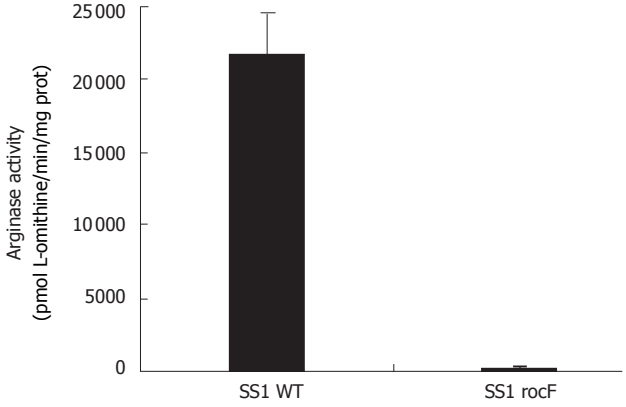

Figure 3 The *rocF* mutant of *Helicobacter pylori* strain SS1 is devoid of arginase activity. Bacteria were harvested in 0.9% NaCl and ice-bath-sonicated (25% intensity, 2 pulses of 30 s each, with 30 s rests on ice between pulses). Following centrifugation (12000 *g*, 2 min, 4℃), supernatants were retained on ice. Equal volumes of extract and 10 mmol/L cobaltous chloride ($CoCl_2$•6 H_2O, final concentration of 5 mmol/L) were preincubated for 30 min at 50-55℃ to activate the enzyme. Arginase buffer (15 mmol/L Tris, pH 7.5, or 15 mmol/L MES, pH 6.0, plus 10 mmol/L L-arginine) was added and incubated at 37℃ for 1 h. The reaction was stopped by addition of 750 μL acetic acid, and the color developed by addition of 250 μL ninhydrin (4 mg/mL) at 95℃ for 1 h and read at 515 nm. The data are presented in units where 1 U is defined as one pmol L-ornithine/min/mg protein + SD. Details of this assay have been reported previously[23].

mozygous arginase Ⅱ knockout mice and genotyped the offspring, similar to that described by Shi *et al*[20]. We successfully obtained homozygous arginase Ⅱ knockout mice (Figure 2). Once the homozygous arginase Ⅱ knockout line was firmly established, we euthanized all heterozygotes from the colony to ensure that no contamination occurred. The homozygous arginase Ⅱ knockout mice were then periodically PCR confirmed for the knockout. Occasionally, we observed reduced fecundity with the arginase Ⅱ knockout colony, although we did not pursue this.

Arginase activity by measurement of ornithine

Arginase activity, determined by ornithine detection in the presence of arginine, was measured from extracts of wild-type SS1 and the isogenic *rocF* mutant grown on CBA plates. No appreciable activity was detected in the mutant, in contrast with the wild-type strain, which had arginase activity (Figure 3). Omission of arginine led to no detectable ornithine, which showed that all of the ornithine that was detected was produced by arginine hydrolysis (data not shown). These results demonstrated that the *rocF* mutant of *H. pylori* had no arginase activity and did not appear to have alternative mechanisms of producing ornithine under these conditions. Using nuclear magnetic resonance, we have previously reported that the *rocF* mutant of SS1 has no arginase activity[14]. There is no evidence for the existence of an alternative urea-generating pathway in *H. pylori*, based on biochemical, genomics and metabolic networking approaches (McGee, unpublished observations)[27], but we cannot completely rule out this possibility.

Arginase Ⅱ knockout mice become infected with arginase mutant H. pylori

We have previously demonstrated that wild-type and *rocF* mutant *H. pylori* strain SS1 colonize wild-type mice[14]. To determine whether arginase Ⅱ knockout mice were colonized by *H. pylori*, these mice were inoculated with wild-type or *rocF* mutant SS1 *H. pylori*. At 2 wk post-infection, there was a trend towards a 1 log_{10} decrease in colonization of the entire stomach by the *rocF* mutant compared with the wild-type strain, but this did not reach statistical significance (Figure 4A). By 10 wk post-infection, there was no difference in colonization between the wild-type and the *rocF* mutant (Figure 4B). We concluded that the *rocF* mutant devoid of arginase activity colonized arginase Ⅱ knockout mice at or near the levels of the wild-type strain of *H. pylori*.

The experiment was repeated, but this time the antrum and body portions were separated, in order to understand whether there might be tropism differences. At 4 wk post-infection, the wild-type and *rocF* mutant both colonized the antrum and body regions of the stomach of arginase Ⅱ knockout mice at similar levels (data not shown).

H. pylori wild-type and *rocF* mutant both induced similar levels of gastritis in the arginase Ⅱ knockout mice (inflammatory score = 2.5 for wild-type; 2.5 for *rocF* mutant, four mice in each group).

BEC decreases in vivo host arginase activity

The *rocF* mutant surprisingly colonized the arginase Ⅱ knockout mice, yet the urea substrate must be available for *H. pylori* urease to function *in vivo*, therefore, we abandoned the hypothesis that arginase Ⅱ generates the essential urea substrate for *H. pylori* urease. Instead, we reasoned that the source of the urea must originate from host arginase Ⅰ. Arginase Ⅰ knockout mice die at neonatal days 10-14, therefore, we could not test the role of arginase Ⅰ directly. Thus, we used the host arginase inhibitor BEC. Preliminary experiments using different concentrations of BEC in mice suggested a concentration of 50 mmol/L would have at least some arginase inhibitory activity in the

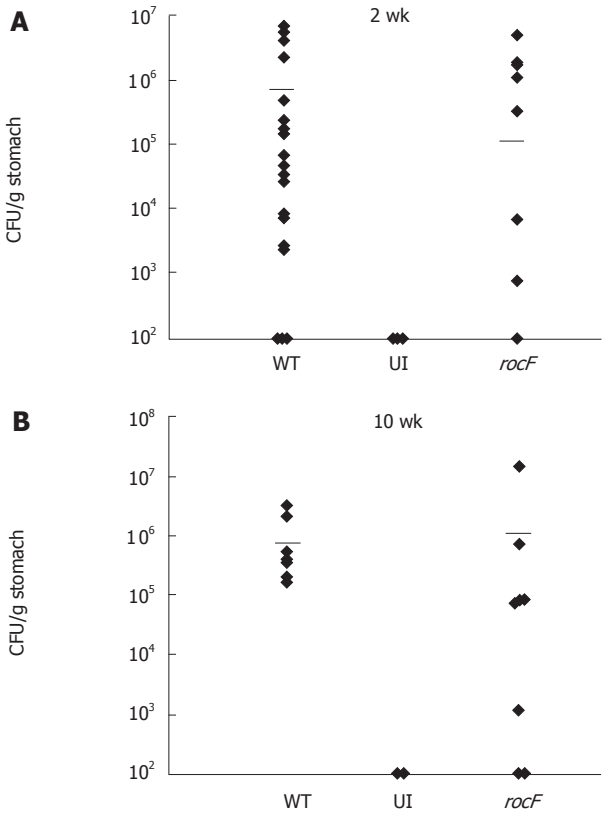

Figure 4 The *rocF* mutant of *Helicobacter pylori* colonizes the stomach of arginase Ⅱ knockout mice. Arginase Ⅱ knockout mice were inoculated with either the wild-type SS1 strain (WT) of *Helicobacter pylori* (*H. pylori*) or the isogenic *rocF* mutant (*rocF*). Number of animals used per group were as follows: 2 wk experiment: 13 for WT, nine for *rocF* mutant; 10 wk experiment: eight for WT, eight for *rocF* mutant. At 2 (A) or 10 (B) wk post-infection, stomachs were removed, completely homogenized, and plated for *H. pylori*. UI, uninfected controls (n = 2 at 2 and 10 wk). Limit of detection: ~100 CFU/g stomach; all animals that lacked *H. pylori* were set to this detection limit. Bar, mean CFU/g stomach. At 2 wk post-infection, P = 0.65 by unpaired two-tailed t test between the mean CFU/g tissue for the wild-type versus the *rocF* mutant. Each symbol represents one mouse. In some cases there are two mice represented by a symbol if the data overlapped.

stomach (data not shown). The expense and concern of toxicity to the animals precluded using higher concentrations. Also, preliminary experiments that compared BEC given *ad libitum* in the drinking water *versus* twice daily by pipetting 20 μL of 50 mmol/L concentration directly into the oral cavity revealed that the latter gave more consistent inhibition of arginase activity (data not shown), and lower quantities of the inhibitor could be used. Thus, oral delivery was used for subsequent experiments.

To demonstrate that BEC inhibited arginase *in vivo*, we fed uninfected arginase Ⅱ knockout mice with BEC by direct oral delivery to inhibit host arginase Ⅰ. The data showed that exceptionally high levels of arginase activity occurred in the liver, as expected for the known location of highest expression of arginase Ⅰ [15]. About 50% of this strong activity was inhibited by BEC (Figure 5A), which indicated that oral BEC delivery had a systemic effect on inhibiting arginase Ⅰ activity. In the kidney, very low levels of arginase Ⅰ activity were detected and this was reduced more than fivefold by BEC (Figure 5B). In the antrum region of the stomach, arginase Ⅰ activity was intermediate to that of the liver or kidney and could be inhibited by more than threefold by BEC (Figure 5C). In contrast, in the body (Figure 5D) and the fundus (data not shown), BEC did not appreciably inhibit the arginase activity, although there was a lot of animal to animal variability. Arginase activity was also much lower in these other gastric regions than in the antrum. We conclude that BEC inhibits at least some of the arginase Ⅰ activity *in vivo*, but residual activity still occurs.

If arginine was omitted from the enzyme buffer, no ornithine was detectable in the stomach (data not shown), which indicated that the tissues did not have appreciable amounts of pre-formed ornithine.

BEC does not inhibit H. pylori arginase activity, but does block arginase Ⅰ activity

BEC is an established, potent eukaryotic arginase inhibitor with a K_i of 400-600 nmol/L[21], therefore, we questioned whether BEC would inhibit *H. pylori* arginase, which shares very limited amino acid homology with human arginases (data not shown). A BEC dose-response curve using extracts from *E. coli* that expressed the *H. pylori rocF* gene (Figure 6A) revealed only partial inhibition of *H. pylori* arginase activity at very high BEC concentrations (≥ 25 μmol/L). Even at 1000 times above the K_i for eukaryotic arginases, 400 μmol/L BEC only inhibited about 50% of the *H. pylori* activity. To show that this lack of inhibition was not due to the complexity of extracts, we purified the *H. pylori* arginase and tested whether BEC would inhibit *H. pylori* arginase activity. BEC used at the K_i for eukaryotic arginases (400 nmol/L) completely failed to inhibit *H. pylori* arginase activity (Figure 6B). To prove that the BEC was working properly under these conditions and also to show directly that BEC can inhibit arginase Ⅰ *in vitro*, we used extracts from *E. coli* that expressed the rat arginase Ⅰ. Rat and mice arginase Ⅰ share 93% amino acid identity and 96% similarity (http://blast.ncbi.nlm.nih.gov/Blast.cgi), therefore, we assume that both enzymes behave in a similar fashion with respect to BEC inhibition. BEC was shown to inhibit 70% of the rat arginase Ⅰ activity at just 80 nmol/L and > 97% at concentrations of ≥ 4 μmol/L (Figure 6C), which was in good agreement with the published K_i values[21]. These results show that BEC strongly inhibits eukaryotic arginase Ⅰ under these enzyme assay conditions, yet BEC is a poor inhibitor of *H. pylori* arginase[21].

rocF mutant of H. pylori colonizes BEC-treated wild-type and arginase Ⅱ knockout mice

Wild-type or arginase Ⅱ knockout mice were pretreated with BEC or water and inoculated with wild-type or *rocF* mutant *H. pylori*. Animals were euthanized at 1 wk post-infection. The *rocF* mutant of *H. pylori* colonized the BEC-treated wild-type mice (Figure 7). Remarkably, the *rocF* mutant also colonized BEC-treated arginase Ⅱ knockout mice (Figure 8) at levels similar to wild-type *H. pylori* (P > 0.05). This similar colonization occurred in all three regions of the stomach (antrum, body and fundus). The experiment was repeated in the arginase Ⅱ knockout mice

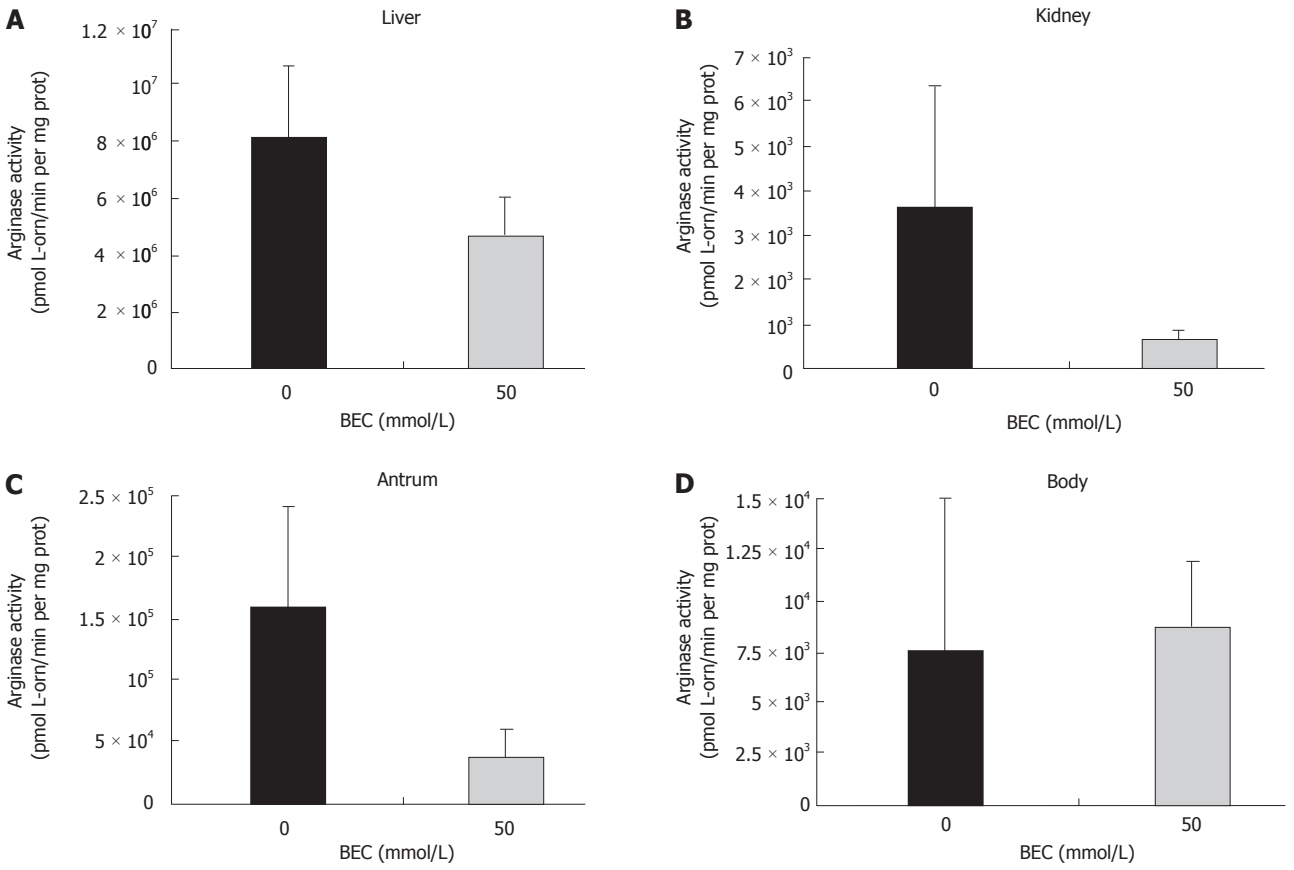

Figure 5 ***Ex vivo*** **arginase activity of organs from arginase Ⅱ knockout mice fed water or the arginase inhibitor S-(2-boronoethyl)-L-cysteine.** Mice were administered 20 μL water or 50 mmol/L S-(2-boronoethyl)-L-cysteine (BEC) once or twice daily for 3 d by direct oral delivery *via* pipetting. Animals were euthanized and organs removed and homogenized. The data represent the average arginase activity of four to five mice ± SD, with each mouse organ measured in duplicate or triplicate using arginine buffer at pH 9.0 in the presence of manganese. A: Liver. Arginase activity was inhibited almost 50% in the liver by BEC. $P = 0.0006$ between the two groups; B: Kidney. Arginase activity was inhibited > 75% in the kidney by BEC. $P = 0.0033$ between the two groups; C: Antrum. Arginase activity was inhibited > 75% in the antrum by BEC. $P = 0.0007$ between the two groups; D: Body. No inhibition was observed in the body ($P > 0.05$).

alone ($n = 5$ or 6 mice per group), and only the antrum and body were processed. Essentially identical results were obtained (data not shown).

DISCUSSION

We demonstrated that the *H. pylori rocF* mutant, which has no detectable arginase activity, colonized host arginase Ⅱ knockout mice. These data are very surprising, because host arginase Ⅱ is expressed in the stomach, and is even upregulated by *H. pylori* infection[16]. Moreover, inhibiting at least some of the host arginase Ⅰ by BEC treatment failed to block the ability of the *rocF* mutant to colonize arginase Ⅱ knockout mice. The data suggest that *H. pylori* must obtain its urea from either the residual arginase Ⅰ that is not inhibitable by BEC, or an alternative pathway of host urea generation, such as agmatinase. The data also suggest that enzymatically active host arginase Ⅰ occurs in the stomach, as has also been suggested previously[28]. A recent study from Wilson and colleagues also have demonstrated that BEC inhibits murine macrophage arginase activity; this leads to an increase in nitric oxide production *via* increased host nitric oxide synthase protein in an arginine-dependent fashion when stimulated by *H. pylori*[29]. Gastric macrophages from *H. pylori*-infected arginase Ⅱ knockout mice that had been given BEC in the drinking water did not produce more nitric oxide than macrophages from WT mice, which suggests that arginase Ⅱ, but not arginase Ⅰ is important for protection of *H. pylori* from nitric oxide[29]. Although arginase Ⅱ clearly plays a crucial role in protection of *H. pylori* from nitric oxide, it is not essential for *H. pylori* colonization of the gastric mucosa, based on the findings reported here.

One other surprise in this study was a lack of substantial inhibition of *H. pylori* arginase by BEC. Only about 50% of the *H. pylori* arginase could be inhibited by very high concentrations of BEC. This finding may be due to the very distant evolutionary relationship between *H. pylori* and host arginases, as well as the finding that *H. pylori* arginase preferentially uses cobalt for enzyme activity[23] *versus* manganese for the host arginases. BEC is known to bind to eukaryotic arginases where the binuclear manganese center is situated[21], and perhaps the presence of cobalt in the *H. pylori* arginase weakens this inhibitory interaction. The very well conserved DAHAD divalent metal binding motif in eukaryotic arginases is completely conserved in *H. pylori*, which suggests that

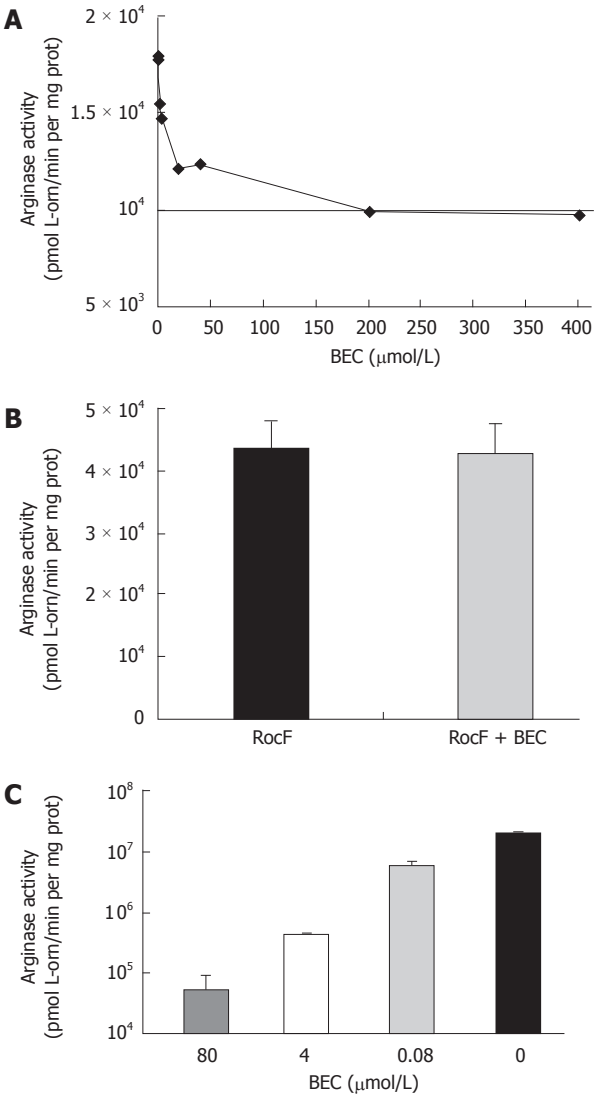

Figure 6 S-(2-boronoethyl)-L-cysteine does not strongly inhibit *Helicobacter pylori* arginase activity *in vitro*. A: Arginase-containing extracts from *Escherichia coli* (*E. coli*) expressing the *Helicobacter pylori rocF* gene on pGEN222. Extracts were incubated in the presence or absence of various concentrations of S-(2-boronoethyl)-L-cysteine (BEC) and measured for arginase activity at pH 6.0 in the presence of cobalt; B: Purified arginase (His6-RocF) was incubated in the presence or absence of BEC (400 nmol/L) and assayed for arginase activity in the presence of cobalt at pH 6.0; C: Extracts from *E. coli* expressing rat arginase Ⅰ were prepared and incubated in the presence of different concentrations of BEC and then assayed for arginase activity in the presence of manganese at pH 9.0. Graph plotted on a logarithmic scale due to magnitude of arginase activity.

other amino acid residues in these proteins are responsible for the differential BEC inhibition observed between eukaryotic and *H. pylori* arginase.

The arginase from *Bacillus anthracis* (*B. anthracis*) has best catalytic activity with nickel[30]. Arginase activity from the purified *B. anthracis* RocF (active site = DAHGD) was not inhibited at all by up to 400 μmol/L BEC (assayed at pH 9.0), and instead, a stimulatory activity was noticed at 40 and 400 μmol/L BEC (Viator and McGee, unpublished observations). Whether the A to G amino acid substitution in the *B. anthracis* DAHGD metal-binding site is responsible for this lack of BEC inhibition is not yet known. Nonetheless, the data suggest that bacterial and eukaryotic arginases have distinct properties with respect to the BEC inhibitor.

The abundant urease must obtain copious amounts of urea for the urease to function efficiently *in vivo*, since urease is absolutely essential for colonization[8,9]. Urea is essential for urease activity, and urease is largely cytosolic in *H. pylori*, therefore, there must be a mechanism to transport urea. Indeed, the *H. pylori* UreI protein, expressed from the urease operon, is an urea transporter[31]. Mutation in *ureI* renders *H. pylori* attenuated in gerbils[32], which illustrates how crucial it is for urea to enter the bacterial cell and allow *H. pylori* to survive at pH < 4.0. Important future experiments are to examine the role of urease in more depth in this arginase Ⅱ knockout mouse background, and to monitor closely intragastric pH and urea concentrations in the different stomach compartments in wild-type and arginase Ⅱ knockout mice.

Although the data showed that BEC could almost completely inhibit arginase Ⅰ *in vitro*, the same was not true *in vivo*. Several possibilities may account for this. First, oral delivery of BEC leads to the dilution of the inhibitor in the gastric juice, which could decrease its effective inhibitory concentration. Second, the acidity of gastric juice could alter inhibitory properties of BEC by protonation of BEC. This may explain why BEC inhibits arginase activity in the antrum, but not the body, where there is much stronger acid production. Third, BEC may not be able to enter the host cell with great efficiency to inhibit arginase Ⅰ, which is found in the host cytosol. In the *in vitro* biochemical experiments, arginase is freed from the cells by a lysis step, which allows BEC direct access to the arginase. Finally, the complexities of the *in vivo* environment may lead to BEC being degraded or sequestered from reaching the arginase Ⅰ. Despite these potential limitations it was demonstrated that oral delivery of BEC could block at least some of the arginase Ⅰ in several tissues examined. However, the residual arginase Ⅰ that remains may have been enough to provide the necessary urea substrate for *H. pylori* urease to utilize. Alternatively, another host urea-generating enzyme, agmatinase[33,34], may be responsible.

Agmatine is prevalent in the stomach[35,36] and 60% of this agmatine goes to the liver[37]. Interestingly, agmatine levels are higher in the gastric juice of *H. pylori*-infected patients versus uninfected patients[36]. Moreover, *H. pylori* has a putative arginine decarboxylase that could theoretically generate the agmatine. There is also the possibility that intestinal normal flora bacteria could provide the urea[38].

Future studies will center on attempting to eliminate host arginase Ⅰ in the stomach. This may pose a challenge, due to the crucial role that arginase Ⅰ plays in mice, which die of hyperammonemia shortly after birth if they are disrupted for arginase Ⅰ[19]. Several possible strategies to inhibit completely host arginase Ⅰ could be inhibitory RNA knockdown experiments, or construction of tissue-specific arginase Ⅰ knockout mice that hopefully would live normally but just fail to produce arginase Ⅰ in the stomach. However, urea could also diffuse to the stomach from arginine hydrolysis in other organs, which might complicate

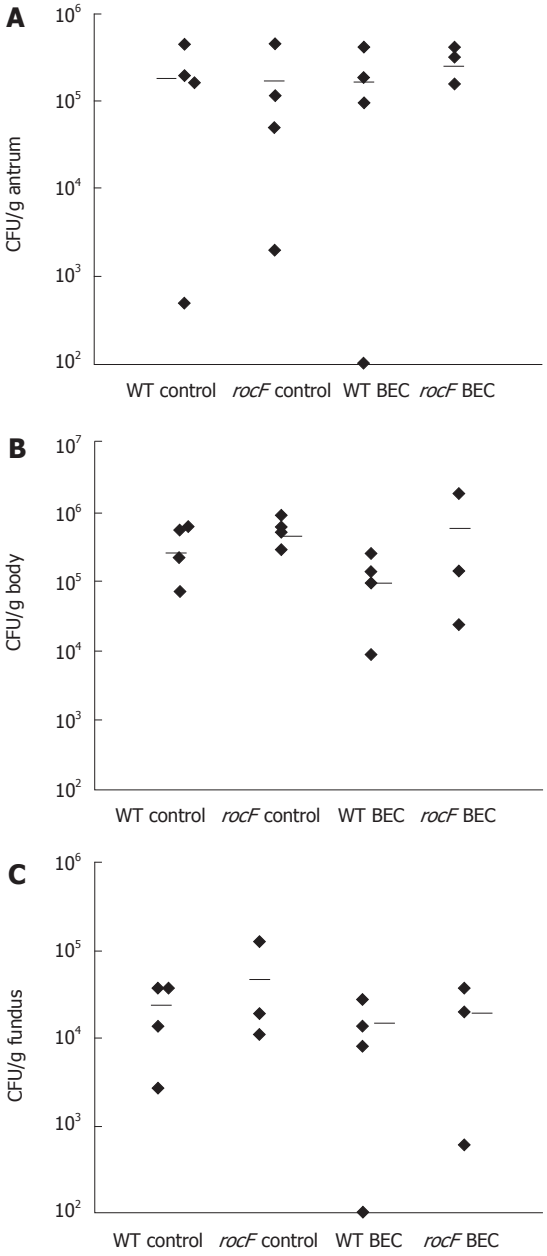

Figure 7 The *rocF* mutant of *Helicobacter pylori* colonizes wild-type mice treated with the arginase inhibitor S-(2-boronoethyl)-L-cysteine. Wild-type (C57 BL/6) mice (n = 3 or 4 per group) were orally fed water or S-(2-boronoethyl)-L-cysteine (BEC) (50 mmol/L) once or twice daily for 3 d before oral inoculation with wild-type or *rocF* mutant *Helicobacter pylori* (*H. pylori*) strain SS1 (~108 CFU). The BEC animal groups continued to receive BEC daily until animals were euthanized. At 1 wk post-infection, animals were euthanized and the stomach dissected into antrum (A), body (B), or fundus (C). Limit of detection was ~100 CFU/g stomach and all animals that lacked *H. pylori* were set to this detection limit. Bar, mean CFU/g tissue. Usually each symbol represents one mouse. In some cases there are two mice represented by a symbol if the data overlapped.

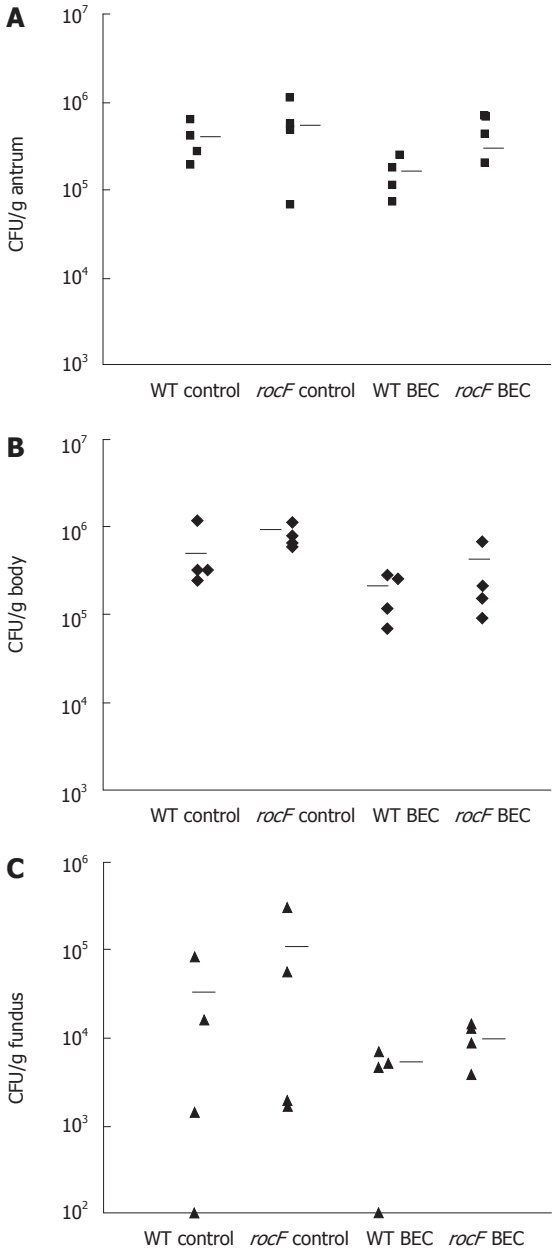

Figure 8 The *rocF* mutant of *Helicobacter pylori* colonizes arginase Ⅱ knockout mice treated with the arginase inhibitor S-(2-boronoethyl)-L-cysteine. Arginase Ⅱ knockout mice (n = 3 or 4 per group) were orally fed water or S-(2-boronoethyl)-L-cysteine (BEC) (50 mmol/L) once or twice daily for 3 d before oral inoculation with wild-type or *rocF* mutant *Helicobacter pylori* strain SS1 (~108 CFU). The BEC animal groups continued to receive BEC daily until animals were euthanized. At 1 wk post-infection, animals were euthanized and the stomach dissected into antrum (A), body (B), or fundus (C). Limit of detection: ~100 CFU/g tissue. Bar, mean CFU/g stomach.

a tissue-specific arginase Ⅰ knockout strategy. The recent study that used an arginase-containing adenovirus delivery system to complement the arginase Ⅰ $^{-/-}$ mouse led to a doubling of survival to 27 d[39]. Further refinements to this exciting model may hold promise for additional exploration of the role of arginase Ⅰ in *H. pylori* pathogenesis.

The *in vivo* source of urea still remains a mystery. However, it is clear that the *in vivo* source of urea for the crucial enzyme urease does not originate from the bacterial arginase or from host arginase Ⅱ. Instead, the data suggest that urea originates from host arginase Ⅰ or an alternative urea-generating pathway.

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COMMENTS

Background

The bacterium *Helicobacter pylori* (*H. pylori*) causes stomach problems, such as ulcers and inflammation and can lead to stomach cancer. One of the important proteins it makes that contributes to disease is called urease, an enzyme that converts urea to ammonia and carbon dioxide. Urease is required for bacteria to cause disease, and thus the substrate urea is also required. In this study, the authors designed experiments to determine from where this urea is coming: bacterial or host sources.

Research frontiers

The most likely enzyme that provides the urea source is arginase and both *H. pylori* and humans have arginases (two in humans, one in *H. pylori*). The authors used a mouse model in which one of the arginases, arginase Ⅱ, was knocked out, along with *H. pylori* wild-type or an arginase knockout bacterium (*rocF*). An arginase inhibitor called S-(2-boronoethyl)-L-cysteine (BEC) was also used to knock down the second mouse arginase (arginase Ⅰ). The authors found that neither the bacterial arginase (*rocF*) nor arginase Ⅱ from the mouse was important to provide the urea, which implies the involvement of either arginase Ⅰ or another pathway (agmatinase).

Innovations and breakthroughs

The study provides the first steps towards the foundation needed to understand where the urea originates for the crucial urease enzyme. Much remains to be done, including completely knocking down arginase Ⅰ in mice and determining whether another enzyme, agmatinase could be involved.

Applications

By understanding where the urea originates, the authors hope to develop novel anti-*Helicobacter* drugs. New therapies are sought because *H. pylori* is becoming resistant to antibiotics.

Terminology

H. pylori causes infection in the stomach, which leads to inflammation, ulcers and cancer. Urease is an enzyme made by *H. pylori* the converts urea to ammonia and carbon dioxide. Arginase is an enzyme that converts arginine to ornithine and urea and is made by humans, mice and *H. pylori*. Knockout refers to a gene that is disrupted and no longer functional.

Peer review

The authors found that the *in vivo* source for the essential urea substrate for the *H. pylori* urease is neither bacterial arginase nor host arginase Ⅱ. Based on these results, they hypothesized that either host urea-generating pathway such as agmatinase or the residual host arginase Ⅰ might be responsible.

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