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Mannheimia haemolytica leukotoxin binds cyclophilin D on bovine neutrophil mitochondria

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ABSTRACT

Mannheimia haemolytica is an important member of the bovine respiratory disease (BRD) complex that causes fibrinous and necrotizing pleuropneumonia in cattle. BRD is characterized by abundant neutrophil infiltration into the alveoli and fibrin deposition. The most important virulence factor of *M. haemolytica* is its leukotoxin. Previous research in our laboratory has shown that the leukotoxin is able to enter into and traffic to the mitochondria of a bovine lymphoblastoid cell line (BL-3). In this study, we evaluated the ability of LKT to be internalized and travel to mitochondria in bovine neutrophils. We demonstrate that LKT binds bovine neutrophil mitochondria and co-immunoprecipitates with TOM22 and TOM40, which are members of the translocase of the outer mitochondrial (TOM) membrane family. Upon entry into mitochondria, LKT co-immunoprecipitates with cyclophilin D, a member of the mitochondria permeability transition pore. Unlike BL-3 cells, bovine neutrophil mitochondria are not protected against LKT by the membrane-stabilizing agent cyclosporin A, nor were bovine neutrophil mitochondria protected by the permeability transition pore antagonist bongkrekic acid. In addition, we found that bovine neutrophil cyclophilin D is significantly smaller than that found in BL-3 cells. Bovine neutrophils were protected against LKT by protein transfection of an anti-cyclophilin D antibody directed at the C-terminal amino acids, but not an antibody against the first 50 N-terminal amino acids. In contrast, BL-3 cells were protected by antibodies against either the C-terminus or N-terminus of cyclophilin. These data confirm that LKT binds to bovine neutrophil mitochondria, but indicate there are distinctions between neutrophil and BL-3 mitochondria that might reflect differences in cyclophilin D.

1. Introduction

Bovine respiratory disease complex is a multi-factorial disease that is caused by several different pathogenic factors, including parasitic, viral, and bacterial agents [1]. The most prevalent organisms include: several viruses including bovine herpes virus type-1, bovine respiratory syncytial virus, parainfluenza type-3, and bovine viral diarrhea virus; and several bacterial species including *Mannheimia haemolytica*, *Histophilus somnii*, *Pasteurella multocida*, and *Mycoplasma bovis* [1]. *M. haemolytica* is arguably the most important bacterial pathogen of the bovine respiratory disease complex, in which it causes a severe fibrinous, necrotizing pleuropneumonia. Because stressors, such as transport of susceptible animals, are a common precursor to disease, *M. haemolytica* infection is often referred to as shipping fever. This pneumonia is morphologically characterized by intense neutrophil (PMN) infiltration into the airways and alveoli, intra-alveolar hemorrhage, fibrin deposition, and necrosis [2,3]. The importance of neutrophils in the production of inflammatory mediators, recruitment of other leukocytes and lung damage [3–6], was demonstrated when PMN-depleted calves were challenged with *M. haemolytica* [3,7]. These calves displayed less lung pathology than control calves infected with *M. haemolytica* [3,7]. From these data, it is clear that PMNs are a key player in the pathology of bovine pleuropneumonia.

An important virulence factor of *M. haemolytica* is its leukotoxin (LKT) [8,9]. LKT is a 104 kDa secreted protein that belongs to the **r**epeats-in-**tox**in (RTX) toxin family of exoproteins produced by a wide variety of gram-negative bacteria [10]. RTX toxins were named because of the C-terminal glycine-rich nonapeptide repeat region, which consists of –G-G-X-G-X-D-X-U-X, where U is

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a hydrophobic residue. The repeat region has been demonstrated to bind calcium, while concomitantly binding a host cell receptor, which tethers the toxin to the host membrane. This interaction is required for cytotoxicity [10,11]. Upon binding of the toxin to the host cell, it is theorized that the N-terminal amino acid residues, which contain amphipathic and hydrophobic domains, are required for pore stabilization and formation, respectively [10]. Because pore formation has been demonstrated for some RTX toxins, it has been theorized that RTX toxins insert into the plasma membrane of target cells, causing lysis and necrotic cell death [10,11].

Using the bovine lymphoblastoid (BL-3) cell line as a model, we have earlier demonstrated that LKT causes a reduction in anti-apoptotic proteins (Bcl-2, Bcl_{xL} and Akt-1), and a concomitant increase in pro-apoptotic proteins (Bax, BAD). These events led to apoptosis via loss of mitochondrial membrane potential, microscopic lesions on the outer membrane, and the release of cytochrome *c* that activates the caspase-9-dependent pathway [12–14]. LKT first binds to CD18, is translocated to lipid rafts and internalized by BL-3 cells in a dynamin-2- and clathrin-dependent manner [14]. Upon internalization, LKT travels to the mitochondria in a dynamin-2-dependent manner, where it binds cyclophilin D on the mitochondria causing the release of cytochrome *c* and a reduction in the mitochondrial membrane potential [12,13]. These processes were prevented by addition of cyclosporin A (CsA), which is a known mitochondrial membrane-stabilization agent [12].

Mitochondria are believed to be endosymbiotic prokaryotes. Through evolution this endosymbiont has lost most of its genetic material, so that 99% of mitochondrial proteins are encoded by nuclear DNA and synthesized using cytosolic ribosomes [15,16]. Cytosolic proteins are believed to be imported into mitochondria via the mitochondrial translocase of the outer membrane (TOM) complex, that recognizes a mitochondrial targeting signal (MTS) and allows entry of proteins into the mitochondria intermembrane space [17]. Further import into the mitochondrial matrix requires the translocase of the inner membrane (TIM) complex [15]. The TOM complex consists of three principal recognition components, one protein conducting channel, and several other accessory proteins. Preproteins or proteins with an N-terminal or internal MTS are recognized by TOM20, TOM22, or TOM70 via hydrophobic or electrostatic interactions. TOM20, and to a lesser extent TOM22, recognize N-terminal MTS, whereas TOM70 recognizes internal MTS. The general belief is that TOM20 or TOM70 recognizes a cytosolic preprotein, and transfers the preprotein to TOM22, where it can be directed to the general import pore formed by TOM40 [15,16]. We have earlier demonstrated that the first 31 amino acids of the LKT N-terminus contains a part of the predicted MTS (amino acids 1–54), that targets LKT to the mitochondria in BL-3 cells and *Cercopithecus aethiops* kidney fibroblast (COS7) cells [18]. Co-immunoprecipitation revealed that LKT bound TOM22 and TOM40, suggesting the TOM complex in BL-3 and COS7 cells is responsible for the entry of LKT into mitochondria [18]. Similar findings were reported for another RTX toxin, the p34 subunit of VacA produced by *Helicobacter pylori*, which utilizes the TOM complex to gain entry into the mitochondria and insert into the inner mitochondrial membrane [19].

Mitochondrial research is classically focused on production of ATP, which is required to support normal cell functions [20]. More recently the mitochondria's ability to regulate cell death via apoptosis has been examined. In particular, the mitochondrial permeability transition pore (mPTP) has received attention for its role in necrosis and apoptosis [20]. The mPTP consists of two core proteins: the adenine nucleotide translocase (ANT; spanning the inner membrane) and cyclophilin D. Other proteins suggested to play a role in the mPTP include the voltage-dependent anion-associated complex (VDAC; spanning the outer membrane) and a mitochondrial phosphate carrier (PiC), although the evidence for their role in mPTP opening has been controversial [21]. CsA is an immunosuppressive drug that binds cyclophilins. CsA inhibits peptidyl-prolyl *cis–trans* isomerases (PPIases), which hinders the interconversion between *cis* and *trans* conformations of peptide bonds adjacent to proline residues [20,22]. Cyclophilin D is a soluble PPIase that, upon oxidative stress or mitochondrial swelling, complexes with ANT leading to *cis–trans* isomerization and greater exposure of ANT to the cytosolic fluid. These events result in channel formation and additional inner mitochondrial membrane swelling, leading to disruption of the outer membrane and mitochondrial lysis [20,23].

Previous research from our laboratory used BL-3 cells as a model system to investigate the cellular microbiology of LKT. BL-3 cells may not reflect the response of phagocytic cells, such as PMNs, that are involved in host defense against *M. haemolytica*. In this study, we investigated internalization of *M. haemolytica* LKT and its transport to the mitochondria of bovine PMNs. As seen previously with BL-3 cells, we observed binding of LKT to cyclophilin D. However, we also identify distinct differences in regulation of LKT binding to mitochondria from bovine PMNs and BL-3 cells.

2. Results

2.1. LKT binds bovine PMN mitochondria in a dose- and time-dependent manner

Previous findings have shown that LKT is transported into BL-3 cells and binds mitochondria [12,14]. In this study, we examined bovine PMNs because of their importance in host defense and immunopathology in *M. haemolytica* infected animals [3–6]. We first incubated purified bovine PMN mitochondria with LKT and found that LKT bound to bovine PMN mitochondria in a time-dependent manner (90% stained positive for LKT within 30 min) (Fig. 1A). We next examined mitochondria isolated from bovine PMNs incubated with various concentrations of LKT for varying lengths of time. LKT was found to bind bovine PMN mitochondria in a dose- and time-dependent manner (Fig. 1A,B). Western blots detected porin, a mitochondrial-specific protein, in LKT containing fractions. However, β-actin was not detected (data not shown), confirming that mitochondria purification did not result in significant contamination by cytosolic proteins. Using flow cytometry and the fluorochrome Mitotracker Red (Molecular Probes) to identify mitochondria, we found that LKT binding to mitochondria peaked at 120 min (Fig. 1C). Later time points were not examined due to eventual LKT-mediated cytotoxicity. Similarly, confocal analysis revealed colocalization of signal for Mitotracker Red and Alexafluor 488 labeled anti-LKT antibody (Fig. 1D).

2.2. LKT interacts with components of the translocase of the outer membrane (TOM) complex

The TOM complex is the entry gate into mitochondria for nearly all mitochondrial precursor proteins [15]. Precursor proteins are recognized by TOM20 and TOM70 and then delivered first to the central receptor TOM22 and then to TOM40, which allows passage of the protein through its protein-conduction channel [24]. We examined TOM22 and TOM40 because these proteins are ultimately required for entry into mitochondria. Previous research using BL-3 and COS7 cell lysates showed that LKT interacts with TOM22 and TOM40 [18]. We wanted to determine if these interactions also occur in bovine PMNs. To answer this question, we incubated LKT with bovine PMN mitochondrial lysates and then immunoprecipitated with anti-TOM22 or anti-TOM40 antibodies. We separated the immunoprecipitates using SDS-PAGE, blotted to PVDF, and probed the blots using an anti-LKT antibody (MM601, kind gift from Dr. Srikumaran). These experiments demonstrated