MAJOR ARTICLE

Angiotensin II Subtype 1 Receptor Blockade Inhibits *Clostridium difficile* Toxin A–Induced Intestinal Secretion in a Rabbit Model

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Angiotensin II (ANG II) has been described in the regulation of intestinal secretion and absorption via angiotensin subtype 1 (AT_1) and AT_2 receptors, respectively, in rats. We investigated the role that ANG II plays in the rabbit ileal-loop model of *Clostridium difficile* infection. Expression of AT_1, the more abundant ANG II receptor, was demonstrated in ileal loops, and an AT_1 receptor blocker, losartan, inhibited hypersecretion induced by *C. difficile* toxin A (mean volume:length ratio, 0.27 ± 0.06 vs. 0.60 ± 0.06 mL/cm in controls). Losartan also decreased production of ANG II in the ileum (0.48 ± 0.06 vs. 0.87 ± 0.12 pg/mg in controls), raising the possibility that ANG II may participate in a positive feedback loop involving the hypersecretory response. Our findings suggest that ANG II plays a significant role in the pathogenesis of *C. difficile* toxin–induced diarrhea.

Toxigenic *Clostridium difficile* is the most important known cause of both antibiotic-associated and nosocomial diarrhea. The clinical manifestations of *C. difficile* colitis are caused by either one or both of the large exotoxins—toxins A and B—secreted by *C. difficile*. Most of the inflammatory and secretory effects of *C. difficile* are due to toxin A, an enterotoxin. Toxin A has been shown to cause the release of inflammatory cytokines, the recruitment of polymorphonuclear cells, and the stimulation of prostaglandin synthesis [1–3]. Toxin B, however, is a cytotoxin that causes cytopathic changes in cell culture. Recent studies have implicated toxin A–negative/toxin B–positive strains of *C. difficile* as a cause of disease outbreaks [4]. Both toxin A and toxin B exert at least some of their effects via the monoglycosylation and inactivation of an intracellular signaling target, rho, which leads to cytoskeletal disruption [5–7].

Angiotensin II (ANG II) receptors exist as subtype 1 (AT_1) and subtype 2 (AT_2) and are known to play a major role in the cardiovascular and renovascular systems that mediate inflammation, cell growth, fibrosis, and vascular tone. There has been considerable interest in the presence of a local angiotensin system in the gastrointestinal tract, including the demonstration of ANG II binding sites, angiotensin-converting enzyme, and AT_1 and AT_2 receptors in the rat jejunum [8–11] and, more recently, in a human intestinal cell line (B.A.C.-F., G.A.C.B., and R.L.G., personal communication). The AT_1 receptor is present throughout the body in most species that have been studied to date [12]. Autoradiography performed on rat intestines detected mostly AT_1 receptors in the mucosa and muscularis of the jejunum, ileum, and colon, whereas only a small population of AT_2 receptors was observed [10]. The AT_2 receptor is more localized in the brain and adrenal glands than in other tissues, and its location may be more variable by species than is that of the AT_1 receptor [12]. Activation of the AT_1 receptor inhibits absorption or stimulates secretion, whereas activation

Received 19 May 2004; accepted 12 January 2005; electronically published 3 May 2005.

Presented in part: 50th Annual Meeting of the American Society of Tropical Medicine and Hygiene, Atlanta, GA, 11–15 November 2001.

Financial support: Fogarty International Center Training and Research in Emerging Infectious Diseases Fellowship (grant 5 D43 TW00909 to C.S.A.).

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The Journal of Infectious Diseases 2005;191:2090–6

0022-1899/2005/19112-0013$15.00

Table 1. Histologic grading.

Score	Description
0	The mucosa is intact, and there is normal cellularity and no vascular congestion.
1	There is focal mucosal disruption, and there is a minimal increase in cellularity or minimal vascular congestion.
2	There is mild mucosal disruption, and there is a mild to moderate increase in cellularity or mild to moderate vascular congestion.
3	There is moderate mucosal disruption, a moderate increase in cellularity, and prominent polymorphonuclear cells or moderate vascular congestion.
4	The mucosal epithelium is mostly denuded, and there is intense inflammatory infiltration and severe vascular congestion.

of the AT_2 receptor causes net absorption of ions and water, although these effects may be species and/or site specific [13–15]. Losartan, a nonpeptide competitive antagonist, selectively blocks AT_1 receptors. In the present study, we investigated whether the same local angiotensin system, through AT_1 receptors, operates in the rabbit model of *C. difficile*–associated diarrhea (CDAD). Furthermore, we investigated the association between ANG II and *C. difficile* toxin A–induced inflammatory and secretory responses with and without AT_1 receptor blockade.

MATERIALS AND METHODS

Rabbit ileal loops. The animal experiment protocol was reviewed and approved by the University of Virginia Center for Comparative Medicine. Twenty-two New Zealand white rabbits weighing 2 kg were fasted overnight. The rabbits were anesthetized with ketamine (60–80 mg/kg) and xylazine (5–10 mg/kg), administered intramuscularly, and a midline abdominal incision was made to expose the small bowel. After the ileum was flushed with 5–10 mL of PBS, 8–14 loops of 4 cm each were ligated using double ties, and a 1-cm interval was left between loops.

We studied 121 ligated ileal loops, of which 68 loops from 13 rabbits served as controls. Each of the control loops was injected intraluminally with a 1-mL solution of PBS with either 10 μg/mL *C. difficile* toxin A (Techlab), 2 μg/mL cholera toxin (CT; ICN Biomedicals), or 20 μg/mL *E. coli* heat-stable toxin (ST; Sigma-Aldrich). Losartan powder (DuPont-Merck Pharmaceutical) was diluted in PBS to concentrations of 10, 3, 1, 0.3, or

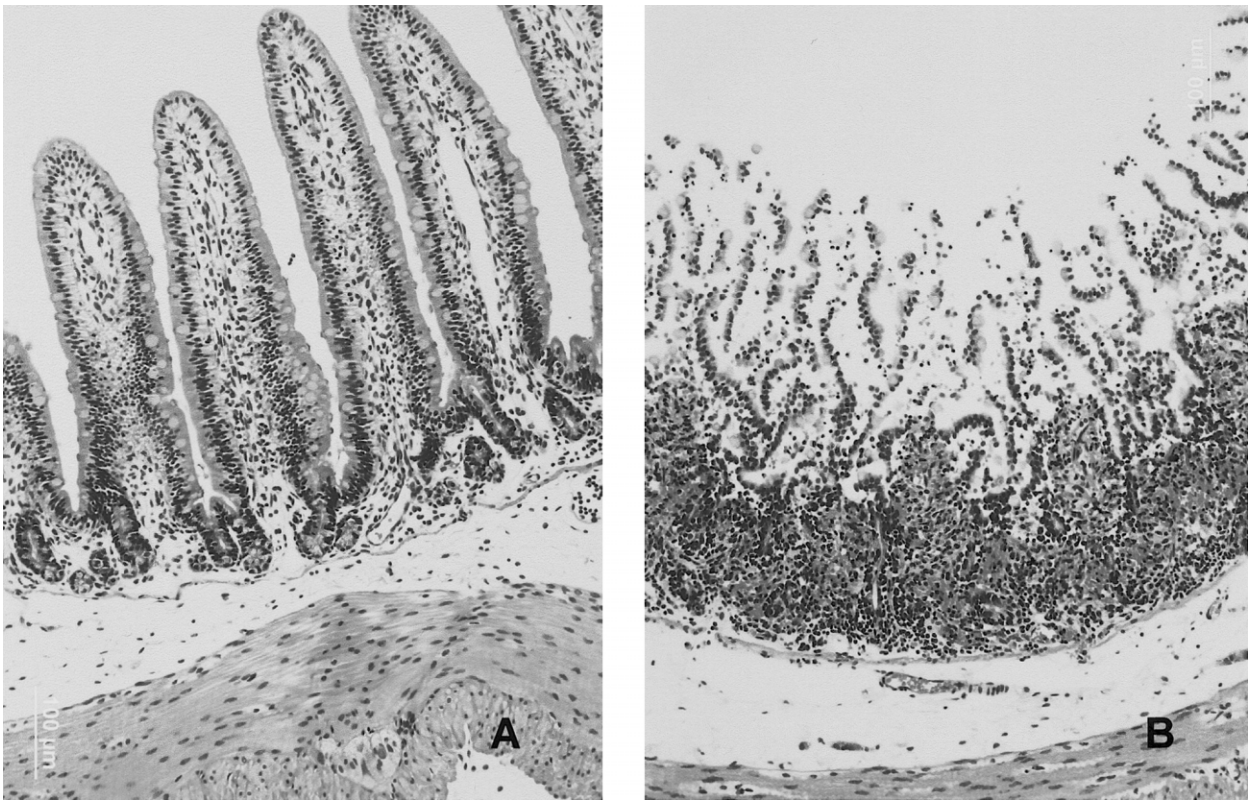

Figure 1. Rabbit ileal loops stained with hematoxylin-eosin revealing mucosal injury, inflammation, and vascular congestion induced by *Clostridium difficile* toxin A *(B)* vs. treatment with PBS alone *(A)* (original magnification ×100).

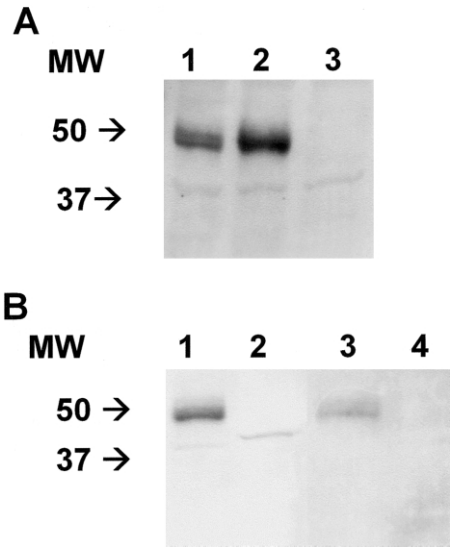

Figure 2. Expression of angiotensin subtype 1 (AT_1) receptor protein of molecular weight (MW) 37–50 in rabbit ileal loops treated with PBS *(A, lane 1)* and *Clostridium difficile* toxin A *(A, lane 2)*. The lower bands in *lanes 1* and *2* correspond with the level of expression of the AT_1 receptor in the vascular smooth muscle *(A, lane 3)*. Preadsorption of the primary antibody (AT_1 [N-10] IgG) with blocking peptide (AT_1 [N-10] peptide) resulted in the disappearance of the AT_1 receptor band in the ileal tissue *(B, lanes 1* and *3)* and PC-12 whole-cell lysate *(B, lanes 2* and *4)*. The upper bands at MW 50 showed fading after neutralization with the primary antibody.

0.1 mg/mL. Immediately before the enterotoxins were administered, 53 loops from another 9 rabbits were treated with losartan. The ileal loops were replaced intraperitoneally, and the abdominal incision was sutured closed. The rabbits were maintained under anesthesia until they were euthanized 5 h later.

Measurement of intestinal secretion. After 5 h of incubation, the ligated ileal loops were removed. The length of each ligated ileal segment was measured. Intraluminal fluid was extracted from each ileal loop and quantified. The volume:length ratio (V:L) was calculated in milliliters per centimeter per loop. The gross description (clear, serous, purulent, or bloody) of the collected fluid was also noted.

Histopathologic assessment. Samples of intestinal tissue from control and treated ileal loops were fixed with 10% formalin and stained with hematoxylin-eosin. Three of the investigators (C.S.A., R.L.G., and G.A.C.B.) randomly read each slide and were blinded to its source. A grading scale was formulated, and each slide was graded from 0 (none) to 4 (worst) on the basis of the degree of mucosal disruption, the increase in cellularity, and the intensity of vascular congestion (table 1). The final histologic score of each slide was the mean of the scores given by the 3 investigators. This grading scale was used in a study published elsewhere on enterotoxin-induced mucosal injury in the same rabbit ileal-loop model [3].

ANG II EIA. Ileal tissues were immediately stored in liquid nitrogen until the EIA was performed. For the ANG II assay, 1 mL of PBS/g of tissue was added. The tissue was homogenized and sonicated. The homogenate was centrifuged at 1500 *g* for 10 min. The supernatant was tested for ANG II by specific EIA (SPI-BIO), in accordance with the manufacturer's instructions. Briefly, the supernatant was dried by vacuum centrifugation. The sample was resuspended in EIA buffer, vortexed, and centrifuged at 3000 *g* at 4°C for 10 min. An ANG II standard and samples were dispensed in duplicate onto plates coated with monoclonal anti–ANG II. After 1 h of incubation at room temperature, glyceraldehydes and borane trimethylamine were successively dispensed onto the plates. The plates were washed with buffer, and an anti–ANG II IgG tracer was dispensed into each well. After an overnight incubation at 4°C, the plates were again washed with buffer, incubated with Ellman's reagent, and read at 405 nm with an ELISA reader (Titertek Multiskan Plus).

Western blot analysis. Ileal tissues were harvested from 2 control rabbits treated with either PBS or toxin A only. The tissue samples were immediately frozen and kept in liquid nitrogen until protein extraction was performed. The samples were homogenized in 1 mL of cold lysis buffer that contained 50 mmol/L HEPES (pH 7.4–8.9), 1% Triton X, and a 10% (wt/vol) protease inhibitor mixture (Sigma). The homogenate was sonicated for 10 s, incubated in ice for 30 min, and centrifuged at 20,000 *g* at 4°C for 10 min. The supernatant was then aliquoted and stored at −70°C until the protein assay was performed. Concentrations of proteins were determined by bioinchoninic acid protein assay (Pierce), in accordance with the manufacturer's instructions.

The samples were diluted in SDS buffer (Sigma-Aldrich) and heated at 100°C for 4 min before being loaded on a 10% Tris-HCL SDS-polyacrylamide gel (Bio-Rad Laboratories). Each lane was loaded with 20-μg samples of protein, and 1 lane of each gel contained prestained molecular weight standards (Bio-Rad Laboratories). Vascular smooth muscle protein extract and PC-12 whole-cell lysate (Santa Cruz Biotechnology) served as positive controls. After electrophoresis, the proteins were transferred to nitrocellulose membranes, which were then placed in Tris-buffered saline with 5% nonfat milk and 0.05% Tween 20. Conjugation of the primary AT_1 (N-10) affinity purified rabbit polyclonal antibody (Santa Cruz Biotechnology) was performed using a Zenon horseradish peroxidase (HRP)–conjugated rabbit IgG labeling kit (Molecular Probes). After an overnight incubation at 4°C, 1 nitrocellulose membrane was incubated with the HRP-conjugated AT_1 antibody (1:500 dilution). For the preadsorption study, another membrane was incubated with an HRP-conjugated primary antibody that was initially mixed for 1 h with a 10-fold molar excess of AT_1 (N-10) peptide (Santa Cruz Biotechnology), a blocking peptide. Immunoreactivity was visualized by enhanced chemiluminescence (ECL Plus; Amersham Biosciences). Quantitative assessment of band densities was

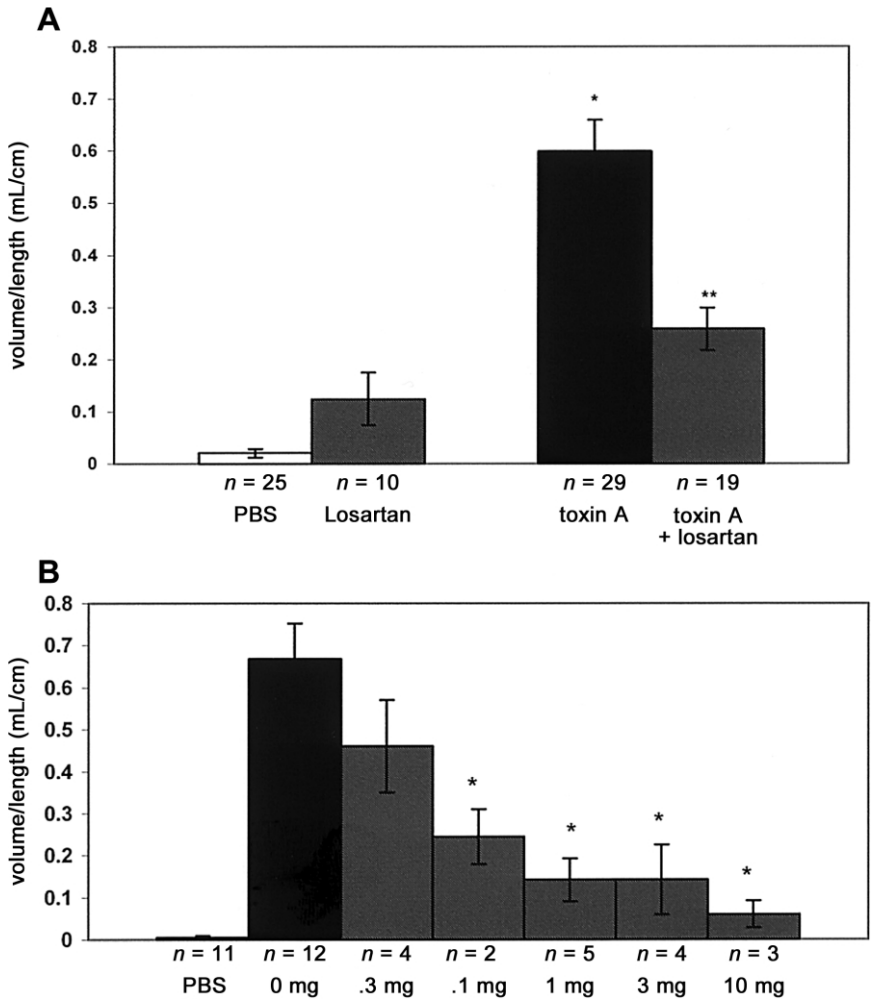

Figure 3. Intraluminal injection of losartan (1 mg/loop) in *Clostridium difficile* toxin A–treated rabbit ileal loops. *A,* Decrease of toxin A–induced secretion by losartan *(gray bars),* compared with loops treated with toxin A *(black bar)* only. *$P < .0001$, PBS vs. toxin A. **$P < .001$, toxin A vs. toxin A plus losartan. *B,* Dose-dependent inhibition of toxin A–induced secretion in tissue treated with PBS only *(white bar),* 0 mg of losartan *(black bar),* and varying concentrations of losartan *(gray bars).* *$P < .01$, toxin A vs. toxin A plus losartan (Student's *t* test). $P < .0005$ for the entire data set (analysis of variance).

performed by scanning densitometry (ImageQuant; Molecular Dynamics).

Statistical analysis. Continuous variables, such as V:L, histologic score, Western blot band densities, and levels of ANG II, are expressed as means ± SE. For statistical comparison of the magnitude of secretion, the amount of inflammation, and protein levels, a 2-tailed Student's *t* test was applied. Analysis of variance (ANOVA) was performed to compare ileal loops treated with varying doses of losartan. $P < .05$ was considered to be statistically significant.

RESULTS

Toxin A–induced hemorrhagic secretion and mucosal injury. Toxin A–treated loops had significantly elevated levels of intestinal secretions (mean V:L, 0.60 ± 0.06 mL/cm), compared with PBS-treated loops (mean V:L, 0.02 ± 0.01 mL/cm; $P < .0001$). Most (89%) of the toxin A–treated loops had blood-tinged or grossly hemorrhagic secretions. Levels of intestinal secretions were also significantly elevated in CT-treated loops (mean V:L, 0.68 ± 0.12 mL/cm; $P = .0007$) and in ST-treated loops (mean V:L, 0.28 ± 0.06 mL/cm; $P = .008$), compared with those in PBS-treated loops (mean V:L, 0.01 ± 0.01 mL/cm). In contrast to the hemorrhagic secretions in toxin A–treated loops, most (86%) CT-treated loops had clear serous fluid, whereas ST-treated loops had mostly cloudy (16%) to blood-tinged (66%) secretions.

Microscopic studies of the intestinal mucosa exposed to toxin A (histologic score, 3.28 ± 0.24 vs. 0.25 ± 0.13 in PBS-treated loops; $P < .0001$) revealed severe epithelial disruption, inflammation, and vascular congestion (figure 1). In contrast, in PBS-, CT-, or ST-treated loops, no gross changes in the epithelial mucosa or differences in the histologic scores were observed.

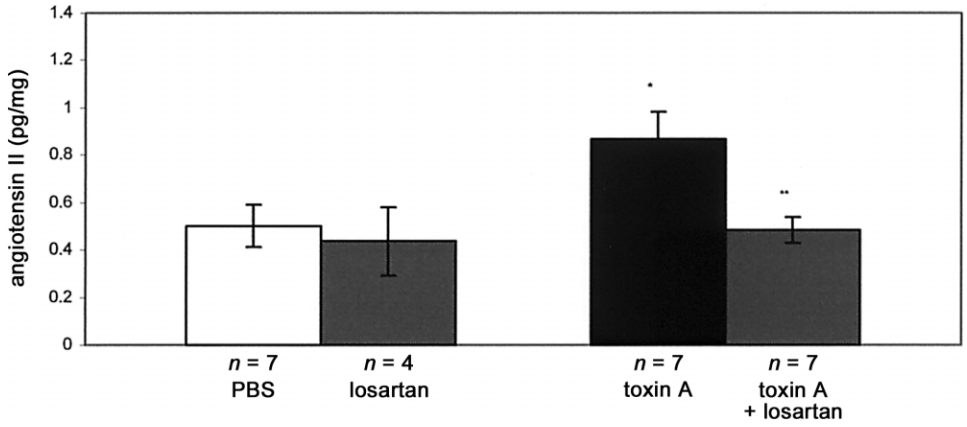

Figure 4. Angiotensin II (ANG II) levels in *Clostridium difficile* toxin A–treated rabbit ileal loops. In the presence of losartan (1 mg/loop; *gray bars*), the level of toxin A–induced secretion of ANG II in the ileal loops was decreased, compared with the levels in loops treated with PBS *(white bar)* or toxin A *(black bar)* only. $*P = .03$, PBS vs. toxin A. $**P = .02$, toxin A vs. toxin A plus losartan (Student's *t* test).

AT_1 receptors expressed in toxin A–treated loops. Western blot analysis using antibody specific for AT_1 receptor proteins recognized 2 bands within the expected molecular weight range of 37–50 in the lanes with rabbit ileal proteins. The protein bands from toxin A–treated loops appeared darker than those from PBS-treated loops (figure 2*A* and 2*B*). Densitometric analysis of bands from toxin A–treated loops showed a mean increase in density volume of 107%, compared with those from PBS-treated loops. The lower band in the lanes with rabbit ileal proteins corresponded to the level of AT_1 receptor expression seen in the positive control, vascular smooth muscle (figure 2*A*). Preadsorption studies demonstrated complete disappearance of the lower bands in rabbit ileal proteins, as was observed in the AT_1 receptor protein band in the positive control, PC-12 whole-cell lysate (figure 2*B* and 2*C*). In the neutralization study, the upper bands demonstrated partial fading. Densitometric analysis of the upper bands showed a mean decrease in density volume of 35% after preadsorption.

AT_1 receptor blockade inhibition of toxin A–induced secretion. All ileal loops treated with both enterotoxin and losartan showed a statistically significant reduction in mean V:L. Toxin A–treated loops had a 57% reduction in their mean secretory response when they were also treated with losartan (mean V:L, 0.27 ± 0.06 vs. 0.60 ± 0.06 mL/cm in loops treated with toxin A alone; $P < .001$) (figure 3*A*). Furthermore, a dose-dependent reduction in secretion occurred as the dose of losartan was increased from 0.1 to 10 mg/mL ($P < .0005$, ANOVA) (figure 3*B*). When each treatment group was compared with the group that was treated with toxin A only, a statistically significant difference was found in all groups (10-mg dose group, $n = 3$ [$P < .0001$]; 3-mg dose group, $n = 4$ [$P = .002$]; 1-mg dose group, $n = 5$ [$P < .0001$]; 0.1-mg dose group, $n = 4$ [$P = .008$]), except in the 0.3-mg dose group. CT- and ST-treated loops showed a reduction in the levels of secretions of 59% ($P = .009$) and 64% ($P = .04$), respectively, when they were also treated with losartan.

Loops treated with losartan at all doses showed a dose-related trend toward inhibition of inflammation and tissue damage ($P = .053$, ANOVA), but a statistically significant difference was observed only at the 10-mg dose ($n = 3$; mean histologic score, 1.67 ± 0.33; $P = .02$). At the 1-mg dose, some loops already showed almost complete inhibition of mucosal injury.

AT_1 receptor blockade inhibition of toxin A–induced secretion of ANG II. ANG II levels were higher in toxin A–treated loops than in PBS-treated loops (0.87 ± 0.12 vs. 0.50 ± 0.09 pg/mg; $P = .03$). Loops treated with both losartan and toxin A showed significantly decreased ANG II levels (0.48 ± 0.06 pg/mg), compared with loops treated with toxin A only (0.87 ± 0.12 pg/mg; $P = .02$) (figure 4).

DISCUSSION

In the rabbit ileum, toxin A induced severe mucosal injury, invoked an intense inflammatory reaction, and augmented intestinal secretion. These destructive changes brought about by toxin A have been well documented in previous studies and may even be consistent with what is observed in severe clinical cases of CDAD [16]. The present study has demonstrated a possible role for the local angiotensin system in CDAD. The rabbit ileal tissue showed increased levels of ANG II in the presence of toxin A. This effect corroborates what has been reported for other inflammatory processes, such as Crohn disease, in which ANG I and ANG II were noted to be elevated [17]. Until now, there had been no information available that implicated ANG II in infectious inflammatory enteritides such as *C. difficile* colitis. Of interest, human neutrophils and mast cells, which are observed to be recruited in toxin A–induced colitis, contain cathepsin G and chymase, respectively, which

are potent enzymes that convert ANG I to ANG II [18–20]. Whether the synthesis of these enzymes is stimulated in CDAD remains to be explored. Elevated levels of ANG II may also be part of a systemic activation of the renin-angiotensin-aldosterone system. Enhanced vascularity secondary to treatment with toxin A may have enhanced the shift of circulating ANG II in the intestinal milieu.

The demonstration of AT_1 receptors in the rabbit ileum further implicates the tissue angiotensin system in the modulation or mediation of inflammatory and secretory processes in the intestinal mucosa. The AT_1 receptor protein bands, which were within the expected molecular weight range, were confirmed by the complete neutralization (lower bands in the Western blot analysis) or decrease in intensity (upper bands in the Western blot analysis) of the bands in the presence of the specific blocking peptide (figure 2). The upper protein bands that were recognized by the specific AT_1 receptor IgG but were partially neutralized by the peptide might represent heavily glycosylated epitopes. Interestingly, toxin A–treated tissues had darker bands, as was confirmed through both visual inspection and densitometry. This difference may indicate that the expression of AT_1 receptors may be up-regulated in inflammatory enteritides, as has been reported in animal studies of other pathological states, such as renal disease and insulin resistance [21, 22].

ANG II levels were elevated in toxin A–treated rabbit ileum, which suggests that the intense mucosal inflammation and accumulation of intraluminal fluid were mediated by ANG II. Blocking the AT_1 receptor with losartan inhibited the secretory response to toxin A. This is consistent with what has been reported in the rat jejunum, where the administration of a high dose of ANG II resulted in intestinal secretion through AT_1 receptors [13–15, 23]. Losartan also caused a concomitant decrease in ANG II levels, and this indicates that ANG II synthesis is regulated by AT_1 receptors. Both CT- and ST-induced secretion were also inhibited by losartan, possibly through a distal common pathway, which suggests that ANG II receptor activation may not be specific to toxin A but can be accomplished by various enterotoxins.

In a previous study, rabbit ileum stimulated with toxin A yielded elevated levels of prostaglandin E_2 (PGE_2) because of the induction of cyclooxygenase 2 expression [3]. AT_1 is also known to stimulate phospholipase A_2, which results in increased levels of PGE_2. Although it was not statistically significant ($P = .053$), a trend toward an effect on inflammation, vascular congestion, and epithelial disruption caused by toxin A was observed in the intestinal mucosa. Other factors in the local tissue angiotensin system may be involved in mucosal integrity. Although it was not demonstrated in the present study, AT_2 receptors have been implicated in the maintenance of mucosal barrier functions. Stimulation of AT_2 receptors has been shown to stimulate the production of nitric oxide, an important mediator of mucosal defense [24, 25]. Whether the ANG II receptors play a role in the alteration of the monoglucosylation of rho substrates in enterocytes in CDAD remains to be proven.

In summary, the findings in the present study suggest that toxin A enhances the expression of ANG II, which, in turn, mediates mucosal injury and intestinal secretion through AT_1 receptors. Blockade of the inflammatory and secretory cascade by use of a pharmacologic approach, such as the inhibition of AT_1 receptors, provides a potentially novel approach to control CDAD and *C. difficile* colitis.

Acknowledgments

We thank Helen McGrath, John Gildea, Charlotte Martin, Nancy Howell, and Ruth Aldridge for their valuable technical support.

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