Chapter 11

Role of Tyrosine Kinase A Receptor (TrkA) on Pathogenicity of *Clostridium perfringens* Alpha-Toxin

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1. Introduction

Clostridium perfringens (*C. perfringens*) is a toxin-producing anaerobic Gram-positive bacterium, which is well known for its role in human tissue infections and food poisoning. It is readily isolated from soil and a component of normal human intestinal and vaginal flora in many individuals. Apart from the classic clostridial myonecrosis of gas gangrene, *C. perfringens* can be responsible for a range of other clinical scenarios including sepsis, aspiration pneumonia, brain abscess, and enteritis necroticans. The potent exotoxins produced by various strains of *C. perfringens* are central to their effectiveness as pathogens, and include four major toxins used in strain classification: a phospholipase C (alpha-toxin, PLC), two pore-forming toxins (beta and epsilon toxins); and an ADP-ribosylation toxin (iota toxin). *C. perfringens* gas gangrene is one of the most fulminant necrotizing infections affecting humans. The infection can become well established in traumatized tissues in as little as 6-8 h and the destruction of adjacent healthy muscle can progress several inches per hour despite appropriate antibiotic coverage. Shock and organ failure occur in 50% of patients, and 40% of these individuals die. Even with modern medical advances and intensive care regimens, the centuries-old practice of radical amputation on an emergent basis remains the single best treatment. Histologically, this infection is characterized by widespread destruction of muscle and the absence of polymorphonuclear leukocytes at the site of infection. Instead, leukocytes accumulate within adjacent vessels.

C. perfringens alpha-toxin is the major virulence factor in gas gangrene with inflammatory myopathies (Williamson and Titball 1993, Awad et al. 1995). The toxin, which exhibits phospholipase C (PLC) and sphingomyelinase activities, causes hemolysis, necrosis, and death, and the activation of neutrophils and release of cytokines (Sakurai, Nagahama and Oda 2004). Bryant reported that the intramuscular injection of alpha-toxin caused a rapid

and irreversible decline in skeletal muscle blood flow due to toxin-induced intravascular aggregates of plates, leukocytes and fibrin (Bryant et al. 2000a, Bryant et al. 2000b). Neutrophils in these aggregates often bordered the endothelium but all remained intravascular (Bryant et al. 2000a). These findings suggested that the large heterotypic aggregates of platelets and leukocytes generated by alpha-toxin also contributed to impairment of the tissue inflammatory response. We have reported that alpha-toxin-induced activation of endogenous PLC and sphingomyelinase via a pertussis toxin (PT)-sensitive GTP-binding protein (Gi) plays an important role in the hemolysis of rabbit and sheep erythrocytes, respectively (Ochi et al. 1996, Ochi et al. 2004, Oda et al. 2008).

Recently, we revealed that the tyrosine kinase A (TrkA) receptor plays an important role in the release of superoxides and cytokines (Oda et al. 2006, Oda et al. 2008). This review will present findings about the signal transduction via TrkA receptor induced by alpha-toxin and summarize information about its likely role in inflammatory disease, especially septic shock.

2. Role of TrkA on a inflammation induced by alpha-toxin

2.1. Signal transduction via TrkA receptor

The TrkA receptor is a 140-kDa transmembrane protein encoded by a proto-oncogene located on chromosome 1 (Martin-Zanca, Hughes and Barbacid 1986). The family of Trk receptor tyrosine kinases consists of TrkA, TrkB and TrkC. While these family members have highly conserved sequences, they are activated by different neurotrophins: TrkA by nerve growth factor (NGF), TrkB by Brain-derived neurotrophic factor (BDNF) or neurotrophin 4 (NT4), and TrkC by NT3. TrkA regulates proliferation and is important for development and maturation of the nervous system (Pierotti and Greco 2006). This receptor comprises a tyrosine-kinase domain in its intra-cytoplasmic region and five extracellular domains, including two immunoglobulin-like domains involved in NGF binding and responsible for the specific selectivity to bind NGF (Wiesmann et al. 1999). In humans, the TrkA receptor is expressed on cells throughout the nervous system (Muragaki et al. 1995) as well as on structural cells and other non-neuronal cells in the immune and neuroendocrine systems (Levi-Montalcini et al. 1995, Aloe et al. 1997, Bonini et al. 2002, Levi-Montalcini 1987). When NGF binds to the TrkA receptor, it induces receptor homodimerization, which initiates kinase activation and transphosphorylation (Kaplan et al. 1991). This kinase activation involves small G proteins (Ras, Rac, Rap-1), PLCγ, protein kinase C (PKC) and phosphatidylinositol-3 kinase (PI3K) in neural cells (Obermeier et al. 1993b, Obermeier et al. 1993a, Melamed et al. 1999, York et al. 2000, Wu, Lai and Mobley 2001). Phosphorylation at Tyr490 is required for association with Shc and activation of the Ras-MAP kinase cascade. Residues Tyr674/675 lie within the catalytic domain, and phosphorylation at this site reflects TrkA kinase activity (Segal and Greenberg 1996, Stephens et al. 1994, Obermeier et al. 1993a, Obermeier et al. 1993b, Yao and Cooper 1995). Point mutations, deletions and chromosomal rearrangements (chimeras) cause ligand-independent receptor dimerization and activation of TrkA.

The mitogen-activated protein kinase (MAPK) pathways are activated next: extracellular-regulated protein kinase (ERK) by the small G proteins; ERK, p38 and JUN-N-terminal kinase (JNK) MAPK by PKC; and p38 and JNK by PI3K (Kaplan and Miller 1997). PI3K in turn induces activation of protein kinase B (PKB or Akt) and PKCξ (York et al. 2000)(Fig. 1).

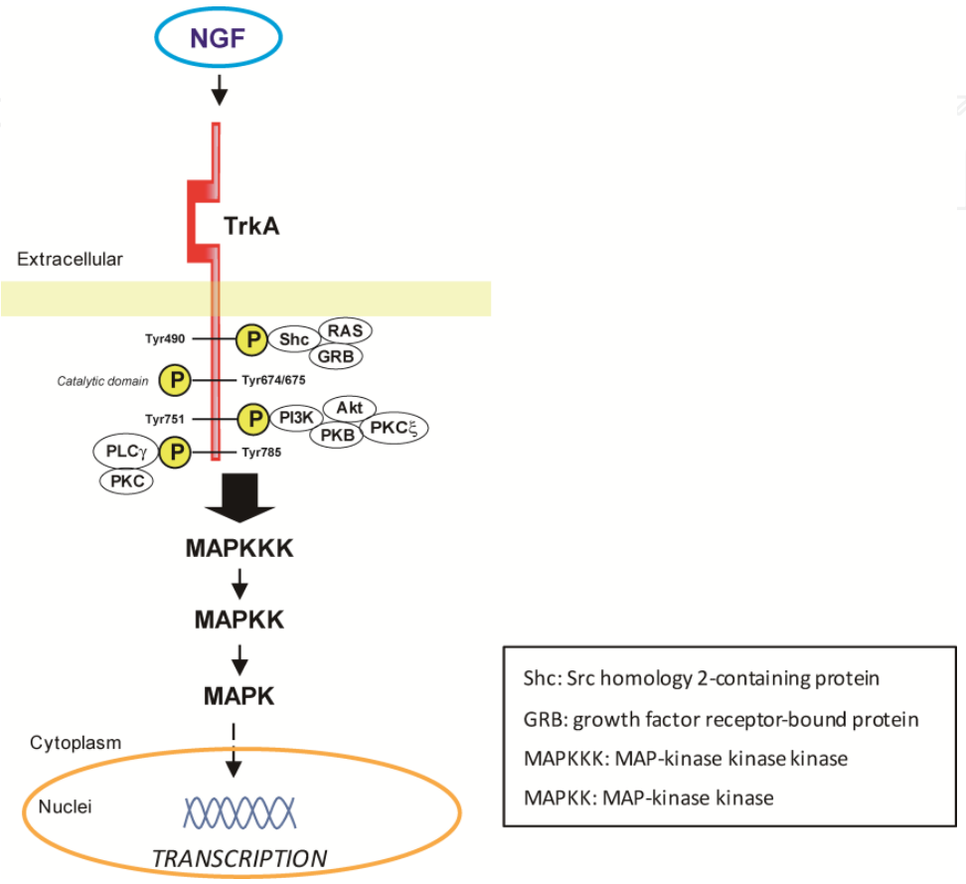

Figure 1. Signal transduction pathways of the TrkA receptor

2.2. Mechanism for the superoxide generation induced by alpha-toxin

The generation of superoxide in neutrophils has been reported to be stimulated by zymosan, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), Ca^{2+} ionophores, and bacterial chemotatic peptides (Babior 1999). The signal transduction process leading to the stimulation has been studied extensively using *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (Kusunoki et al. 1992), platelet-activating factor (Yasaka, Boxer and Baehner 1982), and TPA (Nick et al. 1997, Pongracz and Lord 1998). It has been reported that these stimuli activated MAPK or PI3K in neutrophils (Shenoy, Gleich and Thomas 2003, Yamamori et al. 2004). Furthermore, these studies have demonstrated that the interaction of the ligands with receptors on neutrophils activates endogenous PLC with the formation of diacylglycerol (DG), which activates PKC, and inositol 1, 4, 5-trisphosphate (IP_3), inducing the release of Ca^{2+} from the endoreticulum, and that these products act synergistically to generate superoxide. Several studies also reported that phosphorylation of tyrosine kinases and activation of phospholipase D (PLD) were closely related to the generation of superoxide in neutrophils stimulated with agonists

(Garland 1992, Mitsuyama, Takeshige and Minakami 1993) and that activation of PLD resulted in the formation of PA, which was linked to the activation of NADPH oxidase (Bellavite et al. 1988, Olson, Tyagi and Lambeth 1990). We revealed that alpha-toxin-induced generation of superoxide is closely related to the activation of endogenous PKCθ via a combination of two events: production of DG on activation of PLC through a PT-sensitive GTP-binding protein and activation of phosphatidylinositide kinase 1 (PDK1) through the TrkA receptor (Oda et al. 2006).

There are three classes of PKC isotypes: classical PKC isotypes (PKCα, -β, and -γ) which have a C1 and C2 domain, bind DG, 1-oleoyl-2-acetyl-3-phosphoglycerol (OAG) and TPA, and are regulated by DG and Ca^{2+}; novel PKC isotypes (PKCδ, -ε, -η, and -θ), which have a C1 domain and novel C2 domain and are regulated by DG but not Ca^{2+}; and atypical isotypes (ζ/λ), which do not bind DG and are not regulated by these classical ligands (Le Good et al. 1998). Alpha-toxin induced phosphorylation of PKCθ and PKCζ/λ, and the generation of superoxide induced by the toxin was inhibited by rottlerin and calphostin C, an inhibitor of PKCθ. We reported that the formation of DG induced by alpha-toxin in rabbit neutrophils plays an important role in the generation of superoxide (Ochi et al. 2002). It therefore appears that the toxin-induced generation of superoxide is dependent on the activation of PKCθ, through binding of PKCθ phosphorylated by PDK1 to DG (Parekh, Ziegler and Parker 2000, Toker and Newton 2000). PKCθ has been reported to play an important role in activation of the protein 1 and NF-κB signaling pathway in T cells, production of interleukin-2, and apoptosis (Altman, Isakov and Baier 2000, Fan et al. 2004, Villalba et al. 1999, Villunger et al. 1999). Our data may provide clues to the role of PKCθ in neutrophils.

We reported that the alpha-toxin-stimulated generation of superoxide was related to the formation of DG through activation of endogenous PLC by a PT-sensitive GTP-binding protein in rabbit neutrophils (Ochi et al. 2002). U73122, an inhibitor of endogenous PLC, blocked the toxin-induced generation of superoxide and formation of DG in the cells, supporting that the toxin-induced increase in superoxide is dependent on the formation of DG by endogenous PLC. However, when the level of OAG incorporated into the cells was the same as the level of DG in the cells treated with 25 nM of the toxin, the level of OAG did not induce superoxide generation in the absence of the toxin but did in the presence of a near threshold dose (2.5 nM) of the toxin which did not induce production of DG. The result shows that the toxin-induced production of superoxide requires not only the formation of DG, but also the activation of other events.

It has been reported that the PI3K signaling pathway has an important role in several effector functions including the generation of superoxide (Yamamori et al. 2004). PI3K is known to generate phosphatidylinositol 3, 4, 5-trisphosphate (PIP_3), which is recognized by a pleckstrin homology domain identified as a specialized lipid-binding module (Le Good et al. 1998). Several papers have reported that PDK1 requires PIP_3 as its activator for effective catalytic activity (Le Good et al. 1998). Le Good et al. reported that there is a cascade involving PI3K, PDK1, and various members of the PKC superfamily in signal transduction (Le Good et al. 1998). Furthermore, the function of PKC family members is reported to

depend on the phosphorylation of an activation loop by PDK1 (Le Good et al. 1998). LY294002 and wortmannin, both PI3K inhibitors, inhibited alpha-toxin-induced generation of superoxide and phosphorylation of PDK1 but did not affect the toxin-induced formation of DG. The result shows that the toxin-induced activation of PI3K occurs upstream of the phosphorylation of PDK1, which is an important step in the toxin-induced generation of superoxide. It is likely that the toxin-induced phosphorylation of PDK1 is a process independent of the toxin-induced formation of DG.

Tyrosine phosphorylation is thought to be crucial to the regulation of effector functions in neutrophils (Rollet et al. 1994). It is known that stimuli that induce tyrosine kinase activity in cells evoke the generation of PIP_1, PIP_2, and PIP_3. This tyrosine kinase activity is linked to the NGF receptors with intrinsic tyrosine kinase activity. Kannan et al. reported that NGF enhances the generation of superoxide induced by TPA in murine neutrophils (Kannan et al. 1991). Ehrhard et al. reported that human monocytes express the *trk* proto-oncogene, encoding the signal-transducing receptor unit for NGF, and that the interaction of NGF with monocytes triggers respiratory burst activity (Ehrhard et al. 1993). NGF, which did not induce the generation of superoxide in rabbit neutrophils, potentiated the events triggered by the toxin and caused superoxide to form in the presence of OAG, suggesting that a combination of the production of DG and stimulation of the NGF receptor induces severe activity in the generation of superoxide. The TrkA receptor was detected in rabbit neutrophils and found to be phosphorylated when the cells were treated with the toxin. Furthermore, immunoprecipitation using the anti-TrkA receptor antibody revealed direct binding of the toxin to the TrkA receptor. In addition, the antibody inhibited the toxin-induced generation of superoxide. These observations indicate that the interaction of alpha-toxin with TrkA receptors is important to the production of superoxide. In rabbit neutrophils, K252a, a TrkA inhibitor, and LY294002 inhibited the toxin-induced generation of superoxide and phosphorylation of PDK1 within specific concentration ranges, but PP2, a Src inhibitor, and AG1478, a epidermal growth factor receptor inhibitor, did not, supporting the finding that the TrkA receptor is involved in the toxin-induced increase in superoxide. The results obtained with the anti-TrkA antibody, LY294002, and K252a show that the activation of PI3K through direct binding of the toxin to the TrkA receptor results in production of PIP_3, which activates PDK1. In addition, PT inhibited the alpha-toxin-induced generation of superoxide and formation of DG, but not phosphorylation of PDK1, suggesting that a PT-sensitive GTP-binding protein plays a crucial role in the coupling to endogenous PLC, but not phosphorylation of PDK1. These observations indicate that the toxin independently induces activation of both endogenous PLC via a PT-sensitive GTP-binding protein and PDK1 via the TrkA receptor.

NGF, which binds to the TrkA receptor, is reported to be required for the differentiation and survival of sympathetic and some sensory and cholinergic neuronal populations (Howe et al. 2001). Furthermore, it has been reported that NGF is involved in inflammatory responses, an increase in mast cells in neonatal rats (Woolf et al. 1996), the degranulation of rat peritoneal mast cells (Woolf et al. 1996), and the differentiation of specific granulocytes (Kannan et al. 1991). The injection of *C. perfringens* cells or alpha-toxin into tissues is known

to cause inflammation. Therefore, it is possible that the activation of the TrkA receptor by alpha-toxin is related to inflammation caused by *C. perfringens* in humans and animals.

H148G induced phosphorylation of PKCθ, but not production of DG, suggesting that the enzymatic activity of the toxin is essential for activation of endogenous PLC, but not activation of the TrkA receptor. It has been reported that binding of the C-domain, which does not contain the enzymatic site, to erythrocytes is important for the hemolysis induced by the toxin (Nagahama et al. 2002). It therefore is possible that the C-domain, the binding domain of alpha-toxin, plays a role in the binding of the toxin to the TrkA receptor and in the activation of signal transduction via the TrkA receptor.

Several studies have reported that the activation of PKC by various stimuli results in the generation of superoxide via the activation of MAPK systems (Coxon et al. 2003, Dewas et al. 2000, McLeish et al. 1998, Zu et al. 1998). K252a and U73122 inhibited the toxin-induced phosphorylation of PKCθ and ERK1/2 and generation of superoxide, suggesting that the toxin-induced production of superoxide is linked to the stimulation of the MAPK system via the activation of PKCθ. The toxin causes phosphorylation of ERK1/2, but not p38 and SAPK/JNK, implying that the process is dependent on a MAPK system containing MEK1/2 and MAPK/ERK1/2, but not systems containing p38 and SAPK/JNK.

It has been reported that PA directly or indirectly activated NADPH oxidase in a cell-free system of neutrophils (Erickson et al. 1999) and that PKCδ regulates phosphorylation of $p67^{phox}$ in human monocytes (Zhao et al. 2005). PKC also has been reported to activate directly NADPH oxidase (Johnson et al. 1998). However, PD98059 almost completely inhibited the toxin-induced production of superoxide near the inhibitory threshold dose of the inhibitor. Thus, it is unlikely that PA and PKC directly activate NADPH oxidase under the conditions used here.

We have shown that alpha-toxin induces formation of DG through the activation of endogenous PLC by a PT-sensitive GTP-binding protein and phosphorylation of PDK1 via stimulation of the TrkA receptor, so that DG and PDK1 synergistically activate PKCθ, and that the activation of PKCθ stimulates generation of superoxide through MAPK-associated signaling events in rabbit neutrophils (Fig. 2).

2.3. Mechanism for the cytokine release induced by alpha-toxin

Cytokines are immunoregulatory peptides with a potent inflammatory action, mediating the immune/metabolic response to an external noxious stimulus and fueling the transition from sepsis to septic shock, multiple organ dysfunction syndromes, and/or multiple organ failure (Tracey et al. 1987, Dinarello 2004, Riedemann, Guo and Ward 2003). It is thought that synergistic interactions between cytokines can cause or attenuate tissue injury (Calandra, Bochud and Heumann 2002). TNF-α, which is released early from neutrophils and macrophages, is one of the important cytokines involved in the pathophysiology of sepsis (Tracey et al. 1987, Lum et al. 1999). TNF-α-induced tissue injury is largely mediated through neutrophils, that respond by producing elastase, superoxide ion, hydrogen

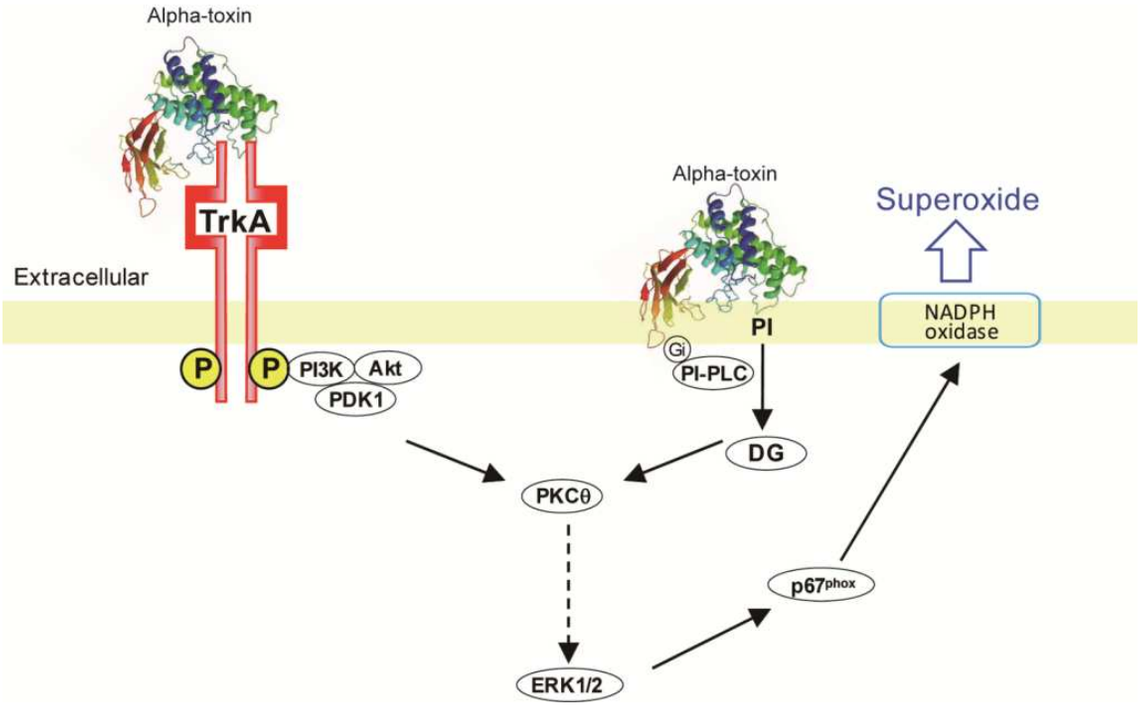

Figure 2. Signaling events involved in alpha-toxin-activated generation of superoxide

peroxide, sPLA2, PAF, leukotriene B1, and thromboxane A2 (Aldridge 2002). IL-1 stimulates the synthesis and release of prostagrandins, elastases, and collagenases and transendothelial microvascular cells, which respond by releasing the powerful neutrophil-stimulating agents, PAF and IL-8 (Leirisalo-Repo 1994). IL-1 and TNF-α are synergistic and share many biological effects in sepsis (Herbertson et al. 1995).

Anti-TNF-α antibody inhibited the death of mice induced by alpha-toxin. Furthermore, TNF-α-deficient mice were resistant to alpha-toxin. These observations suggest that the lethal effect of alpha-toxin is closely related to the release of TNF-α into the bloodstream. Stevens et al. and Bunting et al. suggested that alpha-toxin contributes indirectly to shock by stimulating production of endogenous mediators such as TNF-α and platelet-activating factor (Bunting et al. 1997, Stevens and Bryant 1997). It therefore appears that TNF-α released by alpha-toxin is important in enhancing the toxic actions of alpha-toxin in vivo. Consequently, inhibitors for release and expression of TNF-α may be worth pursuing as a novel therapeutic approach to the treatment of gas gangrene and sepsis caused by *C. perfringens*.

Cytokines such as the pro-inflammatory TNF-α, interleukin-1β (IL-1β) or transforming growth factor-β (TGF-β), increase the synthesis of NGF in airway structural cells. This stimulation has been evidenced in vitro in human pulmonary fibroblasts (Olgart and Frossard 2001, Micera et al. 2001), A549 epithelial cells (Pons et al. 2001) and bronchial smooth muscle cells (Freund et al. 2002). Studies also show that pro-inflammatory cytokines can act in concert to stimulate additional NGF secretion: TNF-α, for example, increases the secretion of NGF induced by IL-1β and interferon γ (IFN-γ) in fibroblasts (Hattori et al. 1994) and by interleukin-4 (IL-4) in astrocytes (Brodie et al. 1998). NGF synthesis in

inflammatory conditions has also been demonstrated in vivo: elevated NGF concentrations are observed in cutaneous inflammation (Safieh-Garabedian et al. 1995) and in asthmatic airways (Olgart and Frossard 2001, Kassel, da Silva and Frossard 2001, Virchow et al. 1998). Taken together, these results suggest that pro-inflammatory cytokines, which are present at high levels in the airways of patients with asthma (Tillie-Leblond et al. 1999), might contribute to the elevated levels of NGF synthesis.

Corticosteroids are well known for their anti-inflammatory properties, particularly in asthmatic airways. Numerous studies report that the glucocorticoids dexamethasone and budesonide affect NGF expression. They cause a significant reduction in the increased NGF expression induced by pro-inflammatory cytokines; in one study, this action was shown to result from the repression of NGF gene transcription in endoneural fibroblasts from the rat sciatic nerve (Lindholm et al. 1990). Olgart and Frossard have reported that glucocorticoid treatment decreases the NGF secretion that the pro-inflammatory cytokines IL-1β and TNF-α stimulate in cultures of human pulmonary fibroblasts (Olgart and Frossard 2001) and in A549 epithelial cells (Pons et al. 2001).

These results suggested that the initial release of pro-inflammatory cytokines induced by alpha-toxin in vivo leads to the production of NGF, and the NGF released synergistically causes systemic inflammation such as sepsis and shock via activation of the TrkA receptor (Fig. 3).

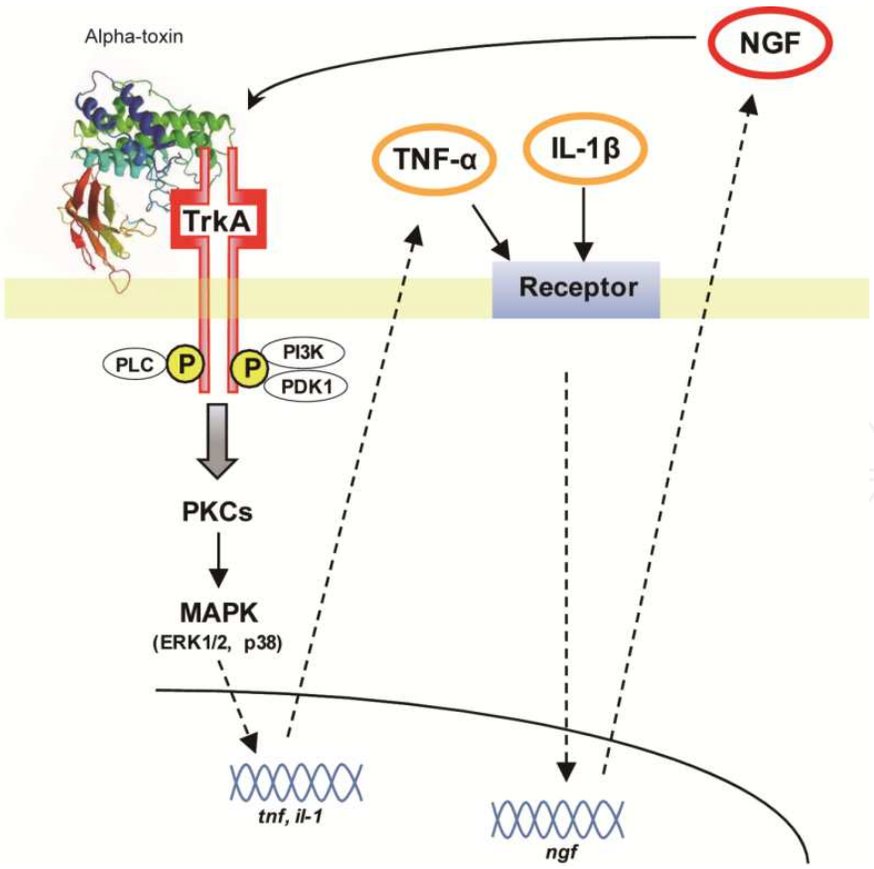

Figure 3. Alpha-toxin-induced release of pro-inflammatory cytokines and NGF

3. Conclusion

C. perfringens alpha-toxin, the main agent involved in the development of gas gangrene and septicemia, induces death, hemolysis, and the activation of macrophages and neutrophils. The toxin activated the MAPK-associated signal transduction from phospholipid metabolism and phosphorylation of TrkA. Penicillin is known to be highly effective in preventing the growth of microorganisms. In conclusion, treatment with TrkA inhibitors (tyrosine kinase inhibitors) and high doses of penicillin would be effective against diseases caused by *C. perfringens*.

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