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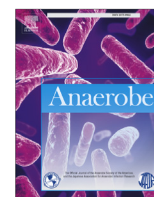

New insights into *Clostridium perfringens* epsilon toxin activation and action on the brain during enterotoxemia

John C. Freedman [a], Bruce A. McClane [a], Francisco A. Uzal [b,*]

[a] *Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA*
[b] *California Animal Health and Food Safety Laboratory, San Bernardino Branch, School of Veterinary Medicine, University of California-Davis, San Bernardino, CA, USA*

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ABSTRACT

Epsilon toxin (ETX), produced by *Clostridium perfringens* types B and D, is responsible for diseases that occur mostly in ruminants. ETX is produced in the form of an inactive prototoxin that becomes proteolytically-activated by several proteases. A recent *ex vivo* study using caprine intestinal contents demonstrated that ETX prototoxin is processed in a step-wise fashion into a stable, active ~27 kDa band on SDS-PAGE. When characterized further by mass spectrometry, the stable ~27 kDa band was shown to contain three ETX species with varying C-terminal residues; each of these ETX species is cytotoxic. This study also demonstrated that, in addition to trypsin and chymotrypsin, proteases such as carboxypeptidases are involved in processing ETX prototoxin. Once absorbed, activated ETX species travel to several internal organs, including the brain, where this toxin acts on the vasculature to cross the blood-brain barrier, produces perivascular edema and affects several types of brain cells including neurons, astrocytes, and oligodendrocytes. In addition to perivascular edema, affected animals show edema within the vascular walls. This edema separates the astrocytic end-feet from affected blood vessels, causing hypoxia of nervous system tissue. Astrocytes of rats and sheep affected by ETX show overexpression of aquaporin-4, a membrane channel protein that is believed to help remove water from affected perivascular spaces in an attempt to resolve the perivascular edema. Amyloid precursor protein, an early astrocyte damage indicator, is also observed in the brains of affected sheep. These results show that ETX activation *in vivo* seems to be more complex than previously thought and this toxin acts on the brain, affecting vascular permeability, but also damaging neurons and other cells.

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1. Introduction

Epsilon toxin (ETX), produced by *Clostridium perfringens* types B and D, is one of the most potent and lethal toxins known [26,32]. The cellular action of ETX begins with binding of the toxin to its receptor, which has not yet been identified. Bound ETX then oligomerizes in a caveolin-1 and -2 dependent manner to form a heptameric prepore [5,26,27]. A recent study by Rumah et al. [29]; demonstrated that myelin and lymphocyte protein (MAL) is also required for the binding and activity of ETX. After oligomerization, the pore-forming domain of the toxin inserts into the membrane of host cells, causing an early loss of intracellular K^+ and an increase in Cl^- and Na^+, with Ca^{++} influx occurring later [25]. Influx of propidium iodide correlates with loss of viability due to pore formation [25].

ETX seems to cause rapid cell death by necrosis, with pyknosis but no DNA fragmentation [25]. While ETX-induced necrosis is not yet completely understood, it involves ATP depletion, AMP-activated protein kinase stimulation, mitochondrial membrane permeabilization and mitochondrial-nuclear translocation of a potent caspase-independent cell death factor named apoptosis inducing factor. The rapid ETX-induced efflux of K^+ is considered the key early event leading to cell necrosis [16]. Additional studies have indicated that ETX stimulates the release of glutamate by targeting glutamatergic neurons [18,21]. Finally, it was recently shown that ETX cytotoxicity activity *in vitro* correlates to its activity *in vivo*, and specifically, the lethal effects of ETX correspond to its ability to cross the blood-brain barrier and exert cytotoxic activity in the kidney [4].

* Corresponding author. California Animal Health and Food Safety Laboratory, San Bernardino Branch, School of Veterinary Medicine, University of California-Davis, 105 W Central Ave, San Bernardino, CA 92408, USA.
E-mail address: fuzal@cahfs.ucdavis.edu (F.A. Uzal).

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ETX producing *C. perfringens* types B and D strains are important for veterinary medicine because they cause highly lethal diseases in ruminants and, occasionally, other animal species [26,34,35]. During enteric infections, ETX is produced as a relatively inactive prototoxin in the small intestine, which (as discussed later) is then activated by several proteases present in the intestinal environment. Once activated, ETX is believed to facilitate its own absorption across the intestinal mucosa and into the systemic circulation, where it reaches several organs, including (but probably not limited to) the brain, lungs and kidneys [14]. In these organs, ETX produces damage by several different mechanisms.

A major effect of ETX in animals is the production of severe neurological alterations [12,13,34,35]. The role of ETX in neurologic disease of ruminants was initially investigated by inoculation of this toxin into several animal species including sheep, goats, cattle, mice and rats [2–4,7–9,30,36,37]. In addition, i.v. administration of ETX prototoxin to rodents prevented or reduced the effects of proteolytically activated ETX [23], suggesting that this prototoxin blocked receptor sites for ETX in the central nervous system [23]. However, final confirmation of a key role for this toxin in the pathogenesis of type D disease was recently obtained using reverse genetic experiments in sheep, goats and mice [12]. These experiments showed that ETX is necessary for type D isolates to induce disease in those animal species [12].

Over the past few years several studies on the mechanism of activation of ETX in the intestine and the pathogenesis of the ETX-induced brain lesions came to light. We therefore review those recent studies here.

2. *Ex vivo* studies of ETX activation

As was discussed earlier, ETX is produced and secreted in the intestine as a prototoxin of 33.05 kDa [11,15,20] that requires activation by intestinal proteases to exert its cytotoxic activity in several organs [40]. Cleavage by purified trypsin, a serine protease, removes the 13 N-terminal amino acids, as well as the 23 C-terminal amino acids, from the ETX prototoxin [15,20,22]. Additionally, cleavage by purified chymotrypsin or lambda protease (produced by some type B and D strains of *C. perfringens*) removes the 29 C-terminal amino acids from the prototoxin, also resulting in activation [20]. Removal of the C-terminus from the prototoxin is the critical step for ETX activation, whereas removal of N-terminal prototoxin amino acids is dispensable for ETX activity [20,22].

Since only purified trypsin, chymotrypsin, and lambda protease of *C. perfringens* had been used to study ETX activation [20,22,40,41], a recent study used goat small intestinal contents to study the activation of ETX *ex vivo* [11]. This work was important because: types B and D strains of *C. perfringens* produce ETX in the lumen of the small intestine in infected goats and sheep [34,35], ETX causes intestinal damage in infected goats [34,35], and the small intestinal environment contains proteases other than trypsin and chymotrypsin [1].

In this *ex vivo* study, purified ETX prototoxin was treated with small intestinal contents from a healthy adult goat for up to 90 min, with samples taken for SDS-PAGE and Western blotting at various time points. This analysis showed that ETX is activated in a step-wise fashion (Fig. 1A), which was not previously appreciated because previous studies had only assessed ETX activation by purified proteases at a fixed time point [11]. This step-wise proteolytic processing of ETX by intestinal contents generated an ETX band of ~27 kDa that was stable for up 120 min in the goat intestinal contents. While initial activation of ETX cytotoxicity by intestinal contents begins at 5 min, the appearance of the ~27 kDa ETX band (at ~30 min) coincides with a small additional increase in cytotoxic activity [11].

The ~27 kDa ETX band was purified and characterized by N-terminal sequencing and mass spectroscopy to determine its precise molecular weight. Interestingly, mass spectroscopy detected the presence of three ETX species, of 27,688.0, 27,801.4, and 27,900 Da, which corresponded to the cleavage of ETX at amino acids N262, N263, and V264, respectively. Note, for reference, that proteolysis of ETX by purified trypsin results in cleavage of ETX at amino acid K273, while digestion by purified chymotrypsin results in cleavage at amino acid Y267 [20]. Thus, the more complex pattern of ETX cleavage observed using caprine small intestinal contents is consistent with further C-terminal truncation beyond the initial C-terminal cleavage caused by trypsin and/or chymotrypsin. This additional trimming of ETX C-terminal sequences is apparently caused by intestinal carboxypeptidases, based upon inhibitor studies (Fig. 1B) [11]. Finally, using recombinant ETX fragments corresponding to the three ETX species generated by caprine small intestinal contents, it was demonstrated that each of these ETX fragments has cytotoxic activity on MDCK cells. Taken together, these *ex vivo* studies of ETX activation strongly suggest that ETX activation *in vivo* is more complex than had been previously appreciated [11].

3. ETX action on the brain

The effect of ETX on the brain and other organs has been studied with multiple animal models using mostly intravenous injection of this toxin and intraduodenal inoculation of *C. perfringens* type D or ETX [2,4,7–9,12,13,36,37]. Those experiments indicate that ETX reaches the brain very soon after it is absorbed in the gut or injected intravenously. Current evidence suggests that most ETX absorption occurs in the small intestine, with ETX then reaching the brain within seconds of absorption [14,19,30].

The first effect of ETX on the brain is the alteration of the brain-blood barrier, which begins with damage to the vasculature, in which endothelial cells show swelling, vacuolation and necrosis. This leads to fluid and protein leakage which produces the proteinaceous perivascular edema characteristic of the disease (Fig. 2) [32,34]. If the animals survive long enough, the edema and associated hypoxia lead to nervous parenchymal necrosis, a lesion grossly known as focal symmetrical encephalomalacia, which is a hallmark of sub-acute and chronic type D disease in sheep [34]. In mice inoculated intravenously with ETX prototoxin or ETX labeled with Green-Fluorescent-Protein, both compounds can be detected bound to the luminal surface of the vascular endothelium [31], suggesting that binding to endothelial epithelium is one of the first steps in the action of ETX on the brain.

The development of nervous tissue damage in ETX-intoxicated animals occurs in a dose- and time-dependent manner [32,33], i.e. i) high doses of ETX cause a wider distribution of brain lesions, while lower doses of this toxin produce smaller, well-circumscribed necrotic foci [32,33], and ii) as the survival time increases, so does the distribution of brain lesions [32,33]. For reasons not yet fully understood, these lesions tend to occur in specific areas of the brain, more prominently in the white matter, which is the site of predilection for vasogenic edema [13]. It has been hypothesized that this white matter preference is the consequence of the lower cell density and wider extracellular spaces in white matter, which would allow for greater and faster fluid accumulation and spread of edema [13].

Although it was always assumed that the hallmark of vascular brain damage in ETX-intoxicated animals is perivascular proteinaceous edema [7,8,34], a recent study in sheep inoculated with cultures of *C. perfringens* type D and its isogenic ETX null mutant [13] showed that ETX-induced edema within the vascular walls of the brain also occurs. This study also demonstrated that the

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