NITRIC OXIDE SYNTHESIS BY CHICKEN MACROPHAGES RESULTS IN
COORDINATED CHANGES IN MULTIPLE ARGININE TRANSPORTERS

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TITLE: Nitric oxide synthesis by chicken macrophages results in coordinated changes in multiple arginine transporters

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ABSTRACT

Nitric oxide synthesis by chicken macrophages results in coordinated changes of multiple arginine transporters

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Arginine transport is primarily mediated by the cationic amino acid transporters (CATs) in mammalian cells, but in aves the y^+ , $b^{0,+}$ and $B^{0,+}$ transport systems have also been observed. Arginine is the limiting catabolic substrate required for the production of nitric oxide (NO), a highly reactive compound that acts as a signaling molecule or killing compound. NO is synthesized by inducible nitric oxide synthase (iNOS) by macrophages for pathogen clearance. In mammals, CAT-2B is responsible for ARG import in the macrophage for NO synthesis, but the chicken CAT-2B isoform does not transport ARG. Therefore the objective of these studies was to identify the CAT(s) involved in mediating ARG uptake during a NO response in the chicken macrophage. Experiments were performed to measure: 1) ARG transporter mRNA and NO production from three sources of macrophages (HD11 cell line, n=6; primary 32d Cobb 500, n=8; Hyline W36, n=7) in response to *Escherichia coli* lipopolysaccharide (LPS); 2) the effect of CAT over-expression on NO production in response to LPS (HD11 cell line; n=8). In response to LPS iNOS mRNA abundance increased ($P<0.05$) 8.5-fold in the HD11 macrophages, 3.22-fold in broiler macrophages and 2.79-fold in layer macrophages. In all cells, CAT-1 was induced and CAT-2A increased ($P<0.05$) between 1.28 and 1.68-fold. CAT-2B was not detected at any time point or treatment condition. In the virally transformed chicken macrophage cell line (HD11) CAT-3 mRNA was induced, but in primary cells CAT-3 increased ($P<0.05$) 1.27-fold in broilers and 1.23-fold in layers. Transiently transfected chicken macrophages produce NO independent

of LPS treatment by 6h, mock transfected controls did not respond by 6h. In the presence of LPS, CAT-1 transfected macrophages produced 50.0% more NO than mock transfected cells ($P<0.05$). CAT-2A and CAT-3 transfected macrophages produced only 17.6% and 72.1% of the total NO produced by controls ($P<0.05$). These results indicate that CAT-1 and CAT-3 are both sufficient to sustain ARG import for NO production in the chicken macrophage, but that CAT-1 produces a maximal response. These results also show that iNOS, despite its name, is constitutively present and can be activated by induction of CATs to import ARG.

DEDICATION

To my advisor and mentor, Brooke Humphrey, for his relentless support and encouragement, this wouldn't have happened without you.

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LIST OF ABBREVIATIONS

ARG	arginine
ASL	argininosuccinate lysase
ASS	argininosuccinate synthetase
AT ^{0,+}	NA-dependent amino acid transporter
BH4	tetrahydrobiopterin
CAA	cationic amino acid
CaM	calmodulin
CAT	cationic amino acid transporter
CD	cluster of differentiation
CIT	citrulline
CPS I	carbamoylphosphate synthetase I
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
h	hour
HD11	chicken monocyte cell line
HLA	human leukocyte antigen
Ig	immunoglobulin
IL	interleukin
IFN	interferon
IRAK	IL-1 receptor activated kinase
LBP	LPS binding protein
LPS	lipopolysaccharide
MC29	avian myelocytomatosis virus
MHC	major histocompatibility complex
NF-κB	nuclear factor κB
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NOS	nitric oxide synthase
ORF	open reading fram
OTC	ornithine transcarbamoylase
PRR	pattern recognition receptor
SLC	solute carrier
T _H	T helper
TGF	transforming growth factor
TLR	toll-like receptor
TNF	tumor necrosis factor
RNS	reactive nitrogen species
ROS	reactive oxygen species
y ⁺ LAT	y ⁺ L type amino acid transporter

CHAPTER 1

Introduction

1.1. Overview of the Immune System

The immune system consists of innate and adaptive branches that provide non-specific, broadly specific and highly specific responses to pathogens and antigens. These different branches are coordinated through cytokine signaling and receptor-mediated immune cell interactions [1, 2]. The innate branch of the immune system is constitutive and the first response to pathogens or antigens. It acts using broadly specific or non-specific responses and recognizes pathogens with via broadly specific pathogen associated molecular patterns [2, 3]. This innate immune recognition does not alter or improve with repeat exposure [1]. In addition to constitutive barriers like the skin and mucous membranes, the innate immune system includes granulocytes (basophils, eosinophils, and neutrophils/heterophils), natural killer cells, dendritic cells and macrophages/monocytes [3, 4]. Granulocytes each target a broad-type response, but they are all phagocytic cells that contain granules of lytic enzymes and anti-microbial molecules. Basophils mediate allergic responses and eosinophils target parasitic infections [3-5]. Neutrophils are involved in bacterial infections and are one of the first cell types to arrive in response to cytokine release [3, 5]. Natural killer cells target a myriad of stressed cell types including virally infected cells, tumor cells and damaged cells [6, 7]. Dendritic cells and macrophages are phagocytic and antigen presenting cells that provide a link between innate and adaptive immune responses [8-10].

The adaptive immune response is characterized by the development of antigen-specific effector and memory cells [11]. It provides cell-mediated and humoral immunity with cytotoxic

T lymphocytes, T helper (T_H) cells and B cells [1, 3, 11]. Cytotoxic T lymphocytes target intracellular pathogens and B cells target extracellular pathogens [3, 5]. B cells produce a wide variety of immunoglobulins (Ig), or antibodies, in response to pathogen recognition or antigen stimulation [3, 10, 12]. IgM is the first Ig secreted by B cells and is secreted as a pentamer [3, 5]. In generating a more targeted immune response, B cells also switch Ig type in addition to undergoing receptor rearrangement [3, 5]. The most common isotype produced by activated mature B cells is IgG, but others include IgA, IgD and IgE [3, 4]. IgA is a secretory antibody produced in mucosal lymphoid tissue as a dimer [4]. IgE is produced in response to parasitic infections and triggers the degranulation of mast cells during an allergic reaction. Many subtypes of T_H cells augment the effector functions of both innate and adaptive immune cells through the production of cytokines [1, 2, 11]. T_H1 cells produce pro-inflammatory cytokines including interferon- γ (IFN- γ) and aid in macrophage activation [13-16]. T_H2 lymphocytes produce interleukin-10 (IL-10) and transforming growth factor- β (TGF- β), cytokines that stimulate B cells and increase antibody production [2, 3, 8]. T_H17 cells are named for their production of IL-17, a cytokine involved in neutrophil recruitment to the site of an infection [3, 5]. Both T_H1 and T_H2 cytokines are cross inhibitory, thus a T_H1 type response prevents T_H2 and T_H17 ; T_H2 inhibits T_H1 and T_H17 activation [3]. Additionally, T_{reg} cells maintain the inactive state of T_H cells through the production of TGF- β until they are down regulated [2, 3].

The tissues of the immune system are classified as primary or secondary lymphoid tissues based on their function. Primary immune tissues are sites of lymphopoiesis and lymphocyte maturation. B cells are produced and develop in the bone marrow of mammals and in the bursa in aves; T cells develop in the thymus [3, 4, 11]. Lymphocytes leave primary lymphoid tissues as self-tolerant mature naïve cells [11, 17]. Secondary lymphoid tissues such as the spleen, lymph

nodes and gut, bronchial, mucosal and cutaneous associated lymphoid tissues are where lymphocytes encounter antigens [4]. These tissues are also the sites of lymphocyte proliferation, Ig class switching and receptor hyper-variability [3, 5].

1.1.1 Macrophages

Macrophages, or activated monocytes, are phagocytic and antigen presenting cells of the innate immune system, but they are also involved in wound healing and tumor suppression [18-20]. Macrophages exist in peripheral blood as monocytes and become active once they leave circulation, or extravasate, and enter tissue [8, 21, 22]. Primary macrophages isolated from tissue and primary monocytes isolated from blood exhibit similar responses when activated. Both types of cell isolates produce reactive nitrogen species, actively phagocytose, synthesize cytokines and increase major histocompatibility II expression [23, 24]. Many specialized macrophages, or macrophage-like cells, exist in numerous tissues including alveolar macrophages in the lungs, Kupffer cells in the liver, microglial cells in the nervous tissue and osteoclasts in bone [25, 26].

Macrophages and macrophage-like cells recognize pathogens through the use of pattern recognition receptors including mannose-binding lectin, the toll-like family of receptors (TLR), antibody-antigen complexes and acute phase protein interactions [9, 10, 27-30]. Primary and cell line macrophages behave as their in vivo counterparts through phagocytosis, antigen presentation and cytokine production [10, 31]. The chicken macrophage cell line (HD11) is an immortalized cell line that was virally transformed by the avian myelocytomatosis virus (MC29) [32]. The MC29 virus creates tumors and immortalizes cells in part by altering the transcription factor *myc* [33]. The differences between primary macrophages and HD11s, as a model for L-arginine

(ARG) dependent nitric oxide (NO) production, remain similar despite 20 years of animal breeding and continual culture [31].

The differences between primary macrophages and cell lines are complex and may or may not accurately represent the *in vivo* immune response. While the HD11 cell line is a pure population of macrophages and thus removes the interactions of contaminating cell types *in vitro*, an *in vivo* immune challenge recruits neutrophils, T_H cells, natural killer cells in addition to circulating antibody and acute phase proteins [6, 34]. However, as a cell line the HD11 macrophages can survive and vigorously respond to simulated immune challenges without co-stimulation or exogenous survival factors. Even using targeted B cell and T cell antibodies and compliment, lymphocyte removal peripheral blood samples is typically only 95% [23]. Semi-pure macrophage populations derived from peripheral monocytes thus may include a variety of T cells and B cells which could more accurately mimic the *in vivo* response *in vitro*. As such in the comparison of a homogenous, transformed cell population and a moderately heterogeneous, wild type cell population, both are incomplete but potentially useful in describing the extremely heterogeneous conditions in the chicken.

1.1.2 Macrophage Activation

Macrophages and monocytes can be activated through a variety of signals and receptor binding ligands. Among these receptors, Toll-like receptor 4 (TLR-4) recognizes lipopolysaccharide (LPS) constitutively found in gram-negative cell walls [35, 36]. Activating macrophages with LPS requires a complex of TLR-4 with CD-14 and MD-2 [35]. LPS-binding-protein (LBP) with bound LPS can also activate macrophages by binding CD-14 [35, 36]. TLR-2

in heterodimers with TLR-1 or TLR-6 can bind a variety of ligands including peptidoglycan, bacterial lipoproteins, lipoarabinomannan and zymosan [29]. In addition TLR-5 binds bacterial flagellin [29]. Intracellular pathogens are detected by TLR-3, TLR-7 and TLR-9 which respond to double stranded RNA, single stranded RNA and CpG DNA in turn [29, 37]. In association with their co-ligands these receptors can each initiate an immune response directed either at intracellular or extracellular pathogens. Macrophages also express a variety of Fc receptors and thus can target antibody-antigen complexes for phagocytosis [8, 12].

Once a macrophage is activated with LPS, this signal is propagated using MyD88 and the serine/threonine kinase IL-1 receptor activated kinase (IRAK) to ultimately activate the transcription factor nuclear factor κ B (NF κ B) [28, 37-40]. The increase in available NF κ B results in a pro-inflammatory immune response with increased transcription of inducible nitric oxide synthase (NOS2 or iNOS) and IL-1 β , IL-6, IL-8, IL-12 and TNF- α production [40-43]. Similar macrophage activation can be achieved using TNF- α alone or in combination with LPS [10, 43, 44]. These cytokines enhance immune function, induce acute phase protein synthesis, serve as chemoattractants for neutrophils, direct a T_H1 type response and aid in extravasation to improve immune cell exfiltration [6, 13, 36].

1.1.3 Macrophage Functions

Once activated, macrophages actively phagocytose and destroy bacteria utilizing a respiratory burst mechanism [10, 15, 45]. The respiratory burst produces bactericidal compounds known as the reactive oxygen species (ROS) and reactive nitrogen species (RNS). The RNS and ROS produced include hydrogen peroxide, superoxide anion, peroxynitrite and nitric oxide (NO)

[46, 47]. All of these compounds are capable of causing oxidative damage to kill invading microorganisms [48, 49]. Even in a culture devoid of immune cells, NO is capable of killing *Leishmania major* [50]. NO contributes to anti-viral immunity by interfering with replication of DNA and protein synthesis [21]. In conjunction with H₂O₂, NO causes double stranded DNA cleavage, depletion of the antioxidant glutathione and increased death in *E. coli* [51]. The stable byproducts of NO, nitrate and nitrite, are also bacteriostatic [46, 48].

NO has other biological uses and can be synthesized by neuronal nitric oxide synthase (nNOS or NOS1) or endothelial nitric oxide synthase (eNOS or NOS3) which are both constitutive Ca²⁺ dependent isoforms [52, 53]. These constitutive forms synthesize NO for neurotransmission (nNOS) or vasoregulation (eNOS) [53]. The isoform utilized by macrophages to synthesize NO, iNOS is Ca²⁺ insensitive and associates with the Ca²⁺ binding protein, Calmodulin (CaM) in Ca²⁺ free media [21, 54]. Macrophages do however undergo rapid oscillations in intracellular Ca²⁺ concentration upon activation and this may provide the minimal amount required to saturate CaM [55]. Sustained production of NO is reliant on intracellular L-arginine (ARG), NADPH and oxygen. D-arginine cannot be utilized for NO production, but neither does it inhibit NO synthesis [21, 31, 56]. The reaction also requires flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH₄), and heme as co-factors [57, 58]. The active NOS enzyme regardless of isoform is a tetramer of two NOS monomers associating with two CaM monomers [57]. Constitutive nNOS requires 200-300 nM free Ca²⁺ to bind CaM and achieve half-maximal NO production [59, 60]. In mice, the CaM-binding region is the sequence residue 501-532 and is responsible for Ca²⁺ independent activation of iNOS [60]. Regardless of isoform, the terminal amino group of ARG is cleaved to produce NO and its co-product l-citrulline (CIT) [56]. Negative feedback from NO production

can occur through the production of stable nitrosyl species. This weakly inhibits iNOS, but has been shown to inactivate as much as 90% of active nNOS [61].

Once pathogens have been destroyed in the phagolysosome by the respiratory burst, they are processed for antigen presentation to by the endocytic pathway. The endocytic pathway processes antigens for presentation on major histocompatibility complex type II (MHC II) [11]. Antigen loaded on MHC II is presented to T_H cells, which are recruited to augment the innate immune response [8, 11]. The process of antigen presentation and recognition is crucial for pathogen clearance and the required for the development of immunological memory [8].

Macrophage cytokine production is important for immune clearance by activating a pro-inflammatory environment and recruiting adaptive immune cells [10]. Macrophages induce T_H cells into a T_H1 type response with the production of IL-12 and IL-18, a response which results in the T_H1 cells producing macrophage augmenting cytokines[10, 62]. The cytokines IL-1 β and IL-6 assist in T cell activation and can induce fever [40, 63]. TNF- α further increases inflammation and can trigger apoptosis in damaged and/or tumor cells through its receptor [13, 40]. With the assistance of T_H1 produced cytokines, notably IFN- γ and the resulting increase in MHC I or human leukocyte antigen (HLA) expression, activated macrophages have improved tumor cell recognition and destruction capacity [20, 64].

Once pathogen clearance has been achieved, macrophages are involved in the subsequent healing of the wounded environment [18, 19]. The enzyme arginase reduces the available substrate for NO synthesis and retards the inflammation [56]. In the mouse macrophage, *Salmonella* has been shown to up-regulate this pathway to survive inside phagocytic compartments [15]. In addition to pathogen clearance, damaged or dead cells are induced to

undergo apoptosis or are scavenged by macrophages [20]. Furthermore, cytokine production recruits additional cells to the wound and improves angiogenesis [18]. The inhibition of macrophages to limit collateral tissue damage and down-regulate activity post-pathogen clearance relies on T_H2 cytokines TGF- β and IL-10 [18, 27].

1.2 Nutrition and the Immune Response

Nutrition, metabolism and the immune response are interdependent and intermodulatory. For example, exposure to LPS induces a febrile response through the production of pro-inflammatory cytokines and reduces nutrient intake [65, 66]. This innate response also modulates nutrient availability on a tissue level by decreases amino acid uptake in skeletal muscle while increasing it in the liver and leukocytes [2]. The extensively studied metabolic hormone insulin regulates energy metabolism and promotes glucose uptake and utilization. Insulin also acts on immune cells such as lymphocytes which preferentially utilize glucose as an energy source [67]. Nutritional status, such as starvation, can result in impaired T lymphocyte function in response to low levels of leptin [68]. Leptin is also a regulatory hormone involved in food intake, basal metabolism and energy expenditure [67, 68].

Nutrition can also modulate immunity and metabolism [69]. Nutrient modulation occurs through numerous mechanisms including increased substrate supply for immune cells (amino acids, Mg, Zn) or pathogens (Fe), protection from immunopathology (Vitamin E, Se, carotenoids), altering signal transduction in immune cells (Vitamins A, D, E), altering gut microbial populations (fiber) and hormonal balance [1, 2, 69, 70].

1.2.1 Arginine Utilization

ARG, as a nutritional substrate for the immune system, has a role in immunomodulation [69]. Depletion of circulating ARG has been shown in abdominal sepsis, trauma, post-surgically and in cancer models [71]. Conversely, supplementation with dietary ARG improves wound healing and improved survival versus tumors in mammals [18, 67, 71]. In addition to utilization by macrophages, ARG is required for T cell proliferation, expression of the T cell receptor complex and the development of memory T cells [71]. In the absence of ARG, CD4⁺ T cells exhibit decreased CD3ζ chain expression, but this effect is reversible by ARG supplementation [72]. This decrease in CD3ζ can be replicated by depleting ARG with arginase I, but not arginase II or iNOS [72]. Increased lymphoid organ weights have also been observed in response to higher dietary levels of ARG in chickens [69]. Additionally high dietary ARG improves T cell proliferation and increases circulating monocyte percentages in growing broiler chicks [73]. Mature B cell proliferation and antibody production is not adversely affected by ARG deprivation or early supplementation [74]. In mice, ARG deprivation during development results in lower B cell numbers in the spleen and lymph nodes [75]. The implications of high dietary ARG in chickens in production may be confounded by differences in production and experimental facilities [69].

1.2.2 Arginine Metabolism

ARG is an essential, or conditionally essential, cationic amino acid depending on species differences in ARG metabolism [56, 76, 77]. These differences correlate with ureotelic and uricotelic nitrogen excretion strategies. ARG is processed through two major metabolic cycles and is a precursor for many other products, the urea cycle and the Citrulline-NO cycle (Figure 1)

[56]. It is also used for protein synthesis and is a precursor of NO, CIT, urea, ornithine, polyamines, proline, glutamate and agmatine [56]. In aves and other uricotelic species, ARG is a dietarily essential amino acid because they lack the complete array of urea cycle enzymes [76].

The complete urea cycle consists of five enzymes: arginase, carbamoylphosphate synthetase I (CPS I), ornithine transcarbamoylase (OTC), argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) [56]. Chickens lack mitochondrial CPS I and have low ASL, ASS and OTC activity [76]. In mammalian, or ureotelic, species the complete urea cycle is present and allows for ARG recycling [56, 77]. In young, growing animals ARG recycling is insufficient to meet metabolic needs and it is dietarily essential [77]. Mature, healthy adults can meet their ARG requirement without supplementation [56]. While ARG synthesis occurs in the liver and kidney, the co-localization of urea cycle enzymes in the liver results in hepatic ARG being utilized for urea synthesis [56, 77]. As such, in ureotelic species most *de novo* synthesized plasma ARG is of renal origin. ARG is thus conditionally essential in ureotelic organisms based on growth, age and health status.

The Citrulline-NO cycle consists of only three enzymes, NOS, ASS and ASL. This can occur, albeit inefficiently, in most NO producing cell types independent of the active NOS isoform [77]. As such, CIT supplementation in uricotelic and ureotelic animals is unable to support high levels of NO synthesis in culture, but chickens can meet their ARG requirements by dietary supplementation [76, 78]. Mammals however, can supply their ARG requirements from ARG, CIT or ornithine supplementation [77]. Despite possessing the necessary enzymes, mammalian macrophages are unable to utilize CIT for NO synthesis [79, 80].

Figure 1. Overview of the Urea and Citrulline-NO cycles. The chicken lacks a complete urea cycles because they lack carbamoylphosphate synthase I and have low activity of ornithine transcarbamoylase (*), but these enzymes are present in ureotelic mammals. Abbreviations: ASL, argininosuccinate lysase; ASS, argininosuccinate synthetase; L-Arg. Succ., L-argininosuccinate; CPS I, carbamoylphosphate synthase I; Carbamoyl P., carbamoyl phosphate; OTC, ornithine transcarbamoylase. Adapted from Sung et al 1991 [31].

1.3 Arginine Transport

Immune cells and tissues, like other tissues, have different nutrient demands and priorities during different physiological states [2]. Macrophages, for example, are a high nutrient priority cell type within the immune system followed by B cells and T cells in decreasing priority [81]. It follows that sepsis and wound healing are nutritionally demanding physiological states [19]. These nutrients are obtained and prioritized by groups of nutrient transporters derived from the solute carrier (SLC) superfamily of genes. Transporters are further divided based on substrate, with three families capable of encoding ARG transporters: the system y^+ cationic amino acid transporters (CAT; SLC7A), systems y^+L and $b^{0,+}$ glycoprotein-associated amino acid transporters (gpaAT; SLC7A and SLC3A) and system $B^{0,+}$ the Na^+/Cl^- dependent transporter (ATB $^{0,+}$; SLC6A) [19]. As a cationic amino acid (CAA), ARG shares these transport systems with other CAAs such as lysine and ornithine [56, 82].

1.3.1 System y^+

System y^+ consists of the glycosylated transmembrane transporters CAT1-4 with 12-14 transmembrane domains and cytosolic N and C-termini (Table 1) [19, 82-84]. CAT-2 is alternatively spliced into CAT-2A and CAT-2B in mammals and also into the truncated CAT-2C isoform in chickens [82, 85, 86]. CAT-4 exhibits approximately 40% homology to the other CATs and has been identified as a CAT in plants, but its function in mammals and aves is not clearly understood [86, 87].

The predominant transporter of the y^+ system in mammals is CAT-1, a high affinity transporter with a K_m range of 0.10-0.16 mM [19, 88]. It is constitutively expressed in most cell types with the notable exception of hepatocytes in adult mammals. Its expression can be

modified by cell proliferation, cytokines, hormones and nutrients [56, 88, 89]. In nutrient replete conditions CAT-1 expression is low, but amino acid deprivation upregulates CAT-1 mRNA [88]. CAT-1 is vital to post-natal growth and development, knockout mice lacking the gene are runts (~25% smaller) and die within 12h of birth [90]. Functional CAT-1 knockout models have been developed using embryonic fibroblasts and while fetal development is impaired, embryonic cells are still viable [90, 91]. In contrast, CAT-2 knockout mice are viable and fertile [45, 92]. CAT-2A is a low affinity transporter with a reported K_m of 2-5 mM and is found primarily in the liver and skeletal muscle [19, 45, 83]. The alternative CAT-2 splicing produces a high affinity isoform, CAT-2B with a K_m similar to CAT-1 and CAT-3 [19, 82, 85, 86]. The expression of CAT-2B in mammals is required for NO production in response to LPS or cytokine stimulation in macrophages, but not in fibroblasts [45, 92]. In other cell types, an absence of CAT-2B can impair NO synthesis to differing degrees [93, 94]. CAT-2B and iNOS are co-induced in mammalian macrophages and CAT-2B preferentially transports ARG utilized by iNOS [45, 89]. The substrate affinity difference in the CAT-2 isoforms is determined by a 41 amino acid sequence in the fourth intracellular region of the protein [19, 83, 86]. The chicken CAT-2B has a crucial amino acid substitution when compared with the mammalian transporter. At the substrate affinity conferring site, residue 369, a glutamine is replaced with lysine [85]. CAT-3 is also a high affinity transporter and is found in the brain and thymus [19, 56, 84, 86]. In CAT-1 deficient models, such as fibroblasts, CAT-3 can functionally restore ARG transport for NO synthesis [91].

Like mammals, chickens express different CAT isoforms depending on the tissue, however the tissue distributions sometimes differs from their mammalian counterparts. CAT-1 is not as ubiquitously expressed as it is in mammals and is found in the liver, skeletal muscle and

bursa but not in the heart, spleen or thymus [17]. CAT-2 is expressed in the liver, skeletal muscle, bursa, heart, spleen and thymus [17]. Like mammals, chickens also splice CAT-2 into multiple transporters, but they produce three variants: CAT-2A, CAT-2B and CAT-2C [85]. However, unlike mammals, CAT-2B is incapable of ARG transport and CAT-2C is a non-functional truncated protein [85]. CAT-3 is expressed in some skeletal muscle (pectoralis) but not in others (gastrocnemius). It is also expressed in the bursa, heart, spleen and thymus, but not in the liver [17]. The expression of the various chicken CATs also depends on physiological conditions including stage of development, health status and nutrient availability. For example, no isoform of CAT mRNA was detectable in the thymus or spleen until day 7 post hatch [17]. An immune challenge with LPS has no effect on CATs in the thymus, but increased CAT mRNA in the liver and bursa [81]. Peripheral blood mononuclear cells responded to surplus dietary ARG with increased expression of CAT-1 [73].

Table 1. Description of γ^+ system transporters

Gene	Tissue Distribution in mammals	Tissue Distribution in chickens	Approximate ARG K_m in mammals	Approximate ARG K_m in chickens	Sequence Homology ²
CAT-1	Ubiquitous, excluding liver	Liver, skeletal muscle and bursa	100-150 μ M	Unknown	94-96%
CAT-2A	Throughout the body, highest in liver	Ubiquitous	2-5 mM	6.5 mM	78-81%
CAT-2B	Immune cells	Bursa, heart, liver and thymus	70-400 μ M	NF	83-85%
CAT-2C	n/a	Ubiquitous	n/a	NF	n/a
CAT-3	Brain and placenta	Pectoralis, bursa, heart, spleen and thymus	40-165 μ M	Unknown	79-83%
CAT-4	Placenta	Unknown	Unknown	Unknown	n/a

¹Abbreviations: ARG, L-arginine; n/a, not applicable; NF, non-functions

²Sequence homology range compared to human, mouse and rat nucleotide sequences with BLAST (<http://blast.ncbi.nlm.nih.gov/>)

1.3.2 Systems y^+L and $b^{0,+}$

The systems y^+L and $b^{0,+}$ include the unglycosylated transmembrane proteins $y^+LAT1/2$ and $b^{0,+}AT$. These transporters form obligate heterodimers with the glycoproteins 4F2hc or rBAT [82, 95]. While $y^+LAT1/2$ primarily associates with 4F2hc and $b^{0,+}AT$ with rBAT, overexpression of either 4F2hc or rBAT can produce different pairings [95]. Both transport systems are high affinity obligate exchangers that exchange neutral amino acids for cationic amino acids, [82, 95]. Both y^+LAT-1 and $b^{0,+}$ are most abundantly expressed in the small intestine and kidney and indicate a role in amino acid absorption or reabsorption [19, 82, 95]. The transporter y^+LAT-2 , much like CAT-1, is ubiquitously expressed in mammalian tissues [19, 82, 95].

1.3.3 System $B^{0,+}$

System $B^{0,+}$ includes the Na^+ and Cl^- dependent transporter $ATB^{0,+}$, a high affinity glycosylated transmembrane transporter [19]. In addition to transporting CAAs $ATB^{0,+}$ is capable of transporting neutral amino acids with an apparent K_m of 0.10-0.15 mM [19]. $ATB^{0,+}$ is also the only Na^+ dependent ARG transporter. In part due to its broad transport capacity, $ATB^{0,+}$ has been a target for transmembrane drug delivery [96]. It has been shown in a wide variety of tissues including the mammary gland, pituitary gland, stomach, colon, and eye, but is most abundant in lungs and salivary glands [17, 19, 96].

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CHAPTER 2

Nitric oxide synthesis by chicken macrophages results in coordinated changes of multiple arginine transporters

Introduction

Macrophages are vital to pathogen clearance, tumor suppression and wound healing [1-3]. They act through phagocytosis, cytokine production, production of toxic molecules and antigen presentation cells [2-4]. They exist as quiescent monocytes in peripheral blood until induced to extravasate and mature into macrophages [5]. Macrophages derived from peripheral blood monocytes exhibit phagocytic behavior, cytokine production, nitric oxide production and major histocompatibility II expression [6, 7]. Macrophage activation can occur through localized tissue damage, stimulation of pattern recognition receptors (PRRs) or chemokine signaling [2, 8]. Commonly, lipopolysaccharide (LPS) or IFN- γ is used to simulate pathogen recognition in macrophages by stimulating either Toll-like receptor 4 (TLR-4) or the IFN- γ receptor [1, 3, 8, 9].

Once activated, macrophages produce bacterially toxic compounds including nitric oxide (NO), the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^\cdot$) and hypochlorite (OCl^-) [3, 10]. These compounds are produced inside endocytic vacuoles and destroy engulfed bacteria using enzymes such as inducible nitric oxide synthase (iNOS or NOS2), NADPH oxidase, superoxide dismutase and other peroxidase enzymes [10, 11]. This process is known as respiratory, or oxidative, burst due to the increase in oxygen consumption to produce microbicidal compounds. While NO production also requires oxygen, it is highly dependent on the presence of the catabolic substrate L-arginine (ARG) [9]. ARG is first oxidized to N-hydroxy-L-arginine, then to L-citrulline (CIT) and NO [12, 13]. The other metabolic fate of

ARG is catabolism by arginase to L-ornithine and urea [12, 14]. This reduces the ARG available for NO synthesis and can be utilized by bacterial species (e.g. *Salmonella*) to avoid destruction [14, 15].

Ureotelic animals, such as mammals, can recycle CIT or L-ornithine in the kidney to produce ARG and it is thus considered a conditionally essential amino acid [12]. While ARG synthesis from CIT has been shown in mouse macrophages, rat alveolar macrophages and chicken macrophages are unable to produce significant quantities of NO in low ARG, high CIT media [16-18]. Thus the capacity for ARG synthesis is not biologically relevant for NO production despite the presence of the requisite enzymes. Uricotelic animals lack carbamoyl phosphate synthase I and ornithine transcarbamoylase and cannot synthesize the metabolic precursors of ARG [19]. Thus in aves, ARG is an essential cationic amino acid because it cannot be synthesized *de novo*. Avian macrophages are thus limited to ARG acquired in the diet for NO synthesis [9].

In mammals, ARG is imported into the macrophage by the cationic amino acid transporter 2B (CAT-2B) [20]. CAT-2B and iNOS mRNA transcription are co-induced in response to cytokine activation or TLR-ligand binding [20, 21]. Mouse macrophages lacking CAT-2B import 95% less ARG and produce 92% less NO [20]. The chicken CAT-2 gene is alternatively spliced to produce three isoforms (CAT-2A, CAT-2B and CAT-2C). Chicken CAT-2A isoform is a high velocity, low affinity transporter that is predominantly expressed in the liver and skeletal muscle [22, 23]. Chicken CAT-2B is the isoform homologous to mammalian CAT-2B; however, chicken CAT-2B does not transport LYS or ARG [22]. This difference has been attributed to a substitution of Lys³⁶⁹ for Glu³⁶⁹ in the affinity-conferring region of the transporter. The chicken CAT-2C isoform results in a non-functional, truncated protein [22].

The other ARG capable transporters present in mammals and aves are CAT-1 and CAT-3., CAT-1 and CAT-3 are high affinity transporters and have variable tissue distributions depending on species [23]. Chicken CAT-1 mRNA is found in the bursa, thymus, skeletal muscle and liver, but not in the heart or spleen [24]. In mammals, CAT-1 is found in nearly all tissues except hepatocytes [25]. Chicken CAT-3 has a slightly lower affinity than CAT-1 and is found in the bursa, thymus, heart and pectoralis [24]. The mammalian CAT-3 is expressed in the thymus, brain, uterus and testis [25]. With CAT-2B not importing ARG for chicken macrophage NO production, another transporter(s) must allow for ARG import. These broad differences in species CAT expression do not indicate a likely candidate for ARG import for NO production in aves.

Due to the absence of a functional homologue to mammalian CAT-2B in the chicken macrophage, CAT-1, CAT-2A and CAT-3 were investigated for their role in ARG transport. Experiments were performed to: 1) identify the changes in ARG transporter mRNA abundance due to activation in primary and immortalized macrophages; 2) determine the effect of transiently over-expressing ARG transporters on NO production.

Materials and Methods

2.1 Cell Culture

HD11 cells obtained courtesy of Dr. Mike Kogut (Southern Plains Agricultural Research Center, TX) were cultured in T75 flasks (Corning, Corning, NY; 430720) in complete medium (IMDM without phenol red [Invitrogen, Carlsbad, CA; 12440], 5% fetal bovine serum [Invitrogen, Carlsbad, CA; 16000], 2% penicillin/streptomycin [Invitrogen, Carlsbad, CA; 15140] and 1% L-glutamine [Sigma, St. Louis, MO; G7513]) at 37° C with 5% CO₂.

2.2 Experimental Design

2.2.1 Experiment 1: Determining Experimental Conditions

HD11 cells for nitric oxide analysis were cultured in 96-well plates (Fisher, Pittsburgh, PA; 353070) at 2×10^5 cells/well (n=4/time point) in 200 μ L complete media containing 0, 0.1, 1 or 10 μ g/mL *Escherichia coli* lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO; L4005). Media was collected at 24, 48 and 72 h for nitric oxide analysis. HD11 cells for multiple time point nitric oxide analysis were cultured in 96-well plates at 4×10^5 cells/well (n=3) in 300 μ L of complete media containing 0 or 1 μ g/mL LPS. Media (50 μ L) was collected from either the same or different set of wells at 6, 18, 24, 36 and 48 h. Nitric oxide concentration from repeatedly sampled wells was corrected for the effect of reducing well volume with the following equation:

$$y_i = x_{i-1} + \frac{x_i - x_{i-1}}{V_0 - (n * (i - 1))}$$

where y_i = corrected nitrite concentration of sample i ; x_i = raw nitrite concentration of sample i ; $x_{i-1} = x_0 = 0 \mu\text{M}$ nitrite; V_{i0} = Initial volume in μL ; n = media volume sampled per time point in μL [26].

2.2.2 Experiment 2: Transporter Identification during a NO Response

HD11 cells were cultured in 6-well plates (Fisher, Pittsburg, PA; 353046) at 4×10^5 cells/well ($n=6$) in complete media containing 0 or 1 $\mu\text{g/mL}$ LPS. Media (500 μl) was collected at 24 and 48 h for nitric oxide analysis and cells were detached using cell scrapers (Fisher, Pittsburg, PA; 353085). Cells were centrifuged for 5 min at 500 x g at 25°C. The media was aspirated and cell pellets were flash frozen in liquid nitrogen and stored at -80°C.

Peripheral blood was collected from Cobb 500 broilers ($n=8$) and Hyline W36 layers ($n=7$) by cardiac puncture into heparinized tubes for isolation of macrophages. Cells were analyzed for viability and the macrophage marker KUL01 by flow cytometry. Macrophages were plated in 6-well plates at 4×10^5 cells/well in complete media containing 0 or 1 $\mu\text{g/mL}$ LPS. Media (500 μl) was collected at 24, 48 and 72 h for nitric oxide analysis. Macrophages were collected at 72 h in RPMI-1640 (Invitrogen, Carlsbad, CA; 11879-020) using cell scrapers and were centrifuged for 5 min at 500 x g at 25°C. The media was aspirated and cell pellets were flash frozen in liquid nitrogen and stored at -80°C.

2.2.3 Experiment 3: Over-expression of CATs by Transient Transfection

Transfected HD11 cells were plated in 96-well plates at 4×10^5 cells/well in 300 μ L IMDM complete media containing 0 or 1 μ g/mL LPS. Supernatant (50 μ L) was collected at 6, 12, 24, 36 and 48 h. The remaining media was aspirated and the cells were detached with trypsin (8.0g Sodium chloride [Fisher, Pittsburgh, PA; S671], 0.4g potassium chloride [Fisher, Pittsburgh, PA; P217], 1.0g glucose [Sigma-Aldrich, St. Louis, MO; G8270], 0.35g sodium bicarbonate [Fisher, Pittsburgh, PA; S233], 0.5g Trypsin [Sigma-Aldrich, St. Louis, MO; T-4799], 0.2g EDTA [Sigma-Aldrich, St. Louis, MO; E8145], 0.06g potassium phosphate monobasic [Fisher, Pittsburgh, PA; P285], 0.09g sodium phosphate dibasic [Fisher, Pittsburgh, PA; S381] in 1L ddH₂O] for 10 min at 37°C with 5% CO₂ and were quenched with complete media. Cells were analyzed by flow cytometry for viability and transfection efficiency.

2.3 Macrophage isolation

In experiment 2, macrophages were isolated from male 32d old Cobb 500 broilers (n=8) or actively laying Hyline W36 (n=7). Broilers and layers were provided access to water and a standard commercial ration *ad libitum*. Whole blood was collected into heparinized (Hospirin, Inc., Lake Forrest IL; 0409-1402-31) syringes via cardiac puncture and stored on ice. Heparinized blood was overlaid onto isovolumetric Histopaque-1077 (Sigma-Aldrich, St. Louis, MO; #10771) and centrifuged for 15 min at 350 x g at 25°C to obtain peripheral blood mononuclear cells. The buffy coat was reconstituted in RPMI-1640 (Invitrogen, Carlsbad, CA; 22400) and centrifuged for 5 min at 500 x g at 25°C. Cell pellets were resuspended in RPMI-1640 complete media (RPMI-1640 containing 5% fetal bovine serum, 2%

penicillin/streptomycin and 1% l-glutamine). The cells were then plated (cells from 1 bird/plate) in 10 cm² plates (Fisher, Pittsburgh, PA; 353003) overnight at 37° C with 5% CO₂. After an overnight adherence, media was aspirated and the plates were washed twice with RPMI-1640 to remove heterophils and erythrocytes. The cells were then collected in RPMI-1640 complete media using cell scrapers and enumerated via hemocytometer.

2.4 Nitric oxide assay

Nitric oxide (NO) was measured in media samples collected from experiments 1-3 using the Griess Reagent System (Promega, Madison, WI; G2930) for optical density at 550 nm. Standard curves were run in triplicate to produce the predictive equation ($R^2 > 0.99$) used to calculate sample concentrations. Sample concentrations containing less than 2.5 µM nitrite were considered to be below the detection limit of the assay.

2.5 Total RNA Isolation

Total RNA was isolated from cell pellets collected in experiment 2 using NucleoSpin Extract II kits (Macherey-Nagel, Bethlehem, PA; 740955) per the manufacturers protocol. Total RNA was quantified at optical density 260 nm and 1 µg total RNA per sample was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA; 170-8891) according to the manufacturers' protocol.

2.6 Quantitative Real-Time PCR

Quantitative real-time PCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA; 4385610), 1 μ L of reverse transcription cDNA product, and 10 μ mol/L of each primer (Table 1). Thermal cycling parameters were 1 cycle of enzyme activation at 95°C for 20 s and 40 cycles of denaturing at 95°C for 3 s and annealing and extending at 60°C for 30 s. After 40 cycles, melting curve analysis was performed at 95°C for 15 s, 60°C for 60 s, followed by a linear temperature increase of 0.5°C/s to 95°C while continuously monitoring fluorescence. Samples containing primer dimers were considered below the level of detection and were excluded from analysis. Relative mRNA abundance was calculated using the modified Δ - Δ equation as previously described [27]. Amplification efficiency was determined with LinRegPCR during the log-linear phase of amplification [28]. Normalization of target mRNA genes was performed by geometric averaging of non-normalized β -2 microglobulin, glyceraldehyde 3-phosphate dehydrogenase and hypoxanthine-guanine phosphoribosyltransferase using GeNorm software [29]. Data are represented as normalized fold abundance relative to 0 μ g/mL LPS for each transporter. Since CAT-1 and HD11 CAT-3 mRNA was below the limit of detection in the 0 μ g/mL LPS treatment data were normalized to y^+ LAT1.

2.7 Transient Transfection

HD11 cells were transiently transfected with expression vectors containing chicken CAT-1, CAT-2A and/or CAT-3 using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA; 11668-019). The open reading frame (ORF) for chicken CAT-1, CAT-2A and CAT-3 were amplified

using Platinum Taq DNA polymerase and were cloned in-frame into pcDNA5/FRT/V5-HIS-TOPO mammalian expression vector (Invitrogen, Carlsbad, CA). The forward primer contained a kozak sequence and the stop codon on the reverse primer was deleted to allow for expression of a C-terminal V5 epitope. A control plasmid provided with the kit contained chloramphenicol acetyltransferase in the pcDNA5/FRT/V5-HIS-TOPO vector was used as an over-expression control. Lipofectamine (0.2 µg/well) was diluted in Opti-Mem (Invitrogen, Carlsbad, CA; 31985) and allowed to conjugate with 0.2 µg plasmid/well in Opti-Mem for 20 min at 25°C. Conjugated plasmids were then added to IMDM incomplete media (no pen/strep) containing 4×10^5 cells/well and incubated for 4 h at 37°C with 5% CO₂.

2.8 Flow Cytometry

Primary macrophage and transfected cell viability were analyzed using the EasyCyte Plus System (Millipore, Danvers, MA) with a 488 nm argon laser with ViaCount Reagent (Millipore, Danvers, MA; 4000) per the manufacturer's instructions. Primary cell isolates were analyzed using mouse monoclonal R-phycoerythrin (R-PE) conjugated antibody (Southern Biotech, Birmingham, AL; 8420-09) KUL01 specific for monocytes and macrophages. Transfection efficiency was analyzed using mouse monoclonal fluorescein isothiocyanate (FITC)-conjugated antibody (Invitrogen Carlsbad, CA; R963-25) specific for the V5 epitope. Cells were washed with DPBS (Invitrogen, Carlsbad, CA; 14040) and permeabilized with 0.2% Triton X-100 (Integra Chemical Company, Kent, WA; T756.30.30) for 10 min. Cells were washed twice with DPBS and blocked with SuperBlock Blocking Buffer (Fisher, Pittsburg, PA; 37515) for 20 min. Excess blocker was removed with two DPBS rinses. Blocked samples were stained with 0.238

µg/mL anti-V5-FITC in DPBS for 1 h at 25°C protected from light. Cells were washed once with DPBS, detached by trypsin addition for 5 min at 37°, and quenched with complete media without phenol red. Cells were then analyzed for FITC or R-PE fluorescence using Guava CytoSoft Software (Millipore, Danvers, MA) on EasyCyte Plus System.

2.9 Statistics

Dependent variables were analyzed by the general linear model (MiniTab Software, Minitab Inc. State College, PA) using either a one-way or two-way analysis of variance (ANOVA). The effect of LPS treatment on HD11 NO production, NO dose response and ARG transporter mRNA abundance were analyzed with a one-way ANOVA. Transfected HD11 NO and monocyte NO assays were analyzed by two-way ANOVA to determine the main effect of LPS treatment, sample origin, and their interaction. Differences were considered significant when $P < 0.05$, and means were compared by student's pair-wise comparison. Transfection efficiency and viability data were arcsin transformed to meet the conditions for ANOVA. Data are shown as untransformed means and pooled standard error.

Table 2. Primers for quantitative real-time PCR^a

Gene		Primer Sequence	Amplicon Length (bp)	Accession Number
CAT1	Sense	5'-ACCTCCATCGTCATCTCCTTC-3'	252	EU360441
	Antisense	5'-AAGTCTTCAATGTGCCACCTATG-3'		
CAT2A	Sense	5'-TGCTTTGTCTACAAGTCTTCTCG-3'	165	EU360448
	Antisense	5'-AATGCCATAATACCAGAGATGACC-3'		
CAT2B	Sense	5'-CTTTGCTTGTCTGCTTCATGG-3'	272	EU360449
	Antisense	5'-CTTCGTTTTTGGAAATTGATTTGAGC-3'		
CAT3	Sense	5'-CCACGGGCACCAAACAGAAG-3'	150	XM_420204
	Antisense	5'-CAGTCAGCACCAACGCAGATG-3'		
y ⁺ LAT1	Sense	5'-GTTGGAGCCAGAGAAGGACATC-3'	165	XM_418326
	Antisense	5'-AAGCCAGTAGTTGAAGCAGTAGTAG-3'		
y ⁺ LAT2	Sense	5'-TTGTTCTCTTATTCTGGTTGGGATAC-3'	100	XM_001231336
	Antisense	5'-TTGGCATAGACACAGCAATAGC-3'		
NOS2	Sense	5'-GCTGTACTCTTTGCGTCATTACTC-3'	90	NM_204961.1
	Antisense	5'-TGATTTCCCAGTCTCGGTTGC-3'		
GAPDH	Sense	5'-GGTGCTGAGTATGTTGTGGAGTC-3'	290	NM_204305.1
	Antisense	5'-GTCTTCTGTGTGGCTGTGATGG-3'		
HPRT-1	Sense	5'-GCCAGACTTTGTTGGATTTGAAG-3'	213	NM_204848
	Antisense	5'-AGAGTTGAAGCCTGTGAGAGATAG-3'		
B2M	Sense	5'-TGGAGCACGAGACCCTGAAG-3'	161	XM_424099.2
	Antisense	5'-TTTGCCGTCATACCCAGAAGTG-3'		

^aAbbreviations: B2M = β -2 microglobulin; CAT = cationic amino acid transporter; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; HPRT-1 = hypoxanthine phosphoribosyltransferase-1; NOS2 = nitric oxide synthase 2 or inducible nitric oxide synthase; y⁺L type amino acid transporter;

Results

2.10 Experiment 1: Determining Experimental Conditions

2.10.1 Timeline and Dose Response

In order to evaluate the conditions for a maximal NO response in the HD11 cell line, a timeline and dose response was performed. Surprisingly, the HD11 response to LPS showed no significant time-dose interaction ($P>0.05$; data not shown) or effect of time ($P>0.05$; data not shown). Maximal nitric oxide (NO) production occurred with 1 $\mu\text{g/mL}$ LPS (Figure 1; $P<0.05$). Subsequent experiments were performed with 1 $\mu\text{g/mL}$ LPS.

2.10.2 Repeat Sampling Conditions

In order to determine if repeat sampling had a biological or merely volumetric effect on NO production, repeat sampling was compared to single sampling and transformed repeat sampling. The HD11s produced no detectable NO by 6 h, regardless of LPS treatment (data not shown). At 18, 24 and 36 h there was no difference between NO concentrations between single-sampled, repeat-sampled wells and corrected repeat-sampled wells (Figure 2A-C; $P>0.05$). By 48 h, the repeat-sampled wells resulted in higher NO concentration compared to controls (Figure 2D; $P<0.05$), but corrected repeatedly-sampled and controls produced the same amount of NO ($P>0.05$). As such repeat sampling had volumetric and not biological effects and subsequent experiments utilizing repeat sampling were adjusted using the concentration correction formula.

Figure 2. Nitric oxide production by HD11 cells (2×10^5 cells/well). Means not sharing a common superscript (a-c) differ within a time point ($P < 0.05$). Means did not differ between time points ($P > 0.05$). Values represent the mean $\pm$ SEM ($n=12$).

Figure 3. Nitric oxide production at 18h (A), 24h (B), 36h (C), and 48h (D) by HD11 cells (4×10^5 cells/well) in response to LPS with or without repeated sampling and adjustment algorithms. Means not sharing a common superscript differ ($P < 0.05$). Values represent the mean $\pm$ SEM ($n=4$).

2.11 Experiment 2: Transporter Identification during a NO Response

2.11.1 Nitric oxide response

Before quantifying the transporters involved in a NO response, the NO response was first verified by quantifying NO and iNOS mRNA. In the HD11 cell line at 24 and 48 h (Figure 3A-B), 1 µg/mL LPS increased NO concentrations above controls ($P<0.05$) and at 48 h LPS increased iNOS mRNA abundance 8.5-fold ($P<0.05$) above controls (Table 2).

After verifying a NO response in the HD11 cell line, the NO response to LPS in primary macrophages (Table 3) was evaluated by measuring NO and iNOS mRNA. The magnitude of NO production in response to LPS differed between layer and broiler macrophages (Figure 4). NO production by layer macrophages was greater than broiler macrophages at 24, 48 and 72 h post-LPS (Figure 4A-C). The broiler macrophage NO response to LPS was similarly increased over controls at all time points ($P<0.05$). In broiler and layer macrophages (Table 2), iNOS mRNA abundance increased ($P<0.05$) 3.22-fold and 2.79-fold, respectively.

2.11.2 Transporter mRNA Abundance

Once a NO response to LPS had been shown in cell line and primary macrophages, ARG capable transporters were quantified in all three cell types. Both CAT-1 and CAT-3 were induced in HD11s in the presence of LPS (Table 2). The low-affinity importer CAT-2A increased 1.68-fold ($P<0.05$) in response to LPS and CAT-2B mRNA was not detected in treatments or controls. In the presence of LPS, y^+ LAT-1 decreased 69.5% ($P<0.05$) and y^+ LAT-2 mRNA abundance was unaltered ($P=0.95$).

Broiler and layer macrophage CAT-1 mRNA was induced in response to LPS (Table 2). The mRNA abundance of CAT-2A and CAT-3 showed a similar pattern of change in both strains in response to LPS. CAT-2A mRNA increased 1.33-fold in broilers ($P<0.05$) and 1.28-fold in layers ($P<0.05$) while CAT-3 mRNA increased 1.27-fold in broilers and 1.23-fold in layers ($P<0.05$). y^+ LAT-1 did not change in either strain in response to LPS ($P>0.05$), but y^+ LAT-2 increased ($P<0.05$) 2.35-fold in broilers and 2.24-fold in layers.

Figure 4. HD11 (4×10^5 cells) nitric oxide production at 24 and 48 h. Asterisks represent significant difference ($P < 0.05$) within a time point. Values represent the mean $\pm$ SEM ($n=6$).

Figure 5. Nitric oxide production by macrophages from 32d old all male Cobb broiler chickens (A; n=8; 4×10^5 cells/well) and actively laying White Leghorns (B; n=7; 4×10^5 cells/well). Means not sharing a common superscript (a-d) differ ($P < 0.05$). ND represents samples that were below the level of detection (< 0.78 mM nitrite). Values represent the mean $\pm$ SEM.

Table 3. Relative mRNA abundance of arginine transporters and inducible nitric oxide synthase in HD11 and primary macrophages from broilers and layers¹

Gene	Cell Source	Relative mRNA Abundance	P-value
CAT-1	HD11	Induced ²	NA
	Broiler	Induced ²	NA
	Layer	Induced ²	NA
CAT-2A	HD11	1.676±0.241	<0.05
	Broiler	1.327±0.058	<0.05
	Layer	1.283±0.129	<0.05
CAT-3	HD11	Induced ²	NA
	Broiler	1.265±0.063	<0.05
	Layer	1.227±0.085	<0.05
y⁺LAT-1	HD11	.3049± 0.186	<0.05
	Broiler	1.052±0.164	0.387
	Layer	1.023±0.304	0.856
y⁺LAT-2	HD11	1.034± 0.812	0.950
	Broiler	2.352±0.115	<0.05
	Layer	2.242±0.267	<0.05
iNOS	HD11	8.514± 0.159	<0.05
	Broiler	3.216±0.247	<0.05
	Layer	2.794±0.168	<0.05

¹values represent fold mRNA abundance relative to 0µg/mL LPS; NA = not applicable; ND = not detected; CAT = cationic amino acid transporter; y⁺L type amino acid transporter; iNOS2 = nitric oxide synthase 2 or inducible nitric oxide synthase.

²Indicates mRNA detected in 1µg/mL LPS and no detectable mRNA in 0 µg/mL LPS.

Table 4. Primary cell isolate viability and KUL01 staining for verification of macrophage phenotype from broilers and layers identified by flow cytometry¹

Cell Source	Viable Cells²	KUL01 Positive Cells³	Cell Count
Broiler	934±32.8.	3052±118	1.49±0.13x10 ^{6a}
Layer	910±26.5	2572±135	2.43±0.16x10 ^{6b}

¹Means not sharing a common superscript differ (P<0.05). Values represent the mean±SEM (n=3)

²Viable cells per 1000 events

³KUL01 positive cells per 5000 events

2.12 Experiment 3: Over-expression of CATs by Transient Transfection

2.12.1 High Affinity Transporter Transient Transfection

The initial transient transfection was performed using the two high affinity ARG importers that were found to be induced or increased in response to LPS, CAT-1 and CAT-3. Transient transfection was performed with CAT-1, CAT-3 or CAT-1/CAT-3 plasmids and utilizing a non-plasmid mock transfection as a control. At 6 h post LPS treatment, no effect of LPS treatment was seen ($P>0.05$), but all transfected cells showed an increase of 15-18 μM NO compared to mock transfected controls (Figure 5A; $P<0.05$). After 12 h, mock transfected controls produced NO in response to LPS at a lower concentration than all plasmid treatments ($P<0.05$; Figure 5B). LPS increased NO production in CAT-1 transfected cells above transfected cells treated with 0 $\mu\text{g/mL}$ LPS and multiple plasmid transfected cells treated with LPS ($P<0.05$). By 24 h, the CAT-1 with 1 $\mu\text{g/mL}$ LPS combination produced the highest concentration of NO ($P<0.05$) followed by mock transfected cells with 1 $\mu\text{g/mL}$ LPS. The mock transfection with LPS produced more NO than non-LPS treated transiently transfected cells ($P<0.05$; Figure 5C). After 36 h, over-expressing CAT-1 in the presence of LPS produced the maximal response of 89.3 μM NO ($P<0.05$; Figure 5D). In the absence of LPS, CAT-1 transfected cells produced the same amount of NO as the transfection control treated with LPS at 48 h ($P>0.05$). Cells transfected with CAT-3 produced the same amount of NO, independent of LPS or CAT-1 co-over-expression, and at a lower concentration than LPS positive controls.

Figure 6. Nitric oxide production at 6h (A), 12h (B), 24h (C), and 36h (D) by HD11 cells (4×10^5 cells/well) transfected with no plasmid, CAT-1 plasmid, CAT-3 plasmid and CAT-1/CAT-3 plasmids. Means not sharing a common superscript (a-d) differ ($P < 0.05$). Values represent the mean $\pm$ SEM (n=8).

2.12.2 Evaluation of Controls for Transient Transfection

After observing the inhibitory response of CAT-3 plasmid in response to LPS compared to mock transfected cells, further transfections were performed to evaluate mock transfection as a control. A second transfection was performed using the mock transfection as one control and a transient transfection with plasmid containing a non-transporter gene (chloramphenicol-acetyltransferase) as a plasmid control. CAT-3 transfection was repeated here as a sample experimental treatment shown to exhibit sub-maximal NO production.

After 6 h, no effect of LPS was observed ($P < 0.05$), but all plasmid transfected cells showed the same increased NO response over the non-plasmid controls ($P < 0.05$; Figure 6A). The non-plasmid control and CAT-3 treatment showed similar NO production in response to LPS at 12 hours (Figure 8B). The control plasmid transfected cells and non-LPS treated transfected cells all produced similar amounts of NO ($P > 0.05$). At 24 h, the non-plasmid control produced the highest concentration of NO at $93.2 \mu\text{M}$ in response to LPS ($P < 0.05$; Figure 6C). The cells over-expressing CAT-3 in the presence of LPS produced more NO ($P < 0.05$) than the plasmid control cells with LPS or non-LPS treated plasmid transfected cells, which all produced similar quantities of NO ($P > 0.05$). At 36 and 48 h, the non-plasmid transfected cells in the presence of LPS produced the highest NO concentration ($P < 0.05$) with over-expressed CAT-3 cells producing the 2nd highest concentration in response to LPS ($P < 0.05$; Figure 6D-E). From 36 h onward CAT-3 transfected cells not treated with LPS produced more NO than the plasmid control ($P < 0.05$), which produced the same quantity of NO regardless of LPS treatment ($P > 0.05$).

Figure 7. Nitric oxide production at 6h (A), 12h (B), 24h (C), 36h (D) and 48h (E) by HD11 cells (n=8; 4×10^5 cells/well) transfected with no plasmid, control plasmid and CAT-3 plasmid. Means not sharing a common superscript (a-e) differ ($P < 0.05$). Values represent the mean $\pm$ SEM (n=8).

2.12.3 Transient Transfection

In order to evaluate effect of over-expression of the CATs and to account for the effect of plasmid-LPS interaction, another transfection was performed. The transfection was performed using both mock transfected and plasmid controls alongside CAT-1, CAT-2A, CAT-3 and CAT-1/2A/3. After 6 h, no effect of LPS was observed ($P>0.05$), but CAT-1 and CAT-1/2A/3 plasmid treatments produced higher concentrations of NO than other transiently transfected cells (Figure 7A). Over-expressing CAT-3 only resulted in moderate amounts of NO production while CAT-2A alone produced the same amount of NO as the plasmid control at 6 h. At 12 h post LPS incubation NO production was observed in mock transfection controls with LPS at higher concentration than in either plasmid controls with LPS or CAT-2A over-expressed cells with LPS ($P<0.05$; Figure 7B). From 12 to 48 h post LPS treatment, over-expressed CAT-1 with LPS produced maximal NO ($P<0.05$; Figure 7B-E). Until 36 h post LPS treatment, the CAT-1/2A/3 treated cells produced more NO in response to LPS than mock transfected controls, but no difference existed after 48 h (Figure 7E). In non-LPS treated cells, over-expression of CAT-1 produced the highest concentration of NO with CAT-1/2A/3 producing slightly lower amounts between 24 and 48 h ($P<0.05$; Figure 7C-E). Mock transfected cells in the presence of LPS equaled NO production from CAT-1/2A/3 without LPS by 24 h ($P>0.05$; Figure 7C), equaled CAT-1 without LPS by 36 h ($P>0.05$; Figure 7D) and exceeded all non-LPS treated combinations by 48 h ($P<0.05$; Figure 7E). Both CAT-2A and the plasmid control induced low amounts of NO production in the absence of LPS at all time points, but in the presence of LPS small increases in NO production were observed ($P<0.05$).

Figure 8. Nitric oxide production at 6h (A), 12h (B), 24h (C), 36h (D) and 48h (E) by HD11 cells (4×10^5 cells/well) transfected with no plasmid, control plasmid, CAT-1, CAT-2A, CAT-3 and CAT-1/CAT-2A/CAT-3 plasmids. Means not sharing a common superscript (a-j) differ ($P < 0.05$). Values represent the mean $\pm$ SEM ($n=8$).

2.12.4 Transfection Verification

After transfecting with dual controls and the full spectrum of CATs, transfected cells were analyzed by flow cytometry to evaluate the potentially detrimental effect of lipofectamine transfection and LPS. Transfected HD11 cell viability did not differ due to plasmid contents or lack of plasmid ($P=0.66$) or LPS treatment ($P=0.13$), though a numerical decrease was observed in treatments with increased quantities of plasmid (Table 4). The total number of cells recovered did not differ by transfection treatment ($P=0.81$) or LPS ($P=0.85$).

The transfected cells were analyzed for successful transfection by V5-epitope staining. The number of the successfully transfected cells producing V5-tagged proteins did not differ by plasmid content ($P>0.05$) or LPS treatment ($P=0.94$) and mock transfection resulted in no evidence of V5-positive transfection product ($P<0.05$).

Table 4. Recovered viable, v5-positive stained and original cells per well from transiently transfected HD11 cells identified by flow cytometry¹

	LPS	Viable Cells²	v5 Positive Cells³	Cells/well
Mock Transfected	0 µg/mL	950±12.9	18±2.8 ^a	3.34±0.15x10 ⁵
	1 µg/mL	969±6.9	26±4.6 ^a	3.34±0.25x10 ⁵
Plasmid Control	0 µg/mL	959±6.9	1652±12.0 ^b	3.36±0.78x10 ⁵
	1 µg/mL	929±12.5	1602±21.6 ^b	3.23±0.16x10 ⁵
CAT-1	0 µg/mL	932±10.5	1607±18.1 ^b	3.22±0.14x10 ⁵
	1 µg/mL	935±27.1	1612±46.7 ^b	3.27±0.26x10 ⁵
CAT-2A	0 µg/mL	938±16.2	1616±27.9 ^b	3.17±0.85x10 ⁵
	1 µg/mL	923±22.0	1591±38.0 ^b	3.26±0.24x10 ⁵
CAT-3	0 µg/mL	927±9.0	1597±15.5 ^b	3.19±0.11x10 ⁵
	1 µg/mL	905±15.3	1559±26.4 ^b	3.16±0.13x10 ⁵
CAT-1/2A/3	0 µg/mL	890±15.7	1534±27.1 ^b	3.03±0.18x10 ⁵
	1 µg/mL	898±21.3	1548±36.7 ^b	3.17±0.23x10 ⁵

¹Means not sharing a common superscript differ (P<0.05), Values represent the mean±SEM (n=4)

²Viable cells per 1000 events

³v5 positive cells per 5000 events

Discussion

The transporters coordinating ARG uptake in the chicken macrophage for a NO mediated immune response have not been fully elucidated. Previous studies indicate that the avian mechanism differs from the mammalian system which relies on CAT-2B because the chicken CAT-2B protein does not transport ARG [20, 22]. These experiments provide evidence that in the absence of a CAT-2B based system, CAT-1 and CAT-3 both act in support of an oxidative burst in the chicken macrophage.

In general chickens are relatively insensitive to LPS, a dose of 1 mg/kg may be required to induce an inflammatory response whereas in humans dosages as low as 2-4 pg/kg can induce an immune response [30]. In production animals, an immune response can be detrimental to weight gain, feed intake and protein accretion, increasing the cost of production [31]. As a result, chicken breeding in favor of production traits has led to animals which are relatively insensitive to LPS [30]. The short generation cycle and human-centered selected is in contrast to humans, where survival is the dominant pressure is survival. These differences in sensitivity and inflammatory response correlate with critical differences in ARG utilization by macrophages of uricotelic species, like chickens, and ureotelic species like mice and humans.

The ARG capable importers show a similar pattern of increase and induction in the broiler and layer macrophage. These mRNA abundance similarities exist despite the confounding effects of age and sex of the birds. This pattern indicates a conserved mechanism for obtaining extracellular ARG for NO production. The high affinity ARG importer CAT-1 was induced in primary macrophages and the immortalized HD-11 macrophage cell line. A second high affinity ARG importer, CAT-3, was increased in primary macrophages and induced in cell line

macrophages. While CAT-2A also increased, it is a low affinity ARG transporter and in mammals is insufficient to increase constitutive NO production [22]. The absence of detectable CAT-2B in these experiments support earlier work showing CAT-2B- not being involved in NO production as it is in mammals [20, 22]. This makes the chicken macrophage a unique model rendering mammalian-based predictions on ARG utilization for NO production tenuous at best.

CAT-1 and CAT-3 are capable of sustaining constitutive NO production when transiently induced in cultured macrophages. Over-expressing CAT-1, but not CAT-3, during a LPS-induced respiratory burst produced maximal NO. However, CAT-3 has also been shown to restore NO production in CAT-1 deficient cells [32]. CAT-2A over-expression increased NO by 34% in response to LPS at 48h, a marginal increase compared to CAT-1, a 1045% increase, or CAT-3, a 450% increase. These data indicate that CAT-1 is the dominant ARG importer for NO production in the chicken macrophage, but that CAT-3 is also sufficient for a NO response. The capacity of these transporters to facilitate a NO response without an immunogen indicates that the limiting factor in immortalized chicken macrophages is not iNOS but the availability of catalytic substrate ARG.

The y^+LAT system of exporters showed an inverse relationship with the magnitude of NO produced in broiler and layer macrophages. Decreased levels of y^+LAT -1 in the HD11 macrophage in response to LPS were observed in conditions that resulted in high levels of NO production ($\sim 100\mu M$). The converse was observed in macrophages from both broiler and layer chickens where y^+LAT -2 increased and maximal levels of NO were ~ 10 -25% of the maximal production observed in HD11s. The increase in y^+LAT 2 may enable ARG export as shown in erythrocytes and fibroblasts but not in other tissues [33, 34]. This increase in y^+LAT 2 may be preparing the cell to down-regulate the NO response after pathogen clearance. Despite the

correlation between the γ^+ LAT type exporter mRNA abundance and NO response, the effect of increased ARG importers by transient transfection indicates importers and not exporters are limiting for NO production. These magnitude differences concur with other studies showing that transformed cell line macrophages are more responsive to LPS than primary macrophages of varying genetic lineage [35].

The relative change in iNOS mRNA abundance showed a positive relationship with the magnitude of the NO response. The enzyme iNOS is typically thought of as an inducible enzyme. The presence of iNOS mRNA without stimulation and the NO response at 6h when the cells were transfected with CAT containing plasmid indicates a constitutive presence of the enzyme. Utilizing siRNA in the HD11 cell line, iNOS mRNA abundance can be reduced as much as 50-fold, but the decrease in NO production ranges 14-28% at 48 h [36]. These data indicate post-transcriptional control, such as substrate availability, in the respiratory burst mechanism of chicken macrophages. The maximal NO concentrations observed were limited by intracellular ARG availability, ARG uptake, or iNOS abundance rather than ARG in the culture medium. ARG in the medium was present at a concentration approximately 4-fold more abundant than the amount utilized and double the 200 μ M threshold at which increased ARG increases NO production [16]. As such, the culture media ARG concentration was not a pertinent factor in observed NO response to LPS.

While genetic similarities between production animals exist despite the confounding factors of age and sex, the magnitude of their NO response was greater than 2-fold different by 72h. The sensitivity of aging individuals and sex-related differences occur across species [37-40]. Senescent mice show higher sensitivity to LPS and have increased NO production mediated by TNF- α [40]. Young mice are less sensitive than senescent animals, but more responsive to

LPS than mature mice [40]. While TNF- α was not investigated in this study, the LPS sensitivity and NO production trend would be similar if we compared young and senescent birds, but is inverted when comparing young and mature birds. Studies in humans show that females have a stronger pro-inflammatory response to LPS than males via increased IL-6 and IL-10 [39]. In female mice, macrophages produce as much as 50% more NO in response to an immune challenge [38]. Male-Female differences have been attributed to sex hormones, notably estradiol, differences in cytokine production and differences in acute phase proteins [37-39]. Female mice produce higher levels of pro-inflammatory cytokines TNF- α , IL-1 β and IL-6 in response to LPS [37, 38]. Serum hormone levels, acute phase proteins and cytokine production were not investigated in these experiments but the trend shows an increased sensitivity to LPS and NO production in female chickens when compared with males. Thus the increases in NO production by older, female chickens are explicable and likely due to parameters beyond the scope of these experiments.

Increasing the abundance of all importers was sufficient for constitutive NO production, but did not to increase LPS-induced NO production. These data indicate that increasing the quantity of plasmid beyond 0.67 $\mu\text{g/mL}$ does not increase the number of recoverable cells positive for V5 epitope tagged protein(s). The amount of V5 tagged protein was not quantified, thus it is possible that increased plasmid can increase protein production or increased plasmid splits the transfection result between the different plasmids. The C-terminus of the transporters containing the V5 epitope are intracellular, but extracellular domains may be degraded by the trypsin solution used to detach the cells from their culture environment [25]. These partially degraded proteins may not be accurately represented by α -V5 epitope antibody tagging. Transfecting with non-transporter containing plasmids resulted in minor amounts of NO

indicating that any plasmid, regardless of contents, may be recognized and induce a separate immune response through TLR9 [41]. A TLR9 mediated response would antagonize the TLR4 response stimulated by LPS by interfering with the MAPKs and NF- κ B signaling pathways [42]. Transfection with foreign sources of DNA or RNA has been shown to silence host transcription and translation [43]. As such, the transient transfections may resemble sole expression of transporter in question and not over-expression, hence why transfection with the all three CATs results in an LPS induced response similar to mock transfection.

These experiments indicate that the respiratory burst mechanism in the chicken macrophage can be mediated by CAT-1 or CAT-3. A maximal NO response is mediated by over-expressing CAT-1 and thus may provide a target for evaluating genetic differences in chicken strains. Selective breeding of chickens for divergent purposes to maximize production capacity has not altered the pattern of CAT changes between breeds observed in a NO response to LPS, but instead shows indications of altering the magnitude of the response. Gram-negative bacteria such as *Salmonella* pose significant financial risk to the poultry industry, especially in flocks of layers. If increases in CAT-1 or CAT-3 can be correlated with increased pathogen clearance by chicken macrophages, they could prove valuable targets for selective breeding.

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CHAPTER 3

Conclusions

5.1 Summary

CAT-1 and CAT-3 are both sufficient to provide for sustained NO production in the absence of an immunogen. CAT-1 is induced in response to a simulated bacterial infection and provides for a maximal NO response when over-expressed. These results indicate that respiratory burst mechanisms in aves are mediated by multiple import-export systems unlike mammals that depend on a single importer.

5.2 Future Studies

To further elucidate the role of each transporter in the complete NO response additional trials need to be conducted using knock-out cells or siRNA to inhibit the importers. Additionally, the biological significance of increasing or decreasing the magnitude of the respiratory burst should be evaluated. The phagocytic and bactericidal effect of differing levels of NO production would show the animal level value in eradicating infections from pathogens like *Escherichia coli* and *Salmonella* spp. While an increased response might increase pathogen clearance, to dramatic an increase in NO production can also cause oxidative damage to the host animal.

To further explore the issue of male-female and age related confounding factors, the mRNA quantification should be replicated in small-scale age matched animals. Young sexed broilers are available and could be raised concordantly with replacement layers and roosters to suss out these interactions.

Arginase II has been shown to regulate pathogenicity of *Salmonella* in mouse macrophages by altering the fate of ARG [1]. Efforts to identify and sequence either arginase I or II in the chicken macrophage have thus far been unsuccessful (data not shown).

5.3 Implications

These results show the potential to identify on a breed and/or gender basis the predicted animal sensitivity and responsiveness to gram-negative bacterial challenges. While increased innate immune activity may increase bird survival, it also negatively impacts performance. Thus the task of identifying optimal immune responsiveness may remain a distant goal.

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APPENDIX

Figure 9. Representative image of ViaCount of HD11 cells on EasyCytePlus Flow cytometer.¹

¹Abbreviations: FSC, forward scatter.

Figure 10. Representative image of KUL01-PE of peripheral blood mononuclear cells on EasyCytePlus Flow cytometer.^{1,2}

¹Grey indicates KUL01-PE stained cells; black indicates cells not stained with KUL01.

²Abbreviations: KUL01-PE, macrophage specific antibody conjugated to R-phycoerythrin.

Figure 11. Representative V5-FITC staining image of transiently transfected HD11 cells on EasyCytePlus Flow cytometer. ^{1,2}

¹Grey indicates V5-FITC stained cells; black indicates cells not stained with V5.

²Abbreviations: V5, transfect target epitope specific antibody conjugated to fluorescein.