Endogenous corticosteroids modulate *Clostridium difficile* toxin A-induced enteritis in rats

Ignazio Castagliuolo, Katia Karalis, Leyla Valenick, Asiya Pasha, Sigfus Nikulasson, Michael Wlk, and Charalabos Pothoulakis

Endogenous corticosteroids modulate *Clostridium difficile* toxin A-induced enteritis in rats. Am J Physiol Gastrointest Liver Physiol 280: G539–G545, 2001.—We examined the role of glucocorticoids in acute inflammatory diarrhea mediated by *Clostridium difficile* toxin A. Toxin A (5 μg) or buffer was injected in rat ileal loops, and intestinal responses were measured after 30 min to 4 h. Ileal toxin A administration increased plasma glucocorticoids after 1 h, at which time the toxin-stimulated secretion was not significant. Administration of the glucocorticoid analog dexamethasone inhibited toxin A-induced intestinal secretion and inflammation and downregulated toxin A-mediated increase of macrophage inflammatory protein-2. Adrenalectomy followed by replacement with glucocorticoids at various doses suggested that intestinal responses to toxin A were related to circulating levels of glucocorticoids. Administration of the glucocorticoid receptor antagonist RU-486 enhanced toxin A-mediated intestinal secretion and inflammation. We conclude that *C. difficile* toxin A causes increased secretion of endogenous glucocorticoids, which diminish the intestinal secretory and inflammatory effects of toxin A.

Hypothalamic-pituitary-adrenal axis; bacterial enterotoxins; macrophage inflammatory protein-2

Toxin A, a 308-kDa enterotoxin released by *Clostridium difficile* (14), mediates fluid secretion and inflammation in animal intestine (28, 44). Toxin A binds to surface enterocyte receptors (34) and is internalized into the cytoplasm where it inactivates the small GTP-binding protein Rho (13, 21). Inactivation of Rho leads to disaggregation of filamentous actin and dysfunction of tight junctions as evidenced by studies in T84 colonic epithelial cells and human colonic mucosal strips placed in Ussing chambers (17, 38). In vivo studies showed that toxin A elicits fluid secretion and mucosal damage with a prominent mucosal neutrophil infiltration that is evident 2–4 h after injection of the toxin in closed intestinal loops (7, 37). We, and others, have shown that the pathogenesis of toxin A-mediated enteritis in rodents involves interactions between sensory nerves, enterocytes, and inflammatory cells of the intestinal lamina propria (7, 8, 10, 27). This leads to release of inflammatory mediators from intestinal epithelial and lamina propria cells (7, 10, 44) and to increased expression of adhesion molecules on endothelial cells and neutrophils [polymorphonuclear neutrophils (PMNs); see Refs. 24 and 26], resulting in PMN recruitment and activation (24). PMN-derived inflammatory mediators act on epithelial cells, causing acute destruction and necrosis of villus enterocytes (24, 45). The importance of PMNs in the inflammatory response to toxin A is supported by recent results showing that administration of toxin A into rat ileum increased mucosal levels of the potent PMN chemoattractant macrophage inflammatory protein-2 (MIP-2), whereas administration of an antibody to MIP-2 reduced toxin A-mediated intestinal responses (8).

Glucocorticoids inhibit the formation of inflammatory exudate and leukocyte infiltration and release of inflammatory mediators in several models of inflammation (6, 15). Endogenous glucocorticoids are released during inflammation after activation of the hypothalamic-pituitary-adrenal (HPA) axis by cytokines and other inflammatory mediators (5, 39). The importance of endogenous glucocorticoids for the control of the inflammatory response is highlighted by studies showing that adrenalectomized animals exhibit a severe and often lethal inflammatory reaction to endotoxins (50).

In this study, we investigated the importance of endogenous glucocorticoids in the control of acute enteritis elicited by *C. difficile* toxin A. In addition, we evaluated the effects of pharmacological doses of glucocorticoids on the development of toxin A-mediated intestinal inflammation. To further understand the ability of glucocorticoids to regulate the inflammatory pathways stimulated by toxin A, we studied the effects of dexamethasone administration on upregulation of the proinflammatory cytokine MIP-2, previously shown to play a major role in this experimental model of enteritis (8).

Received 17 September 1999; accepted in final form 25 October 2000.
MATERIALS AND METHODS

Materials

Male adult Wistar rats weighing 200–250 g (Charles River Breeding Laboratories, Wilmington, MA) were housed in groups of two under controlled illumination (lights on 0700 and out at 2100) and humidity conditions. The animals were acclimated for at least 3 days before any experimental manipulation, and they had free access to water and food. Rats were fasted 18 h before the experiments, but free access to water was retained. Toxin A was purified to homogeneity from culture supernatants of C. difficile strain 10,463 as previously described (36). [3H]mannitol (30 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Dexamethasone phosphate, purchased from Sigma Diagnostics (St. Louis, MO) and dissolved in ethanol (1 mg/ml), was administered intraperitoneally 30 min before formation of ileal loops as described in Rat ileal loop model. The glucocorticoid antagonist RU-486, a generous gift from Roussel-Uclaf (Paris, France), was dissolved in DMSO (1 mg/ml). RU-486 was administered intraperitoneally either at a single dose of 1 mg/kg 30 min before ileal loops were formed or two times, first before fasting as above and then 30 min before loops were formed at the same dose. Protein concentrations were determined by the bicinchoninic acid assay method (Pierce Laboratories, Rockford, IL).

Methods

Rat ileal loop model. Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg; Abbott, Chicago, IL); a laparotomy was then performed, and two 5-cm-long ileal loops were formed (7, 10). Both renal pedicles were tied to prevent urinary excretion of [3H]mannitol, and 10 μCi of [3H]mannitol were injected into the inferior vena cava. Each loop was then injected with either 0.4 ml of 50 mM Tris buffer (pH 7.4) containing 5 μg toxin A or buffer alone (control). Previous studies showed that 5 μg of toxin A were effective in inducing a time-dependent increase in rat ileum in vivo (7, 37). The abdomen was then closed, and animals were maintained under general anesthesia with pentobarbital sodium (10 mg·kg⁻¹·h⁻¹ ip). Thereafter, rats were placed on a heating pad for the duration of the experiment to keep the body temperature at ~37°C. After 30 min, 1 h, 2 h, or 4 h, animals were killed, and the following parameters, previously shown to be associated with the in vivo enterotoxic effects of toxin A, were measured: 1) fluid secretion, estimated as the loop weight-to-length ratio (7, 10), 2) mucosal permeability to mannitol, determined by counting [3H]mannitol in the loop fluid and expressed as disintegrations per centimeter of loop (dpm/cm; see Refs. 7 and 10), 3) myeloperoxidase (MPO) activity, measured in aliquots of intestinal luminal fluid as an estimate of neutrophil infiltration (7, 10) and expressed as units per centimeter of loop, and 4) tissue damage determined on ileal sections fixed in formalin and stained with hematoxylin and eosin. Histological damage was graded by a gastrointestinal pathologist (S. Nikulasson, who was not informed of the identity of the samples, by evaluating 1) epithelial cell damage, 2) hemorrhagic congestion and mucosal edema, and 3) neutrophil infiltration, as previously described (7). A score of 0–3 denoting increasingly severe damage was given to each of these parameters.

Adrenalectomy and corticosterone replacement. Rats were adrenalectomized under general anesthesia by the retroperitoneal route as previously described (18). Animals were left to recover for 6 days after adrenalectomy. Corticosterone/cholesterol pellets were made and implanted subcutaneously as previously described (18, 19) to provide the desired plasma corticosterone levels in adrenalectomized rats of this size. It has been previously demonstrated that 100-mg pellets containing 30% corticosterone-70% cholesterol provided approximately physiological corticosterone replacement in 225-g rats, as shown by inhibition of adrenalectomy-induced morning plasma ACTH levels and no reduction of thymus weight (2). In our studies, rats were replaced with 100-mg pellets containing 15% corticosterone-85% cholesterol [shown to provide resting plasma corticosterone levels similar to those of the sham-operated animals (19)], 30% corticosterone-70% cholesterol, and 60% corticosterone-40% cholesterol, representing ~1×, 2×, and 4×, respectively, the physiological replacement (1×). Adrenalectomized animals were given 0.9% NaCl instead of tap water to compensate for the excess salt loss. Control (sham-operated) rats underwent the same surgical procedure, but without removal of the adrenals. No placebo pellets were implanted in sham-adrenalectomized or adrenalectomized nonreplaced rats, since no effect of pellet implantation has been shown on the regulation of the HPA axis (19).

Effect of toxin A-induced enteritis on plasma corticosterone levels. Wistar rats were housed in the room where the experiment was performed and were fasted for 18 h. Animals were anesthetized by intraperitoneal pentobarbital sodium and left in their cages for 30 min before handling. Ileal loops were then prepared and injected with either buffer or 5 μg of toxin A. Blood samples (0.2 ml) were collected from the inferior vena cava just before (time 0) or 1 and 2 h after toxin A or buffer injection, as previously described (10). Blood samples were centrifuged (800 g for 10 min at 4°C), and plasma aliquots were stored at −20°C until corticosterone levels were measured by an RIA kit (ICN Biomedicals, Costa Mesa, CA), as previously described (11).

Total mucosal RNA extraction and RT-PCR amplification. Ileal loops were injected with either buffer or toxin A, and 2 h later animals were killed, loops were removed, opened, and washed in ice-cold sterile PBS, and the mucosa was scraped with RNase-free glass slides (Fisher). Total mucosal RNA was isolated by the cesium chloride extraction method as previously described (33), and RNA integrity was confirmed by 1% agarose formaldehyde gel electrophoresis. cDNA was prepared from 1 μg of total RNA, and RT-PCR reaction was performed at a final 1× PCR buffer containing 1 μM dNTPs, 1.2 μM each of 5’- and 3’-primers, 1.5 units Ampli Taq DNA polymerase, and 0.25 μl of α-[32P]dCTP (3,000 Ci/mmol; New England Nuclear) in a total volume of 50 μl using a Perkin-Elmer (Norwalk, CT) thermal cycler as previously described (8). The MIP-2 and glyceraldehyde 3-phosphate dehydrogenase primers and the conditions for the PCR reaction were as previously described (8). Ten microliters of the amplification reactions were analyzed directly on a 5% polyacrylamide gel (8). The MIP-2 and glyceraldehyde 3-phosphate dehydrogenase primers and the conditions for the PCR reaction were as previously described (8). Ten microliters of the amplification reactions were analyzed directly on a 5% polyacrylamide gel (8).
The mucosa was then homogenized (20 s) and centrifuged (11,000 g for 10 min), and the supernatants were filtered (4.5 μm filter; Gelman Sciences, Ann Arbor, MI). MIP-2 levels (pg/mg protein) were assayed by a commercially available immunoassay kit (Biosource, Camarillo, CA).

Statistical analyses. Results were expressed as means ± SE. Data were analyzed using the Sigma-Stat professional statistics software program (Jandel Scientific Software, San Rafael, CA). ANOVA with protected t-test was used for intergroup comparisons.

RESULTS

Plasma Corticosterone Levels are Increased During Toxin A-Mediated Enteritis

Plasma corticosterone levels were assessed at 0, 1, and 2 h after injection of purified toxin A in rat ileum. Previous studies indicated that fluid secretion and ileal permeability in response to toxin A were only evident 2 h after toxin administration (7, 37). As shown in Fig. 1, plasma corticosterone levels were increased by ~2-fold 1 h after toxin A injection, with no further increase after 2 h compared with the 0-h time point. Time 0 corticosterone levels were elevated to levels similar to those seen during stress (19). Similar corticosterone levels have been reported recently in fasted rats 16–18 h after food removal (12). Prolonged fasting of the animals in our studies was necessary for the subsequent experimental manipulations. No significant change of plasma corticosterone levels over the 2-h duration of the experiment was found in animals with buffer-injected loops (Fig. 1).

Administration of RU-486 Enhances the Enterotoxic Effects of Toxin A in Rat Ileum

To assess the importance of the increased levels of endogenous glucocorticoids in response to toxin A administration, we treated a group of animals with 1 mg/kg of the glucocorticoid receptor antagonist RU-486 30 min before administration of toxin A. Previous experiments showed that 1 mg/kg of RU-486 augmented inflammatory responses in rats in vivo (22). As has been previously reported (7, 8, 10), injection of 5 μg of toxin A into ileal loops of vehicle-treated rats caused increased fluid secretion (Fig. 2A), [3H]mannitol permeability (Fig. 2B), and MPO activity (Fig. 2C) in the luminal fluid compared with buffer-injected loops. Histological examination of tissues from loops exposed to toxin A showed severe epithelial cell necrosis with villi destruction and mucosal neutrophil infiltration (data not shown), as previously reported (7, 8, 10). Administration of RU-486 enhanced toxin A-induced intestinal secretion by 20% (P < 0.01, Fig. 2A), [3H]mannitol permeability (Fig. 2B), and MPO activity (Fig. 2C) in the luminal fluid compared with buffer-injected loops. RU-486 significantly enhanced toxin A-induced fluid secretion (A), [3H]mannitol permeability (B), and MPO activity (C). Data are means ± SE of 8–10 loops/group. *P < 0.05 and **P < 0.01 compared with toxin A alone.
where RU-486 was administered only one time 30 min before loop formation. RU-486 given two times, as described above, had no significant effect on basal fluid secretion levels in vehicle-injected animals (n = 4, data not shown).

Effect of Adrenalectomy and Glucocorticoid Replacement on Toxin A-Induced Ileal Responses

Exacerbation of toxin A-mediated intestinal responses by RU-486 administration (Fig. 2) suggested the importance of endogenous glucocorticoids for controlling the ileal responses elicited by toxin A. We next extended our studies in adrenalectomized rats, which, as shown in Fig. 3, A and B, have increased toxin A-induced fluid secretion and mucosal permeability by 26 and 48%, respectively, compared with the sham-operated animals (P < 0.01 for both). Fluid secretion and mucosal permeability were similar in ileal loops of sham-operated or adrenalectomized rats injected with buffer (Fig. 3). To evaluate the glucocorticoid level required to control toxin A-induced enterotoxicity, we implanted corticosterone-containing pellets (1, 2, and 4×, as described in MATERIALS AND METHODS) in adrenalectomized rats 5 days before administration of toxin A. As described above, this treatment maintains constant circulating corticosterone levels ranging from similar (1× pellet) to up to 4–6 times (4× pellet) the resting low physiological level (19). Our findings (Fig. 3, A and B) showed that adrenalectomized rats that were either not replaced or replaced with a low dose of corticosterone (1×) exhibited significantly increased intestinal responses to toxin A (Fig. 3, A and B). Administration of glucocorticoids at a level similar to that considered to be physiological replacement (approximate to the 2× replacement dose; see Ref. 2) resulted in an inflammatory response similar to that of the sham-operated rats (Fig. 3). Finally, high-dose corticosterone replacement (4×) resulted in a significant reduction of both toxin A-induced inflammatory markers, i.e., secretion (by 50%, P < 0.01) and mucosal permeability (by 60%, P < 0.01; Fig. 3, A and B). Thus the magnitude of toxin A-mediated enteritis in adrenalectomized rats was inversely correlated to the levels of corticosterone given via pellet replacement. Interestingly, adrenalectomized rats were more susceptible to the anti-inflammatory effects of glucocorticoids, as suggested from their response to exogenous glucocorticoid at levels lower than those achieved by inflamed sham-operated animals (Fig. 3). It might be possible that prolonged lack of circadian variation in corticosterone release in adrenalectomized, corticosterone-replaced rats may be one of the underlying reasons for this effect. Detailed studies on the effect of chronic glucocorticoid treatment on the inflammatory responses are needed to address this issue.

Glucocorticoids Inhibit Toxin A-Induced Synthesis of MIP-2 in the Ileal Mucosa

We next examined whether the anti-inflammatory effect of glucocorticoids in this model is exerted by regulating the expression of MIP-2, known to be increased by toxin A (8). We found that, 2 h after injection of toxin A in ileal loops, MIP-2 protein and mRNA levels were significantly increased in the mucosa compared with buffer-treated loops, whereas administration of dexamethasone (50 μg/kg) completely abolished the stimulation of MIP-2 gene and protein expression (Table 1 and Fig. 4). Previous studies have shown that similar doses of dexamethasone also reduced the inflammatory exudate in the air pouch model of carrageenin-induced inflammation in rats (22). Dexamethasone also inhibited toxin A-mediated intestinal fluid secretion (by 57%), mucosal mannitol permeability (by 66%), and histological damage (by 45%; n = 6, P < 0.01 in all parameters).
Data represent means ± SE of 6–8 experiments, each with duplicate determinations. Two hours after injection of either buffer (control) or toxin A (5 μg) in rat ileal loops, animals were killed and mucosa was scraped and homogenized. After centrifugation, macrophage inflammatory protein (MIP)-2 levels were measured by RIA and expressed as pg/mg protein. *P < 0.01 vs. control. †P < 0.01 vs. toxin A.

DISCUSSION

The main observation of this study is that activation of the HPA axis occurs early during C. difficile toxin A-mediated enteritis, leading to increased plasma glucocorticoids (Fig. 1), and that glucocorticoids are required to regulate the intestinal inflammatory response to toxin A. We show that administration of the glucocorticoid receptor antagonist RU-486 enhances the secretory and inflammatory effects of toxin A (Fig. 2) as previously shown in another model of inflammation (22), suggesting that endogenous glucocorticoids control the progress of toxin A-mediated enteritis. Furthermore, administration of RU-486 two times before fasting and 30 min before toxin A administration resulted in an additional increase of the toxin A-induced secretory response because of its blockade of the action of endogenous corticosteroids. In agreement with the above, adrenalectomy enhanced the secretory and inflammatory responses to toxin A, whereas glucocorticoid replacement of the adrenalectomized animals reversed this effect in a dose-dependent manner (Fig. 3).

Several studies have suggested that HPA axis activity is stimulated by inflammatory mediators (29, 39), which most likely stimulate secretion of corticotropin-releasing factor (39). Direct stimulation of adrenal glucocorticoid release by inflammatory mediators has also been suggested (3, 48). As we have shown in previous studies (8, 9, 45), toxin A, as soon as 30 min after its injection in rodent intestine, causes release of tumor necrosis factor-α, PGE₂, and MIP-2, which might stimulate glucocorticoid release. Our findings suggest that activation of the HPA axis occurs early during toxin A-mediated enteritis as shown by the increased plasma glucocorticoid levels (Fig. 1), most probably due to the stimulatory effect of the proinflammatory mediators on hypothalamus, pituitary, and/or adrenals (3, 39). The importance of endogenous glucocorticoid secretion during stress has been indicated in previous studies on adrenalectomized animals (31). Adrenalectomized mice also have increased lethality after lipopolyssacharide administration (50). Moreover, adrenalectomy enhances pancreatic inflammation in two different experimental models of acute pancreatitis, and hydrocortisone replacement reverses these effects (1). Similarly, endogenous glucocorticoids have been shown to suppress neutrophil recruitment in a model of cholestasis in rats (43) and apoptosis of pancreatic acinar cells during acute pancreatitis (25). Our current findings of increased toxin A-mediated intestinal secretion and inflammation in the absence of endogenous glucocorticoids due to adrenalectomy (Fig. 3) further support the importance of intact endogenous glucocorticoid secretion in regulating the intestinal inflammatory processes.

Smith et al. (41) showed that glucocorticoid deprivation achieved by adrenalectomy increased dorsal root ganglia content of the proinflammatory neuropeptide substance P (SP) and calcitonin gene-related peptide (CGRP). We have previously reported increased release of SP and CGRP from the cell bodies of the dorsal root ganglia shortly after toxin A administration in rat ileum (23, 33). Thus it is likely that glucocorticoids might regulate toxin A-induced secretion and inflammation by inhibiting SP and CGRP release after toxin A administration. Previous results also suggested that mast cells play a proinflammatory role in toxin A-mediated enteritis in rats (35) and mice (47) and that the mechanism of this response involves mast cell-neurone interactions (10). Other investigators also demonstrated that dexamethasone pretreatment in rats resulted in engulfment and destruction of intestinal mast cells (42) and that exposure of activated mast cells to dexamethasone in vitro reduced interleukin-4 production and intercellular adhesion molecule expression in these cells (49). Thus endogenous corticosteroids might exert their anti-inflammatory effects in the experiments described in the current study by inhibiting mast cell activation, known to occur during the course of toxin A-mediated enteritis.

Recent results indicate that the proinflammatory cytokine MIP-2 is an important early mediator in neutrophil recruitment and secretion and in inflammation mediated by toxin A in rat ileum (10). Glucocorticoids modulate the expression of several proinflammatory factors such as neuropeptides and cytokines. We have shown in this study that dexamethasone-treated rats have reduced toxin A-mediated intestinal secretion and mucosal permeability to mannitol and attenuated...
MIP-2 synthesis in the ileal mucosa (Table 1 and Fig. 4). Consistent with our observations, previous studies also reported that levels of MIP-2 in the lung during ozone damage could be reduced by dexamethasone administration (16). Although the pathway(s) by which glucocorticoids reduce toxin A-induced MIP-2 expression have not yet been clarified, it is possible to involve activation of nuclear factor-κB (NF-κB), a crucial transcriptional activator of proinflammatory cytokine genes (20, 30) shown to be downregulated by glucocorticoids (4, 40). Interestingly, in a preliminary report, we have recently shown that toxin A activates NF-κB in human monocytes in vitro, and this is followed by increased interleukin-8 production from these cells (46).

In summary, our results show that endogenous glucocorticoids modulate the secretory and inflammatory effects of C. difficile toxin A in rat ileum. Our findings also suggest that the anti-inflammatory effects of glucocorticoids in toxin A enteritis involve inhibition of toxin A-induced expression of proinflammatory cytokines such as MIP-2.

This work was supported by grants from the Colon’s and Colitis Foundation of America (C. Pothoulakis, K. Karalis, and I. Castagliuolo) and by National Institute of Diabetes and Digestive and Kidney Diseases grant DK-47343 (C. Pothoulakis).

REFERENCES


