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Characterization of turkey inducible nitric oxide synthase and identification of its expression in the intestinal epithelium following astrovirus infection

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ABSTRACT

The inducible nitric oxide synthase (iNOS) enzyme has long been recognized as a key mediator of innate immune responses to infectious diseases across the phyla. Its role in killing or inactivating bacterial, parasitic, and viral pathogens has been documented in numerous host systems. iNOS, and its innate immune mediator NO has also been described to have negative consequence on host tissues as well; therefore understanding the pathogenesis of any infectious agent which induces iNOS expression requires a better understanding of the role iNOS and NO play in that disease. Previous studies in our laboratory and others have demonstrated evidence for increased levels of iNOS and activity of its innate immune mediator NO in the intestine of turkeys infected with astrovirus. To begin to characterize the role iNOS plays in the innate immune response to astrovirus infection, we identified, characterized, developed tkiNOS specific reagents, and demonstrated that the intestinal epithelial cells induce expression of iNOS following astrovirus infection. These data are the first to our knowledge to describe the tkiNOS gene, and demonstrate that astrovirus infection induces intestinal epithelial cells to express iNOS, suggesting these cells play a key role in the antiviral response to enteric infections.

1. Introduction

Inducible nitric oxide synthase (iNOS) is recognized as an evolutionarily conserved enzyme that plays a key role in the innate immune response to infectious diseases [1]. It is distinct among the three isoforms of NOS, as it is not constitutively expressed but rather up-regulated following stimulation through pathogen pattern receptors and/or cytokines [1,2]. In addition, iNOS is capable of producing considerably more NO than either endothelial or neuronal NOS (eNOS and nNOS) primarily through the high affinity association it forms with calmodulin which allows it to function independent of calcium levels [3].

The expression of iNOS and its subsequent increase in NO activity has been reported to play a role in the host response to multiple viral families, and in various host species [4]. In the majority of reports the presence of NO activity is associated with decreasing viral disease [5–8], however some reports suggest that NO activity can also exacerbate pathological changes [9–11]. In addition to its antiviral properties, NO has been described to modulate intestinal barrier function, gut motility, ion transport [12,13], and has been implicated in numerous infections and non-infectious diseases in the intestine

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[13–15]. Several laboratories have demonstrated that mucosal epithelial cells are capable of expressing iNOS under different infectious and non-infectious disease conditions [16–18]. The ability of these barrier cells to respond to stimuli with innate immune responses illustrates the dual physiological and defense functions of these cells. Recent studies by Borghan et al. suggest that intestinal epithelial cells produce iNOS following exposure to the rotavirus enterotoxin NSP4, and propose this response is involved in the development of diarrhea [19].

Studies in our laboratory have investigated the role of iNOS in the response to another enteric virus, astroviruses. Initial studies demonstrated that macrophages from astrovirus-infected turkeys produced more nitric oxide (NO) following *ex vivo* stimulation with lipopolysaccharide (LPS) than cells from mock-infected control [20]. Additionally, treatment of the macrophage cell line HD11 with purified TAstV-2 resulted in increased NO activity [20]. Subsequent experiments demonstrated that pretreatment of virus with NO donor compounds significantly suppressed replication, while infecting embryos with TAstV-2 in the presence of an inducible NO synthase (iNOS) inhibitor resulted in increased replication [20].

These studies suggest astroviruses can induce expression of iNOS and its replication can be modulated by NO activity when experimentally manipulated; however there was no direct evidence of increased iNOS expression in the intestine of infected animals, as the turkey iNOS gene had not been identified or reagents developed to characterized its role in this or other diseases.

To better understand the role of the innate immune response to viral infections in turkeys, our laboratory has worked to identify innate immune factors up-regulated in the intestine following infection. The present study investigated how astrovirus infection affected the *in vivo* expression of iNOS and identified the cells in the intestinal epithelium involved in this aspect of the innate response.

2. Materials and methods

2.1. Sequencing and molecular characterization

The full NOS coding region of multiple species [NM_001003186 (canine iNOS), NM_001081769 (equine iNOS), NM_001076799 (bovine iNOS), XM_001148238 (chimpanzee iNOS), NM_00625 (human iNOS), NM_010927 (murine iNOS), X76881 (black rat iNOS), NM_012611 (Norway rat iNOS), NM_204961 (chicken iNOS), AY904361 (cat shark iNOS), NM_001124359 (rainbow trout iNOS), NM_001104937 (zebrafish iNOS), AY904362 (goldfish iNOS), NM_008712 (murine nNOS), NM_000620 (human nNOS), NM_008713 (murine eNOS), NM_000603 (human eNOS)] were aligned using ClustalW2 (http://www.clustal.org/). Regions of high sequence homology among iNOS genes were identified, and chicken iNOS-specific primers synthesized (Integrated DNA Technologies, Inc.). These primers were used to produce initial PCR amplicons from a cDNA library generated from primary turkey embryo fibroblast cells stimulated for 24 h with 100 ng *E.* coli LPS (Sigma–Aldrich). Turkey iNOS amplicons were then produced using the high fidelity Phusion polymerase (Finnzymes), cloned into pGEM Easy T vector (Promega), and isolated clones subjected to cycle sequencing (GENEWIZ). The resulting sequence was then used to design additional primers producing overlapping amplicons, as well as a series of 3′ and 5′ RACE reactions (Life Technologies). All sequence analysis was performed use Vector NTIv10 (Life Technologies). The tkiNOS sequence reported in this manuscript has been submitted to GenBank and assigned the accession number GQ184820.

Sequence alignment of tkiNOS and human iNOS reductase and calmodulin complex (Protein Data Bank code 3HR4) was used to generate a structural alignment in Swiss PDB viewer v4.0.1 [21]. Mean force potential energy was computed within the Swiss PDB viewer to validate predicted structure of the tkiNOS calmodulin-binding domain. Following validation, essential residues for tight binding of tkiNOS to calmodulin were identified based on previous observations made on huiNOS [3], and visualized using PyMOL Viewer v1.1 (The PyMOL Molecular Graphics System, Version 1.1, Schrödinger, LLC).

2.2. Anti-tkiNOS polyclonal antibody

The predicted tkiNOS amino acid sequence was analyzed to identify candidate oligopeptides based on surface probability and antigenicity (Vector NTIv10, Life Technologies). The oligopeptide CYTSDDSWNPKKHRI was synthesized, conjugated to KLH, and injected into naïve goats to generate protein G purified polyclonal anti-tkiNOS peptide anti-sera (Genscript).

2.3. Expression of recombinant tkiNOS

The full coding region of tkiNOS was cloned into both a bacterial and eukaryotic expression vector. For bacterial expression of tkiNOS the full coding region was amplified by PCR using tkiNOS/pEcoliNterm Lt Fwd (AAAGAGCTGCTGTGCCCATGGCAGTTTGCATTC) and tkiNOS 3669 Rev (CCCATGGTTGCAAATCTCTTATGTTC) primers. The tkiNOS amplicon was then cloned into pEcoliNterm (Clontech), following linearization by inverse PCR [pEcoliNterm Right primer (ATTTGCAACCATGGGCCACCGCTGAGCAATAACTAGCAT) and pEcoliNterm Left primer (GCACAGCAGCTCTTTGTCGACAGAGGCCTTATCATCGTC)] Phusion DNA polymerase (Finnzyme), followed by cloning using the InFusion cloning system (Clontech). Clones were screened by restriction digest and confirmed by sequencing. The resulting pEcoliNterm/tkiNOS was then expressed in BL21(DE3) *E. coli* following the manufacturer's instructions (Clontech). Cells were resuspended in lysis buffer (TBS + 0.5% SDS and 0.75 mg/ml lysozyme), disrupted by sonication, clarified, and His-tagged tkiNOS protein purified using TALON cobalt resin following the manufacturer's instructions (Clontech). Expression of the 6x-HN-tagged recombinant tkiNOS was then detected by western blot using a rabbit anti-6x-HN antibody, or a goat anti-tkiNOS polyclonal antibody.

Additionally, the full coding region of tkiNOS was cloned into a bicistronic lentivirus vectored expression system (pLVX-ZsGreen1, Clontech) for eukaryotic