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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, paracetic, 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.

Keywords

Astrovirus; enterocyte; turkey; inducible nitric oxide; innate immunity

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

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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 [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 TAsV-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 TAsV-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 hrs

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 using 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 (Xia et al.), 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 (AAAGAGCTGCTGTGCCCCATGGCAGTTTGCATTC) and tkiNOS 3669 Rev (CCCATGGTTGCAAATCTCTTATGTTTC) primers. The tkiNOS amplicon was then cloned into pEcoliNterm (Clontech), following linearization by inverse PCR [pEcoliNterm Right primer (ATTTGCAACCATGGGCCACCGCTGAGCAATAACTAGCAT) and pEcoliNterm Left primer (GCACAGCAGCTCTTTGTGTCGACAGAGGCCTTATCATCGTC)] 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 0.75mg/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 expression. The tkiNOS coding region was PCR amplified using Phusion DNA polymerase (Finnzyme) with pLVX-tkiNOS Fwd (TATTTCCGGTGAATTGTGTACAAAATGCTGTGCCCCAT) and pLVX-tkiNOS Rev (TAGTCTCGAGGAATTCCCCATGGTTGCAAATCTCTTATGTTTC) primers, then cloned into EcoRI-digested pLVX-ZsGreen1 using the In-Fusion Advantage PCR cloning system (Clontech). Clones were screened by restriction digest and confirmed by sequencing. The resulting pLVX-ZsGreen1/tkiNOS construct was then used to transduce 293T cells by transfecting cells with the Lenti-X expression system (Clontech) following manufacturer's instructions. 293T cells were transduced with either the bicistronic pLVX-

IRES-ZsGreen1/tkiNOS or empty plasmid to produce 293T/tkiNOS or 293T/Zs cells. The two recombinant cell types were cultured to confluency on glass coverslips, fixed using ice cold acetone-methanol (1:1), and expression of tkiNOS detected using goat anti-tkiNOS polyclonal antibody followed by universal biotinylated secondary antibody (Vectastain Universal ABC kit, Vector Laboratories) and Streptavidin Alexa Fluor 488 (Molecular Probes).

2.4 Nitrite assay

293T/Zs and 293T/tkiNOS cells were seeded (3×10^5 cells/well) in 5 replicate wells of a 96-well plate and incubated in DMEM+10% FBS at 37° C + 5% CO₂ for 48 hrs. Following incubation, the level of ZsGreen expression was assessed using a fluorescent plate reader (Ex: 485/Em: 538, Fluoroskan, Thermo). The supernatants were then removed and assayed for the presence of nitrite by Griess assay, as previously described [20]. The relative amount of recombinant tkiNOS activity was then calculated (μ M nitrite/relative fluorescence units).

2.5. Animals

Fifty 1-day-old unvaccinated turkey poultts were obtained from a commercial hatchery and randomly assigned to one of two 934-1-WP isolators (L. H. Leathers Inc, Athens GA) with free access to feed and water. After an acclimation period of 3 days, control animals were sham inoculated with sterile phosphate-buffered saline (PBS), infected animals were orally inoculated with 0.2 μ m filtered infectious material, containing $\sim 10^6$ genomic units of TAsV-2 (TAsV-2/NC/99) in 100 μ l of phosphate-buffered saline (PBS), as previously described [22–24]. Viral inoculum previously demonstrated to be devoid of other known infectious agents [22, 25] was prepared as previously described [26, 27] in embryonating turkey eggs, homogenized, clarified, and passed through a 0.2 μ m filter. All animal procedures were approved by the North Carolina State University Institutional Animal Care and Use Committee.

2.6. Indirect immunofluorescence

Jejunum and cecum samples were collected at 4 dpi, embedded in OCT media (Tissue Tek, Sakura Finetek), frozen, and sectioned at 5 μ m. Tissue sections were then incubated with goat anti-tkiNOS IgG and rabbit anti-TAsV-2 nonstructural protein (anti-TAsV-2 NSP) as previously described [24]. Targets were detected using a mixture of donkey anti-goat-Alexa Fluor 488, donkey anti-rabbit-Alexa Fluor 594, and nuclear stain DAPI (Invitrogen). Slides were mounted in fluorescent mounting media (DakoCytomation) and examined with a Vanox AHS-3 Photomicroscope linked to a Spot RT Slider cooled charge-coupled device digital camera.

3. Results

3.1. Identification and characterization of turkey iNOS

PCR primers were designed based on conserved regions of the iNOS mRNA identified using alignments of all available iNOS sequences. A panel of overlapping amplicons was produced and the sequence of the 4,346 bp turkey iNOS (tkiNOS) cDNA was determined. The 3,411 ORF nucleotide sequence was then compared to iNOS, nNOS, and eNOS sequences from multiple species using ClustalW2. The resulting phylogeny suggested tkiNOS is more closely related to other iNOS sequences as compared to the other NOS isoforms (Fig. 1A). Similarly, comparison of the tkiNOS predicted amino acid sequence with other NOS enzymes identified conserved regions of the iNOS functional domains (Fig. 1B), and the specific residues described to be important for high affinity binding of iNOS to calmodulin (Fig. 1C) thereby conferring its Ca²⁺ independent enzyme activity [3].

To further characterize the putative tkiNOS cDNA, the full coding region was transduced into 293T cells using a bicistronic lentivirus expression vector pLVX-IRES-ZsGreen (Clontech) and assayed for NOS activity. Comparing the amount of nitrite produced by 293T/tkiNOS per relative fluorescence units to that of 293T/Zs cells demonstrated a 325-fold increase in the amount of NO activity in the cells expressing the tkiNOS cDNA over that of the empty plasmid control cells (Fig 2A).

3.2. Characterization of anti-tkiNOS polyclonal sera

Following molecular and functional characterization of the tkiNOS gene, the predicted amino acid sequence was used to select a candidate peptide that was then used to generate an anti-tkiNOS polyclonal antibody (pAb). The specificity of the anti-tkiNOS pAb was assessed using recombinantly expressed tkiNOS protein. The anti-tkiNOS antibody was found to specifically detect both the *E. coli* expressed 129kDa His-tagged tkiNOS protein by western blot (Fig. 2B); as well as the lentiviral expressed tkiNOS by immunofluorescent antibody (IFA) analysis in the recombinant 293T/tkiNOS cells (Fig 2C).

3.3. Astrovirus infection induced expression of iNOS by enterocytes

Naïve turkey poultts were orally inoculated with TAsV-2 or PBS, then monitored for both clinical signs and virus replication. Within 3 dpi all infected animals demonstrated signs of severe diarrhea, which persisted until 6 dpi before beginning to abate. Microbiological analysis of intestinal contents found no bacterial species present known to be associated with disease in turkeys (data not shown). Tissue sections from astrovirus-infected intestines were assayed for the presence of iNOS protein and compared to uninfected control tissues. Immunofluorescent examination of these tissues demonstrated iNOS expression in the intestinal epithelium following TAsV-2 infection (Fig. 3). This result was observed in both the small and large intestine, and was associated with the presence of replicating virus (Fig 3).

4. Discussion

The inducible isoform of the NOS enzyme is a key component of innate immunity, with recognized roles in anti-cancer, anti-parasitic, anti-bacterial, and antiviral responses [28]. iNOS activity is largely regulated at the transcriptional level, and once expressed it converts L-arginine into citrulline and NO [28]. The reactive NO molecule produced then interacts with superoxide to form peroxynitrite which is largely responsible for the toxic activity attributed to NO [29]. Unlike the other NOS isoforms (endothelial and neuronal), iNOS forms a tight association with calmodulin which allows it to function in the absence of free calcium ions [3, 28]. This allows iNOS to produce more NO than the other isoforms and is responsible for its pathogen-cidal properties [28]. Identifying and characterizing this innate immune mediator in various species is important to our understanding of its role in the immune response to infectious diseases across phyla.

Previous studies of iNOS demonstrated that its sequence is conserved across vertebrate species [30]. Comparison of the turkey iNOS sequence reported in this study supports these earlier observations, demonstrating higher similarities among iNOS sequences from different species than among NOS isoforms within a given species (Fig. 1). Collectively, the overall sequence conservation, and the identification of the characteristic iNOS functional domains within the putative tkiNOS led us to conclude that this is the first description of the sequencing and characterization of the full turkey iNOS cDNA; an important first step towards understanding its role in the response to viral enteritis in the immunologically immature or impaired host.

Previous studies have demonstrated that treating astrovirus with NO donor compounds inactivated the virus, while inhibiting the iNOS enzyme *in ovo* resulted in increased virus replication [20]. These results suggest that iNOS and NO are capable of regulating astrovirus replication; however the only evidence of changes in iNOS expression or NO activity following astrovirus infection comes from experiments investigating the macrophage response. These reports have demonstrated increased expression of iNOS and NO following exposure *in vitro* with purified infectious or inactivated astrovirus particles [20], or recombinant astrovirus capsid protein (unpublished observation). Furthermore, primary macrophage cells isolated from infected animals demonstrated increased NO production following *ex vivo* stimulation as compared to uninfected controls. Collectively these data suggest that host cells, specifically macrophages, recognize and respond to astroviruses; however given the observations reported by ours and other laboratories of no significant influx of immune cells to the sites of astrovirus replication it was still unclear if iNOS was expressed in the intestine following infection and if so by what cells.

The data presented in this study demonstrates that iNOS expression is upregulated following astrovirus infection. Furthermore, these data suggest that the intestinal epithelial cells (Fig. 3) are responsible for producing iNOS. This is the first report, to our knowledge, of any antiviral host factors produced *in vivo* following astrovirus infection, suggesting the intestinal epithelial cells produce innate antiviral factors and provide their own defense in the absence of responding immune cells.

It is possible that the epithelial-derived NO participates in viral clearance through both direct viral inhibition and indirectly by inducing diarrhea to flush the pathogen from the intestine. Additional studies are needed to appreciate the relative contributions these two mechanisms may play in clearance of astrovirus infections.

5. Conclusions

Collectively, these studies are the first to our knowledge to identify and characterize the iNOS gene of turkeys, as well as investigate the spatial expression of iNOS in astrovirus infected intestines. The observation that the intestinal epithelial cells express iNOS following TASTV-2 infection suggests these cells are capable of producing factors leading to viral clearance. Additional studies are required to determine if the epithelial cells produce other innate antiviral mediators, and to what extent each plays in the overall anti-astrovirus response.

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References

1. Gonzalez-Domenech CM, Munoz-Chapuli R. Molecular evolution of nitric oxide synthases in metazoans. *Comp Biochem Physiol Part D Genomics Proteomics*. 2010; 5:295–301. [PubMed: 20884305]
2. Aktan F. iNOS-mediated nitric oxide production and its regulation. *Life Sci*. 2004; 75:639–53. [PubMed: 15172174]
3. Xia C, Misra I, Iyanagi T, Kim JJ. Regulation of interdomain interactions by calmodulin in inducible nitric-oxide synthase. *J Biol Chem*. 2009; 284:30708–17. [PubMed: 19737939]

4. Reiss CS, Komatsu T. Does nitric oxide play a critical role in viral infections? *J Virol.* 1998; 72:4547–51. [PubMed: 9573217]
5. Lin YL, Huang YL, Ma SH, Yeh CT, Chiou SY, Chen LK, et al. Inhibition of Japanese encephalitis virus infection by nitric oxide: antiviral effect of nitric oxide on RNA virus replication. *J Virol.* 1997; 71:5227–35. [PubMed: 9188590]
6. Tucker PC, Griffin DE, Choi S, Bui N, Wesselingh S. Inhibition of nitric oxide synthesis increases mortality in Sindbis virus encephalitis. *J Virol.* 1996; 70:3972–7. [PubMed: 8648734]
7. Akarid K, Sinet M, Desforges B, Gougerot-Pocidalo MA. Inhibitory effect of nitric oxide on the replication of a murine retrovirus in vitro and in vivo. *J Virol.* 1995; 69:7001–5. [PubMed: 7474119]
8. Klingstrom J, Akerstrom S, Hardestam J, Stoltz M, Simon M, Falk KI, et al. Nitric oxide and peroxynitrite have different antiviral effects against hantavirus replication and free mature virions. *Eur J Immunol.* 2006; 36:2649–57. [PubMed: 16955520]
9. Akaike T, Noguchi Y, Ijiri S, Setoguchi K, Suga M, Zheng YM, et al. Pathogenesis of influenza virus-induced pneumonia: involvement of both nitric oxide and oxygen radicals. *Proc Natl Acad Sci U S A.* 1996; 93:2448–53. [PubMed: 8637894]
10. Raber J, Toggas SM, Lee S, Bloom FE, Epstein CJ, Mucke L. Central nervous system expression of HIV-1 Gp120 activates the hypothalamic-pituitary-adrenal axis: evidence for involvement of NMDA receptors and nitric oxide synthase. *Virology.* 1996; 226:362–73. [PubMed: 8955056]
11. Aldridge JR Jr, Moseley CE, Boltz DA, Negovetich NJ, Reynolds C, Franks J, et al. TNF/iNOS-producing dendritic cells are the necessary evil of lethal influenza virus infection. *Proc Natl Acad Sci U S A.* 2009; 106:5306–11. [PubMed: 19279209]
12. Mourad FH, Turvill JL, Farthing MJ. Role of nitric oxide in intestinal water and electrolyte transport. *Gut.* 1999; 44:143–7. [PubMed: 9895365]
13. Xu DZ, Lu Q, Deitch EA. Nitric oxide directly impairs intestinal barrier function. *Shock.* 2002; 17:139–45. [PubMed: 11837790]
14. Nadler EP, Ford HR. Regulation of bacterial translocation by nitric oxide. *Pediatr Surg Int.* 2000; 16:165–8. [PubMed: 10786973]
15. Upperman JS, Potoka D, Grishin A, Hackam D, Zamora R, Ford HR. Mechanisms of nitric oxide-mediated intestinal barrier failure in necrotizing enterocolitis. *Semin Pediatr Surg.* 2005; 14:159–66. [PubMed: 16084403]
16. Vareille M, Rannou F, Thelier N, Glasser AL, de Sablet T, Martin C, et al. Heme oxygenase-1 is a critical regulator of nitric oxide production in enterohemorrhagic *Escherichia coli*-infected human enterocytes. *J Immunol.* 2008; 180:5720–6. [PubMed: 18390757]
17. Daniels I, Cavill D, Murray IA, Long RG. Elevated expression of iNOS mRNA and protein in coeliac disease. *Clin Chim Acta.* 2005; 356:134–42. [PubMed: 15936309]
18. Salzman AL, Eaves-Pyles T, Linn SC, Denenberg AG, Szabo C. Bacterial induction of inducible nitric oxide synthase in cultured human intestinal epithelial cells. *Gastroenterology.* 1998; 114:93–102. [PubMed: 9428223]
19. Borghan MA, Mori Y, El-Mahmoudy AB, Ito N, Sugiyama M, Takewaki T, et al. Induction of nitric oxide synthase by rotavirus enterotoxin NSP4: implication for rotavirus pathogenicity. *J Gen Virol.* 2007; 88:2064–72. [PubMed: 17554041]
20. Koci MD, Kelley LA, Larsen D, Schultz-Cherry S. Astrovirus-induced synthesis of nitric oxide contributes to virus control during infection. *J Virol.* 2004; 78:1564–74. [PubMed: 14722310]
21. Guex N, Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis.* 1997; 18:2714–23. [PubMed: 9504803]
22. Behling-Kelly E, Schultz-Cherry S, Koci M, Kelley L, Larsen D, Brown C. Localization of astrovirus in experimentally infected turkeys as determined by in situ hybridization. *Vet Pathol.* 2002; 39:595–8. [PubMed: 12243474]
23. Koci MD, Moser LA, Kelley LA, Larsen D, Brown CC, Schultz-Cherry S. Astrovirus induces diarrhea in the absence of inflammation and cell death. *J Virol.* 2003; 77:11798–808. [PubMed: 14557664]

24. Nighot PK, Moeser A, Ali RA, Blikslager AT, Koci MD. Astrovirus infection induces sodium malabsorption and redistributes sodium hydrogen exchanger expression. *Virology*. 2010; 401:146–54. [PubMed: 20219227]
25. Koci MD, Seal BS, Schultz-Cherry S. Molecular Characterization of an Avian Astrovirus. *J Virol*. 2000; 74:6173–7. [PubMed: 10846102]
26. Schultz-Cherry S, Kapczynski DR, Simmons VM, Koci MD, Brown C, Barnes HJ. Identifying agent(s) associated with poult enteritis mortality syndrome: importance of the thymus. *Avian Dis*. 2000; 44:256–65. [PubMed: 10879904]
27. Schultz-Cherry S, King DJ, Koci MD. Inactivation of an astrovirus associated with poult enteritis mortality syndrome. *Avian Dis*. 2001; 45:76–82. [PubMed: 11332502]
28. MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. *Annu Rev Immunol*. 1997; 15:323–50. [PubMed: 9143691]
29. Akaike T, Maeda H. Nitric oxide and virus infection. *Immunology*. 2000; 101:300–8. [PubMed: 11106932]
30. Reddick JI, Goostrey A, Secombes CJ. Cloning of iNOS in the small spotted catshark (*Scyliorhinus canicula*). *Dev Comp Immunol*. 2006; 30:1009–22. [PubMed: 16876672]
31. Lin AW, Chang CC, McCormick CC. Molecular cloning and expression of an avian macrophage nitric-oxide synthase cDNA and the analysis of the genomic 5'-flanking region. *J Biol Chem*. 1996; 271:11911–9. [PubMed: 8662618]

Fig. 1A

Fig. 1B

Fig. 1. Identification and characterization of turkey iNOS

(A) The nucleotide sequence of the putative tkiNOS cds was aligned with previously reported iNOS, nNOS, and eNOS sequences from various species using ClustalW2. A phenogram was constructed using the neighbor joining method and 1000 bootstrap replicates were calculated. (B) The predicted amino acid sequence of the putative tkiNOS was aligned with chicken, human, and mouse iNOS, along with human and mouse nNOS and eNOS using ClustalX. Identical and similar residues to a consensus sequence were shaded in black or gray, respectively, using BoxShade v3.21. Functional domains of the NOS isoforms [31] were located and indicated as shown. Heme, heme binding site; Calmodulin, calmodulin binding site; FMN, FMN binding site; F, FAD binding site; N, NADPH binding site. The residues within the calmodulin binding site previously described to be essential for tight, Ca^{2+} -independent binding of calmodulin [3] are denoted by chevrons. The amino acid sequence used for peptide antigens to generate the goat-anti-tkiNOS poly clonal Abs is denoted by the dashed box. (C) The tkiNOS and huiNOS amino acid sequences were aligned based on the primary sequence, and a structural alignment generated, followed by threading of tkiNOS to huiNOS. The panels depict the calmodulin binding domain of iNOS (gray) complexed to calmodulin (green) based on that previously described for huiNOS [3]. Residues R⁵⁰⁸ to K⁵³⁵ are depicted for tkiNOS while the corresponding region (R⁵¹¹ to R⁵³⁸) is depicted from huiNOS. In each panel the residues responsible for tight binding between iNOS and calmodulin are indicated by stick structures. The specific iNOS residues

are labeled for both tkiNOS (K⁵¹⁷, L⁵²⁰, L⁵²⁴, and K⁵²⁸) and huiNOS (K⁵²⁰, L⁵²³, M⁵²⁷, and K⁵³¹). The predicted interactions between the iNOS and calmodulin residues are depicted by dashed lines and interacting oxygen (red), nitrogen (blue) and sulfur (yellow) atoms.

Fig. 2. Characterization of recombinantly expressed turkey iNOS and anti-tkiNOS polyclonal antibody

(A) 293T cells transduced with tkiNOS (293T/tkiNOS) or empty plasmid control (293T/Zs) were seeded in 5 replicate wells of a 96-well plate (3×10^5 cells/well) incubated for 48hrs. Cells were analyzed for ZsGreen1 fluorescence, then the supernatants were collected and assayed for the presence of nitrite. The relative amount of recombinant iNOS activity was then calculated (nM nitrite/relative fluorescence units). Bars represent the average of replicate wells. * denotes means determined to be significantly different ($p < 0.05$, student's t-test). Data are representative of five independent experiments. (B) His-tagged tkiNOS was expressed in BL21(DE3) *E. coli*, disrupted by sonication, clarified, and His-tagged tkiNOS protein purified using an activated cobalt resin. Protein from whole cell lysates from either uninduced or IPTG-induced BL21(DE3) *E. coli* cells, affinity column wash flow through, or affinity elution were loaded on SDS-PAGE gels, transferred onto nitrocellulose membranes, and blotted with either an anti-6xHN antibody or the anti-tkiNOS polyclonal antibody. (C) 293T/tkiNOS or 293T/Zs cells, described above, were cultured to confluency on glass coverslips. Following fixation in ice cold acetone-methanol (1:1), cells were stained using anti-tkiNOS, and detected using a biotinylated secondary antibody and avidin-Alexa Fluor 488.

Fig. 3. Expression of iNOS by intestinal epithelial cells following TAsTV-2 infection

Jejunum and cecum sections were collected from TAsTV-2 infected and control animals at 4 dpi. Tissues were stained for tkiNOS (green), TAsTV-2 nsp (red), and nuclei (blue). White bars represent 25 μ m. Panels are representative of a minimum of 5 fields per section.