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Clostridial toxins

Sensing a target in a hostile gut environment

Numan Oezguen,[1] Trevor D. Power,[2] Petri Urvil,[1] Hanping Feng,[3] Charalabos Pothoulakis,[4] Jonathan S. Stamler,[5] Werner Braun[2] and Tor C. Savidge[1,*]

[1]Department of Internal Medicine; University of Texas Medical Branch; Galveston, TX USA; [2]Department of Biochemistry & Molecular Biology; University of Texas Medical Branch; Galveston, TX USA; [3]Department of Microbial Pathogenesis; University of Maryland Dental School; Baltimore, MD USA; [4]Inflammatory Bowel Disease; Division of Digestive Diseases; University of California at Los Angeles; Los Angeles, CA USA; [5]Institute for Transformative Molecular Medicine; Department of Medicine; University Hospitals; Case Western Reserve University; Cleveland, OH USA

The current global outbreak of *Clostridium difficile* infection exemplifies the major public health threat posed by clostridial glucosylating toxins. In the western world, *C. difficile* infection is one of the most prolific causes of bacterial-induced diarrhea and potentially fatal colitis. Two pathogenic enterotoxins, TcdA and TcdB, cause the disease. Vancomycin and metronidazole remain readily available treatment options for *C. difficile* infection, but neither is fully effective as is evident by high clinical relapse and fatality rates. Thus, there is an urgent need to find an alternative therapy that preferentially targets the toxins and not the drug-resistant pathogen. Recently, we addressed these critical issues in a *Nature Medicine* letter, describing a novel host defense mechanism for subverting toxin virulence that we translated into prototypic allosteric therapy for *C. difficile* infection. In this addendum article, we provide a continued perspective of this antitoxin mechanism and consider the broader implications of therapeutic allostery in combating gut microbial pathogenesis.

Keywords: toxin, *C. difficile*, S-nitrosylation, allostery, inositol phosphate, dietary supplement

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*Correspondence to: Tor C. Savidge; Email: tcsavidg@utmb.edu

Cysteine proteases degrade polypeptides via a mechanism that normally involves a reactive cysteine thiol in a catalytic motif.[1] This prevalent enzyme class regulates many important cellular activities in gut host cells and their associated microflora.[1,2] It has been recently appreciated that several gut pathogens produce toxins that also depend upon a conserved cysteine protease for virulence.[3-6] The autocatalytic cysteine protease of these large, secreted bacterial proteins is required for intoxication of target host cells and includes the disease-inducing clostridium glucosylating toxins, as well as RTX, α-hemolysin, FrpC, and adenylate cyclase pore-forming toxins from *Vibrio cholerae*, *Escherichia coli, Neisseria meningiditides* and *Bordetella pertussis*, respectively.[3-6] Although not yet demonstrated for all of the above bacterial exoproteins, cellular intoxication by the clostridium glucosylating toxins and *Vibrio cholerae* RTX_{VC} toxin also depend on host-derived inositol phosphate cofactors.[7-14] These regulatory cofactors bind to an allosteric site on the toxin after its insertion into the plasma membrane, activating the autocatalytic cysteine protease to facilitate toxin self-cleavage (**Fig. 1**). A smaller toxin effector domain is then injected into the cytosol where it inactivates Rho GTPases in target cells.[5-7] The Rho family members act as molecular switches in a number of important cell signaling pathways associated with actin polymerization, inflammation and cell death.

The dramatic increase in severity of *C. difficile*-associated disease in North America and Europe over the last decade highlights the clinical prominence of *C. difficile's* glucosylating toxins, and is partially due to the spread of new epidemic-associated strains, for example BI/NAP1/027 that produce high amounts of these toxins.[15] Accompanying this surge in disease severity is a rise in recurrent clinical episodes in up to 35% of patients

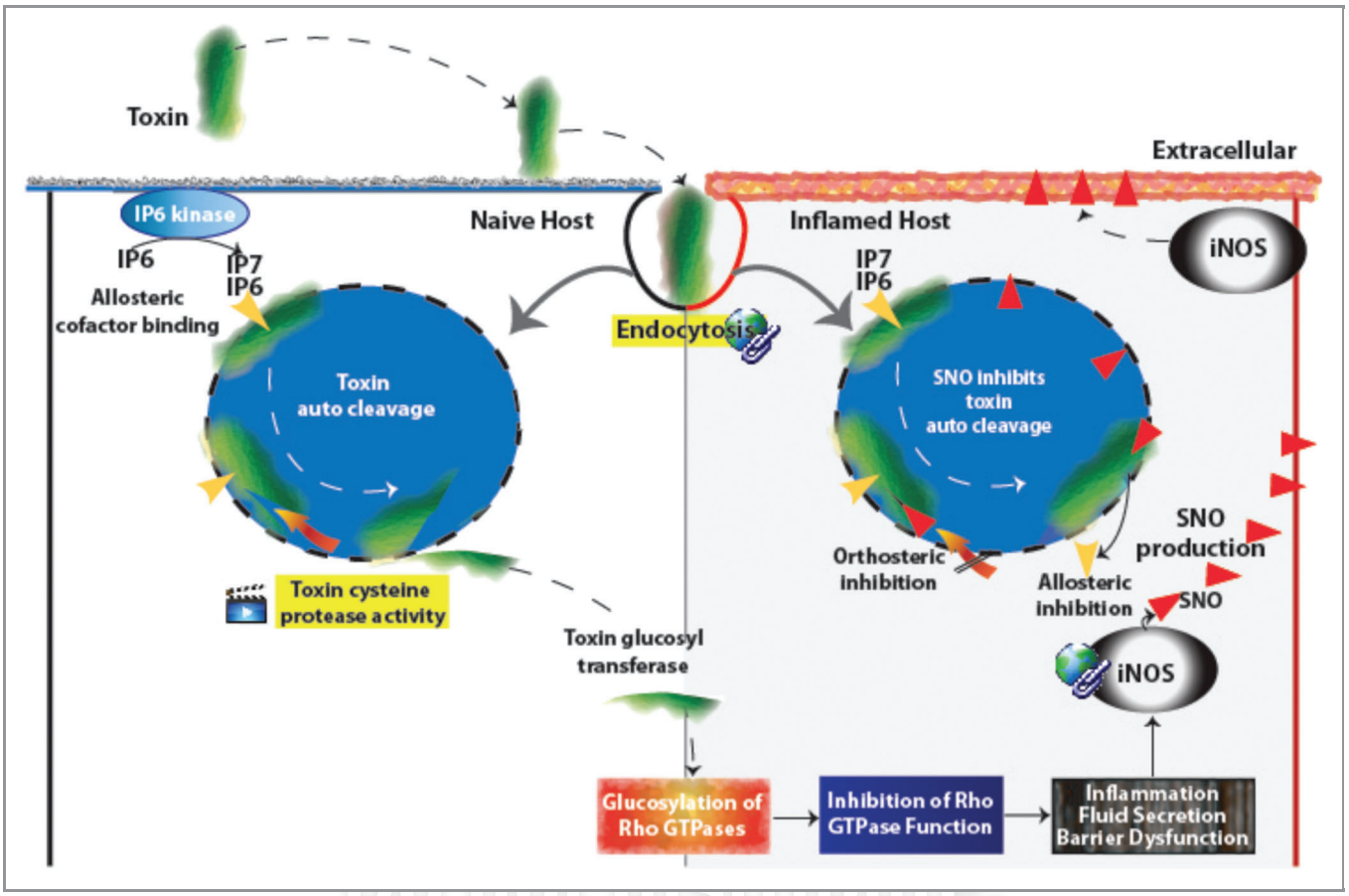

Figure 1. Summary figure with video links of the toxin allosteric mechanism.

with symptomatic *C. difficile* infection (CDI).[15] These unmet clinical issues represent a significant medical and financial challenge to health care systems, and have rekindled interest in improving therapy against this increasingly prevalent pathogen. Fidaxomicin has shown promise in reducing CDI relapse, but this new antibiotic appears less effective against the epidemic strain BI//NAP1/027.[16] Adjuvant antitoxin immunotherapy has also shown promise in preventing CDI relapse, but the economic costs are potentially high.[17] These issues highlight the complexity of CDI management, and emphasize the need to identify susceptible patients and alternative approaches to therapy.

A Novel Toxin Sensor for Evading Dietary Antitoxins

A majority of the *C. difficile* bacterial strains that cause disease in humans secrete two large toxins, TcdA (308 kDa) and TcdB (270 kDa). There is little ambiguity that these pathogenic toxins are the major cause of CDI since toxin-deficient clinical isolates are avirulent and may form a new line of clinical therapy by competing with pathogenic strains.[15] Microbial genetic manipulation studies have highlighted the disease-inducing potential of both toxins but implicate TcdB as the primary virulence factor in CDI,[18,19] supporting earlier unequivocal reports that TcdB is the major enterotoxin in the human colon.[20,21] This notion is supported by a recent clinical study reporting that antibodies against TcdB (but not TcdA) are associated with asymptomatic hospital acquired *C. difficile* colonization.[22] Nevertheless, novel antitoxin-based therapy should neutralize both toxins since each has the capacity to induce disease.

TcdA and TcdB are structurally similar, with functional domains that are now reasonably well defined.[5-7] The C-terminus receptor binding domain is involved in toxin attachment to the host cell membrane. The transmembrane and cysteine protease domains are involved in toxin entry into target cells, and the N-terminus is a catalytic glucosyltransferase domain. Interactions between the C-terminus binding domain and host cell receptors initiate receptor-mediated endocytosis (**Fig. 1**). Although the precise intracellular mode of action remains unclear, the toxins undergo a conformational change in the endosome, leading to membrane insertion. A cytosolic virulence cofactor, *myo*-inositol *hexakis*phosphate ($InsP_6$), is then required to trigger an allosteric structural change that activates the cysteine protease domain (CPD) to induce toxin self-cleavage and release of the glucosyltransferase domain into the cytosol (**Fig. 1**).[7-14] In this instance, allosteric regulation or allostery refers to a change in the shape and activity of the toxin cysteine protease that results from molecular binding with an inositol phosphate regulatory factor at a site other than the enzymatically active one (orthosteric site). Once in the cytosol, this effector domain

mono-O-glucosylates and inactivates small Rho GTPases, leading to alterations in the actin cytoskeleton, diarrhea, inflammation, and necrosis of the colonic mucosa.

Cysteine-dependent cleavage is a key regulatory mechanism for *C. difficile* virulence since it facilitates entry of the glucosyltransferase domain into target cell cytosol. Allosteric coupling by $InsP_6$ activates the toxin cysteine protease catalytic reaction to facilitate toxin self-cleavage. Specific inhibition of this cleavage reaction by mutagenesis or alkylation of the active site cysteine,[5,7,12,13] or by competitive peptide inhibition,[23] significantly attenuates cytotoxicity. Although irreversible chemical modifiers of cysteine thiol and peptide inhibitors of the cysteine protease active site are known to inhibit toxin virulence with great sensitivity, poor selectivity for microbial over human cysteine proteases remains a potential concern. Also, it is desirable to design non-peptide-based reversible inhibitors for oral therapy so as to minimize the potential toxicity that can be observed with irreversible inhibitors.

High resolution CPD crystal structures for TcdA and TcdB closely align with the CPD crystal structure for RTX_{VC} (**Fig. 2A**), and show a well-defined catalytic cleft that is structurally distinct from a positively charged $InsP_6$ binding pocket abutting a flexible β-hairpin fold (β-flap).[12,22,24] A potential deficiency of these TcdA and TcdB CPD crystal structures is the lack of their native N-terminus cleavage fragment that may significantly alter the ordering of the substrate cleft and catalytic triad. To gain further structural insight into the toxin allosteric mechanism, we used the coordinates of the CPD crystal structures for TcdA and TcdB, and the N-terminus from RTX_{VC}[11] to generate native structural models for TcdA and TcdB that included the uncut N-terminus substrate within the catalytic cleft. These toxin CPD homology models spanned CPD residues V534-S801 in TcdA and K535-T799 in TcdB (**Fig. 3**). Because it has not yet been possible to generate crystal structures of the native $InsP_6$ unbound toxin CPD configuration, we performed molecular dynamics (MD) structural simulations[25,26] to better understand the structural basis of the $InsP_6$ allosteric mechanism. These simulation models consistently demonstrated two distinct allosteric mechanisms that were highly dependent on whether the uncut N-terminus substrate is absent (short model simulation) or present (long model simulation) in the catalytic cleft. In the short model simulations (essentially

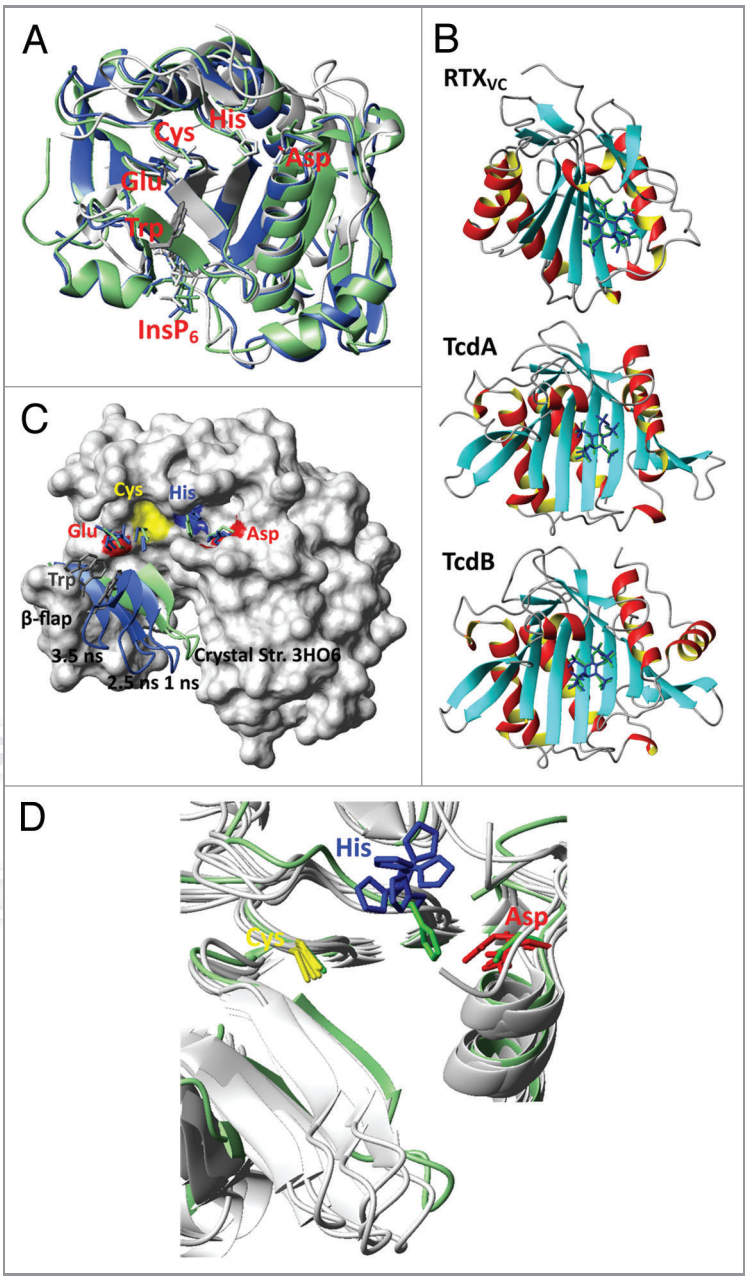

Figure 2. (A) The β-strand core region, the active site and the $InsP_6$ binding site together with the β-flap are conserved and align well for CPD's of RTX_{VC} (gray), TcdA (green) and TcdB (blue). Shown are the crystal structures with the PDB-codes 3EEB (RTX_{VC}), 3OH6 (TcdA) and 3PA8 (TcdB). They all have $InsP_6$ bound and the substrate is cleaved. Therefore, there are no peptides bound in the active site (we refer to these structures as short). In TcdB the shown residues represent: C698, H653, D587, E743 and W761. (B) Re-docking of $InsP_6$ to the crystal structures of RTX_{VC} (3EEB), TcdA (3HO6) and TcdB (3PA8) with Autodock (blue) shows excellent agreement with the original position of $InsP_6$ (green). (C) β-flap movement of the $InsP_6$ free TcdA CPD (short form 3HO6) during a MD-simulation. Shown residues are C700, H655, D589, E745 and W763. (D) Along with the β-flap the Asp (D589) and His (H655) in the catalytic triad move drastically during an $InsP_6$ free MD-simulation of TcdA CPD (3HO6). Different snapshots at 3.5, 4.0, 5.0, 5.5 and 7.0 ns are compared with the crystal structure positions (green).

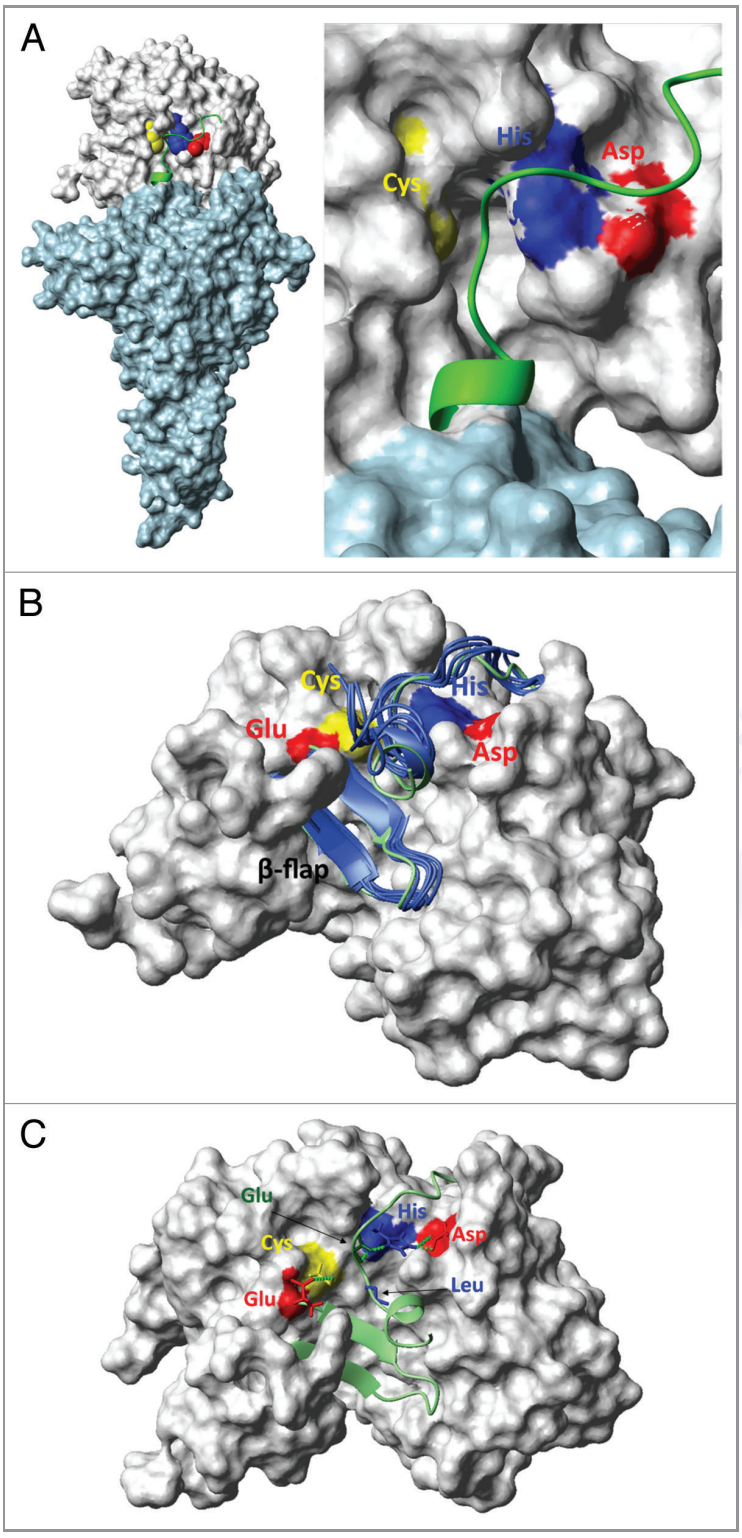

Figure 3. (A) Model of the TcdB glucosyltransferase (light blue) and cysteine protease domains (gray). Residues C698, H653 and D743 are shown in yellow, blue and red, respectively. (B) β-flap does not move during a MD-simulation of the $InsP_6$ free longer TcdB CPD (long form based on the crystal structures 3PA8 and 3FYZ). Starting conformation is shown in green and snapshots at 1, 2, 3, 4 and 5 ns are shown in blue. Residues E761 (red), C698(yellow), H653 (blue) and D587 (red) are highlighted at the surface. (C) Active site of the $InsP_6$ free longer TcdB CPD. Asparagine (D587 in red) and histidine (H653 in blue) stabilize the substrate via hydrogen bonds (dotted green). The cysteine (C698 in yellow) is inhibited by another hydrogen bond to the glutamic acid (E743 in red).

using CPD crystal structures lacking the N-terminus substrate), in silico docking of $InsP_6$ bound accurately to the allosteric pocket (**Fig. 2B**; **Supplemental Material**), and induced conformational changes that facilitated substrate access to the active site cysteine by leveraging the flexible β-flap away from catalytic cleft (**Fig. 2C**; **Vids. 1 and 3**). Furthermore, $InsP_6$ induced allostery may bolster cysteine thiol reactivity toward the substrate by promoting spatial proximity and alignment of catalytic residues (**Fig. 2D**). Nevertheless, even in the $InsP_6$ bound crystal configuration, the catalytic cysteine thiolate and the histidine imidazolium remain > 6Å apart, raising the question as to whether the toxin CPD functions as a conventional cysteine protease. In the related clan CD cysteine proteases, which includes the caspases, the histidine normally lies within a 5Å radius of its catalytic dyad partner to impart significant cysteine thiol nucleophilicity.[1,2] Thus, our short model simulations show that $InsP_6$ allostery promotes both accessibility and catalytic reactivity toward substrate, and in general this is in agreement with a series of elegant experiments that have recently defined the allosteric circuit in the short CPD domain of TcdB.[24]

In our *Nature Medicine* letter we propose an alternate mechanism for the $InsP_6$ induced allostery based on our CPD structural homology models that contain the N-terminus glucosyltransfease domain (**Fig. 3A**). It appears clear from these studies that the toxin catalytic dyad does not function via a conventional enzymatic mechanism. Our long model simulations show comparatively little evidence of leveraging of the flexible β-flap away from catalytic cleft as is evident in the short CPD simulations (**Fig. 3B**; **Vid. 2**). Furthermore, following conformational coupling by $InsP_6$, no interaction between the catalytic cysteine and histidine is evident because the N-terminus cleavage substrate is positioned between these dyad residues. Alternatively, the catalytic histidine appears to play a major role in guiding and orienting the cleavage substrate within the catalytic cleft (**Fig. 3C**). Our long models also show that hydrogen bonding exists between the catalytic cysteine and a juxtaposed glutamic acid

in the simulated $InsP_6$ unbound CPD structure, and this interaction likely inhibits the cysteine protease activity in the inactive state. Thus, conformational coupling by $InsP_6$ regulates CPD hydrogen bond interactions that on the one hand suppress catalytic cysteine thiol reactivity, while on the other align the N-terminus substrate within the catalytic cleft (**Fig. 3**; **Vids. 2 and 4**). Experimental site-directed mutagenesis studies using TcdB confirmed that toxin mutants lacking the catalytic cysteine (residue 698) or histidine (residue 653) become enzymatically dead, whereas toxin mutants lacking the juxtaposed glutamic acid (residue 743) exhibit greatly enhanced catalytic activity in response to allosteric coupling by $InsP_6$.[27] This toxin mutant is also prone to spontaneous autocatalytic cleavage, indicating that the regulatory glutamic acid governs the equilibrium between catalytically active and inactive states.

Because this cysteine protease catalytic motif is structurally conserved among microbial CPDs, we have proposed that the above structural features function as a general allosteric switch mechanism to prevent premature toxin self-cleavage by $InsP_6$ in the extracellular gut environment. Extracellular $InsP_6$ concentrations in blood and plasma are generally too low (< 1 nM) to facilitate toxin self-cleavage.[28] However, $InsP_6$ can reach much higher concentrations in the gut lumen from dietary sources (micromolar range),[29] where it may play a protective role in some patients by prematurely inactivating the toxin. Dietary $InsP_6$ (phytate or phytic acid) is the principle storage form of phosphorous in many plants, especially in bran and seeds.[29] It is also available as a dietary nutritional supplement. Thus, the Achilles heel of the *C. difficile* toxins may be their reliance on allosteric $InsP_6$ as a virulence sensor. Indeed, if this is the case, then dietary $InsP_6$ supplementation may confer clinical benefits to symptomatic CDI patients. This notion is supported by a recent metabolomics approach that we have used to demonstrate that dietary $InsP_6$ bioavailablity is markedly diminished in stool specimens from symptomatic CDI patients. As a result, we are currently in the process of initiating a clinical trial to test the efficay of dietary $InsP_6$ supplementation in symptomatic CDI patients (http://www.its.utmb.edu/mtts/clostridial_difficile_infection.html).

Host S-Nitrosylation: A Gut Defense Mechanism for Subverting Toxin Virulence

Other gut defense mechanisms that might be employed to protect against CDI are not well defined, although the toxins induce potent mucosal antibody and nitric oxide responses that may explain why this disease is self-limited in some patients. However, the precise protective mechanisms are only just being defined. Elevated nitric oxide production has been shown to induce anti-inflammatory transcription factor activity and inhibits leukocyte homing in murine toxigenic ileal loop models of CDI.[30,31] Using a metabolomics approach, we have confirmed in patient stool specimens that biochemical molecules associated with nitric oxide synthesis are significantly elevated in symptomatic CDI (**Fig. 4**). In our Nature Medicine letter we additionally report that foreign microbial protein, notably toxin itself, is subject to functional regulation by elevated nitric oxide production in the infected host.[27]

It is increasingly appreciated that many diverse signaling cascades associated with nitric oxide production are attributed to S-nitrosothiol species that act via covalent modification of cysteine thiol groups in target molecules (S-nitrosylation), and that aberrant S-nitrosylation plays a major role in disease-etiology.[32] Because cysteine residues are often key regulators of protein function, S-nitrosylation represents a physiologically important signaling mechanism that regulates virtually all known cellular signaling pathways. The emerging mechanism for regulation of protein function by S-nitrosylation is that it is governed by structural motifs that are targeted by nascent nitrosylases.[33] The recent discovery of specific nitrosylases that transduce nitric oxide action places S-nitrosylation firmly on the path of being the nitric oxide signaling equivalent of phosphorylation and ubiquitylation (regulated by protein kinases and ubiquitin E3 ligases, respectively).[33,34]

We have reported that S-nitrosylation may also function as a gut defense mechanism for subverting microbial pathogenesis in CDI.[27] Small peptide S-nitrosothiols (the most significant being S-nitrosoglutathione or GSNO) are endogenous inhibitors of *C. difficile* toxin action, acting in significant part by S-nitrosylation of the cysteine protease active site thiol. This active site cysteine forms part of a novel microbial S-nitrosylation-catalytic motif that co-serves as a regulator of $InsP_6$ induced toxin self-cleavage (**Fig. 3**). Physiological context is provided by showing that $InsP_6$ and inositol pyrophosphate ($InsP_7$) are specificity-determinants of toxin S-nitrosylation. Further, because plasma membrane-associated $InsP_7$ is the more cogent allosteric activator of the toxin cysteine protease, this phylogenically ancient family of inositol phosphates may constitute the preferred specificity-determinant for toxin virulence. Thus, GSNO attenuates the *C. difficile* toxins by a novel, dual orthosteric and allosteric mechanism of action: $InsP_6$ enables S-nitrosylation of the toxin cysteine protease active site, which then displaces the allosteric activator (**Fig. 1**). S-nitrosylation of the active site cysteine may itself alter the allosteric transition of the toxin cysteine protease by masking existing (or revealing new) binding sites, or by changing surface charge distributions in the catalytic cleft.

Perspectives: New Therapeutic Concepts for Gut Microbial Pathogenesis

Host S-nitrosylation is not a random event, but is most often governed by consensus motifs that encompass the cysteine residue targeted for posttranslational modification.[32,33] Protein S-nitrosylation is often also subject to regulation by host allosteric cofactors. The novelty of our work may be viewed in the broader context in which hypo- and hyper-nitrosylation of specific bacterial proteins represent disease-modifying events. We demonstrate that a structurally conserved microbial catalytic motif is targeted for inactivation by host nitrosylases, and that endogenous inositol phosphate cofactors act as specificity determinants in the S-nitrosylation

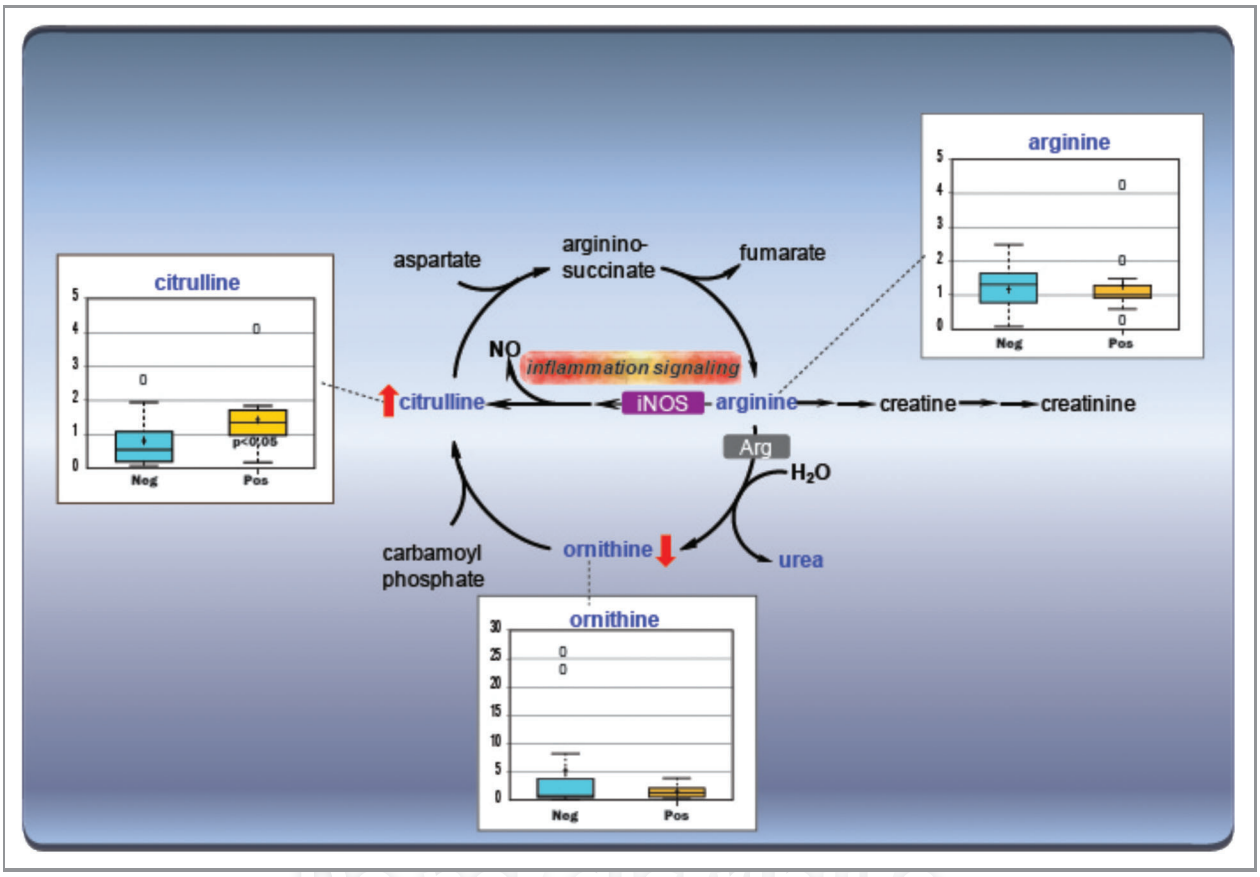

Figure 4. Arginine Metabolism. *C. difficile* is known to be the primary causative agent for pseudomembranous colitis and indicators of inflammation including significantly elevated levels of citrulline in stool of infected patients compared with non-infected patients with antibiotic-associated diarrhea.

action. A physiological correlate may be drawn from the structurally related caspase family of allosterically regulated cysteine proteases, as these may be maintained in a constitutively S-nitrosylated and inactive state in the inner mitochondrial membrane.[32] Our studies show that under certain pathophysiological conditions, the plasma membrane compartment may also facilitate privileged access of host nitrosylases and allosteric cofactors to exogenous microbial proteins in order to maintain these in an avirulent state. Because the structurally conserved bacterial catalytic-S-nitrosylation motif is found in abundantly diverse disease-associated cysteine proteases, we have proposed that host S-nitrosylation may play a universal role in subverting gut microbial pathogenesis.[27]

As a direct counter measure to evade host S-nitrosylation defenses, bacterial nitric oxide detoxification strategies appear to have evolved in several gut pathogens to inhibit cellular S-nitrosothiol formation.[35] Therapeutic strategies that elevate S-nitrosothiol bioavailability may therefore enhance the clearance of certain gut bacterial infections. GSNO is well tolerated in humans, and is already known to be a multifaceted protective agent that exhibits broad-spectrum anti-microbial activity.[32] Studies by our group and other investigators have demonstrated that exogenous GSNO provides potent disease-attenuating signals in the gastrointestinal tract.[36-38] A primary goal in nitric oxide therapeutics is to identify the nitrosylation state of proteins that are identified with pathophysiology, and to selectively and specifically control this modification. We have shown that GSNO can function as the physiological corollary of therapeutic inhibitors currently being developed to treat CDI. Furthermore, allosteric regulation of S-nitrosylation by inositol phosphate cofactors (which has not been previously demonstrated), suggests new therapeutic approaches to regulate the S-nitrosylation state of specific disease-related targets. Therapeutic context for the principle that allosteric modulation of S-nitrosylation can be employed to treat CDI is demonstrated by the efficacy of exogenous GSNO and $InsP_6$–separately or in a combined form — in treatment of experimental CDI.

Acknowledgments

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Supplemental Material

Supplemental material and Videos 1–4 can be downloaded here:
www.landesbioscience.com/journals/gutmicrobes/article/19250

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