ABSTRACT

Title of Document: ENERGY METABOLISM IN DEVELOPING CHICKEN LYMPHOCYTES DURING THE EMBRYONIC TO POSTHATCH TRANSITION

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In chickens, the primary energy substrate is lipid during embryogenesis and carbohydrate after hatch. Accordingly, chicks adapt their metabolism to utilize glucose after hatch; however, little is known about metabolic adaptation in developing lymphocytes. Therefore, the objective of this dissertation was to examine metabolic adaptation in developing lymphocytes and the associated impact on their development. The first objective examined energy substrate utilization in bursacytes and thymocytes during the embryonic to posthatch transition. Glucose metabolism increased in both lymphocyte populations during the first two weeks posthatch due to increased glucose transporter-3 mRNA abundance, glucose uptake and hexokinase activity. Additionally, some of these metabolic markers were positively correlated with the serum glucose concentration. Glutamine metabolism increased in bursacytes only, and lipid metabolism was unaltered in both populations. Collectively, glucose is a preferred energy substrate for lymphocytes

posthatch, and glucose utilization by developing lymphocytes may be related to the serum glucose concentration. The second objective determined the effect of glucose availability on thymocyte metabolism, energy status and survival. Embryonic thymic lobes were grown in culture in media containing varying glucose concentrations. Thymocyte glucose metabolism and mitochondria membrane potential were highest in 15 mM glucose and apoptosis was highest in 5mM glucose. Collectively, glucose availability regulates glucose metabolism in thymocytes, and these changes in glucose metabolism were related to thymocyte energy status and survival. The third objective determined the effect of glucose availability on T cell development. Thymocyte Interleukin-7Rα (IL-7Rα) mRNA abundance and $CD4^+$ T cell numbers over the culture period were dependent upon glucose availability. Between 12 and 24 h, thymocyte IL-7Rα mRNA abundance increased in 5 mM increased 1.74-fold, while it decreased in 15 mM by 58.6%. $CD4^+$ numbers decreased with time in 5 mM, whereas they increased with time in 15 mM. T cell receptor (TCR) β excision circles were higher in 15 mM compared to 5 mM at 12 h. Glucose availability alters TCR β rearrangement, IL-7Rα gene expression and $CD4^+$ T cell development, which may influence naïve T cell generation. As thymocytes develop in a low glucose environment *in ovo*, this may be one factor that limits T cell development until hatch.

ENERGY METABOLISM IN DEVELOPING CHICKEN LYMPHOCYTES DURING THE EMBRYONIC TO POSTHATCH TRANSITION.

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Table of Contents

List of Tables

List of Figures

INTRODUCTION

In chickens (*Gallus gallus domesticus*), the primary macronutrient supply is altered from lipids to carbohydrates during the embryonic to posthatch transition. The avian embryo derives energy from egg nutrients that are high in protein (48%) and lipid (46%), and low in carbohydrate (4%). At hatch, chicks are fed seed-based diets that are high in carbohydrate (60%) and low in lipid (6%). Consequently, developing chicks adapt their energy metabolism to the altered dietary macronutrient supply during the embryonic to posthatch transition (Hamdy et al., 1991; McKee et al., 1997). It is well established that several developing tissues adapt their metabolism to utilize carbohydrates as the major energy source and this metabolic transition occurs during the first week posthatch (Humphrey et al., 2004; Noy and Sklan, 1996; Noy and Sklan, 1999; Sklan and Noy, 2000; Zhang and Hillgartner, 2004). However, not much is known about metabolic adaptations in developing immune tissues.

In chickens, lymphocyte development occurs during the embryonic to posthatch transition which involves metabolic adaptation to altered macronutrient supply. T cell progenitors enter the thymus in three waves during embryonic life (e6.5, e12 and e18). T cell progenitors from each wave undergo a period of extensive proliferation within the thymic cortex that lasts up to three weeks (e7 - day7 posthatch, e12 - d12, and e18- d18) (Cooper et al., 1991). These waves give rise to three distinct populations of T cells, which express $\gamma\delta$, $\alpha\beta_1$ and $\alpha\beta_2$ T cell receptor (TCR), respectively. In contrast, commitment to the B cell lineage and expansion occurs only during embryogenesis (e15-21) and the first few weeks posthatch (Ratcliffe et al., 1996). Thus, the antigen-independent proliferative

phase of B and T lymphocyte development coincides with the change in macronutrient supply associated with the embryonic to posthatch transition.

Lymphocyte development in primary immune tissues is integrated with their bioenergetic status. Developing lymphocytes proliferate extensively in primary immune tissues. Proliferation requires ATP and intermediary metabolites of energy metabolism that serve as precursors for macromolecule biosynthesis (Ardawi and Newsholme, 1985; Buttgereit and Brand, 1995; Buttgereit et al., 1992; Greiner et al., 1994). To meet ATP and precursor needs of biosynthesis, proliferating lymphocytes must coordinate their nutrient metabolism (Bauer et al., 2004; Ciofani and Zuniga-Pflucker, 2005; Fox et al., 2005; Frauwirth and Thompson, 2004; Vander Heiden et al., 2001; Yu et al., 2003). Thus, the development of lymphocytes in primary immune tissues is tightly coupled to their ability to metabolize energy substrates. The ability of developing lymphocytes to coordinate their metabolism during the embryonic to posthatch transition has not been studied. Hence, the overall objective was to determine the metabolic adaptation in developing lymphocytes during the embryonic to posthatch transition and the associated impact on their development.

The rationale for conducting this research project was that understanding metabolic adaptation in developing lymphocytes during the embryonic to posthatch transition may provide clues to advance the establishment of the peripheral lymphocyte pool that can recognize a broad-array of antigens. Therefore, the following objectives explored the possibilities to advance lymphocyte development during the period surrounding hatch, which has practical relevance to the poultry industry to enhance the efficacy of in-ovo vaccines and to prevent early mortality due to infectious diseases.

The first objective was to determine whether or not developing lymphocytes adapt their energy metabolism during the embryonic to posthatch transition. The hypothesis for this objective was that the embryonic to posthatch transition induces changes in energy substrate utilization in developing lymphocytes. This was tested by measuring the expression of genes and the activity of enzymes that are involved in glucose, glutamine and fatty acid metabolism to meet cellular energy and substrate needs for biosynthesis.

The second objective was to determine the effects of glucose availability on thymocyte metabolism, energy status and survival. The hypothesis for this objective was that glucose availability induces changes in thymocyte metabolism, which alters thymocyte energy status and survival. To study this we used thymic organ culture as a model because it supports lymphocyte development in vitro and allows for manipulation of the glucose concentration in the culture media. This hypothesis was tested by measuring glucose transporter gene expression, glucose uptake and hexokinase enzyme activity as markers of glucose metabolism, mitochondrial membrane potential as a marker of energy status and phosphatidyl serine exposure on the cell surface as a marker of apoptosis.

The third objective was to determine the effect of glucose availability on the ability of developing thymocytes to differentiate in the thymic microenvironment. The hypothesis was that altered glucose availability induced changes in thymocyte energy status affect their ability to develop in the embryonic thymus. This was tested by measuring the expression of IL-7R α receptor and TCR rearrangement, which guide T cell differentiation in the thymus, and by profiling T cell populations based on $CD4^+$ and $CD8^+$ expression.

CHAPTER 1

LITERATURE REVIEW

A. MACRONUTRIENT METABOLISM IN THE DEVELOPING EMBRYO AND POSTHATCH CHICK.

1. Introduction

The development of the avian embryo is dependent upon the supply of adequate nutrient stores within the egg at the time of lay. The macronutrient composition of an egg at the time of lay consists of 12-13% protein, 10-11% fat and low amounts of carbohydrate (1%). These nutrient stores are rapidly mobilized to support the rapid rate of chick growth during embryogenesis. Accordingly, the decrease in egg nutrient stores is accompanied by an increase in the type and size of tissues within the chick (**Figure 1.1**). Furthermore, the embryonic tissues adapt metabolically to changing macronutrient supply during embryogenesis.

2. Macronutrient metabolism in the developing avian embryo

a) LIPID METABOLISM

The yolk sac membrane (YSM) distributes yolk lipids to the developing chicken embryo. The YSM surrounds the egg yolk early in embryogenesis. YSM endodermal cells engulf and package yolk lipid into lipoproteins for secretion into the embryonic circulation. The total quantity of yolk lipid transferred to embryonic tissues is low (7-8 % of total lipids) during the first two weeks of embryogenesis and is maximum (67 % of total lipids) during the last few days of embryogenesis (**Figure 1.2**) (Noble and Cocchi, 1990; Noble et al., 1986; Speake et al., 1998).

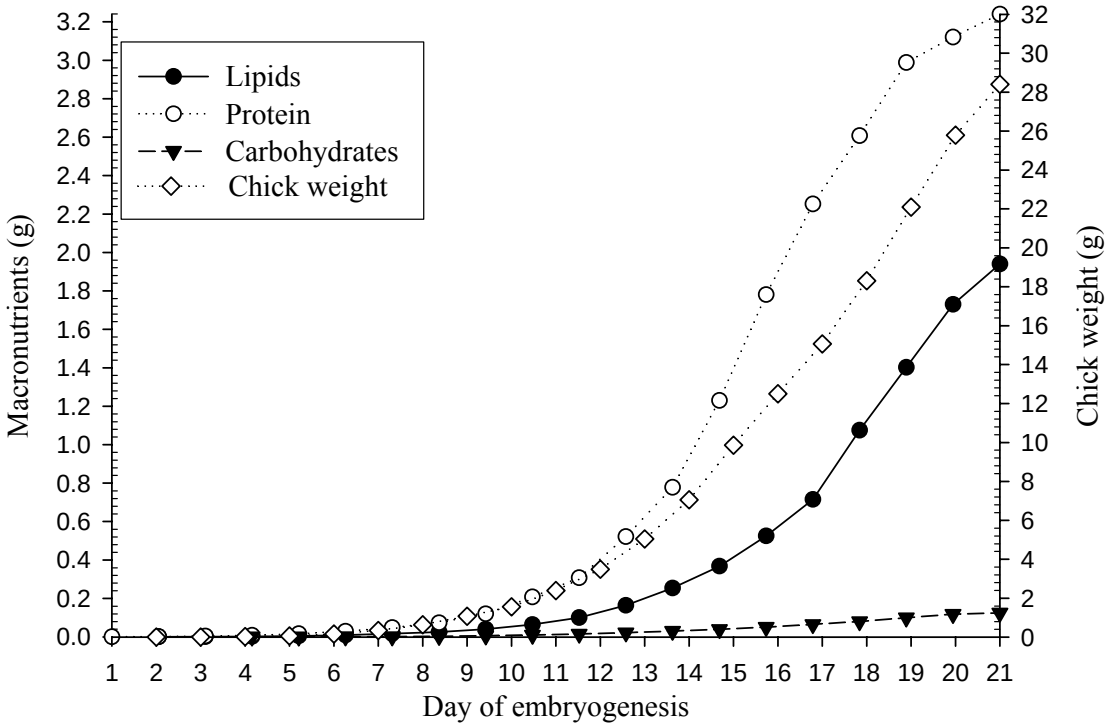

Figure 1.1 A comparision between macronutrient acquisition by the avian embryo and its weight during embryogenesis. Data adapted from Romanoff (1967).

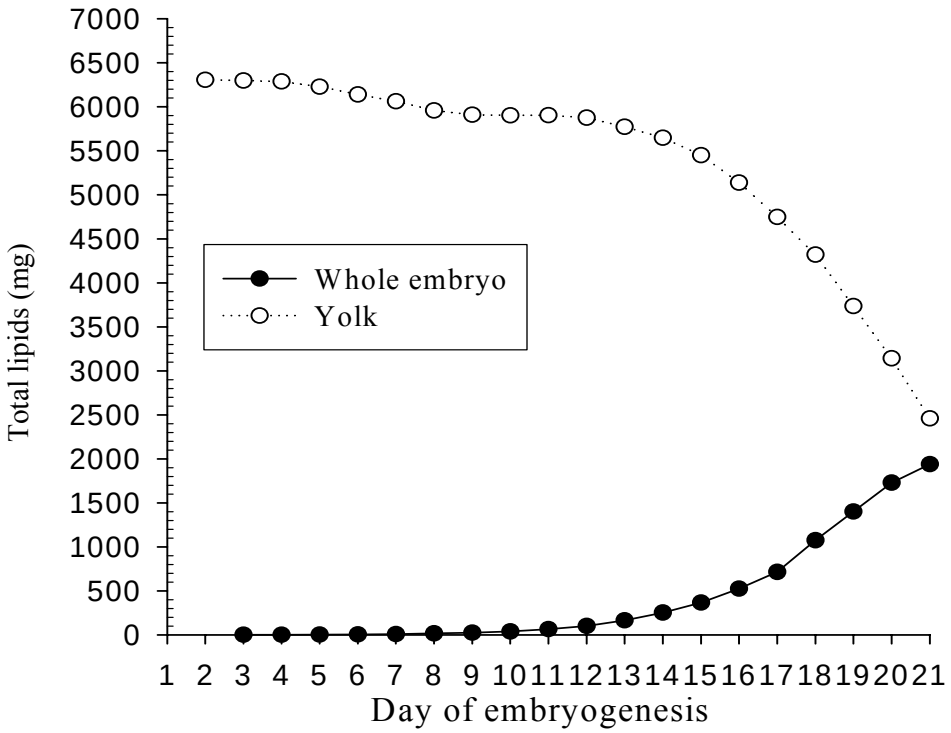

Figure 1.2 Lipid mobilization from the yolk to the developing avian embryo. Data adapted from Romanoff (1967).

Lipoproteins that enter the embryonic circulation are modified by lipoprotein lipase (LPL) to distribute fatty acids to the developing tissues. Fatty acids from yolk lipids are extensively deposited in the adipose tissue of the embryo and to a lesser extent in the heart, muscle, liver and brain tissues of the developing embryo (Speake et al., 1993). It is likely that adipose stores in the embryo serve as a fatty acid source for energy in the developing embryonic tissues. Fatty acids are oxidized in the embryonic tissues, such as heart, muscle, liver and brain, to provide 90% of the energy needs of the developing chick (Noble and Cocchi, 1990; Pugh and Sidbury, 1971).

b) CARBOHYDRATE METABOLISM

Carbohydrate stores in the egg contribute little to the energy needs of the developing chick relative to lipid (~10 kJ from glucose vs. ~250 kJ from lipid in an egg weighing 60g). Glucose stores in the egg are primarily oxidized to CO_2 via the tricarboxylic acid (TCA) cycle since the activity of this pathway increases throughout embryogenesis. Glucose flux through the pentose phosphate pathway occurs early in embryogenesis and declines from E15 to hatch (Pearce and Brown, 1971).

The embryonic tissues differ in their ability to acquire glucose as shown by differential expression of facilitative glucose transporters (Glut). Glut-1 is expressed in brain, heart, muscle and liver, whereas Glut-3 is expressed in brain only (Carver et al., 2001).

The low abundance of carbohydrate reserves in the egg and the rapid oxidation of glucose during the first week of embryogenesis require glucose to be synthesized from other substrates. The liver and kidney are the two primary tissues in the chick involved

in gluconeogenesis. These tissues differ in the substrates used for gluconeogenesis due to differences in the location of phosphoenolpyruvate carboxykinase. The two rate-limiting gluconeogenic enzymes, pyruvate carboxylase and phosphoenolpyruvate carboxykinase, increase activity in the liver and the kidney during the second week of embryogenesis to provide glucose to developing tissues (Pearce and Brown, 1971). Despite the liver being gluconeogenic, this tissue also utilizes glucose to synthesize glycogen and to increase hepatic glycogen reserves.

The developing embryo is subjected to hypoxic stress associated with the onset of pulmonary respiration during E17-18 (**Figure 1.3)** (Romanoff, 1967; Vleck and Vleck, 1987). A limited supply of oxygen requires the embryo to utilize the non-oxidative glycolytic pathway. This also occurs at hatching since skeletal muscle activity associated with eggshell pipping increases the rates of non-oxidative metabolism. This indicates that there is a period surrounding hatch where developing chicks are heavily dependent upon glucose as an energy source since energy derived from lipid and amino acid is oxidative. The hepatic glycogen stores serve as a source of glucose for developing tissues and the amount of liver glycogen decreases approaching hatch (Freeman, 1965).

c) AMINO ACID METABOLISM

Proteins, along with lipids, form one of the largest macronutrient stores in the chicken egg at lay. Unlike lipids, an equal quantity of protein is found in both egg yolk and albumen. Most amino acids in these egg stores are used for protein synthesis, while some amino acids are also used as gluconeogenic substrates. Amino acid stores in egg

albumen are almost completely utilized by E18, while amino acids in yolk are utilized up to several days following hatch.

3. Macronutrient Metabolism in the posthatch chick

a) CHANGE IN NUTRIENT SUPPLY

The egg yolk and albumen stores have adapted the developing chick to lipid and protein substrates, respectively. After hatching, however, chicks are fed a seed-based diet that is relatively low in lipid (6%) and high in protein (22%) and carbohydrate (60%). Consequently, the embryonic to posthatch transition requires metabolic adaptations due to the sharp contrasts in the dietary macronutrient composition.

4. Nutrient metabolism posthatch

a) LIPID METABOLISM

The internalized yolk remnant comprises 20% of hatchling body weight at hatch and mainly consists of fatty acids (33%) and proteins (60%) (Romanoff, 1967). Yolk lipid is mobilized from the yolk remnant and deposited into either the circulation or intestinal lumen for absorption (Noy and Sklan, 1996). Yolk lipids continue to serve as the major energy source immediately posthatch and are completely depleted by 8 days posthatch (**Figure 1.4**). Fatty acid absorption is high (80%) at hatch and remains unchanged with age (Noy et al., 1996).

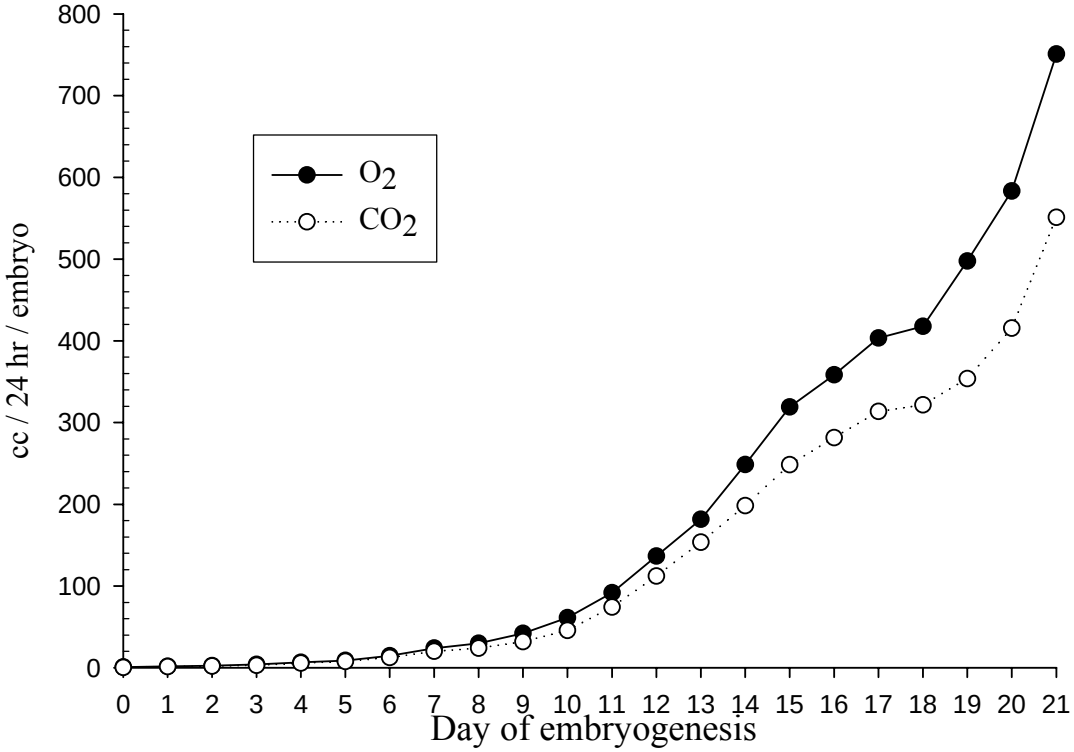

Figure 1.3 Oxygen and carbon dioxide exchange in the developing avian embryo. Data adapted from Romanoff (1967).

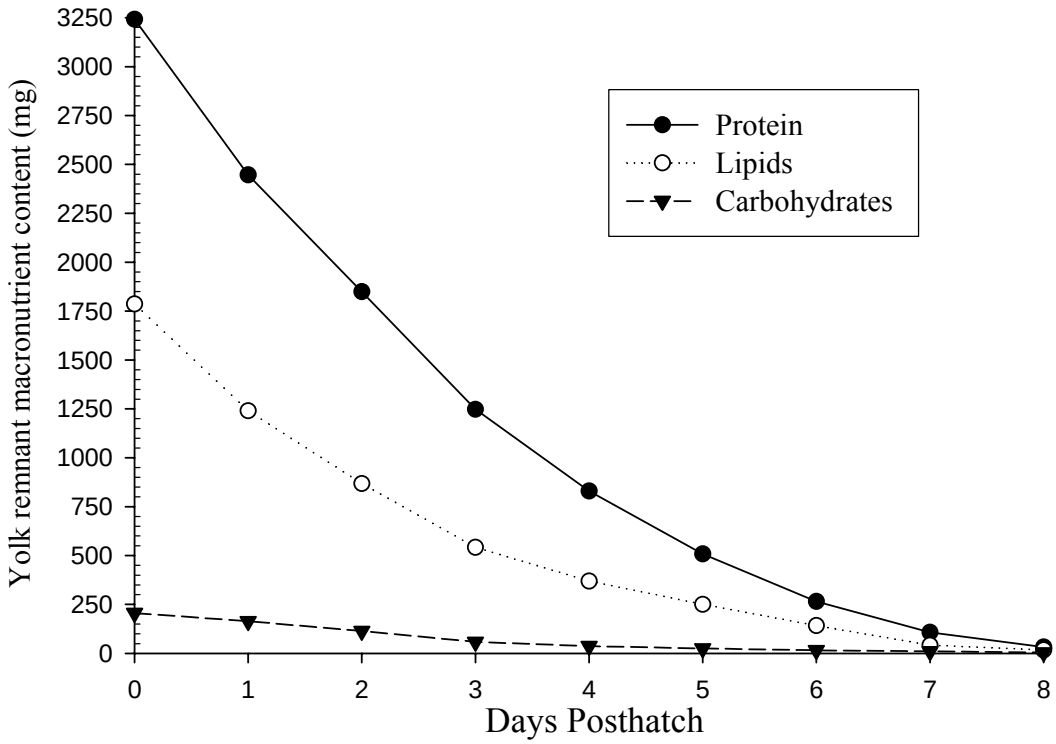

Figure 1.4. The change in macronutrient content of the yolk remnant following hatch. Data adapted from Romanoff (1967).

b) CARBOHYDRATE METABOLISM

Carbohydrate absorption is low at hatch (<50%) due to continued mobilization of lipid from the yolk remnant. Secretion of yolk lipid into the intestinal lumen creates a hydrophobic environment that is unfavorable for glucose absorption (Sklan and Noy, 2000). However, glucose absorption is 59% by four days posthatch and is maximum (80%) by one week posthatch (Noy and Sklan, 1999; Noy and Sklan, 2001; Noy et al., 1996).

Glucose oxidation increases during the first week posthatch due to a steady increase in the activity of glycolytic (phosphofructokinase, pyruvate kinase and hexokinase) and TCA cycle (succinate dehyodrogenase) enzymes (Pearce and Brown, 1971). During the posthatch phase, tissues such as heart, liver, muscle and bursa express higher levels of glucose transporter (GLUT) on day 7 and 14 posthatch compared to day 1 posthatch (Humphrey et al., 2004). The respiratory quotient of hatchlings changes from 0.72-0.74 to 0.9-1.0 by nine day posthatch (Hamdy et al., 1991; McKee et al., 1997), which indicates a shift from fat oxidation to glucose oxidation. This suggests that posthatch chicks undergo metabolic adaptation to utilize glucose as the major energy source as yolk lipids are depleted in the first week posthatch.

c) AMINO ACID METABOLISM

In the early posthatch phase, yolk remnants also provide protein along with seed-based diets. The yolk contains ~3g protein, almost all of which is utilized by day eight posthatch (Figure 1.4). Similar to glucose, amino acid absorption is low (43%) at hatch, increases (58%) by four day posthatch and is maximum (80%) by one week posthatch

(Noy and Sklan, 1999). Reasons for the low uptake of amino acids in the immediate posthatch phase are similar to that of glucose.

5. Summary

Yolk lipid is the primary energy source for embryogenesis. Yolk lipid mobilization and oxidation is maximum during E15-19. Low glucose stores in the egg are exhausted early in embryogenesis. Consequently, rates of gluconeogenesis increase during embryogenesis and glucose is utilized as the main energy source to fuel the events of hatching. In posthatch chicks, yolk lipid is the primary energy source up to three days and glucose becomes the primary energy source around seven days posthatch.

B. ONTOGENY OF AVIAN LYMPHOCYTE DEVELOPMENT.

1. Introduction

The immune system defends the body against pathogens. The vertebrate immune system recognizes and eliminates antigens via innate and adaptive immune responses. The innate immune system provides protection during the early stages of infection, and the adaptive immune system provides long-term protection to the host through the generation of immunological memory. The adaptive immune system consists of antigen specific B and T lymphocytes that protect the host from pathogens. B and T lymphocytes develop within primary lymphoid tissues. T lymphocytes develop in the thymus, and in birds, B lymphocyte development occurs in the bursa. The development of lymphocytes within primary lymphoid tissues is essential for enabling the host to generate an immune response to a wide range of antigens.

2. B Lymphocyte development in the bursa

The bursa of Fabricius is a gut associated primary lymphoid tissue (GALT) which is colonized by lymphoid progenitor cells during embryogenesis and is essential for the normal progression of B lymphocyte development (Ratcliffe et al., 1996). B lymphocyte development in the bursa continues for several weeks posthatch until sexual maturity is reached. The bursa arises on embryonic day 5 (e5) as a dorsal diverticulum of the cloaca. The bursa is a compartmentalized organ consisting of an outer cortex and inner medulla and contains approximately 10,000 lymphoid follicles (Olah and Glick, 1978). Avian B cell development can be divided into three phases: (1) colonization of the bursa by

precursor cells committed to the B cell lineage, (2) gene conversion and clonal expansion, and (3) emigration of immature B cells from the bursa to the periphery.

a) Colonization of the bursa by precursor cells

Lymphoid progenitors committed to the B cell lineage, also known as prebursal stem cells, colonize the bursa over a span of one week beginning on E8 (Houssaint et al., 1976). Prebursal stem cells are restricted to the embryonic phase and are first located in the yolk sac, blood, spleen and bone marrow (Moore and Owen, 1967; Pink et al., 1987; Ratcliffe et al., 1986; Ratcliffe et al., 1996). Prior to entering the bursa, prebursal stem cells rearrange their immunoglobulin (Ig) genes and express surface Ig (sIg) on their plasma membrane. Only 40-50% of the prebursal stem cells productively rearrange their Ig genes to seed the bursa and the remaining prebursal cells die in the periphery (McCormack et al., 1989). Expression of sIg on the plasma membrane is necessary for prebursal stem cell entry into the bursa. A total of 20,000-40,000 prebursal stem cells enter the bursa and colonize epithelial buds and begin to rapidly proliferate. These clusters of proliferating cells form the estimated 10,000 lymphoid follicles of the bursa (Pink et al., 1985).

b) B lymphocyte gene conversion and clonal expansion

Prebursal stem cells that colonized bursal follicles begin to proliferate and generate Ig diversity through gene conversion. These cells are referred to as bursal

cells as defined by their anatomical location and ability to reconstitute lymphocyte numbers in bursae of B lymphocyte depleted chicks (Pink et al., 1987).

As mentioned previously, prebursal stem cell Ig genes have been rearranged prior to their entry into bursal follicles. These cells express B cell receptor (BCR) that consists of Ig heavy-chain (H) associated with invariant Igα and Igβ molecules, but this intermediate BCR generally lacks Ig light-chain (L) (Monroe, 2004). Since the chicken H and L loci have a single V (variable) and a single J (joining) gene, germline rearrangement of these genes with D (diversity) gene generates V(D)J segment with a limited diversity. The Ig repertoire is generated by diversifying H and L through a unique molecular mechanism called gene conversion. The rearranged H and L loci have a single functional V(D)J gene which is diversified in the bursa through somatic gene conversion using a pool of pseudogenes (~25-80) as donors (Maizels, 1987; Reynaud et al., 1987; Reynaud et al., 1992). This step is essential in the normal progression of B cell development in birds, as the generation of Ig repertoire occurs only in the bursa (Glick et al., 1957; Van Alten et al., 1968).

The proliferative events of bursal cells within bursal follicles are quite extensive. The two to four prebursal stem cells that originally colonized each bursal follicle by E8-15 proliferate to produce a total of 10^5 B cells per follicle. The bursa contains approximately 10^4 lymphoid follicles, so the proliferative events within each bursal follicle results in the total production of approximately 10^9 B cells (Pink et al., 1985). B cell numbers proliferating within the bursa double once a day between E17-E21 and following hatch (Ekino, 1993; Reynolds, 1987).

c) Emigration of B lymphocytes to the periphery

B cells begin to emigrate from the bursa to peripheral tissues a few days prior to hatch. These emigrating B cells represent a small percentage (5%) of the total proliferating B cells within the bursa. Most of the proliferating B cells (95%) undergo apoptosis due to unproductive Ig rearrangements (Lassila, 1989; Motyka and Reynolds, 1991). Recent bursal emigrants express high levels of surface IgM and major histocompatibility complex (MHC) class II on their plasma membrane (Paramithiotis and Ratcliffe, 1993). The emigration rate of immature B cells to the periphery in an hour is approximately 0.84 and 0.96% of the blood lymphocyte and splenic B cell pool, respectively (Paramithiotis and Ratcliffe, 1993). Recent B cell immigrants constitute 35% of the total peripheral blood B cell population. These B cells are not dividing and have a half-life of 12 days. The majority of peripheral blood B cells (60%) have a half-life of 30 hours. The remaining peripheral blood B cell pool (5%) is also short-lived and is continuously generated from postbursal stem cells (Paramithiotis and Ratcliffe, 1993). Postbursal stem cells are defined by their ability to restore functional immunity in B cell depleted hosts, however these cells are unable to reconstitute the bursa of B cell depleted hosts (Pink et al., 1987). Postbursal stem cells are predominantly found in the blood and spleen a few days prior to hatch, with few numbers present in the thymus, bone marrow and GALT.

3. T Lymphocyte development

a) Thymocyte precursors: Origin and thymus homing

Thymocyte precursors are derived from hematopoietic stem cells (HSC) located in the thoracic aorta. The HSC precursors emerge as early as E4 and immediately begin to colonize the spleen, yolk sac and bone marrow (Cooper et al., 1991). Thymocyte precursors colonize the thymus in three distinct waves beginning at E6.5, E12 and E18 (Le Douarin et al., 1984). Each wave lasts for one or two days and is orchestrated by thymic stromal cells via chemoattraction of thymocyte precursors (Coltey et al., 1989; Coltey et al., 1987).

b) T cell development in the thymus: repertoire generation and selection

The avian thymus consists of 5-7 lobes along each side of the neck. Each lobe is divided into multiple lobules consisting of an outer cortex and an inner medulla. T cell precursors that have homed to the thymus proliferate in the thymic cortex. A thymus lobe contains approximately $3x10^4$ thymocytes on E10, and these thymocytes proliferate more than 100-fold in each thymus lobe by E20 (Davidson et al., 1992).

Each successive wave of thymocyte precursors gives rise to a distinct population of T lymphocytes. The first thymocyte precursor wave gives rise to the T cell receptor 1 (TCR1) population of T cells, while the second and third thymocyte precursor waves give rise to TCR2 and TCR3 cells, respectively. TCR1 cells express the γ and δ-subunits of the TCR, whereas TCR2 and TCR3 cells express the α and β

TCR subunits. TCR2 and TCR3 differ in their β-isoform. TCR1, TCR2 and TCR3 proliferate in the cortex to become predominant subpopulations by E15, E17-18 and E20-21, respectively (Bucy et al., 1990; Cooper et al., 1991; George and Cooper, 1990).

The total number of TCR specificities available to the host is known as the T cell repertoire. Like the B cell repertoire, T cell diversity is generated during development within primary lymphoid tissue. This involves stromal cell-thymocyte interactions that induce the expansion of the TCR repertoire and the selection of T lymphocytes that are non-reactive to self. These gene rearrangement events occur in T cells expressing both CD4 and CD8 surface molecules (Bucy et al., 1990). The T cell repertoire is generated via V (D) J combinatorial and junctional diversification, unlike the gene conversion mechanism to generate antibody repertoire. T cell antigen receptors that react favorably with self ligands are selected for survival and begin to proliferate within the thymic cortex. These proliferating T cells express either $CD4^+$ or $CD8^+$ to become single-positive thymocytes. Developmental differences in T cells expressing either the αβ or γδ TCR have been demonstrated in mammals, however this comparison has not been extensively studied in birds (George and Cooper, 1990; Kisielow and von Boehmer, 1990).

Mature thymocytes exit into the periphery in the same order of their development and expansion in the thymus. The TCR1, TCR2 and TCR3 cells begin to migrate to spleen around E16, E19 and one day posthatch, respectively. T lymphocytes are also found proliferating in the bursa and intestine prior to hatching (Cooper et al., 1991).

4. Lymphocyte development in secondary lymphoid organs

a) Spleen

The spleen is the largest secondary lymphoid organ in chickens and is the major site of antigen processing and antibody production after hatching. B and T cells begin to seed the spleen from E12 and E16, respectively. αβ T cells are predominantly seen in germinal centers whereas γδ T cells are primarily located in the red pulp (Kon-Ogura et al., 1993). B cells are found in germinal centers and peri-ellipsoid lymphocyte sheaths of the spleen. Germinal centers are the site of proliferation of activated B cells in the spleen. B lymphocyte activation requires T cell-help in adaptive immune responses against protein antigens. Such T-dependent B cell activation and proliferation occurs in germinal centers (Toivanen et al., 1974), (Toivanen and Toivanen, 1977). The late events of T cell-dependent antibody responses, such as affinity maturation and B memory cell generation, occur in germinal centers.

b) Harderian gland

The harderian gland (HG) is a secondary lymphoid tissue concentrated in the oculonasal region of birds. B lymphocytes from the bursa seed the HG prior to hatching (Baba et al., 1988). These B cells terminally differentiate into plasma cells that primarily produce IgA under the HG microenvironment (Baba et al., 1990; Gallego and Glick, 1988). T lymphocytes are observed in the HG around one-week-

posthatch and their numbers increase until four-week-posthatch (Maslak and Reynolds, 1995).

c) Intestinal lymphoid tissue

The avian intestinal lymphoid tissue is characterized by diffuse mucosal lymphoid infiltration, which consists of cecal tonsils, Peyer's patches and Meckel's diverticulum. These lymphoid tissues have T cell zones, lymphoid follicles with germinal centers, and a distinct lymphoid-epithelium with microfold cells (Befus et al., 1980). A few days prior to hatching, B and T lymphocytes colonize the GALT to provide IgA and cell-mediated immunity, respectively. T lymphocytes are found in the epithelium and lamina propria of the intestine with γδ cells being found predominantly in the epithelial layer (Cooper et al., 1991).

5. Summary

B and T lymphocytes acquire the capacity to respond to different antigens by developing their repertoire within primary immune tissues. However, both B and T lymphocyte repertoire generation is inefficient and results in large-scale apoptosis due to unproductive gene rearrangements. Since such a small percentage of developing lymphocytes is released into the periphery, lymphocytes proliferate extensively while generating Ig repertoire. B and T lymphocyte proliferation is high during the embryonic development (E15-21 for B cell; E7-21 for T cell) and the first few weeks posthatch.

C. LYMPHOCYTE METABOLISM

1. Introduction

Lymphocytes provide adaptive immunity against diverse pathogens. This requires antigen specificity which is achieved via the generation of immunoglobulin (Ig) and T cell receptor repertoire in B and T lymphocytes, respectively. Lymphocytes generate their antigen specificity in primary immune tissues, and this process is characterized by rapid rates of lymphocyte proliferation. Lymphocyte proliferation involves coordinated metabolic events as these cells increase nutrient uptake and metabolism. Thus, the development of lymphocytes in primary immune tissues is tightly coupled to their ability to metabolize nutrients, and is regulated by cytokine and endocrine factors that control metabolic pathways.

2. Energetics of lymphocyte development and function

Lymphocyte activities such as proliferation, maintenance and effector functions require energy (**Table 1.1**). The most energy-consuming reactions in activated lymphocytes include macromolecule biosynthesis and transport processes, e.g. Na^+K^+-ATPase, Ca^{2+}-ATPase and H^+-ATPase (Brown, 1992; Buttgereit and Brand, 1995; Buttgereit et al., 1992). These ATP-consuming processes are crucial for lymphocyte development and function since energy depletion alters cytokine synthesis, cytokine receptor expression and affinity (Karlsson and Nassberger, 1992; Sanchez-Alcazar et al., 1995).

Table 1.1 Energy requiring processes associated with immune function (Buttgereit et al., 1992).

Function	ATP-consumers	Importance
Proliferation	Protein, DNA & RNA synthesis; Na^+K^+-ATPase; Ca^{2+}-ATPase	These process are associated with cell proliferation and secretory functions
Migration	Actomyosin-ATPase	ATP is required for cytoskeletal rearrangement via the actomyosin system
Antigen processing	H^+-ATPase	Requires ATP for proteasome degradation and H^+-ATPase to generate peptides in endocytic vesicles
Antigen presentation	ATPase	Transporter associated with antigen processing (TAP) is ATP dependent
Cytotoxicity	H^+-ATPase	Requires H^+-ATPase to maintain acidic pH in lytic vesicles

Lymphocytes metabolize glucose, glutamine and fatty acids to generate the energy required for their development and function. Like most cells, lymphocytes generate ATP via substrate level phosphorylation, i.e. glycolysis, and oxidative phosphorylation, i.e. tricarboxylic acid cycle (TCA). Resting lymphocytes use reducing equivalents from TCA to derive most of their energy via oxidative phosphorylation. Glucose, glutamine and fatty acid oxidation contribute approximately 40, 30 and 30% of total energy to resting lymphocytes, respectively. In activated lymphocytes glucose is the primary energy source (Ardawi and Newsholme, 1985).

3. Glucose metabolism in lymphocytes

Lymphocytes do not have large internal glycogen stores and therefore must constantly acquire extracellular glucose. Cellular glucose uptake and release is catalyzed by members of the SLC-2 family of glucose transporters (GLUT) (Uldry and Thorens, 2004). Glucose transport across the plasma membrane is the first regulatory step of

glucose utilization. Resting lymphocytes express low levels of GLUT-1 and GLUT-3 mRNA, however the mRNA levels of these isoforms increase following activation (Fu et al., 2004; Rathmell et al., 2000). The increase in GLUT isoforms in activated lymphocytes is presumably due to an increased demand for glucose.

Glucose is essential for proliferating lymphocytes (Greiner et al., 1994). Lymphocyte proliferation requires biosynthesis of nucleotides, proteins and membrane lipids. Rapid glycolysis is essential to provide precursors for nucleotide and membrane lipid synthesis. Additionally, glucose oxidation generates ATP via substrate-level phosphorylation which is necessary to maintain redox homeostasis in proliferating lymphocytes. Activated lymphocytes increase oxidation of glucose to lactate in a manner independent of oxygen availability, termed 'aerobic glycolysis' (Cooper et al., 1963; Culvenor and Weidemann, 1976). In activated lymphocytes, 86% of the glucose is converted to lactate via aerobic glycolysis (Brand and Hermfisse, 1997; Greiner et al., 1994). Increased rates of glucose flux to lactate in activated lymphocytes may maintain higher levels of glycolytic intermediates which serve as precursors for macromolecule synthesis (Hume et al., 1978). Furthermore, aerobic glycolysis aids in the regeneration of the cellular NAD^+ pool (Greiner et al., 1994) which is critical to maintain rapid flux of glucose via glycolysis. Finally, increased aerobic glycolysis reduces the synthesis of reactive oxygen species (ROS) which can result in increased oxidative damage and interfere with metabolism (Brand and Hermfisse, 1997). For example, enhanced ROS formation in lymphocytes inhibits glycolytic enzyme induction as well as proliferation (Aulwurm and Brand, 2000; Hamm-Kunzelmann et al., 1997). The formation of ROS varies with the type of substrate used as an energy source, and this is related to a

substrate's ability to generate reducing equivalents. Lipids are the most reduced energy substrate used by lymphocytes and therefore have the greatest potential to generate ROS. Amino acids are second to that of lipids, while glucose is the least, especially since lymphocytes employ aerobic glycolysis (Brand and Hermfisse, 1997). Therefore, metabolizing glucose to lactate is one strategy utilized by proliferating lymphocytes to reduce ROS production.

Lymphocytes metabolize glucose via the pentose phosphate pathway to generate pentose sugars and NADPH for nucleic acid and reductive biosynthesis, respectively. Glucose utilization by lymphocytes correlates with the activity of glucose-6-phosphate dehydrogenase (Pithon-Curi et al., 2004). Proliferating lymphocytes have a greater demand for pentose sugars and NADPH compared to resting lymphocytes. Lymphocyte activation results in maximal activity of the pentose phosphate pathway by 48 h post-mitogen stimulation, which coincides with the DNA synthesis in S phase of the cell cycle (Sagone et al., 1974).

4. Lipid metabolism in lymphocytes

Fatty acids (FA) are important energy substrates and are also required for many biological functions. Thus, FA entry into a cell needs to be highly regulated to ensure normal cellular function. Fatty acids cross the plasma membrane by diffusion and protein-mediated processes (Kalant and Cianflone, 2004). Several transporters are involved in FA uptake, including fatty acid translocase (FAT/CD36), plasma membrane fatty acid binding protein ($FABP_{pm}$) and the fatty acid transport proteins (FATP1 to FATP6) (Bonen et al., 2004; Kalant and Cianflone, 2004). The importance of these

transporters in FA uptake and their subsequent metabolism in lymphocytes has not been investigated.

Fatty acid binding proteins regulate the partitioning of FA towards β-oxidation. AcylCoA synthases are cytosolic FABPs that direct FA into β-oxidation for ATP production. A key step in β-oxidation is the transport of FA across the inner membrane of mitochondria, and is mediated by carnitine acyltransferase I (CAT-I). Once in the mitochondria, acylCoA dehydrogenase catalyzes the first step of β-oxidation. Compared to non-lymphoid cells, the activity of CAT-I in lymphocytes is low. In contrast to glucose and glutamine utilization, lymphocyte activation increases FA uptake but does not increase FA oxidation (Ardawi and Newsholme, 1985). Perhaps the increase in FA uptake by proliferating lymphocytes is related to the increase in plasma membrane lipid formation.

5. Glutamine metabolism in lymphocytes

Glutamine is conditionally essential for lymphocyte proliferation (Ardawi and Newsholme, 1983; Newsholme et al., 2003). Glutamine metabolism in proliferating lymphocytes is characterized by rapid glutamine uptake and glutaminolysis. The rapid breakdown of glutamine results in buildup of intracellular glutamate pool in proliferating cells. It is hypothesized that a glutamate gradient facilitates amino acid transport via the systems X^-_c, L and ASC (Aledo, 2004). Glutamine is transported across the plasma membrane by several transport systems with distinct substrate specificity and transport properties (**Table 1.2**).

Table 1.2 Glutamine transporters: transport mechanisms and specificity.

System	Protein	Gene	Mechanism	Notes
I. Neutral amino acid transporters: Sodium-dependent				
A	SAT1 SAT2	SLC38A1 SLC38A2	$1Na^+$/AA cotransport	Ubiquitous expression. Sensitive to low pH.
ASC	ASCT2	SLC1A5	Na^+-dependent antiporter	High-affinity AA exchanger. Ubiquitous expression.
B^0	ASCT2	SLC1A5	Na^+/AA cotransporter	Broad substrate specificity. Expressed on apical surface of many epithelium.
II. Neutral amino acid transporters: Sodium-independent				
L	LAT1 LAT2	SLC7A5 SLC7A8	Antiporter	Gln is a week substrate.
III. Neutral amino acid transporters: Sodium-dependent				
$B^{0,+}$	ATB(0,+)	SLC6A14	$2Na^+/1Cl^-$ /AA cotransporter	Broad specificity for neutral and cationic AA.
Y^+L	Y+LAT1 Y+LAT2	SLC7A7 SLC7A6	Na^+-dependent antiporter	Na^+-dependent cationic/neutral AA exchanger.
IV. Neutral amino acid transporters: Sodium-independent				
$B^{0,+}$	b(0,+)AT	SLC7A9	Antiporter	Broad specificity cationic/neutral AA exchanger.

The capacity of glutamine transport is augmented in lymphocytes stimulated by a mitogen (Piva et al., 1992). Systems ASC and L transport glutamine in proliferating cells (Bode et al., 2002; Wasa et al., 2002). System A maintains cytosolic levels of non-essential amino acids, such as glutamine (Broer et al., 2002). Bovine lymphocytes have system ASC and L activities (Piva et al., 1992). Therefore, the coordinated function of system ASC, L and A play an important role in the acquisition and the possession of glutamine by lymphocytes.

Lymphocytes primarily metabolize glutamine to glutamate, aspartate, lactate, ammonia, pyruvate and alanine, while 25% of glutamine is completely oxidized to CO_2 for energy generation (Ardawi and Newsholme, 1983). Glutaminase catalyzes glutamine to glutamate and the activity of this enzyme is correlated with glutamine utilization in

lymphocytes (Ardawi and Newsholme, 1983). Glutamate metabolism results in the production of NADPH by malic enzyme. NADPH is required for biosynthetic reactions and glutathione reductase activity. In leukocytes, NADPH production from malic enzyme is approximately equal to that of the pentose phosphate pathway (Costa Rosa et al., 1995), suggesting that NADPH production from glucose and glutamine are similar. In addition to NADPH synthesis, glutamine is a donor of amino groups required for purine nucleotide synthesis.

6. Regulation of lymphocyte metabolism

a) Effects of cytokines

Cytokines are capable of regulating lymphocyte growth and function by altering nutrient transport and enzyme levels and activities. For example, cytokines are capable of regulating cellular glucose and amino acid uptake via an mTOR-dependent pathway (Edinger and Thompson, 2002; Rathmell et al., 2000). In addition to nutrient transport, these cytokines also regulate enzymes that catalyze glycolysis, such as hexokinase and PFK-1, and oxidative phosphorylation (Vander Heiden et al., 2001). Specifically, IL-2, IL-3 and IL-7 act upon developing and proliferating T and B lymphocytes to increase their glucose uptake, glycolytic enzyme activities and lactate production (Bauer et al., 2004). Furthermore, signal transduction via CD28, an accessory molecule required for T cell activation, increases glucose uptake and glycolytic rate in T lymphocytes (Frauwirth et al., 2002). Taken together, altering

lymphocyte metabolism is an important mechanism used by cytokines to regulate lymphocyte development and function.

b) Hormonal Regulation

Hormones play a central role in directing the partitioning of nutrients to tissues. The effect of nutritional stressors, such as protein-energy malnutrition and nutrient deficiencies, activates the hypothalamic-pituitary-adrenal axis leading to elevated levels of plasma glucocorticoids. Elevated glucocorticoids reduce pre-T and pre-B cells undergoing proliferative events within their primary immune tissue, consequently leading to the atrophy of these organs (Fraker and King, 2001). These developing lymphocytes also express low levels of Bcl-2, an anti-apoptotic protein, compared to cells from normal-fed animals (Fraker and King, 2004). In addition to reducing Bcl-2 expression, glucocorticoids decrease glucose uptake and hexokinase activity (Hallahan et al., 1973; Munck, 1968; Tome et al., 2004). As naïve lymphocytes in primary immune tissues migrate to the periphery to protect against diverse pathogens, a possible adverse outcome of elevated glucocorticoids is altered recent emigrants in the periphery. This has been confirmed in a study in chickens where steroid treatment reduced the supply of naïve T cells to the periphery (Kong et al., 2002).

7. Summary

Lymphocyte development involves extensive proliferation and this process requires energy. Lymphocytes use glucose, glutamine and fatty acids to generate energy

to fuel their development and proliferation. In addition to serving as energy substrates, these nutrients, particularly glucose and glutamine, also provide precursors of macromolecule biosynthesis which is essential for lymphocyte proliferation. Cytokines regulate lymphocyte proliferation by modifying nutrient uptake and the activities of enzymes in metabolic pathways. Accordingly, the adaptation in lymphocyte metabolism during development and proliferation is associated with altered nutrient transporter levels and enzyme activities. Therefore, adaptations in lymphocyte energy metabolism to the altered dietary macronutrient supply can be evaluated by monitoring changes in the expression of genes and proteins that acquire and channel glucose, glutamine and FA to meet cellular energy and substrate needs for biosynthesis during the embryonic to posthatch transition.

CHAPTER 2

DEVELOPING CHICKEN LYMPHOCYTES ALTER THEIR ENERGY METABOLISM DURING THE EMBRYONIC TO POSTHATCH TRANSITION.

Abstract

Adequate energy status in lymphocytes is vital for their development. The ability of developing chicken lymphocytes to acquire and metabolize macronutrients was determined during embryonic (e) and neonatal (d) life when primary energy-substrate metabolism is altered at the whole-animal level. In three experiments, bursacytes and thymocytes were isolated on e17, e20, d1, d3, d7 or d14 to analyze markers associated with glucose, glutamine and lipid metabolism. Bursacyte glucose transporter-3 (Glut-3) mRNA abundance increased from d1 to d14, and hexokinase-1 (HK-1) mRNA abundance was maximum on e20 (P<0.05). Thymocyte glucose transporter-1 (Glut-1), Glut-3 and HK-1 mRNA abundance increased from e17 to d14 (P<0.05). HK enzyme activity increased from e20 to d3 in bursacytes and d3 to d7 in thymocytes (P<0.05). Glucose uptake by bursacytes and thymocytes was greater on d14 compared to d1 and d7 (P<0.05). Bursacyte and thymocyte sodium-coupled neutral amino acid transporter-2 (SNAT-2) and glutaminase (GA) mRNA abundance increased from e20 to d7 (P<0.05). GA enzyme activity increased from e20 to d7 in bursacytes (P<0.05) and did not change in thymocytes. Carnitine palmitoyl transferase enzyme activity did not change over time in either cell type. These studies suggest that developing B and T lymphocytes adapt their metabolism during the first two weeks after hatch. Developing lymphocytes increase glucose metabolism with no change in fatty acid metabolism, and bursacytes, but not thymocytes, increase glutamine metabolism. Understanding the factors that regulate lymphocyte development in neonatal chicks may help promote their adaptive immune responses to pathogens in early life.

Keywords: chicken, energy, lymphocyte, metabolism

Introduction

Lymphocytes exist in a wide range of activity states with resting lymphocytes being among the least active cells and developing or activated lymphocytes being some of the most metabolically active and rapidly proliferating cells in the body. Energy generation is essential for lymphocyte proliferation and their macronutrient metabolism must be regulated to meet these associated metabolic demands. This is in part mediated by trophic signal transduction pathways which coordinate lymphocyte macronutrient metabolism and ATP generation to permit lymphocyte survival, proliferation, and development (Bauer et al., 2004; Ciofani and Zuniga-Pflucker, 2005; Fox et al., 2005; Frauwirth et al., 2002; Jacobs and Rathmell, 2006; Rathmell et al., 2001; Rathmell et al., 2000; Vander Heiden et al., 2001). For example, ligation of the T cell co-stimulatory CD28 receptor or binding of antigen by the B cell antigen receptor activates the phosphatidylinositol 3-kinase/Akt signaling pathway that induces glucose uptake and glycolytic metabolism (Doughty et al., 2006; Frauwirth et al., 2002). In developing lymphocytes, hematopoietic cytokines and developmental ligands, such as interleukin-7 (IL-7)[2] and Notch-1, respectively, coordinate lymphocyte development in part by altering their metabolism (Ciofani and Zuniga-Pflucker, 2005; Yu et al., 2003). Consequently, macronutrient metabolism and energy generation are requisite for the proliferative events associated with lymphocyte development and effector function.

Alterations in energy status at the whole-animal level affects lymphocyte function, proliferation and development (Hosea et al., 2004; Poetschke et al., 2000; Reddy Avula et al., 1999). Cellular anabolic processes associated with lymphocyte proliferation, such as protein synthesis, nucleotide synthesis and cation transport, are

ATP-demanding (Buttgereit et al., 1992) and it has been shown that protein synthesis and nucleotide synthesis are most sensitive to ATP supply (Buttgereit and Brand, 1995). Glycolysis is the major ATP-generating pathway in proliferating lymphocytes (Ardawi and Newsholme, 1985; Greiner et al., 1994) and inhibiting ATP-generation via glycolysis adversely affects lymphocyte proliferation (Miller et al., 1994). Glutaminolysis and β-oxidation of fatty acids also provide energy in proliferating lymphocytes (Ardawi and Newsholme, 1985). Since protein synthesis is an energy-consuming process, depleted energy status in lymphocytes results in reduced membrane bound IL-2 receptor (IL-2R) expression and soluble IL-2R secretion (Karlsson and Nassberger, 1992). This highlights the need for lymphocytes to maintain adequate energy supplies to sustain development and function when metabolic perturbations occur at the organism level.

In chickens, primary energy-substrate metabolism is altered at the whole-animal level during the embryonic to posthatch transition. During embryogenesis, lipid serves as the primary energy source, whereas at hatch, feeding a carbohydrate rich grain-based diet induces metabolic adaptations over the first two weeks posthatch that promote carbohydrate utilization (Hamdy et al., 1991; Humphrey et al., 2004; McKee et al., 1997; Noy and Sklan, 1996; Noy and Sklan, 1999; Sklan and Noy, 2000; Zhang and Hillgartner, 2004). Development of the adaptive immune system is also critical during this period since chicken lymphocyte development in primary immune tissues is initiated during embryogenesis and continues for several weeks after hatch (Cooper et al., 1991; Ratcliffe et al., 1996). Developing B lymphocytes proliferate extensively in the bursa of Fabricius during the first few weeks after hatch and migrate to peripheral tissues to form a self-renewing B cell pool (Ratcliffe et al., 1996). In contrast, T cell progenitors enter

the thymus in three waves during the embryonic life (e 6.5, e12 and e18) where they proliferate up to three weeks within the thymic cortex prior to their emigration to peripheral tissues (Cooper et al., 1991; Davidson et al., 1992). The effect of altered primary energy-substrate metabolism, at the whole-animal level, on developing B and T lymphocyte metabolism is unknown. Therefore, the objective of these experiments was to determine the ability of developing chicken B and T lymphocytes to acquire and metabolize glucose, glutamine and fatty acids to generate energy. Three experiments were performed to measure: (a) the mRNA abundance for genes involved in glucose, glutamine and fatty acid metabolism in developing B and T lymphocytes; (b) the activity of enzymes that catalyze glycolysis, glutaminolysis and β-oxidation in developing B and T lymphocytes; (c) serum glucose, glutamine and NEFA concentrations; and (d) glucose uptake by developing B and T lymphocytes.

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[2]Abbreviations: ACC-α, acetyl CoA carboxylase-α; β_2-M, β_2-microglobulin; CPT-1, carnitine palmitoyl transferase-1; FAT, fatty acid translocase; Glut-1 and -3, glucose transporter-1 and -3; GA, glutaminase; GAPDH, glyceraldehyde phosphate dehydrogenase; HK-1, hexokinase-1; HPRT-1, hypoxanthine phosphoribosyltransferase-1; IL-7, interleukin-7; 2-NBDG, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose; NEFA, non-esterified fatty acid; SNAT-1 and -2, sodium coupled neutral amino acid transporter-1 and -2; SREBP-1, sterol regulatory element binding protein-1; TBP, TATA box binding protein.

Materials and Methods

Animals and diet. Fertile chicken eggs of the Ross strain (Allen's Hatchery) were incubated at 37.5°C and 65% humidity. Hatched chicks were raised in brooder batteries (Petersime Inc) and provided ad libitum access to water and a diet formulated to meet National Research Council reference diet specifications for a growing broiler chick (NRC, 1994). All animal procedures were approved by the University of Maryland Animal Care and Use Committee.

Experimental design. Three experiments were designed to characterize glucose, glutamine, and fatty acid metabolism in bursacytes and thymocytes from embryonic and neonatal chicks. On the day of incubation, eggs were randomly assigned to each sampling time point to achieve similar average egg weights. In all experiments, cells were isolated from the bursa and thymus at selected time points from embryonic day (e) 17 to neonatal day (d) 14. In experiment one, mRNA abundance of genes involved in glucose, glutamine and fatty acid metabolism in bursacytes and thymocytes were measured on e17, e20, d1, d7 and d14 (n = 5/time point). In experiment two, mRNA abundance and enzyme activity in bursacytes and thymocytes were measured on e20, d1, d3 and d7 (n = 6/time point). In experiment three, glucose uptake by bursacytes and thymocytes was measured on d1, d7 and d14 (n = 6/time point).

Lymphocyte isolation. The bursa and the thymus were isolated from each chick and minced with forceps in RPMI 1640 and strained through a 70 μm nylon cell strainer (BD

Falcon). Lymphocyte purity of bursacyte and thymocyte populations was analyzed using a FACSCalibur flow cytometer (BD Biosciences). FITC-conjugated mouse anti-chicken λ chain and FITC-conjugated mouse anti-chicken ChT1 (SouthernBiotech) were used as markers for B and T lymphocytes, respectively. Lymphocyte viability was determined by propidium iodide staining. Bursacyte and thymocyte aliquots (2×10^6 in experiment one; 5×10^6 in experiment two) were immediately snap-frozen in liquid nitrogen and stored at -80°C until further analysis. In experiment three, bursacytes and thymocytes (1×10^6) were cultured in RPMI 1640 to measure glucose uptake.

Quantitation of mRNA by real-time PCR. In experiments one and two, total RNA was isolated from bursacytes and thymocytes using the NucleoSpin RNA II Total RNA Isolation Kit (Macherey-Nagel), and optical density at 260 nm was used to determine RNA concentrations. Total RNA (0.5 μg) was reverse transcribed (RT) as described (Humphrey et al., 2004). Intron-spanning gene specific primers were designed to either cloned or predicted chicken genes (**Table 2.1**) using the Beacon Designer 4 software (Premier Biosoft International). Polymerase chain reaction (PCR) products were separated by agarose gel electrophoresis, gel purified using the MinElute gel extraction kit (Qiagen) and sequenced (Gene Gateway) to ensure amplification specificity. Quantitative real-time PCR was performed using the iQ SYBR Green Supermix (Bio-Rad). Duplicate reactions for each sample contained 1μL of the 1:2 diluted RT reaction and 0.3 μmol/L of each gene specific primer in a total reaction volume of 12.5 μL. Amplification and detection of specific PCR products was performed using the iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad). Thermal cycling conditions

for real-time PCR were 95°C for 3 min followed by 40 cycles of denaturing at 95°C for 15 s, annealing for 30 s and extension at 72°C for 30 s. The optimum annealing temperature for each primer pair is provided in Table 2.1. After 40 cycles, the specificity of each PCR reaction was confirmed by melt-curve analysis. Thermal cycling conditions for melt-curve analysis were 95°C for 1 min, 55°C for 1 min followed by a linear increase in temperature of 0.5°C/10 s up to 95°C while continuously monitoring fluorescence. The change in mRNA abundance from e17 was calculated using the delta-delta equation (Livak and Schmittgen, 2001) with certain modifications. The amplification efficiency of each reaction was obtained using the LinRegPCR software (Ramakers et al., 2003). Target gene mRNA abundance was normalized by geometric averaging of HPRT, GAPDH, TBP, β2-M, β-actin, ACC and FAT raw-non-normalized values using the geNorm software (Vandesompele et al., 2002). Data are presented as the normalized fold-change in mRNA abundance of a gene relative to its mRNA abundance on e17 or e20 for experiment one or two, respectively.

Enzyme assays. In experiment two, bursacytes and thymocytes (5×10^6) were lysed using a cell-lysis buffer with non-ionic detergent (1 % v/v NP-40 and 0.9 % w/v NaCl in phosphate buffered saline). Cell lysate protein content was measured using the Quick Start Bradford Protein Assay kit (Bio-Rad). HK activity was measured as described (Bergmeyer et al., 1983). CPT-1 activity was measured as described (Bieber et al., 1972) with minor modifications. Assay conditions were: 116 mmol/L tris pH 8.0, 1.1 mM EDTA, 0.05mmol/L palmitoyl-CoA, 7.5 mmol/L carnitine, 0.12 mmol/L DTNB, 50 µL cell lysate in a reaction volume of 200 µL. GA activity was measured as described

(Graham and Aprison, 1969) with minor modifications. Assay conditions were: 80 mmol/L glutamine, 0.5 mmol/L potassium phosphate, 10 µL of cell lysate, 100 µL tris-hydrazine buffer pH 8.5 (100 mmol/L tris-base, 5 mmol/L EDTA, 10 mmol/L $MgCl_2$, 400 µmol/L hydrazine dihydrochloride), 28 mmol/L NAD, 3 units of glutamate dehydrogenase in a reaction volume of 150 µL. Results of all enzyme assays are presented as nmoles of substrate produced per mg protein per minute.

Serum substrate concentration. In experiment two, blood was collected by cardiac puncture on e20, d1, d3 and d7. Blood was allowed to clot at 25°C for 90 min, centrifuged at 4,000 x g for 5 min at 4°C, and then serum was collected and stored at -80°C until further analysis. Serum glucose was measured using the GAHK-20 glucose assay kit (Sigma). Serum glutamine was measured as described (Lund, 1986). Serum non-esterified fatty acid (NEFA) concentration was measured using the NEFA C assay kit (Wako Chemicals).

Glucose uptake. In experiment three, glucose uptake by bursacytes and thymocytes was measured using 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG; Molecular probes). Bursacytes and thymocytes (10^6) were cultured in RPMI 1640 + 10% fetal bovine serum with or without 20 µmol/L 2-NBDG for 0, 30 and 60 min at 37°C with 5% CO_2. Samples incubated without 2-NBDG were used to measure background fluorescence. B and T lymphocyte glucose uptake was determined by measuring fluorescence of λ chain and ChT1 positive cells, respectively. Mean fluorescence intensity (MFI) due to 2-NBDG uptake was measured for 10,000 gated

events for each replicate using a FACSCalibur flow cytometer (BD Biosciences). Background fluorescence was subtracted from MFI of each sample. Data are presented as background-corrected MFI.

Statistical analysis. Dependent variables were analyzed by general linear model procedure (JMP) using a one-way ANOVA. Prior to analysis, data on mRNA abundance was assessed for homogeneity of variance by Levene's test and was log-transformed when significant ($P<0.05$). Data are reported as non-transformed means and pooled standard errors. When main effects were significant ($P<0.05$), means were compared by Tukey's means comparison. Data on glucose uptake within an incubation time point were compared by LSmeans contrasts. Pearson correlation coefficients between selected dependent variables were calculated by the multivariate procedure using JMP.

Results

*Lymphocyte purity***.** The λ chain and ChT1 antigens were used as lineage-specific markers for developing B and T cells, respectively (Chen et al., 1984; Chen et al., 1982). Flow cytometric analysis of bursacyte and thymocyte populations showed that 90% of bursacytes were λ chain positive and 95% of thymocytes were ChT1 positive. The viability of both cell populations exceeded 98%.

Gene expression. The mRNA abundance of genes involved in glucose, glutamine and fatty acid metabolism changed in both bursacytes and thymocytes during the first two weeks after hatch. Bursacyte Glut-3 mRNA abundance increased 1.5-fold from d1 to d14 while Glut-1 mRNA abundance decreased 60 % from e17 to e20 ($P<0.05$; **Fig. 2.1A**). Bursacyte HK-1 mRNA abundance reached maximum levels on e20 (Fig. 2.1B). Thymocyte Glut-1 and Glut-3 mRNA abundance increased after hatch ($P<0.05$; Fig. 2.1C), and HK-1 mRNA abundance increased 2.5-fold from d7 to d14 ($P<0.05$; Fig. 2.1D). Bursacyte SNAT-2 mRNA abundance increased 1.5-fold from e20 to d7 ($P<0.05$; **Fig. 2.2A**) and GA mRNA abundance increased 2.5-fold from e17 to d7 ($P<0.05$; Fig. 2.2B). However, bursacyte SNAT-1 mRNA abundance did not change over time. Thymocyte SNAT-2 mRNA abundance increased 4-fold from d1 to d7 ($P<0.05$), SNAT-1 mRNA abundance increased 2-fold from e20 to d7 ($P<0.05$) and GA mRNA abundance increased 4-fold from e17 to d7 ($P<0.05$; Fig. 2.2C and 2.2D). Bursacyte CPT-1 mRNA abundance decreased 75 % from e17 to e20 ($P<0.05$; **Fig. 2.3A**). Thymocyte CPT-1 mRNA abundance increased 4-fold from e17 to d1 and decreased 50% from d1 to d7

(P<0.05; Fig. 2.3B). In experiment two, the mRNA abundance of genes (same as in experiment one) did not change significantly from e20 to d7 in either bursacytes or thymocytes (**Table 2.2**).

Serum substrate concentration. Serum glucose concentrations increased 191% between e20 and d7 (P<0.05; **Table 2.3**). Serum glucose concentration was positively correlated (P<0.05) with thymocyte Glut-3 mRNA abundance (r = 0.75), HK-1 mRNA abundance (r = 0.66) and bursacyte HK-1 mRNA abundance (r = 0.52). Serum NEFA concentration increased 223% between e20 and d1 (P<0.05), and was similar between d1 and d7. Serum glutamine concentration remained constant between e20 and d7. Serum NEFA and glutamine concentrations were not correlated with the mRNA abundance of metabolic genes involved in their metabolism.

Enzyme activity. Bursacyte HK enzyme activity reached maximum levels by d3. Bursacytes had 168% higher HK activity on d3 compared to e20 (P<0.05; Table 2.3). Thymocyte HK activity reached maximum levels by d7. Thymocytes had 139% higher HK activity on d7 compared to d3 (P<0.05). GA enzyme activity increased 204% from d3 to d7 (P<0.05) in bursacytes and did not change in thymocytes. CPT-1 enzyme activity did not change in either bursacytes or thymocytes. Serum glucose concentration was positively correlated (P<0.05) with HK enzyme activity in bursacytes only (r=0.52). Serum NEFA and glutamine concentrations were not correlated with the activity of enzymes involved in their metabolism.

Glucose uptake. Glucose uptake by bursacytes and thymocytes increased with each neonatal day to maximum levels on d14 ($P<0.05$). In bursacytes, glucose uptake on d14 was higher than uptake on d1 and d7 at 30 and 60 min of incubation ($P<0.05$; **Fig. 2.4A**). In thymocytes, glucose uptake was higher on d7 than on d1 at either 30 or 60 min of incubation ($P<0.05$; Fig. 2.4B). Similarly, glucose uptake by thymocytes on d14 was higher than on d7 at either 30 or 60 min of incubation ($P<0.05$).

Discussion

The objective of these experiments was to study glucose, glutamine and fatty acid metabolism in developing chicken lymphocytes during the embryonic to posthatch transition. Metabolic markers associated with these pathways were determined at time points when either lipid (e17, e20, d1) or carbohydrate (d3, d7, d14) is the primary energy source at the whole-animal level (Hamdy et al., 1991; McKee et al., 1997; Romanoff, 1967). These studies show that macronutrient metabolism in developing lymphocytes is altered after hatch and that this adaptation is nutrient and cell type specific.

Developing B and T lymphocytes showed increased transcription of glucose transporter genes beginning at the time of hatch. Glut-3 mRNA abundance, an inducible isoform in chickens (Wagstaff et al., 1995), increased from d1 to d14 in B and T lymphocytes. Glut-3 protein levels were not determined in these experiments since an antibody is not yet available to this chicken protein, but the increase in glucose uptake and Glut-3 mRNA abundance with age suggests that Glut-3 may be the primary transport protein responsible for increasing glucose uptake in developing B and T lymphocytes after hatch. In developing T lymphocytes, however, Glut-1 mRNA abundance also increased after hatch, and this isoform is also likely to play an important role in developing T lymphocyte glucose metabolism. The increase in transcription of both Glut-1 and Glut-3, two high affinity glucose transporters (Uldry and Thorens, 2004), suggests that developing T lymphocytes may have an increased metabolic need for glucose beginning at hatch. In the thymus, high Glut expression and glucose uptake is characteristic of proliferating $CD4^+CD8^+$ thymocytes (Swainson et al., 2005; Whitesell

and Regen, 1978). Therefore, the apparent increased metabolic need for glucose by developing B and T lymphocytes after hatch suggests increased rates of cell proliferation.

Hexokinase-1 (HK-1) mRNA abundance and total HK activity increased in both developing B and T lymphocytes following hatch. Upon entry into the cell, glucose can enter into one of several alternative metabolic pathways and this is regulated by different HK isozymes. HK-2 provides glucose-6-phosphate for glycolysis, while HK-2 and HK-3 provide glucose-6-phosphate for either glycogen synthesis or the pentose phosphate pathway (Wilson, 2003). There are several lines of evidence to suggest that increased HK activity in developing lymphocytes is associated with increased rates of glycolysis in developing chicken lymphocytes. First, lymphocytes have low activities of glycogen synthase, suggesting low to no internal glycogen stores and, consequently, incorporation of glucose carbon into glycogen in this cell type is minimal (Ardawi and Newsholme, 1982). Second, malic enzyme provides the majority of reducing equivalents required for fatty acid synthesis in chickens due to an inactive pentose phosphate pathway at hatch (Pearce, 1972). Taken together, it is likely that the increased HK activity in developing B and T lymphocytes after hatch reflects an increased flux of glucose carbon through the glycolytic pathway for energy generation.

Some of the metabolic markers for glucose transport and metabolism in developing lymphocytes were correlated with the serum glucose concentration. Glucose availability is sensed by cells via kinases that regulate the transcription of glycolytic and lipogenic genes (Girard et al., 1997). Glucose sensing is well studied in lipogenic tissues, such as liver and adipose, where increased glucose availability results in the activation of carbohydrate response element binding protein (ChREBP), a transcription factor, which

induces the transcription of lipogenic genes (Towle, 2005). The mRNA abundance of ACC-α and SREBP-1, two lipogenic genes that are transcriptional targets of ChREBP, did not change in developing B and T lymphocytes (**Figure 2.5**). This suggests that ChREBP is unlikely to integrate the increased serum glucose concentration to glucose metabolism in developing lymphocytes. Similar to ChREBP, AMP activated protein kinase (AMPK) is responsive to glucose availability and regulates glucose metabolism in mammals (Jones et al., 2005), and AMPK subunit genes are expressed in developing chicken B and T lymphocytes (Richards, Growth biology laboratory, USDA-Beltsville, personal communication). It remains to be determined whether the AMPK has a role in glucose-mediated adaptation in glycolytic metabolism of developing chicken B and T lymphocytes.

Glutamine is a preferred substrate for the system A-encoding isoforms SNAT-1 and SNAT-2 (Mackenzie and Erickson, 2004), and the level of activity of these transporters is high in proliferating cells. During embryogenesis and for several weeks posthatch, developing B and T lymphocytes proliferate extensively within their respective primary immune tissue, doubling approximately twice daily (Davidson et al., 1992; Pink et al., 1985). In the present study, SNAT-2 mRNA abundance increased in developing B lymphocytes while both SNAT-1 and SNAT-2 mRNA abundance increased in developing T lymphocytes, suggesting heightened metabolic activity for both populations (Piva et al., 1992; Schroder et al., 1990). Consequently, the increase in transcription of SNAT isoforms and the direct correlation of SNAT-2 mRNA abundance with system A activity (Jones et al., 2006) suggests that developing B and T lymphocytes increase their ability to acquire glutamine and other neutral amino acids after hatch.

Glutaminase (GA) catalyzes glutamine to glutamate and ammonia, and the activity of this enzyme is correlated with glutamine utilization in lymphocytes (Newsholme et al., 2003). Glutamate is partially oxidized via the citric acid cycle to provide energy or is metabolized to provide precursors for protein, fatty acid and nucleic acid biosynthesis (Ardawi and Newsholme, 1983). About 40% of glutamate produced by GA is oxidized to CO_2 via the tricarboxylic acid cycle in resting and proliferating lymphocytes (Ardawi and Newsholme, 1982; Brand, 1985). Increased GA enzyme activity in developing B lymphocytes suggests that glutamine is an important energy source for chicken B lymphocytes during the first week after hatch. Indeed, feeding neonatal chicks diets supplemented with glutamine enhances bursa maturation (Dibner, 1998). In contrast, developing T lymphocyte GA activity did not change despite an increase in GA mRNA abundance. Glutamine has been shown to be an important energy substrate for T lymphocytes (Ardawi and Newsholme, 1982; Brand, 1985), so perhaps glutaminolysis in this cell type has already reached maximum by hatch.

In these experiments, there were no significant changes in the transcription of lipogenic and lipolytic genes, nor did CPT-1 enzyme activity change in either lymphocyte population. However, serum NEFA concentrations increased three-fold by one week posthatch and this is likely due to the rapid absorption of lipids from both the diet and yolk sac remnant (Noble and Cocchi, 1990). Though the NEFA concentration increased in serum, lymphocytes did not appear to increase their ability to utilize lipid as an energy source, suggesting that increased β-oxidation is not associated with developing lymphocyte metabolism posthatch. This is similar to fatty acid metabolism in mammalian proliferating lymphocytes, since these cell types do not increase rates of β-oxidation

despite an elevated serum NEFA concentration caused by fasting (Ardawi and Newsholme, 1985). However, activated mammalian lymphocytes increase fatty acid uptake (Ardawi and Newsholme, 1985), and this is related to an increased NEFA demand for plasma membrane synthesis (Calder et al., 1994). In these experiments, fatty acid transporter mRNA abundance did not change (Figure 2.5) and markers associated with plasma membrane synthesis were not analyzed, so the utilization of NEFA for plasma membrane synthesis could not be evaluated.

Several hematopoietic cytokines and developmental ligands associated with lymphocyte development regulate their energy metabolism, particularly glucose metabolism. For example, IL-7 and Notch-1 increase glucose metabolism in differentiating thymocytes (Ciofani and Zuniga-Pflucker, 2005; Yu et al., 2003). This suggests that changes in either IL-7, Notch-1 or other cytokines and ligands may be contributing to the changes in developing B and T lymphocyte energy metabolism. Indeed, IL-7 receptor mRNA abundance increases after hatch in developing lymphocytes (**Figure 2.6**), indicating that these cells may be more sensitive to IL-7 availability during this period of increased glucose metabolism. In addition, endocrine factors, such as insulin, also regulate lymphocyte metabolism (Matarese and La Cava, 2004), and their levels are altered during the embryonic to posthatch transition (Cogburn, 2000; Tokushima et al., 2003). Therefore, it is likely that cytokines, developmental ligands and endocrine factors are contributing to altered energy metabolism in developing lymphocytes after hatch.

Developing lymphocytes increase their glucose transport and metabolism to generate energy during the first two weeks posthatch. Similarly, developing non-immune

tissues adapt to oxidize carbohydrates by increasing hexose transport (Noy and Sklan, 1996; Noy and Sklan, 2001) and the transcription of genes involved in carbohydrate metabolism (Humphrey et al., 2004; Sklan et al., 2003). This suggests that metabolic responses in B and T lymphocytes are similar to those occurring at the whole-animal level during this life-stage transition. Consequently, factors that regulate metabolic adaptation at the whole-animal level may also regulate developing lymphocyte energy metabolism. Identifying these regulatory mechanisms may help to promote lymphocyte development and maturation, which is vital for adaptive immune responses to pathogens in neonatal chicks.

Table 2.1 Gene-specific primers used to quantify mRNA abundance by real-time PCR[1].

Gene	Accession number[2]	Primer sequence (5' → 3')	Orientation	Annealing (°C)	Product size (bp)
Glut-1	NM_205209	GCAGGAGATGAAGGAGGAGAGC	F	56	352
		CAGCAGCGTCAGAGCAATGG	R		
Glut-3	NM_205511	AGCAGTTCGCCGCAGAGATG	F	58	324
		AAGCCAAGACATTCACCAACAGC	R		
HK-1	NM_204101	CGCCTGGTGCCTGACTCG	F	58	282
		TCCGTTCTCTGTGCCATCCG	R		
SNAT-1	CB270839	GGATGGGCGGATTCTTGTTGTTG	F	58	286
		GAAAGCGATGGTGGGCAAAGC	R		
SNAT-2	CB270839	ACTGTCTACGCTGTCCCAATCC	F	60	365
		TGACGCCACCAACTGAACTCC	R		
GA	NM_001031248	GTTCCTGAATGAAGATGACAAGCCC	F	57	373
		GCCACCGTTAGCCAGAGTTG	R		
FAT	AI981815	CTTAGAAGTCACCCTCCATACAGC	F	57	231
		ATCTCCAACTGGTATCAGAACTCC	R		
CPT-I	AY675193	GGCTACTTGGAAGACGGACACTG	F	60	488
		CAGAACAGATGGCGGTCAATGC	R		
ACC α	NM_205505	TGCGTCGTCAATAGTGGCTCAG	F	60	231
		ATTCCCTTCCCTCCTCCTCCTTC	R		
SREBP-1	NM_204126	CGCTACCTCTCATCCATCAAC	F	60	270
		CTCCATCACCTCCTCCTTCG	R		
β-actin	NM_205518	CCCCAGCCATGTATGTAGCC	F	59	199
		TCTGTCAGGATCTTCATGAGGTAG	R		
GAPDH	NM_204305	GGTGCTGAGTATGTTGTGGAGTC	F	59	290
		GTCTTCTGTGTGGCTGTGATGG	R		
HPRT-1	NM_204848	GGGAAACAGAGAGGGTTGGAGTG	F	57	213
		TCCGCCCGTCTTTCTAACATCC	R		
β_2-M	Z48921	TGGAGCACGAGACCCTGAAG	F	59	161
		TTTGCCGTCATACCCAGAAGTG	R		
TBP	NM_205103	TTTAGCCCGATGATGCCGTATG	F	58	196
		CTGTGGTAAGAGTCTGTGAGTGG	R		

[1]Abbreviations used: ACC-α, Acetyl CoA carboxylase-α; β_2-M, β_2-Microglobulin; CPT-1, Carnitine palmitoyl transferase-1; FAT, Fatty acid translocase; Glut-1 and -3, glucose transporter-1 and -3; GA, Glutaminase; GAPDH, Glyceraldehyde phosphate dehydrogenase; HK-1, Hexokinase-1; HPRT-1, Hypoxanthine phosphoribosyltransferase-1; SNAT, SNAT-1 and -2, sodium coupled neutral amino acid transporter-1 and -2; SREBP-1, Sterol regulatory element binding protein-1; TBP, TATA box binding protein.
[2]Genbank sequences for either cloned or predicted chicken gene sequences that contain the PCR products listed.

Table 2.2 Metabolic gene mRNA abundance in developing chicken lymphocytes during the embryonic to posthatch transition[1].

Gene[2]	e20	d1	d3	d7
		B lymphocytes		
Glut 1	1.0 ± 0.23	0.85 ± 0.23	1.52 ± 0.23	0.77 ± 0.23
Glut 3	1.0 ± 0.50	1.93 ± 0.50	1.90 ± 0.50	2.21 ± 0.50
Hexokinase	1.0 ± 0.16	1.28 ± 0.16	1.47 ± 0.16	1.54 ± 0.16
SNAT 1	1.0 ± 0.22	1.24 ± 0.22	1.39 ± 0.22	1.14 ± 0.22
SNAT 2	1.0 ± 0.37	1.22 ± 0.37	2.46 ± 0.37	1.78 ± 0.37
Glutaminase	1.0 ± 0.23	1.02 ± 0.23	1.87 ± 0.23	1.69 ± 0.23
CPT 1	1.0 ± 0.23	1.29 ± 0.23	1.60 ± 0.23	1.14 ± 0.23
		T lymphocytes		
Glut 1	1.0 ± 0.38	1.58 ± 0.38	1.42 ± 0.38	2.05 ± 0.38
Glut 3	1.0 ± 0.40	2.12 ± 0.40	2.15 ± 0.40	2.69 ± 0.40
Hexokinase	1.0 ± 0.25	1.59 ± 0.25	1.51 ± 0.25	1.67 ± 0.25
SNAT 1	1.0 ± 0.33	2.01 ± 0.33	1.40 ± 0.33	1.70 ± 0.33
SNAT 2	1.0 ± 0.44	1.65 ± 0.44	1.11 ± 0.44	2.89 ± 0.44
Glutaminase	1.0 ± 0.30	1.46 ± 0.30	1.33 ± 0.30	1.63 ± 0.30
CPT 1	1.0 ± 0.28	1.49 ± 0.28	1.25 ± 0.28	1.26 ± 0.28

[1]Values are relative to e20 mRNA abundance. Values are mean ± SEM, n = 6. Means without a common superscript differ ($P < 0.05$).
[2]Abbreviations used: CPT-1, Carnitine palmitoyl transferase-1; Glut-1 and -3, glucose transporter-1 and -3; GA, Glutaminase; HK-1, Hexokinase-1; SNAT-1 and -2, sodium coupled neutral amino acid transporter-1 and -2.

Table 2.3 Serum substrate concentrations and enzyme activity in bursacytes and thymocytes from embryonic day (e) 20 to neonatal day (d) 7[1].

	e20	d1	d3	d7	SEM
Serum concentration			*mmol/L*		
Glucose	5.32[c]	11.82[b]	13.62[ab]	15.50[a]	0.52
Glutamine	0.82	0.78	0.84	0.63	0.15
NEFA	0.17[b]	0.55[a]	0.48[a]	0.49[a]	0.06
Bursacytes			*nmoles substrate / mg protein · min*		
HK	6.91[b]	13.30[ab]	18.52[a]	13.73[ab]	2.90
GA	1.19[b]	3.71[b]	2.87[b]	8.73[a]	0.74
CPT-1	0.24	0.15	0.12	0.21	0.07
Thymocytes					
HK	12.88[ab]	13.55[ab]	8.42[b]	20.11[a]	2.61
GA	2.74	2.34	3.14	3.06	0.85
CPT-1	0.41	0.42	0.23	0.38	0.07

[1]Values are mean ± SEM, n = 6. Means within a row without a common superscript differ, $P<0.05$.
[2]Abbreviations: CPT-1, carnitine palmitoyl transferase-1; GA, glutaminase; HK, hexokinase; NEFA, non-esterified fatty acid;

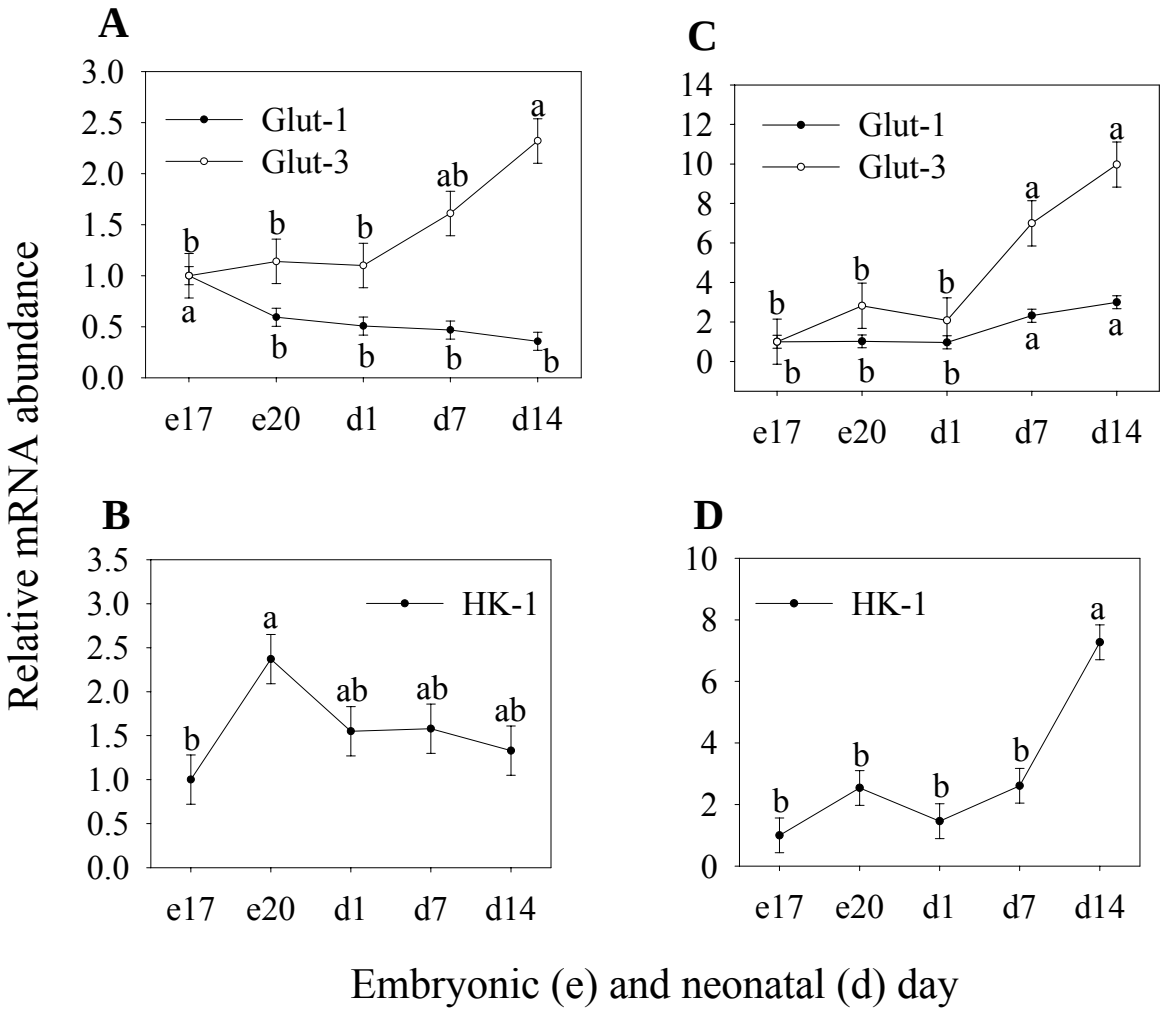

Figure 2.1 Glucose transporter (Glut) and hexokinase-1 (HK-1) mRNA abundance in bursacytes and thymocytes from embryonic day (e) 17 to neonatal day (d) 14. A) Bursacyte Glut-1 and 3; B) Bursacyte HK-1; C) Thymocyte Glut-1 and 3; D) Thymocyte HK-1. Data are presented as the normalized fold-change in mRNA abundance of a gene relative to its mRNA abundance on e17. Values are mean ± SEM, n = 5. Means within an isoform without a common letter differ, $P<0.05$.

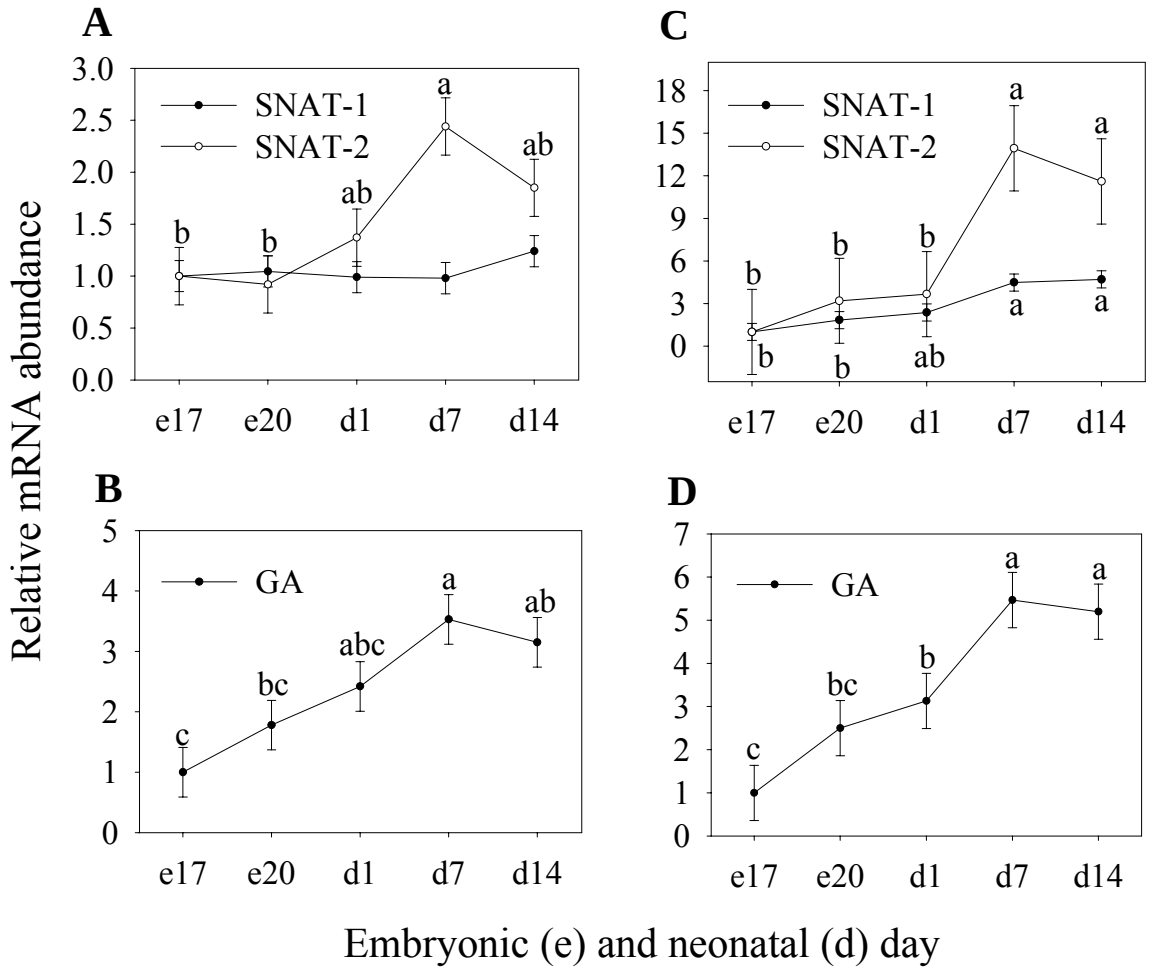

Figure 2.2 Sodium coupled neutral amino acid transporter (SNAT) and glutaminase (GA) mRNA abundance in bursacytes and thymocytes from embryonic day (e) 17 to neonatal day (d) 14. A) Bursacyte SNAT-1 and 2; B) Bursacyte GA; C) Thymocyte SNAT-1 and 2; D) Thymocyte GA. Data are presented as the normalized fold-change in mRNA abundance of a gene relative to its mRNA abundance on e17. Values are mean ± SEM, n = 5. Means within an isoform without a common letter differ, $P<0.05$.

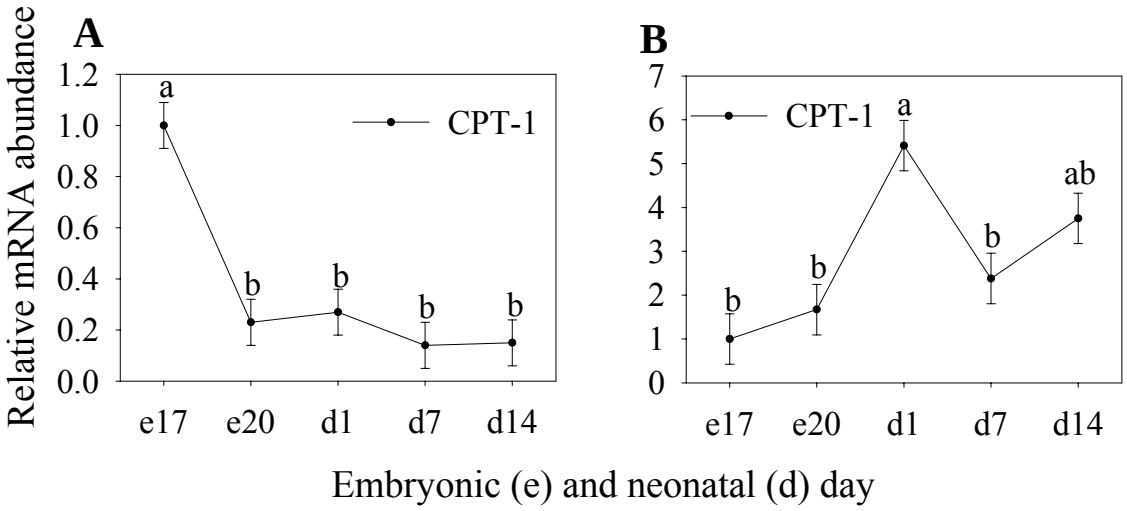

Figure 2.3 Carnitine palmitoyl transferase-1 mRNA abundance in A) bursacytes and B) thymocytes from embryonic day (e) 17 to neonatal day (d) 14. Data are presented as the normalized fold-change in mRNA abundance of a gene relative to its mRNA abundance on e17. Values are mean ± SEM, n = 5. Means without a common letter differ, $P<0.05$.

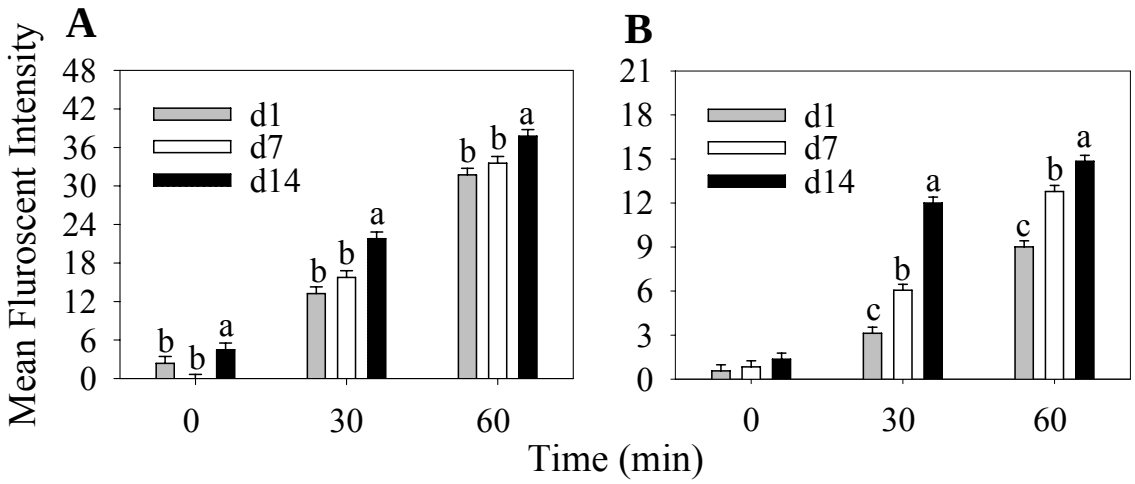

Figure 2.4 Mean fluorescence intensity (MFI) of 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NDBG) in A) bursacytes and B) thymocytes on neonatal d1, d7 and d14. MFI values are corrected for background fluorescence by subtracting MFI from samples incubated without 2-NBDG. Values are mean ± SEM, n = 6. Means within a time point without a common letter differ, $P<0.05$.

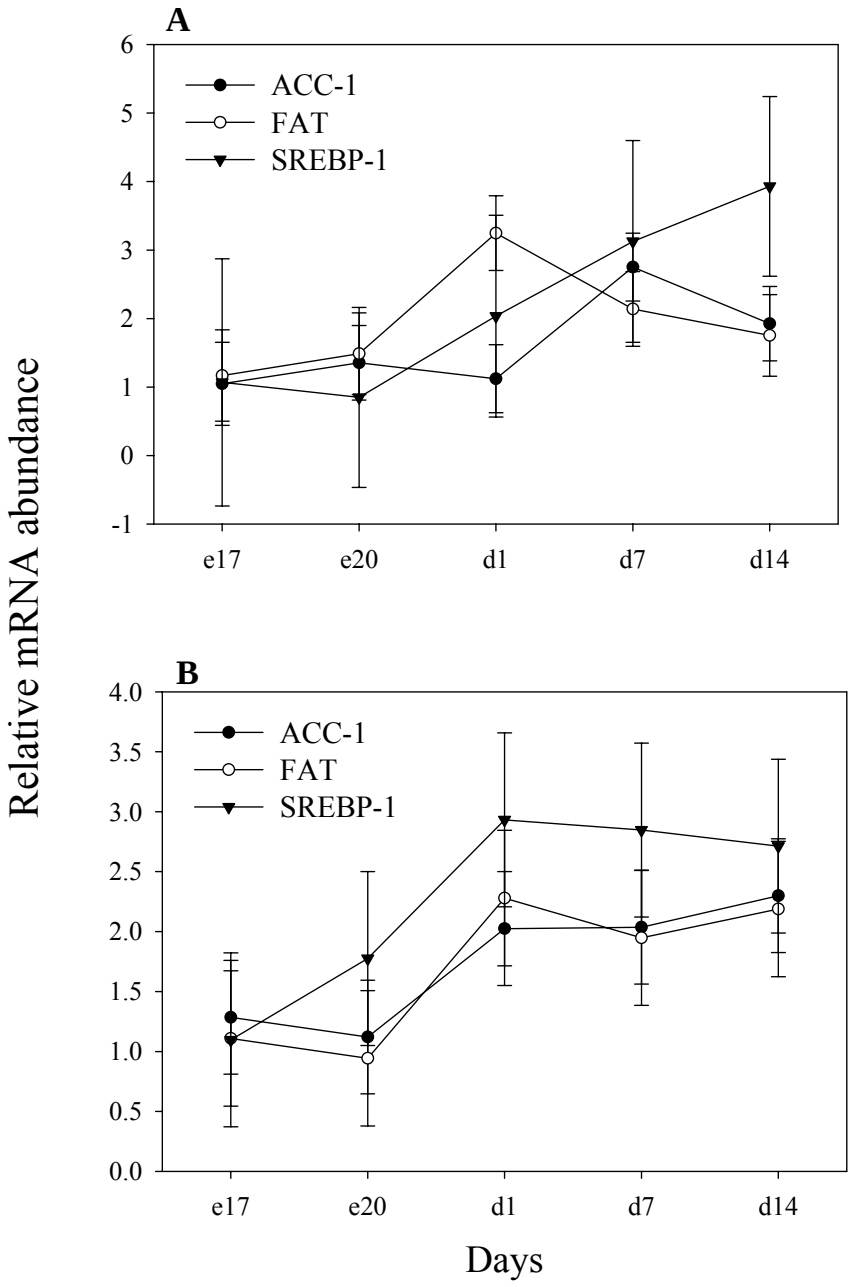

Figure 2.5 Acetyl CoA carboxylase-1, Fatty acid translocase and Sterol regulatory binding protein-1 mRNA abundance in A) thymocytes and B) bursacytes from embryonic day (e) 17 to neonatal day (d) 14. Data are presented as the normalized fold-change in mRNA abundance of a gene relative to its mRNA abundance on e17. Values are mean ± SEM, n = 5. Means without a common letter differ, $P<0.05$.

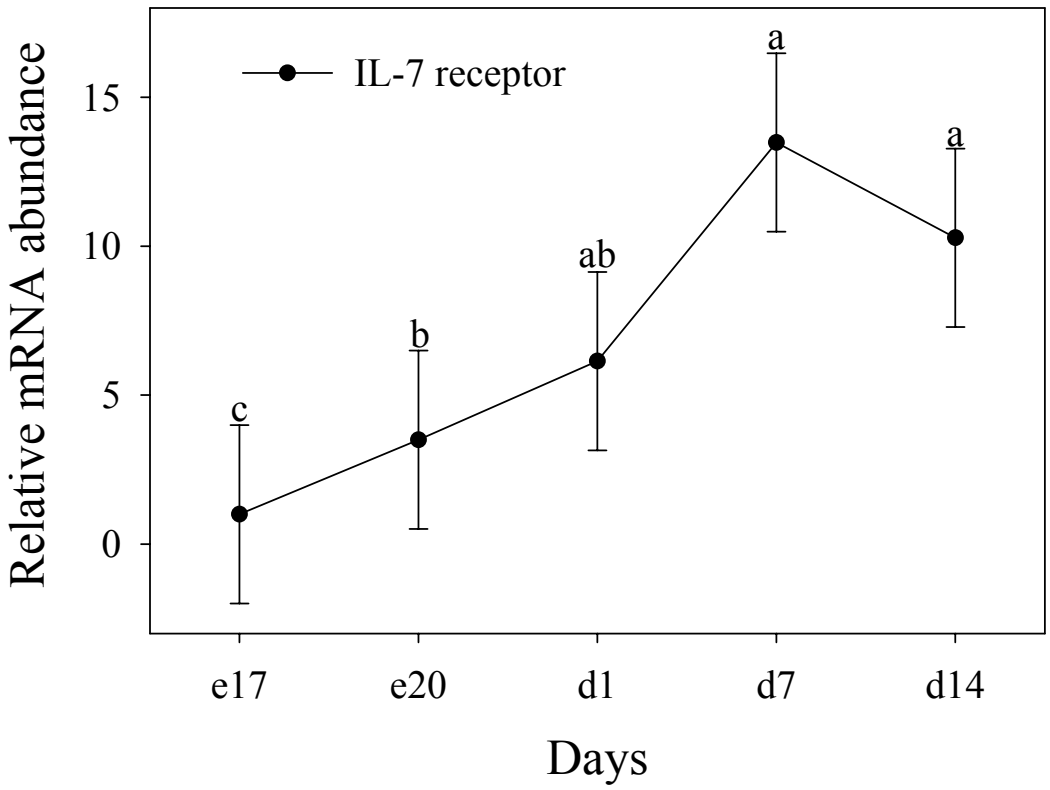

Figure 2.6 Interleukin-7 receptor α mRNA abundance in thymocytes from embryonic day (e) 17 to neonatal day (d) 14. Data are presented as the normalized fold-change in mRNA abundance of a gene relative to its mRNA abundance on e17. Values are mean ± SEM, n = 5. Means without a common letter differ, $P<0.05$.

CHAPTER 3

GLUCOSE AVAILABILITY REGULATES THYMOCYTE METABOLISM, ENERGY STATUS AND APOPTOSIS IN CHICKEN EMBRYONIC THYMUS.

Abstract:

Glucose metabolism in thymocytes is coupled to their development and selection in the thymus. In chickens, thymocytes develop in a low glucose concentration (5 mM) *in ovo* and a high glucose concentration (15 mM) after hatch. To determine the effect of glucose availability on thymocyte metabolism, energy status and apoptosis, embryonic thymic lobes were cultured in media containing 0 to 20 mM glucose and thymocytes were isolated for analysis. Thymocyte glucose metabolism was related to glucose availability. Glucose transporter-1 mRNA abundance was 3-fold higher in thymocytes from 20 mM compared to 0 mM glucose by 12 h ($P<0.05$). Glucose transporter-3 mRNA abundance was at least 2-fold higher in thymocytes from 10 and 20 mM compared to 0 mM glucose by 12 h ($P<0.05$). Glucose uptake was greatest in thymocytes from 20 mM glucose ($P<0.05$). Thymocytes from 0 and 20 mM glucose had 185% greater hexokinase activity compared to 10 mM glucose ($P<0.05$). Enhanced glucose metabolism improved thymocyte energy status and survival. Thymocyte mitochondria membrane potential was higher in thymocytes from 15 mM glucose compared to 5 mM glucose ($P<0.05$). Thymocyte viability was higher in 10 and 15 mM compared to 5 mM glucose ($P<0.05$). Thymocyte apoptosis was higher in 5mM compared to 15 mM glucose ($P<0.05$). Glucose availability induced metabolic changes in thymocytes and altered their energy status and survival. Consequently, these data indicate that glucose availability may influence the development of naïve T cells in the embryonic thymus.

Keywords: apoptosis, energy, glucose, lymphocyte, metabolism

Introduction:

T cell development in the thymus is a highly regulated process. The expression of developmental ligands and the rearrangement of T cell antigen receptors (TCR) have long been recognized as factors regulating T cell development. Recent studies in lymphopoiesis have implicated a role for metabolism, particularly bioenergetics, in T cell development. The proliferative events associated with T cell development following either positive or negative selection in the thymus in part depend upon T cell bioenergetics (Ciofani and Zuniga-Pflucker, 2005; Swainson et al., 2005). In fact, different T cell maturation stages, from the double negative[1] (DN; $CD4^-CD8^-$) to the single positive stage (SP; $CD4^+$ or $CD8^+$), differ in their metabolic phenotype (Swainson et al., 2005; Whitesell et al., 1977; Whitesell and Regen, 1978; Yu et al., 2003). Consequently, T cells at different developmental stages differ in their ability to acquire and metabolize nutrients, particularly glucose, which serves as a major energy substrate and precursor for macromolecule synthesis in developing T cells (Ardawi and Newsholme, 1982; Ardawi and Newsholme, 1985). For example, glucose metabolism in developing T cells is elevated during TCR-β rearrangement (Ciofani and Zuniga-Pflucker, 2005), in which outcome of expression serves as a checkpoint for DN T cell progression to the DP T cell stage (Ciofani et al., 2004; Michie and Zuniga-Pflucker, 2002). Additionally, a subset of double positive (DP; $CD4^+CD8^+$) T cells in early post-β selection are actively dividing (Whitesell and Regen, 1978) and have high rates of glucose transport and glycolysis in the thymus (Swainson et al., 2005). Taken together, these data indicate that glucose metabolism in developing T cells is integrated with their selection and differentiation in the thymus.

Glucose metabolism in T cells is closely regulated with their differentiation, a highly ordered process (Cooper, 2002; Kang and Der, 2004), by cytokines and developmental ligand pathways that are part of the thymic microenvironment. For example, interleukin-7 increases glucose transport and metabolism in developing T cells to promote their differentiation from the DP to $CD8^+$ developmental stage (Ciofani and Zuniga-Pflucker, 2005; Yu et al., 2003). Notch signals also regulate glucose metabolism in differentiating T cells and serve to rescue these cells from apoptosis and permit progression to β selection (Ciofani and Zuniga-Pflucker, 2005; Jacobs and Rathmell, 2006). However, our understanding of thymocyte metabolism has in large part accumulated from studies conducted in single cell suspensions or reaggregate cell culture models (Ardawi and Newsholme, 1985; Buttgereit and Brand, 1995; Buttgereit et al., 2000) that lack these and other hematopoietic cytokines and developmental ligands known to regulate developing T cell metabolism (Hare et al., 1999). The embryonic thymic organ culture (ETOC) system supports thymocyte development *in vitro* (Boyd et al., 1993; Jenkinson and Anderson, 1994) and maintains the thymic microenvironment and its associated hematopoietic cytokines and developmental ligands that regulate developing T cell metabolism. Therefore, the ETOC system is an ideal alternative model system to single cell suspensions for the study of thymocyte glucose metabolism and development. The use of an ETOC system to study the effect of nutrient availability on T cell development has been underutilized and in the case of examining the effect of glucose availability on T cell development has not been reported.

In chickens, T cell development is initiated during embryogenesis and continues for several weeks posthatch. We previously showed that developing T cells increase their

ability to acquire and metabolize glucose during the embryonic to posthatch transition and that serum glucose concentration is low during embryogenesis and increases three-fold by one week posthatch (Rudrappa SG, 2007; Rudrappa and Humphrey, 2007). Since glucose metabolism in T cells is closely integrated with their development in mammals, the objective of these studies was to determine the effect of glucose availability on thymocyte glucose metabolism in chickens. Here, we measure mRNA abundance of glycolytic genes, glucose transport, hexokinase enzyme activity, mitochondria membrane potential ($\Delta\psi$) and apoptosis in thymocytes to answer whether or not glucose availability affects developing T cell metabolism, energy status and survival.

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[1]Abbreviations: $\Delta\psi$, mitochondria membrane potential; β2-M, β2-Microglobulin; DN, Double negative; DP, Double positive; ETOC, Embryonic thymic organ culture; Glut-1 and 3, Glucose transporter 1 and 3; GAPDH, Glyceraldehyde phosphate dehydrogenase; HK-1, Hexokinase-1; HPRT-1, Hypoxanthine phosphoribosyltransferase 1; 2-NBDG, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose; SP, single positive; TBP, TATA box binding protein.

Materials and Methods:

Animals. Fertile chicken eggs of the Ross strain (Allen's Hatchery, Seaford, DE) were incubated at 37.5°C and 65% humidity to obtain embryos. All animal procedures were approved by the University of Maryland Animal Care and Use Committee.

Embryonic thymic organ culture (ETOC). Thymic lobes were cultured on transwell inserts (Corning Inc., Corning, NY) as previously described (Bendelac et al., 1992) with modifications. Thymic lobes were harvested on embryonic day (e) 15 and were placed on a polycarbonate membrane (12 mm diameter; 0.4 μm pore size). Replicates consisted of four thymic lobes per embryo per transwell insert. In all experiments, RPMI 1640 with glutamine (Invitrogen) containing 10% fetal bovine serum and 1% Penicillin-Streptomycin (ETOC media) was used to achieve final glucose concentrations ranging from 0 mM to 20 mM. Either glucose deplete (0 mM) RPMI 1640, glucose replete (10 mM) RPMI 1640 or their combination was used to achieve 0, 5 and 10 mM final glucose concentrations and D-glucose (Sigma) was added to glucose replete (10 mM) RPMI 1640 to achieve 15 and 20 mM final glucose concentrations. ETOC media was added to each well (900 μl) and ETOCs were maintained at 37°C with 5% CO_2.

Lymphocyte isolation. Thymocytes were isolated from thymic lobes by mincing and straining as previously described (Rudrappa and Humphrey, 2007). Lymphocyte viability was determined by trypan blue exclusion using a hemocytometer or propidium iodide staining using FACSCalibur flow cytometer (BD Biosciences). For total RNA and

protein isolation, thymocyte pellets were immediately snap-frozen in liquid nitrogen and stored at -80°C until further analysis. For all other assays, isolated thymocytes were immediately used at the indicated cell number.

Quantification of mRNA abundance by real-time PCR. Thymocyte Glut-1, Glut-3 and HK-1 mRNA abundance was determined after 12 and 24 h of culture in ETOC media containing 0, 10 and 20 mM glucose (n = 6 per treatment). Total RNA was isolated from 2.5 x 10^6 thymocytes using the Trizol reagent (Invitrogen, Carlsbad, CA), and optical density at 260 nm was used to determine total RNA concentrations. Total RNA (0.5 μg) was reverse transcribed using the iScript Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA) and oligo$(dT)_{20}$ primer according to the manufacturer's instructions. Real-time PCR primers, cycling conditions and analysis were performed as previously described (Rudrappa and Humphrey, 2007) with minor modifications. Target gene mRNA abundance was normalized by geometric averaging of HPRT-1, GAPDH, TBP and β2-M raw-non-normalized values using the geNorm software (Vandesompele et al., 2002). Data are presented as the normalized fold-change in mRNA abundance of a gene relative to its mRNA abundance at 0 h.

Glucose uptake. Thymocyte glucose uptake after 24 h of culture in ETOC media containing 0, 10 and 20 mM glucose (n = 6 per treatment) or after 6 and 12 h of culture in 5 and 15 mM glucose (n = 5 per treatment) was measured as previously described (Rudrappa and Humphrey, 2007). Mean fluorescence intensity (MFI) due to 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) uptake was

measured for 10,000 ChT1 positive gated events for each replicate using a FACSCalibur flow cytometer (BD Biosciences). Background fluorescence was subtracted from MFI of each sample. Data are presented as background-corrected MFI.

Hexokinase activity. Thymocyte HK activity after 24 h of culture in ETOC media containing 0, 10 and 20 mM glucose (n = 6) or after 6 and 12 h of culture in 5 and 15 mM glucose (n = 5) was measured as previously described (Rudrappa and Humphrey, 2007). HK activity is presented as nmoles of NADPH produced per mg protein per minute.

Mitochondria membrane potential. Thymocyte mitochondria membrane potential ($\Delta\psi$) after 24 h of culture in ETOC media containing 5, 10 and 15 mM glucose (n = 4) was determined by staining with tetramethylrhodamine ethyl ester perchlorate (TMRE; Molecular Probes). Thymocytes (10^6) were incubated in their respective ETOC media containing 100 nM TMRE for 30 min at 37°C with 5% CO_2. To determine whether TMRE staining is sensitive to $\Delta\psi$, the $\Delta\psi$ was depolarized by adding 50 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP; Molecular Probes). To determine thymocyte mitochondrial biomass, thymocytes were stained with 50 nM 10-nonyl acridine orange (NAO; Molecular Probes). Fluorescence was measured for 10,000 ChT1 positive gated events for each replicate using a FACSCalibur flow cytometer (BD Biosciences). Non-specific fluorescence in TMRE and CCCP treated samples was subtracted from fluorescence of TMRE stained samples. Fluorescence in NAO and CCCP samples was not different from NAO stained samples (**Figure 3.1**); therefore, fluorescence due to

NAO was not corrected for non-specific fluorescence. Data on $\Delta\psi$ are presented as a fold-change in fluorescence relative to 5 mM glucose.

Apoptosis. Thymocyte apoptosis after 24 h of culture in ETOC media containing 5, 10 and 15 mM glucose (n = 4) or after 6 and 12 h culture in 5 and 15 mM glucose (n = 5) was determined by staining with FITC-conjugated annexin V (Molecular probes). Thymocytes (10^6) were incubated in 100 µl 5X annexin binding buffer containing 5 µl FITC-annexin V and 1ng/µl PI for 15 min at 37°C with 5% CO_2. Fluorescence was measured for 10,000 ChT1 positive gated events for each replicate using a FACSCalibur flow cytometer (BD Biosciences). Thymocytes were gated into apoptotic or dead populations based on annexin V and PI fluorescence.

Statistical analysis. Dependent variables were analyzed by general linear model procedure (JMP, Cary, NC). A one-way analysis of variance (ANOVA) was used to determine the main effect of glucose on thymocyte mRNA abundance, glucose uptake, mitochondrial membrane potential, viability and apoptosis and means were compared by Tukey's means comparison. Prior to analysis, data were assessed for homogeneity of variance by Levene's test and when significant ($P<0.05$) were log-transformed to attain homogeneity of variance. Data are reported as non-transformed means and pooled standard errors. A two-way ANOVA was used to determine the main effect of glucose, time and their interaction on thymocyte hexokinase activity, glucose uptake, viability and apoptosis and main effects were compared by Tukey's means comparison. Differences between means or interactions were considered significant at $P<0.05$.

Results:

Effect of glucose availability on thymocyte viability.

Thymocyte viability was used as a marker to optimize culture conditions for the ETOC system. First, preliminary experiments determined that the addition of ETOC media (10 mM glucose; 50 µl) onto the polycarbonate membrane and replacing half of the media in the well every 12 h improved thymocyte viability (**Figure 3.2**). Second, a time by glucose availability interaction ($P<0.05$) showed that thymocyte viability decreased between 24 and 48 h of culture in 0 and 10 mM glucose, but not 20 mM glucose ($P<0.05$; **Figure 3.3**). Therefore, ETOC media was changed at the times and volumes indicated, and ETOC were maintained for a maximum of 24 h in all experiments.

Effect of glucose availability on thymocyte glucose transporter mRNA abundance, glucose uptake and hexokinase activity.

Glut-1, Glut-3 and HK-1 mRNA abundance was measured to determine the effect of glucose availability on the transcription of genes involved in glucose uptake and metabolism. Thymocytes cultured in 20 mM glucose had 3-fold greater Glut-1 mRNA abundance compared to 0 mM glucose at 12 h ($P< 0.05$; **Figure 3.4A**). Glut-3 mRNA abundance was 2-fold greater in thymocytes from 10 and 20 mM glucose compared to 0 mM glucose at 12 h ($P< 0.05$; Figure 3.4B). Thymocyte HK-1 mRNA abundance did not differ between glucose concentrations at either 12 or 24 h (**Figure 3.5**). Thymocyte glucose uptake was measured to determine if changes in Glut-1 and Glut-3 mRNA

abundance resulted in differences in glucose uptake. Glucose uptake was greatest in thymocytes cultured in 20 mM glucose compared to thymocytes cultured in either 0 or 10 mM glucose ($P< 0.05$; **Figure 3.6A**). Thymocyte HK enzyme activity was measured to determine the effect of glucose availability on glycolysis. HK activity was 185% higher in thymocytes from 0 and 20 mM glucose compared to 10 mM at 24 h ($P< 0.05$; Figure 3.6B).

Effect of glucose availability on thymocyte mitochondria membrane potential ($\Delta\psi$), viability and apoptosis.

Thymocyte $\Delta\psi$ was measured to determine the effect of glucose availability on thymocyte energy status. However, in these studies, the glucose concentrations used were 5, 10 and 15 mM to better reflect *in ovo and in vivo* serum glucose concentrations during the embryonic to posthatch transition, respectively (Rudrappa and Humphrey, 2007). Thymocyte $\Delta\psi$ in 15 mM glucose was 2-fold higher than 5 mM glucose at 24 h ($P< 0.05$; **Figure 3.7A**). Thymocyte mitochondria biomass did not differ between glucose concentrations **(Figure 3.8)** indicating that changes in mitochondria membrane potential were not due to differences in mitochondria number. Thymocyte apoptosis was measured to determine the effect of glucose availability on survival. Thymocytes cultured in 5 mM glucose had lower viability compared to thymocytes cultured in 10 or 15 mM glucose ($P< 0.05$; Figure 3.7B). The percentage of thymocytes undergoing apoptosis was higher in 5 mM glucose compared to 15 mM glucose ($P< 0.05$; Figure 3.7C).

Effect of glucose availability and time on thymocyte glucose uptake, hexokinase activity and apoptosis.

A time-course experiment measured thymocyte glucose uptake, HK activity and apoptosis to determine the onset of glucose-induced changes in thymocyte metabolism and apoptosis. Thymocytes cultured in either 5 mM or 15 mM glucose did not differ in their glucose uptake between 6 and 12 h (**Table 3.1**). Both glucose availability and time tended to affect thymocyte HK activity (P=0.07; Table 3.1). Thymocyte HK activity decreased 37% in 5 mM glucose and increased 20 % in 15 mM between 6 and 12 h (Table 3.1). Thymocytes cultured in either 5 mM or 15 mM glucose did not differ in their rates of apoptosis between 6 and 12 h (Table 3.1). The percentage of dead thymocytes increased in 5 and 15 mM glucose between 6 and 12 h (P=0.01; Table 3.1). The percentage of dead thymocytes were higher in 5 mM glucose compared to 15 mM at 6 and 12 h (P=0.02; Table 3.1).

Discussion:

In these studies, an ETOC system was utilized to determine the effect of glucose availability on T cell metabolism. Thymus size has been shown to be a major factor limiting the duration of ETOC (Jenkinson and Anderson, 1994), and typically e10 thymic lobes are utilized in the ETOC system and can be maintained in culture up to 8 days before the initiation of cell death and necrosis of thymic lobes (Davidson et al., 1992). In our studies, e15 lobes were used in the ETOC system and could be maintained in culture media containing glucose up to 48 h before the initiation of cell death, which is attributed to limited thymus size and the inability of this organ to export T cells *in vitro* (Hare et al., 1999; Jenkinson and Anderson, 1994). Consequently, our results are in agreement with others that the maximum age of thymic lobes suitable for the ETOC system is e17-e18. Results from these studies show that high media glucose concentrations, comparable to posthatch serum glucose concentrations, improve thymocyte energy status and reduce apoptosis.

Thymocytes adapted their metabolism to glucose availability by altering Glut-1 and Glut-3 gene expression, glucose transport and hexokinase activity. Rat thymocytes in single cell suspension increase glucose metabolism, glycolytic gene expression and enzyme activity in response to high glucose concentrations (Aulwurm and Brand, 2000; Moreno-Aurioles et al., 1996). Chicken thymocytes in these studies increased their glucose metabolism in relation to media glucose concentration, which suggests that these cells are able to sense glucose availability. However, specific glucose sensing mechanisms and pathways in thymocytes are yet to be elucidated in detail. Eukaryotic cells take cues from glycolytic and pentose phosphate pathway metabolites to sense

glucose availability through the action of protein kinases and transcription factors. In leukocytes, stimulatory protein-1 (Sp1) (Towle, 2005; Vaulont et al., 2000) and AMP-activated kinase (AMPK) (Jones et al., 2005) participate in glucose sensing. Sp1 links glucose availability to the transcription of glycolytic genes and to the activity of glycolytic enzymes in thymocytes (Aulwurm and Brand, 2000; Schafer et al., 1997). In thymocytes, high glucose concentrations dephosphorylate Sp1 and increase the binding of this transcription factor to promoter regions of glycolytic genes to increase their transcription (Schafer et al., 1997; Schafer et al., 1996). AMPK (Itani et al., 2003; Jones et al., 2005) has been shown to link glucose availability in the extracellular environment to cellular metabolism in non-immune cells. AMPK is expressed in chicken thymocytes (Rudrappa and Humphrey, 2007), functional in mammalian T lymphocytes (Jhun et al., 2006) and regulates energy metabolism in lymphocytes to meet their ATP demand (Tamas et al., 2006). Collectively, AMPK and Sp1 may be important molecular determinants in thymocytes that increase the transcription of genes to facilitate glucose uptake and metabolism in response to elevated glucose availability.

Glucose transport and metabolism in mammalian lymphocytes contributes to their $\Delta\psi$ (Rathmell et al., 2000). Cell $\Delta\psi$ is a measurement of the proton gradient across the inner mitochondria membrane (IMM), and this serves as a marker of cell energy status since $\Delta\psi$ is directly related to ATP production potential (Chen, 1988). Thymocytes cultured in high glucose concentrations had increased $\Delta\psi$, suggesting greater ATP producing potential and consequently an elevated cell energy status. The increase in $\Delta\psi$ in response to elevated glucose availability could be due to increased substrate level phosphorylation of ADP to ATP via glycolysis since lymphocytes metabolize most of

their glucose carbon to lactate via glycolysis (Ardawi and Newsholme, 1985; Greiner et al., 1994; Guppy et al., 1993). Consequently, $\Delta\psi$ is maintained since cytosolic ADP to ATP ratios decrease and inhibit further proton dissipation from the IMM. On the other hand, low $\Delta\psi$ in glucose depleted thymocytes could be due to a combination of low cellular ATP levels associated with glucose deprivation (Gonin-Giraud et al., 2002) and/or loss of outer mitochondria membrane (OMM) integrity leading to proton leakage. The latter is consistent with previous reports demonstrating that decreases in glycolysis due to glucose depletion increase OMM permeability and serve as the first step in the activation of apoptosis (Gottlob et al., 2001; Majewski et al., 2004; Vander Heiden et al., 2001). Overall, glucose availability regulates the energy status of chicken thymocytes and indicates that changes in glucose availability or glucose metabolism will affect energy-dependent processes, such as differentiation associated with T cell receptor development.

Thymocytes developing in low glucose concentrations (5 mM) had elevated rates of apoptosis and thymocyte HK activity tended to decrease before the activation of apoptosis. This suggests that altered glycolytic metabolism is an upstream stress factor leading to apoptosis. Apoptosis in T lymphocytes can be initiated via the death ligand-receptor regulated extrinsic pathway or a Bcl-2 family protein regulated intrinsic pathway (Zhang et al., 2005). Inhibiting glycolysis by depleting media glucose concentrations releases cytochrome c and other pro-apoptotic factors from the mitochondria (Plas et al., 2002; Plas et al., 2001; Vander Heiden et al., 2001). Recent evidence suggests that glucose availability regulates lymphocyte survival by altering the expression of Noxa, a Bcl-2 family protein with pro-apoptotic function, and Mcl-1, a Bcl-2 analogue with pro-survival function (Alves et al., 2006). Glucose deprivation increases Noxa levels which

interact with Mcl-1 to reduce its pro-survival activity resulting in apoptosis. Glucose repletion inhibits apoptosis via decreased Noxa activity and increased Mcl-1 activity. Furthermore, intermediary metabolites of glucose metabolism regulate apoptosis by altering the activity of caspase enzymes, which cleave the key cytosolic and nuclear proteins to activate apoptosis. Glucose-6-phosphate or NADPH inhibit caspase-2 activation resulting in cell survival (Nutt et al., 2005). Since thymocyte glucose metabolism was related to glucose availability, it is likely that either or both of these intrinsic pathways may mediate the initiation of apoptosis in glucose depleted chicken thymocytes.

Glucose deprived (0 mM) thymocytes increased glucose transporter gene expression between 12 and 24 h and HK activity was similar to 20 mM glucose after 24 h of culture. This could be a part of a salvage response in cells since increased thymocyte glucose transporter gene expression and metabolism is generally associated with high glucose concentrations (Ardawi and Newsholme, 1985; Aulwurm and Brand, 2000; Greiner et al., 1994; Hume et al., 1978; Moreno-Aurioles et al., 1996). Nutrient depletion induces salvage responses in cells that produce efforts to increase the expression and the activity of proteins required for nutrient acquisition and metabolism (Hammerman et al., 2004). Glucose deprivation activates AMPK, which induces glucose transport and glycolytic enzyme activity to improve cellular energy status (Carling, 2004; Jones et al., 2005). However, long-term expression of AMPK leads to breakdown of salvage efforts and the activation of apoptosis (Jones et al., 2005; Meisse et al., 2002). It is possible that glucose deprived thymocytes may increase their ability to acquire and metabolize glucose between 12 to 24 h in order to prevent the activation of apoptosis.

Glucose availability determined thymocyte energy status in the presence of the other important energy substrate glutamine, which underscores the important role of glucose in chicken thymocyte bioenergetics. Elevated thymocyte energy status, in response to a high-glucose environment, decreased apoptosis in these cells. Since apoptosis has a key role in thymocyte selection and differentiation (Zhang et al., 2005) and low glucose concentrations used in these studies were similar to embryonic serum glucose concentrations, it is possible that glucose availability may play a crucial role in their development during embryogenesis. Therefore, it is important to study whether or not the low-glucose environment present during embryogenesis enhances thymocyte susceptibility to apoptosis. There is substantial interest to manipulate the *in ovo* environment to promote the ability of newly hatched chicks to grow and adapt posthatch (Halevy et al., 2006; Rozenboim et al., 2003; Rozenboim et al., 2004). Therefore, manipulating thymocyte differentiation by increasing the *in ovo* availability of glucose, or other trophic factors that promote T cell glucose metabolism, may help promote the development of the adaptive immune system in early life.

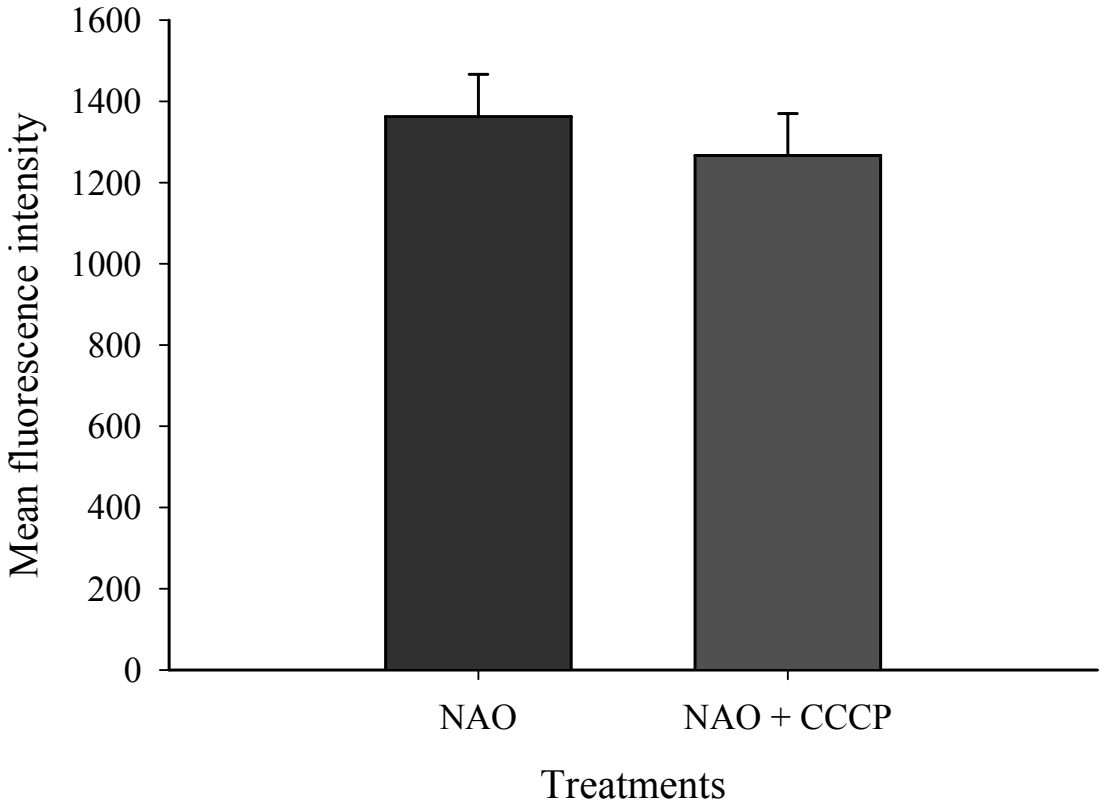

Figure 3.1 Effect of carbonyl cyanide m-chlorophenylhydrazone (CCCP) on mitochondria biomass in thymocytes. Thymic lobes from embryonic day 15 were cultured in ETOC media. Thymocytes were isolated after 24 h of culture and mitochondria biomass was determined by incubating with 50 nM 10-nonyl acridine orange (NAO). Values are means ± SEM, n = 12 per treatment. Means not sharing a common letter differ, $P<0.05$.

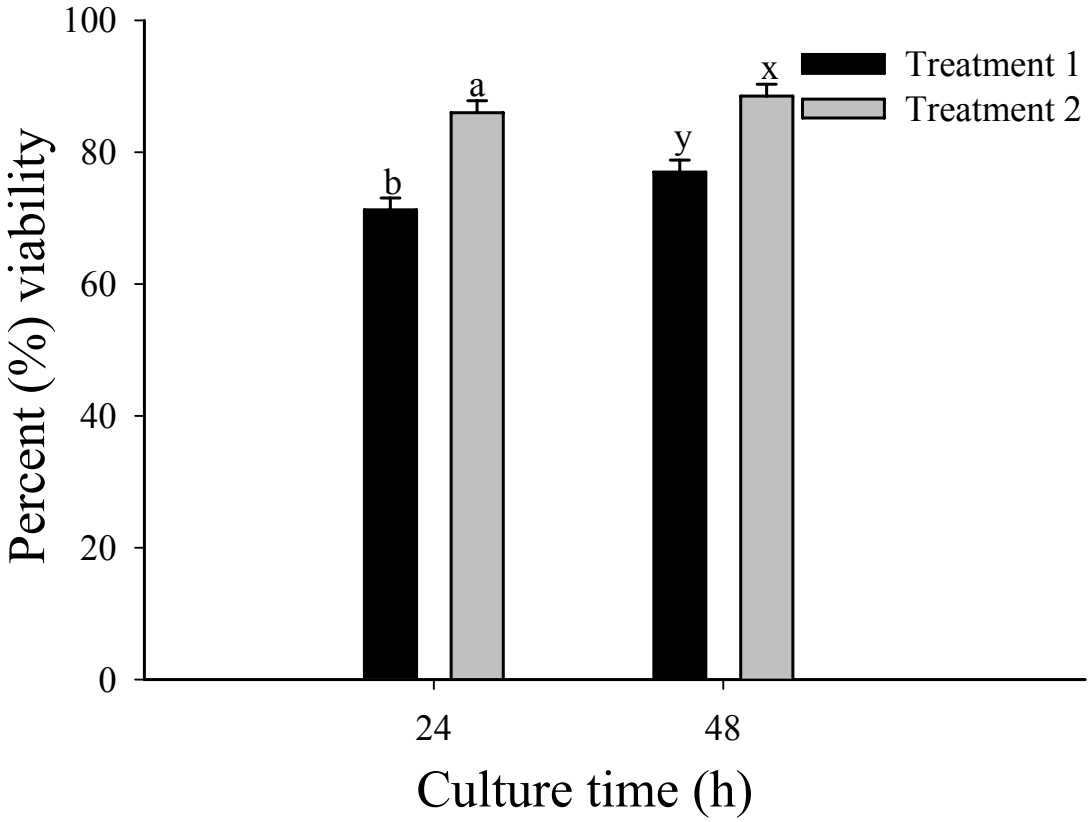

Figure 3.2 Effect of ETOC media replacement on thymocyte viability. Thymic lobes from embryonic day 15 were cultured in ETOC media containing 10 mM glucose. Thymocytes were isolated after 24 and 48 h of culture and viability was determined by trypan blue exclusion. Media was not changed in Treatment 1 and half of the media (500 μl) was replaced every 12 h in Treatment 2. Values are means ± SEM, n = 4 per treatment. Means not sharing a common letter differ, $P<0.05$.

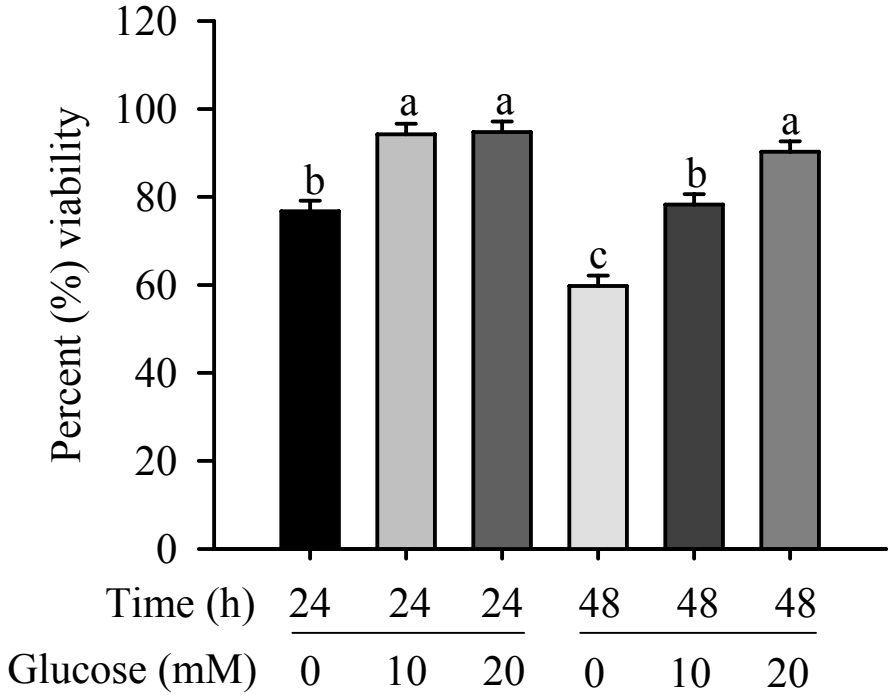

Figure 3.3 Effect of glucose availability on thymocyte viability. Thymic lobes from embryonic day 15 were cultured in ETOC media containing 0, 10 and 20 mM glucose. Thymocytes were isolated after 24 and 48 h of culture and viability was determined by trypan blue exclusion. Results shown are representative of at least 5 experiments. Values are means ± SEM, n = 4. Means not sharing a common letter differ, $P<0.05$.

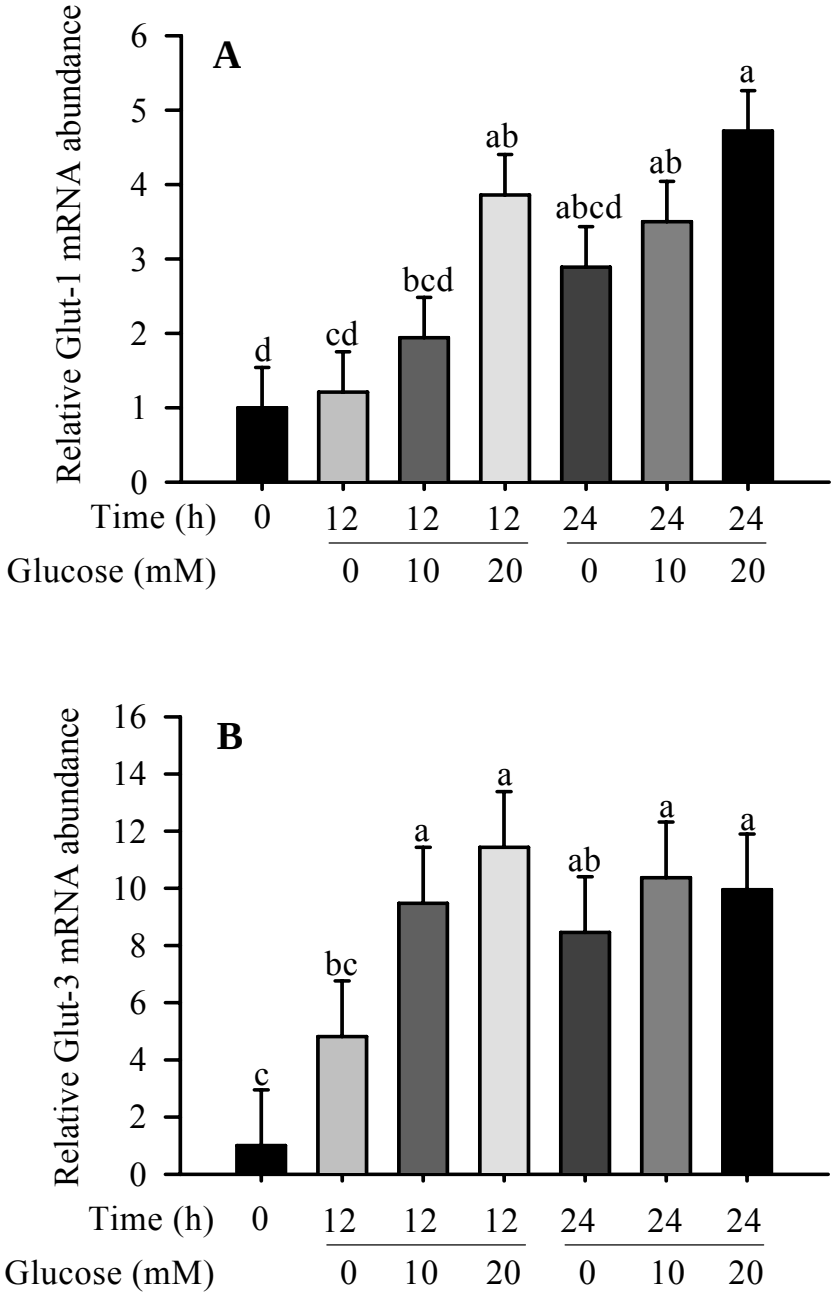

Figure 3.4 Effect of glucose availability on thymocyte (A) glucose transporter-1 (Glut-1) and (B) Glut-3 mRNA abundance. Thymic lobes from embryonic day 15 were cultured in ETOC media containing 0, 10 and 20 mM glucose. Thymocytes were isolated after 12 and 24 h of culture. Data are presented as the normalized fold-change in mRNA abundance of a gene relative to 0 h time point. Values are means ± SEM, n = 6. Means not sharing a common letter differ, $P<0.05$.

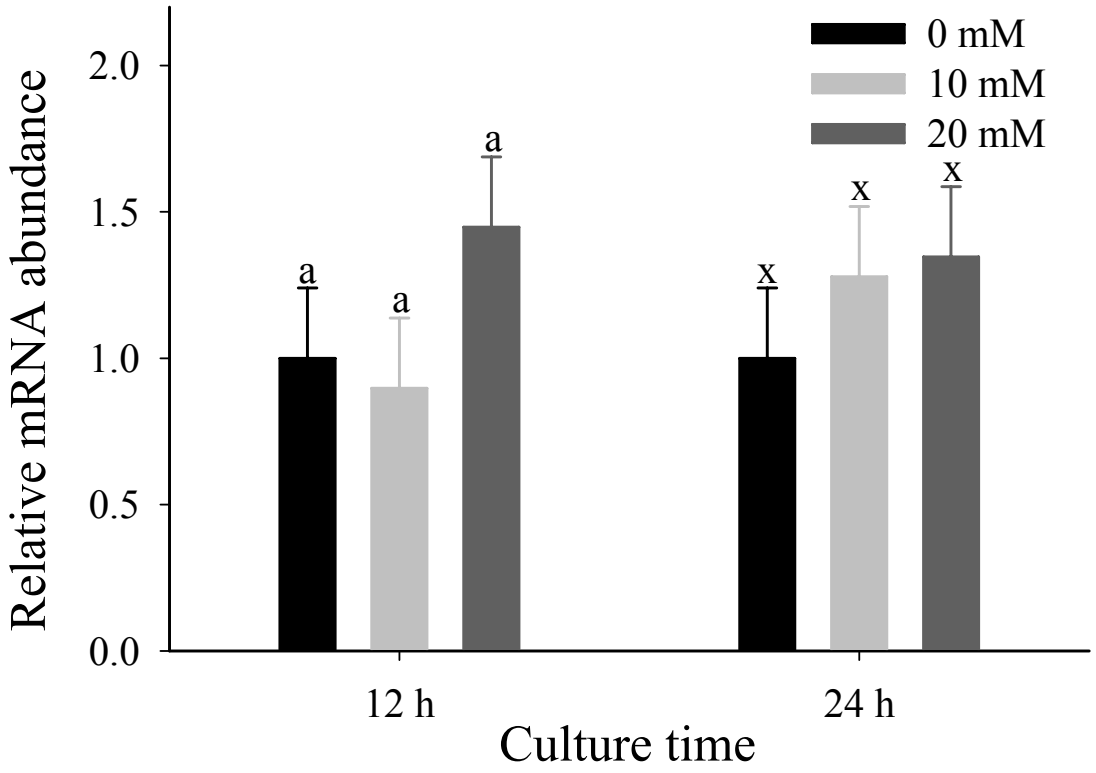

Figure 3.5 Effect of glucose availability on hexokinase-1 (HK-1) mRNA abundance in thymocytes. Thymic lobes from embryonic day 15 were cultured in ETOC media containing 0, 10 or 20 mM glucose. Thymocytes were isolated after 12 and 24 h of culture. Data are presented as the normalized fold-change in mRNA abundance of a gene relative to 0 h time point. Values are means ± SEM, n = 6. Means not sharing a common letter differ, $P<0.05$.

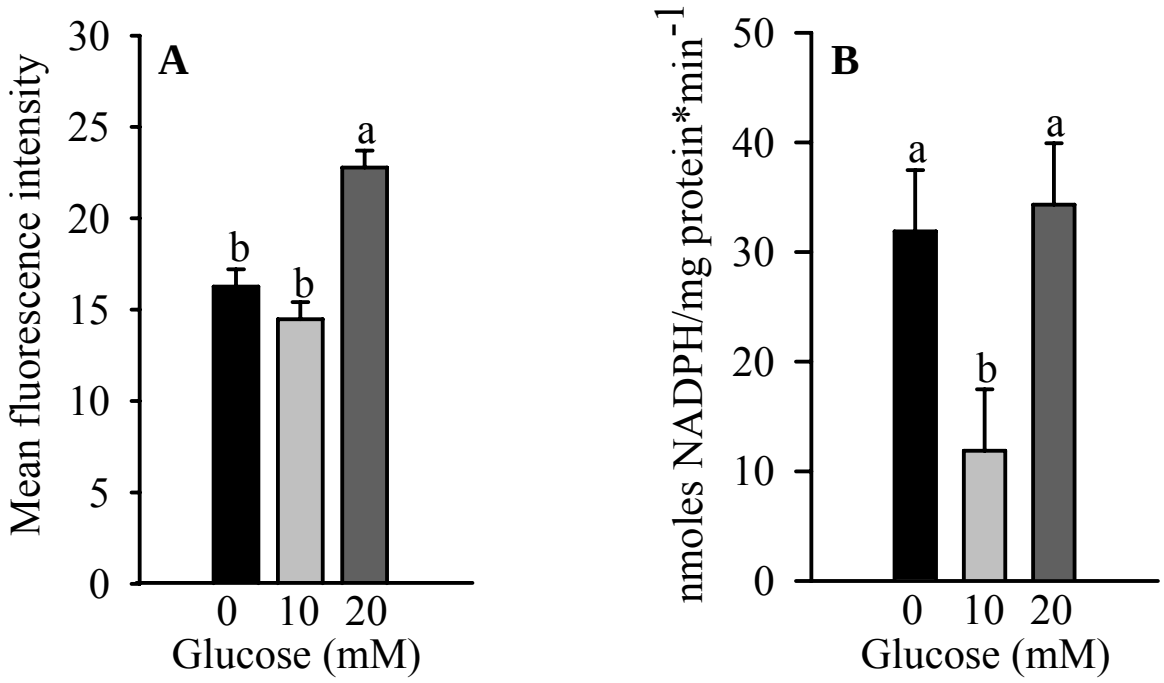

Figure 3.6 Effect of glucose availability on thymocyte (A) glucose uptake and (B) hexokinase activity. Thymic lobes from embryonic day 15 were cultured in ETOC media containing 0, 10 and 20 mM glucose. Thymocytes were isolated after 24 h of culture. Thymocyte glucose uptake was measured after 30 min of incubation with 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NDBG). Mean fluorescence intensity values are corrected for background fluorescence. Values are means ± SEM, n = 6. Means not sharing a common letter differ, $P<0.05$

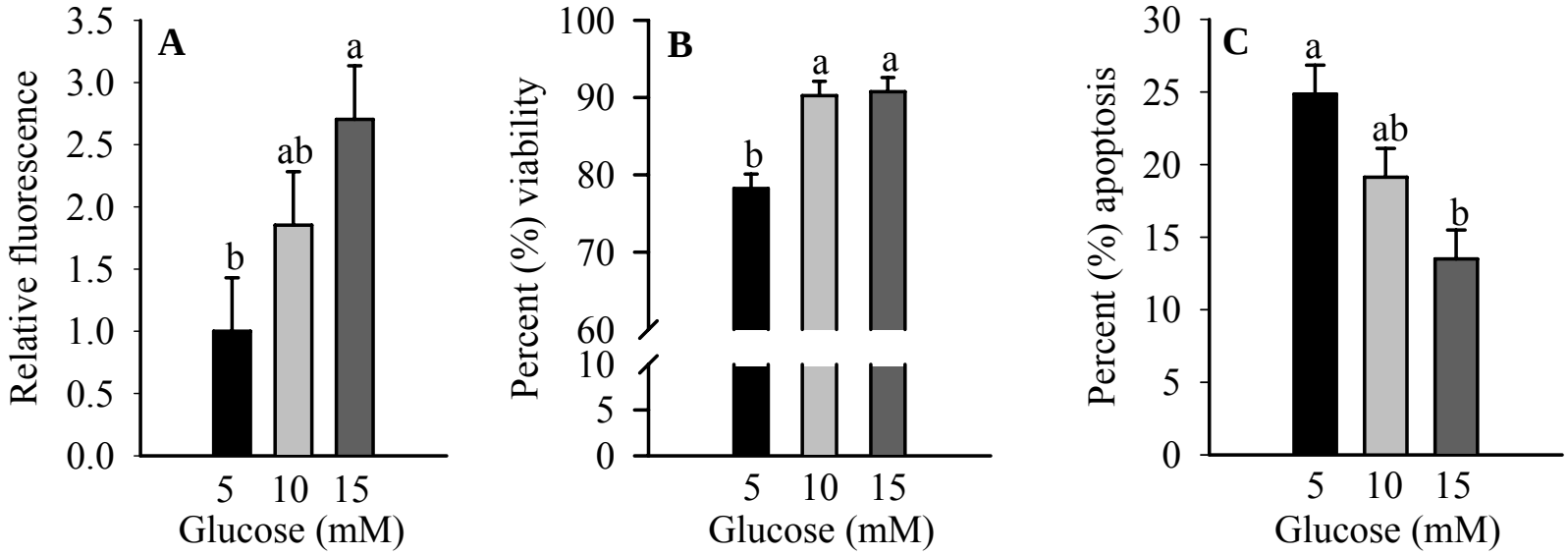

Figure 3.7 Effect of glucose availability on thymocyte (A) mitochondria membrane potential, (B) viability and (C) apoptosis. Thymic lobes from embryonic day 15 were cultured in ETOC media containing 5, 10 and15 mM glucose. Thymocytes were isolated after 24 h of culture. Values are means ± SEM, n = 4. Means not sharing a common letter differ, $P<0.05$.

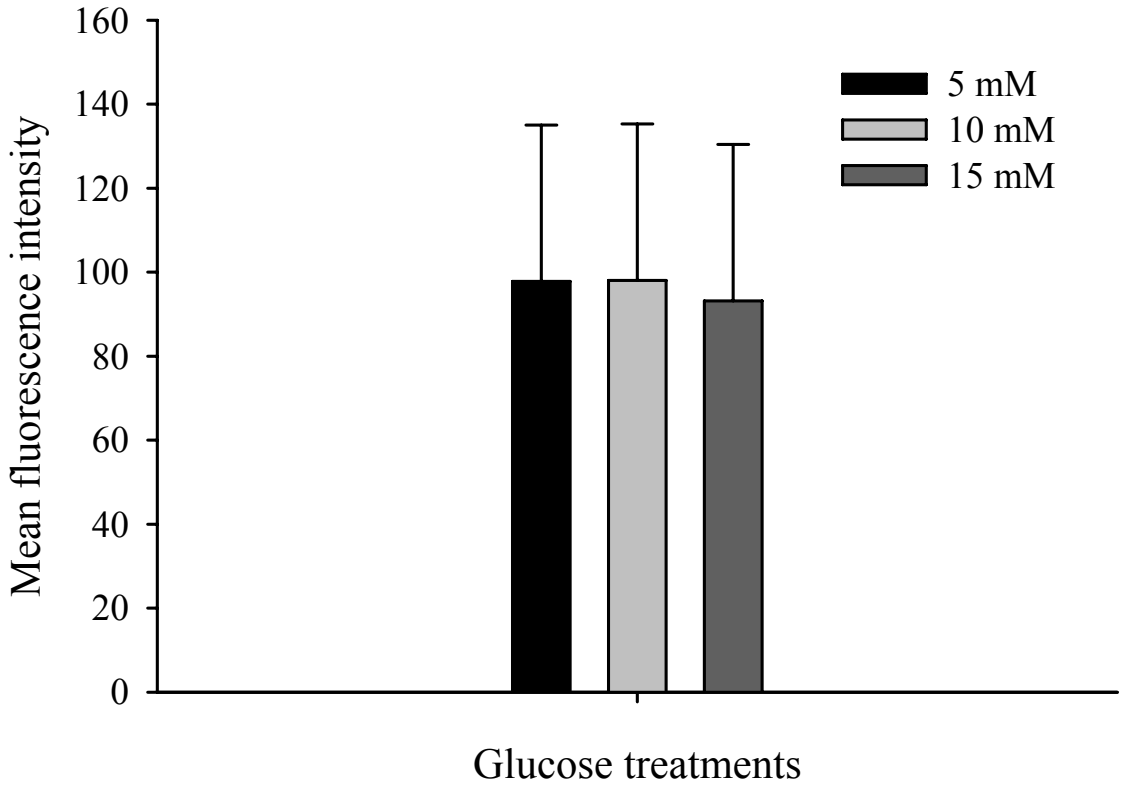

Figure 3.8 Effect of glucose availability on thymocyte mitochondria biomass. Thymic lobes from embryonic day 15 were cultured in ETOC media containing 5, 10 or 15 mM glucose. Thymocytes were isolated after 24 h of culture. Values are means ± SEM, n = 4. Means not sharing a common letter differ, $P<0.05$.

Table 3.1 Effect of glucose availability on thymocyte apoptosis, dead cells, glucose uptake and hexokinase activity[1].

	Apoptosis[2] (%)		Dead[3] (%)		Glucose uptake[4]		Hexokinase activity[5]	
	6 h	12 h	6 h	12 h	6 h	12 h	6 h	12 h
5 mM glucose	10.8	10.9	1.4	2.2	15.05	15.55	59.6	37.7
15 mM glucose	11.4	8.7	0.3	1.7	16.24	16.76	47.0	56.6
Pooled SEM	0.82		0.30		3.02		8.01	
Time *P*-value	0.13		0.01		0.87		0.46	
Glucose *P*-value	0.37		0.02		0.70		0.70	
Time X Glucose *P*-value	0.11		0.35		0.99		0.07	

[1]Values are means ± SEM, n = 5.
[2]Thymocytes gated as annexin-V positive and propidium iodide negative cells.
[3]Thymocytes gated as propidium iodide positive and annexin-V negative cells.
[4]Background-corrected mean fluorescent intensity of 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) uptake.
[5]nmoles of NADPH produced per mg protein per minute.

CHAPTER 4

GLUCOSE AVAILABILITY ALTERS CHICKEN THYMOCYTE INTERLEUKIN-7 RECEPTOR GENE EXPRESSION, T CELL RECEPTOR REARRANGEMENT AND T CELL DIFFERENTIATION.

Abstract:

Glucose availability regulates thymocyte energy status and survival in the chicken embryonic thymus. Since thymocytes develop in a low glucose environment during embryogenesis, the objective of these experiments was to determine the effect of glucose availability on thymocyte development using an embryonic thymic organ culture system. The consequences of altered glucose availability were determined by measuring thymocyte interleukin-7 receptor α (IL-7Rα) mRNA abundance, T-cell receptor (TCR) rearrangement and T cell population profiles in embryonic thymic organ culture. Thymocyte IL-7Rα mRNA abundance over the culture period was dependent upon glucose availability ($P<0.05$). Between 12 and 24 h, thymocytes cultured in 5 mM glucose increased IL-7Rα mRNA abundance 1.74-fold while thymocytes cultured in 15 mM decreased IL-7Rα mRNA abundance by 58.6%. Glucose availability affected TCR β chain rearrangement. TCR β excision circles were higher in thymocytes cultured in 15 mM glucose compared to 5 mM glucose at 12 h of culture ($P<0.05$). The number of unrearranged TCR β chains, TCR γ excision circles and unrearranged TCR γ chains in thymocytes decreased with time in both glucose treatments ($P<0.05$). The percentage of $CD4^+$ T cells over the culture period was dependent upon glucose availability ($P<0.05$). The $CD4^+$ T cell population decreased with time in 5 mM glucose, whereas it increased with time in 15 mM glucose. The $CD8^+$ T cell population increased with time ($P<0.05$) in both glucose treatments. Taken together, glucose availability modifies TCR β rearrangement, IL-7Rα gene expression and regulates $CD4^+$ T cell development. Based on these results, we speculate that the low glucose environment during embryogenesis

may limit thymocyte differentiation. Therefore, this warrants further investigation in physiologically complex settings such as *in ovo* or neonatal chick models.

Keywords: Glucose, Interleukin-7 receptor α, T cell receptor, T cell receptor excision circles, thymocytes

Introduction:

The thymic microenvironment is vital for naïve T lymphocyte generation. Signals from the thymic microenvironment to thymocytes are mediated via the interleukin-7 receptor (IL-7R)[1] and the T cell receptor (TCR) (Anderson et al., 2000), which regulate T cell survival and differentiation (Aifantis et al., 2006; Akashi et al., 1998; Haks et al., 1999), entry into the cell cycle (Aifantis et al., 2006; Hare et al., 2000) and ability to pass critical developmental checkpoints within the thymus (Laouar et al., 2004; Malissen and Malissen, 1996). IL-7R is highly expressed in T cell progenitors and double negative (DN) T cells and provides these cells with survival and proliferation signals (Hofmeister et al., 1999; Mazzucchelli and Durum, 2007). In IL-7R$^{-/-}$ deficient mice, absence of the IL-7R signal inhibits common lymphoid progenitor T cell lineage commitment, DN T cell proliferation and TCR rearrangement (Khaled et al., 2002; Maraskovsky et al., 1997). Pre-TCR or TCR are expressed in double positive (DP) and single positive (SP) T cells and are required for their further selection and differentiation into naïve T cells (Michie and Zuniga-Pflucker, 2002). Both IL-7R and TCR expression by thymocytes are either induced or inhibited in response to changes in cytokines (Doan et al., 2003; Park et al., 2004) as well as hormones and developmental ligands secreted and presented by thymic stromal cells (Balciunaite et al., 2005; Franchimont et al., 2002; Lee et al., 2005). Thus, both IL-7R and TCR expression by thymocytes are dynamically regulated to integrate signals from the thymic microenvironment that support and guide T cell development.

T cell development in the thymus is extremely sensitive to nutritional status (Hasselbalch et al., 1996; Mittal et al., 1988) and physiological changes associated with altered nutritional status (McDade et al., 2001; Moore et al., 2006; Savino, 2002; Winick

and Noble, 1966). Common functional consequences of such nutrition-induced changes on T cell development in the thymus include altered T cell population profiles and reduced naïve T cell generation (Fraker and King, 2004; King et al., 2002). For example, decreasing energy availability reduces thymus size and cellularity, particularly DN thymocytes (Poetschke et al., 2000). Similar responses have also been reported in response to altered availability of nutrients, such as zinc (Fraker and King, 2004). Though such findings are commonly associated with an altered nutritional environment, information pertaining to the impact of nutritional environment on the expression of ligand-receptor interactions that are vital T cell development is poorly defined.

The bioenergetic status of T cells is one factor that regulates their developmental progression in the thymus (Ciofani and Zuniga-Pflucker, 2005; Jacobs and Rathmell, 2006). In chickens, thymocytes develop during a period of metabolic adaptation. Glucose is the major energy source for mammalian thymocytes (Ardawi and Newsholme, 1982; Ardawi and Newsholme, 1985), and chicken T cells develop in a relatively low glucose environment during embryogenesis (Rudrappa and Humphrey, 2007). Limiting glucose availability to developing T cells decreases their energy status and ability to survive (Rudrappa and Humphrey, unpublished); however, the effect of glucose availability on T cell development in chickens is not known. Therefore, the objective of these studies was to determine the effects of glucose availability on T cell development using an embryonic thymic organ culture system. The consequences of altered glucose availability on T cell development were determined by measuring changes in the mRNA abundance of IL-7Rα, amounts of TCR gene rearrangements and T cell developmental populations.

[1]Abbreviations: DN, Double negative; DP, Double positive; ETOC, Embryonic thymic organ culture; IL-7, Interleukin-7; IL-7Rα, Interleukin-7Rα; Pre-TCR, Pre-T cell receptor; SP, Single positive; TCR, T cell receptor; TREC, T cell receptor excision circles; β_2-M, β_2-Microglobulin; GAPDH, Glyceraldehyde phosphate dehydrogenase; HPRT-1, Hypoxanthine phosphoribosyltransferase-1; TBP, TATA box binding protein.

Materials and Methods:

Animals. Fertile chicken eggs of the Ross strain (Allen's hatchery, Seaford, DE) were incubated at 37.5°C and 65% humidity to obtain embryos. All animal procedures were approved by the University of Maryland Animal Care and Use Committee.

Embryonic thymic organ culture (ETOC). Thymic lobes were cultured on transwell permeable supports (Corning Inc., Corning, NY; 3401) as described by Bendelac et al. (Bendelac et al., 1992) with modifications. Thymic lobes were harvested on embryonic day (e) 15 and were placed on a polycarbonate membrane (12 mm diameter; 0.4 μm pore size). Replicates consisted of four thymic lobes per embryo per transwell insert. In all experiments, RPMI 1640 with glutamine (Invitrogen, Carlsbad, CA; 11875-085) containing 10% fetal bovine serum and 1% penicillin-streptomycin (ETOC media) was used. Glucose deplete (0 mM) RPMI 1640 and glucose replete (10 mM) RPMI 1640 were mixed to achieve 5 mM final glucose concentrations and D-glucose (Sigma, St. Louis, MO; G-8270) was added to glucose replete RPMI 1640 to achieve 15 mM final glucose concentrations. ETOC media was added to each well (900 μl) and ETOCs were maintained at 37°C with 5% CO_2. Previous ETOC studies using glucose deplete ETOC media showed that thymocytes can be maintained until 24 h with greater than 80% viability (Rudrappa and Humphrey, unpublished). Previous studies also showed that thymocyte metabolism, energy status and survival differ between 5 and 15 mM glucose (Rudrappa and Humphrey, unpublished), which is similar to changes in serum glucose

concentration from e20 to d7 posthatch. Therefore, ETOC were maintained for 24 h in ETOC media that contained either 5 or 15 mM glucose.

Lymphocyte isolation. Thymocytes were isolated from thymic lobes by mincing and straining as previously described (Rudrappa and Humphrey, 2007). For total RNA and DNA isolation, thymocyte pellets were immediately snap-frozen in liquid nitrogen and stored at -80°C until further analysis. For all other assays, isolated thymocytes were immediately used at the indicated cell number.

Quantification of mRNA abundance by real-time PCR. Thymocyte IL-7Rα mRNA abundance was determined after 12 and 24 h of culture in ETOC media containing 5 and 15 mM glucose (n = 6). Total RNA was isolated from 2.5 x 10^6 thymocytes using the Trizol reagent (Invitrogen, Carlsbad, CA; 15596-018), and optical density at 260 nm was used to determine total RNA concentrations. Total RNA (0.5 μg) was reverse transcribed using the iScript Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA; 170-8896) and oligo$(dT)_{20}$ primer according to the manufacturer's instructions. PCR product of IL-7Rα gene specific primers (**Table 4.1**) were sequenced (GENEWIZ, Inc., North Brunswick, NJ) to ensure amplification specificity. Real-time PCR, cycling conditions and analysis were performed as previously described (Rudrappa and Humphrey, 2007) with minor modifications. Target gene mRNA abundance was normalized by geometric averaging of HPRT, GAPDH, TBP and β2-M raw-non-normalized values using the geNorm software (Vandesompele et al., 2002). Data are presented as the normalized fold-change in mRNA abundance of a gene relative to its mRNA abundance at 0 h.

T cell receptor (TCR) rearrangement. Primers were designed for Vβ1-dβ and Vγ1-Jγ1 T cell receptor excision circles (TREC) as described by Kong et al. (Kong et al., 1999) (Table 4.1). The Vβ1-dβ and Vγ1-Jγ1 primers detect rearrangement between Vβ1/Vβ2 gene segment and dβ segment on TCR β locus, and rearrangement between Vγ1/Vγ2/Vγ3 and Jγ1 segment on TCR γ locus, respectively. Unrearranged TCR dβ and Vγ1 loci were measured to obtain additional data on TCR rearrangement. The TCR dβ and Vγ1 forward primers were designed to overlap the dβ or Vγ1 gene-segment boundary, respectively, and the reverse primers were designed within the immediate downstream introns (**Figure 4.1**). If there is no rearrangement in TCR β chain, primers would bind to specific sites spanning the dβ segment and the intron, which results in the amplification of this region of unrearranged DNA. Since TCR β contains only one d segment, the dβ primer pair amplification is a measure of all unrearranged TCR β loci. The TCR γ chain utilizes only one of three Vγ segments, and only one Vγ segment combines with the Jγ1 segment and the intervening sequence between the Vγ segment and the Jγ1 segment is deleted. Since Vγ1 is downstream of Vγ2 and Vγ3, this region will be deleted when there is rearrangement between either of these Vγ segments and Jγ1 segment. Therefore, if Vγ1, Vγ2 or Vγ3 are not combined with the Jγ1 segment during rearrangement, the Vγ1 region primer pairs would amplify this unrearranged region of DNA and is a measure of all unrearranged TCR gamma γ loci. These specific primers and genomic DNA were used to amplify TRECs and gene segments by PCR. PCR products were inserted into a plasmid TOPO® vector and transformed into TOP10 *E. coli* strain using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA; K4660-01). Plasmid DNA was extracted using

S.N.A.P Miniprep Kit (Invitrogen, Carlsbad, CA; K1900-01) and sequenced using M13 forward primers (GENEWIZ, Inc., North Brunswick, NJ) to ensure the insertion of desired PCR products.

Genomic DNA was isolated from thymocytes (2×10^6) using the Nucleospin tissue DNA isolation kit (Macherey-Nagel, Easton, PA; 740952.50). Threshold cycle (Ct) values were obtained for each sample using the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA; 170-8882) by iCycler-iQ Multicolor Real-Time PCR detection system (Bio-Rad, Hercules, CA). Reactions of each sample contained 1 µl of genomic DNA and 0.3 µmol/L of forward and reverse primer in a total reaction volume of 12.5 µl. Thermal cycling conditions for real-time PCR were 95°C for 3 min followed by 50 cycles of denaturing at 95°C for 15 s, annealing for 30 s and extension at 72°C for 30 s. The optimum annealing temperature for each primer pair is provided in Table 4.1. After 50 cycles, the specificity of each PCR reaction was confirmed by melt-curve analysis as described previously (Rudrappa and Humphrey, 2007). Standard curves for Vβ1-dβ TREC, Vγ1-Jγ1 TREC, dβ and Vγ1segements were prepared using 1 in 10 serial dilutions of known amounts of plasmid DNA and were analyzed in parallel with experimental samples. TREC or gene segment copy number in a ng of plasmid DNA was calculated by dividing 9.1×10^{11} bp / ng DNA by the number of nucleotides in each cloned TCR gene product (Rutledge and Cote, 2003). Regression plots of log copy number vs Ct values were obtained using the iCycler iQ Real-Time PCR Detection System Software, which provides the intercept, the slope and the PCR efficiency for each standard curve (Bio-Rad, Hercules, CA; **Table 4.2**). Absolute copy number for each sample was calculated using the standard curve and was normalized for total DNA in

each sample. Data are presented as the number of Vβ1-dβ TREC, Vγ1-Jγ1 TREC, dβ and Vγ1 TCR gene segments per ng of total cellular DNA.

T cell population profiles. Thymocytes (10^6) from ETOC after 12 and 24 h culture were incubated with FITC-conjugated mouse anti-chicken CD4 (SouthernBiotech, Birmingham, AL; 8210-02) and R-PE-conjugated mouse anti-chicken CD8α (SouthernBiotech, Birmingham, AL; 8220-09) for 30 min at room temperature. Thymocyte $CD4^+$ and $CD8^+$ expression was measured using a FACSCalibur flow cytometer (BD Biosciences). Thymocytes (10,000) were gated into $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^-CD8^+$ and $CD4^+CD8^-$ populations based on $CD4^+$ and $CD8^+$ expression. Data are presented as the percentage of T cells in each population.

Statistical analysis. Dependent variables were analyzed by the general linear model procedure (JMP, Cary, NC). A two-way ANOVA was used to determine the main effect of glucose (5 and 15 mM), culture time (12 and 24 h) and their interaction on mRNA abundance, copy number of Vβ1-dβ TREC, Vγ1-Jγ1 TREC, dβ and Vγ1 TCR gene segments and T cell populations. When glucose availability effects were significant ($P<0.05$) and no significant interaction, treatment means within a time point were compared using LS means contrasts ($P<0.05$). Data on T cell populations were arcsin transformed to stabilize variances associated with small or large percentage values. Data are reported as non-transformed means and pooled standard errors.

Results:

IL-7 receptor α (IL-7Rα) mRNA abundance.

Thymocyte IL-7Rα mRNA abundance over the culture period was dependent upon glucose availability ($P<0.05$; **Figure 4.2**). Between 12 and 24 h, thymocytes cultured in 5 mM glucose increased IL-7Rα mRNA abundance 1.74-fold while thymocytes cultured in 15 mM decreased IL-7Rα mRNA abundance by 58.6%.

T cell receptor (TCR) rearrangement.

Glucose availability alters TCR β excision circle (β TREC) number. The number of β TRECs was higher in 15 mM glucose compared to 5 mM glucose at the 12 h time point ($P<0.05$; **Figure 4.3A**). β TREC copy numbers and other data on TCR rearrangement at the 0 h time point is provided as a reference, and were not included in the statistical analysis. The number of unrearranged TCR β chains in thymocytes cultured in 5 and 15 mM glucose decreased with time in both glucose treatments ($P<0.05$; Figure 4.3B). The number of TCR γ excision circles (γ TREC) decreased with time between 12 and 24 h in both glucose treatments ($P<0.05$; **Figure 4.4A**). The number of unrearranged TCR γ chains in thymocytes decreased with time in both glucose treatments ($P<0.05$; Figure 4.4B).

T cell population profiles.

$CD8^+$ T cells increased with time ($P<0.05$) in 5 and 15 mM glucose treatments. Double negative T cells tended ($P = 0.07$) to decrease with time in both 5 and 15 mM glucose treatments. The $CD4^+$ T cell population over the culture period was dependent

upon glucose availability ($P<0.05$; **Table 4.3**). The $CD4^+$ T cells population decreased with time in 5 mM glucose, whereas it increased with time in 15 mM glucose (**Figure 4.5**).

Discussion:

Our previous studies showed that glucose availability affects thymocyte metabolism and energy status in the chicken embryonic thymus. Thymocyte energy status is linked to their growth and proliferation. Therefore, the objective of these experiments was to study the effects of glucose availability on T cell development in the embryonic thymus. First, IL-7 receptor α (IL-7Rα) mRNA abundance was measured as this receptor is vital for thymocyte development along with the TCR. Second, unrearranged TCR and TRECs were quantified to assess TCR rearrangement. Finally, T cell populations were quantified based on CD4 and CD8 expression to assess their developmental progression in response to glucose availability. These data show that glucose availability modifies thymocyte IL-7Rα mRNA abundance, TCR β rearrangement and the development of $CD4^+$ T cells within the thymus.

Thymocyte IL-7Rα gene expression increased with time when glucose availability was low. IL-7 and IL-7Rα ligand-receptor interaction is necessary for thymocytes to overcome developmental checkpoints and to differentiate into naïve T cells in the thymus (Laky et al., 2006; Mazzucchelli and Durum, 2007). IL-7Rα promotes thymocyte differentiation by providing signals for survival (Khaled et al., 2002; Maraskovsky et al., 1997; Rathmell et al., 2001) in double negative and single positive thymocytes (Sudo et al., 1993), by regulating cell cycle progression (Swainson et al., 2006) and by providing signals for TCR rearrangement in DN T cells (Berg and Kang, 2001; Candeias et al., 1997). Thus, IL-7 and IL-7Rα ligand-receptor axis provides diverse signals to different thymocyte subsets. These effects on thymocyte differentiation are partly due to its regulation of glucose metabolism via the PI3K pathway (Ciofani and Zuniga-Pflucker,

2005; Swainson et al., 2007; Yu et al., 2003). Our previous studies showed that glucose availability altered thymocyte mitochondria membrane potential, a measure of cell energy status, in a glucose concentration dependent manner (Rudrappa and Humphrey, unpublished). Glucose deprivation has been shown to regulate cell cycle arrest via AMPK-p53 signaling to conserve energy and to promote cell survival (Jones et al., 2005). Collectively, increased IL-7Rα expression in low-glucose conditions may be due to several reasons. First, double negative and single positive cells may receive additional survival signals. Second, DN thymocytes, the most abundant cell population, rearrange their TCR in low glucose conditions to differentiate into double positive (DP) T cells. Lastly, a combination of both aforementioned causes in response to low glucose availability may have occurred. Information on how IL-7Rα is regulated during transient stages of metabolic adaptation is limited and the results from these studies provide evidence that IL-7 signaling through IL-7Rα may play a key role in thymocyte survival and differentiation while adapting to metabolic perturbations, such as during the embryonic to posthatch transition in chickens.

The absolute number of unrearranged TCR β and β TREC numbers were numerically higher compared to unrearranged TCR γ and γ TREC numbers, respectively. This may be due to ontogeny of T cell development in the chicken thymus where the second wave of T cell progenitors become the predominant subpopulation between embryonic day 15 to 18 and a majority of these cells differentiate into αβ TCR T cells (Cooper et al., 1991). TCR β chain rearrangement was increased with high glucose availability. TCR β chain rearrangement and its expression with the common α receptor as the pre-TCR are essential for thymocyte differentiation and are regulated by cytokines

and developmental ligands (Ciofani et al., 2004). TCR rearrangement and selection occurs during the DN stage of T cell development and are energy intensive (Ciofani and Zuniga-Pflucker, 2005; Jacobs and Rathmell, 2006). Consequently, glucose metabolism is induced by cytokines, such as IL-7, and developmental ligands, such as Notch1 receptor-ligands, to maintain an adequate bioenergetic state to rescue DN cells from cell death by pro-apoptotic proteins, such as p53 and TNFR (Jiang et al., 1996; Newton et al., 2000). Our previous studies showed that low-glucose availability results in decreased energy status in thymocytes (Rudrappa and Humphrey, unpublished). Therefore, it is likely that low glucose availability reduces T cell energy status and reduces TCR β chain rearrangement. The information on effects of altered cellular glucose metabolism on TCR rearrangement and expression are limited; however, TCR–antigen interaction increases glucose metabolism to meet the energy and metabolite needs of these activated T cells (Frauwirth and Thompson, 2004; Tamas et al., 2006). Upon successful TCR rearrangement and the expression of pre-TCR, developing T cells interact with MHC expressed by stromal cells in the thymic microenvironment while undergoing selection. Consequently, this antigen-independent signal increases glucose metabolism in these proliferating thymocytes. Therefore, glucose availability is important for maintaining the energy status of developing T cells and for their differentiation into the next developmental stage.

The development of $CD4^+$ T cells was affected by glucose availability. The changes $CD4^+$ T cells could be due to their altered rates of apoptosis, proliferation, progression from the DP stage, or to a combination of these factors. Results from previous studies showed that low glucose availability increased thymocyte apoptosis

(Rudrappa and Humphrey, unpublished). Therefore, $CD4^+$ T cells may be susceptible to apoptosis when glucose availability is limited. At the whole-animal level, reducing energy status reduces thymocyte number and differentiation (Fraker and King, 2004; King et al., 2002) and increases apoptosis in specific developing T cell populations (Fraker and King, 2001; King et al., 2002; Poetschke et al., 2000). This suggests that thymocyte development in glucose limiting conditions may hinder the generation of naïve $CD4^+$ T cells and their emigration from the thymus.

Thymocyte subset ($CD8^+$ and DN cells) proportions were influenced by time. Culturing chicken thymic lobes for one week resulted in the decreased DN cells and the accumulation of $CD8^+$ cells (Davidson et al., 1992). Glucose-independent changes in thymocyte subsets during culture could be due to their differentiation into the subsequent stage of development or a change in a particular thymocyte subset as a result of cell death or proliferation. In our studies, DN T cells tended to decrease in both glucose treatments while $CD8^+$ T cells increased. This may be due to the differentiation of DN cells to DP T cells and then to $CD8^+$ T cells. Unlike $CD4^+$ cells, $CD8^+$ cell differentiation was not affected by glucose availability, which suggests that $CD8^+$ T cell development is independent of glucose availability. It can be speculated that glutamine, another important energy substrate for lymphocytes, may be used by $CD8^+$ cells to maintain their energy status when glucose availability is limiting and may explain why $CD8^+$ T cell populations did not differ between low and high glucose treatments.

Glucose availability regulated the expression of receptors required for thymocyte differentiation. Similarly, glucose has been shown to regulate the expression of cytokines, such as IFN γ, cytokine receptors, such as IL-2R, in effector T cells and their function

(Cham and Gajewski, 2005; Stentz and Kitabchi, 2005). Glucose availability determines T cell energy status, which controls the rate of various energy-consuming processes, such as transcription and translation, resulting in the inhibition of entry into the cell cycle (Buttgereit and Brand, 1995; Buttgereit et al., 1992; Buttgereit et al., 2000). Accordingly, functional responses in developing thymocytes due to changes in glucose availability are likely due to altered thymocyte energy status. Therefore, high glucose availability should promote thymocyte differentiation after overcoming developmental checkpoints. In addition, these results show that glucose-induced alterations in T cell development occur in the context of the thymic microenvironment. Hence, it may be possible to manipulate thymocyte energy status and the expression of developmental receptors in the thymus via nutritional supplementation. This is a useful tool to manipulate thymocyte development when there is change in supply of energy-yielding nutrients or changes in energy metabolism at the whole-animal level due to physiological changes associated with life-stage transitions.

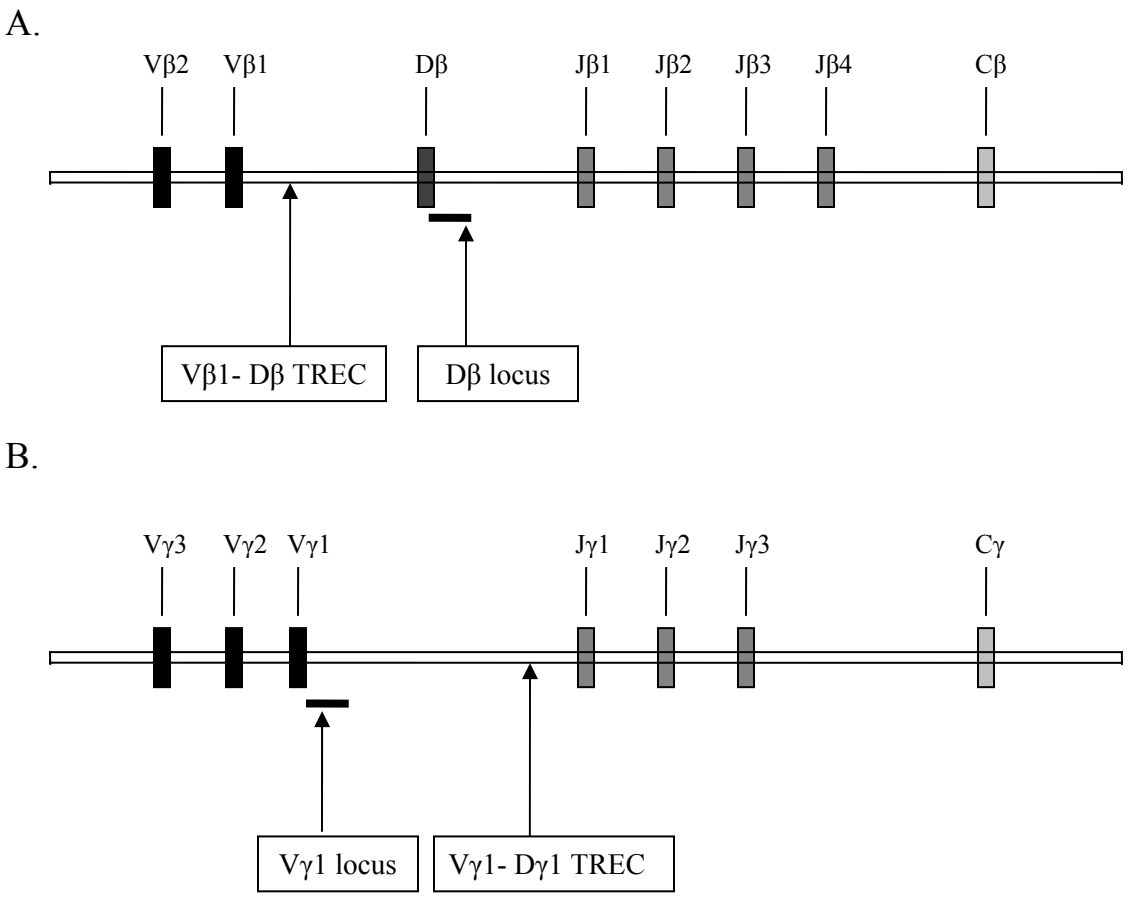

Figure 4.1 A schematic diagram showing the location of specific primers used to amplify T cell receptor excision circles and gene-segments on TCR β (A) and TCR γ (B) chain locus.

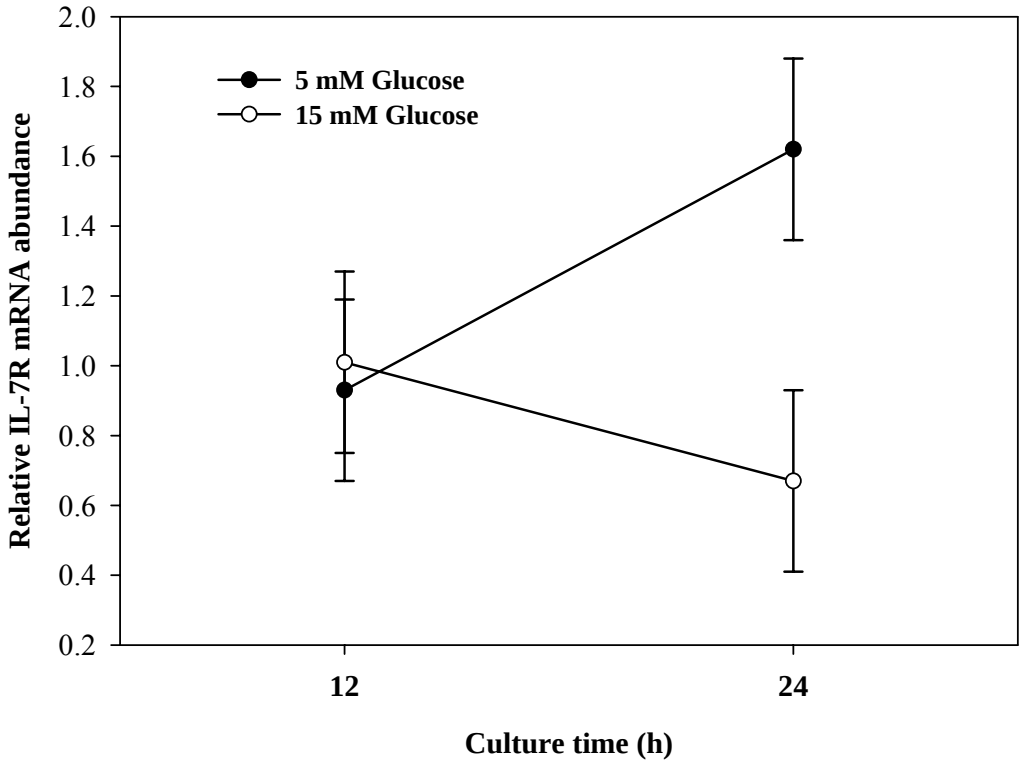

Figure 4.2 Effect of glucose availability on IL-7 receptor α mRNA abundance in thymocytes. Thymic lobes from embryonic day 15 were cultured in ETOC media containing 5 and or 15 mM glucose. Thymocytes were isolated after 12 and 24 h of culture. Data are presented as the normalized fold-change in mRNA abundance of a gene relative to 0 h time point. Values are means ± SEM, n = 6.

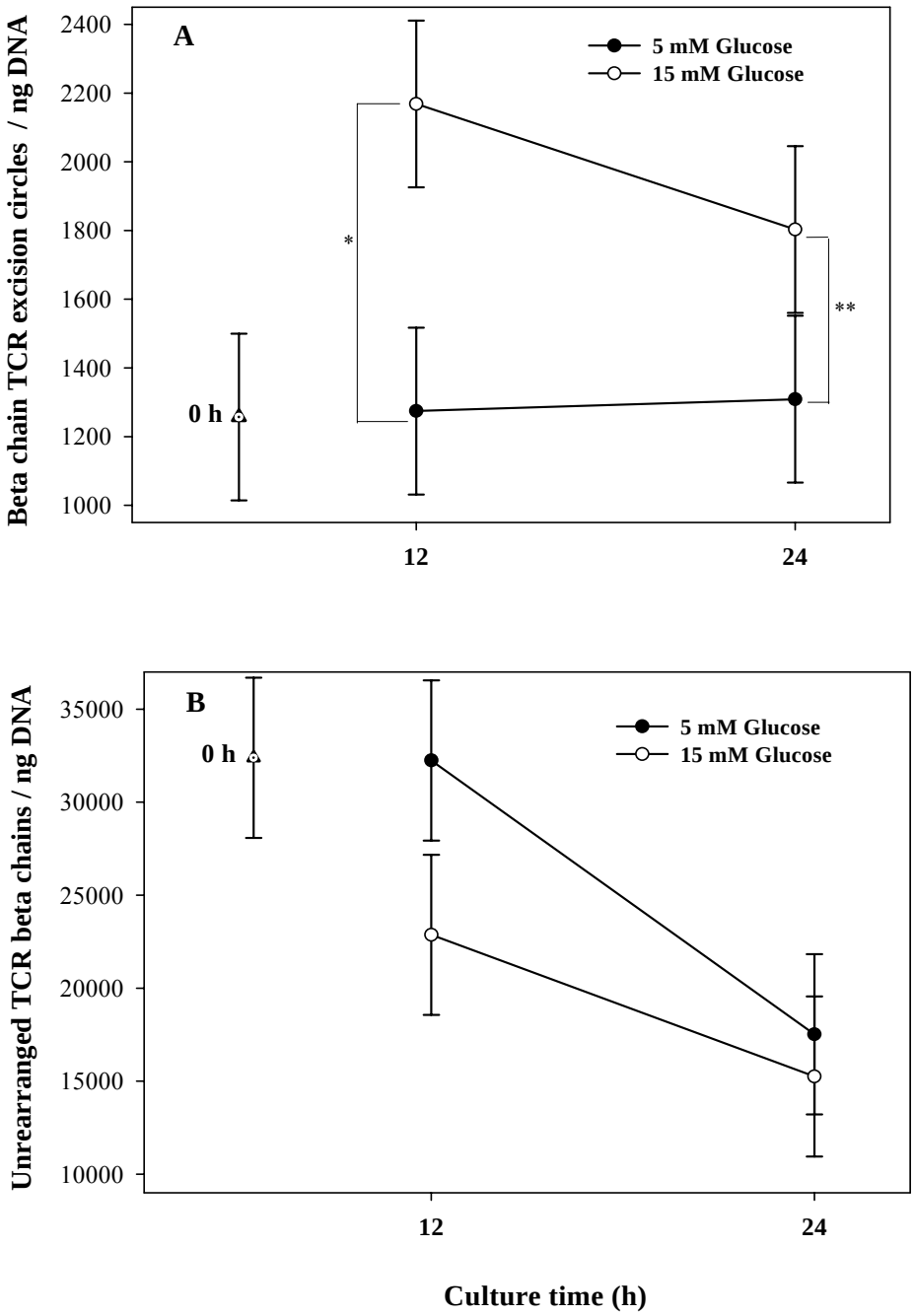

Figure 4.3 Effect of glucose availability on TCR β rearrangement in thymocytes. (A) TCR β excision circles; (B) unrearranged dβ locus. Thymic lobes from embryonic day 15 were cultured in ETOC media containing 5 and 15 mM glucose. Thymocytes were isolated after 12 and 24 h of culture. Data are presented as number of TCR β excision circles or unrearranged TCR β per ng DNA. The average of 0 h time point is provided for reference. Values are means ± SEM, n = 6. P values for TCR β excision circles: * < 0.02; **<0.17. P values for unrearranged dβ locus: Time < 0.02.

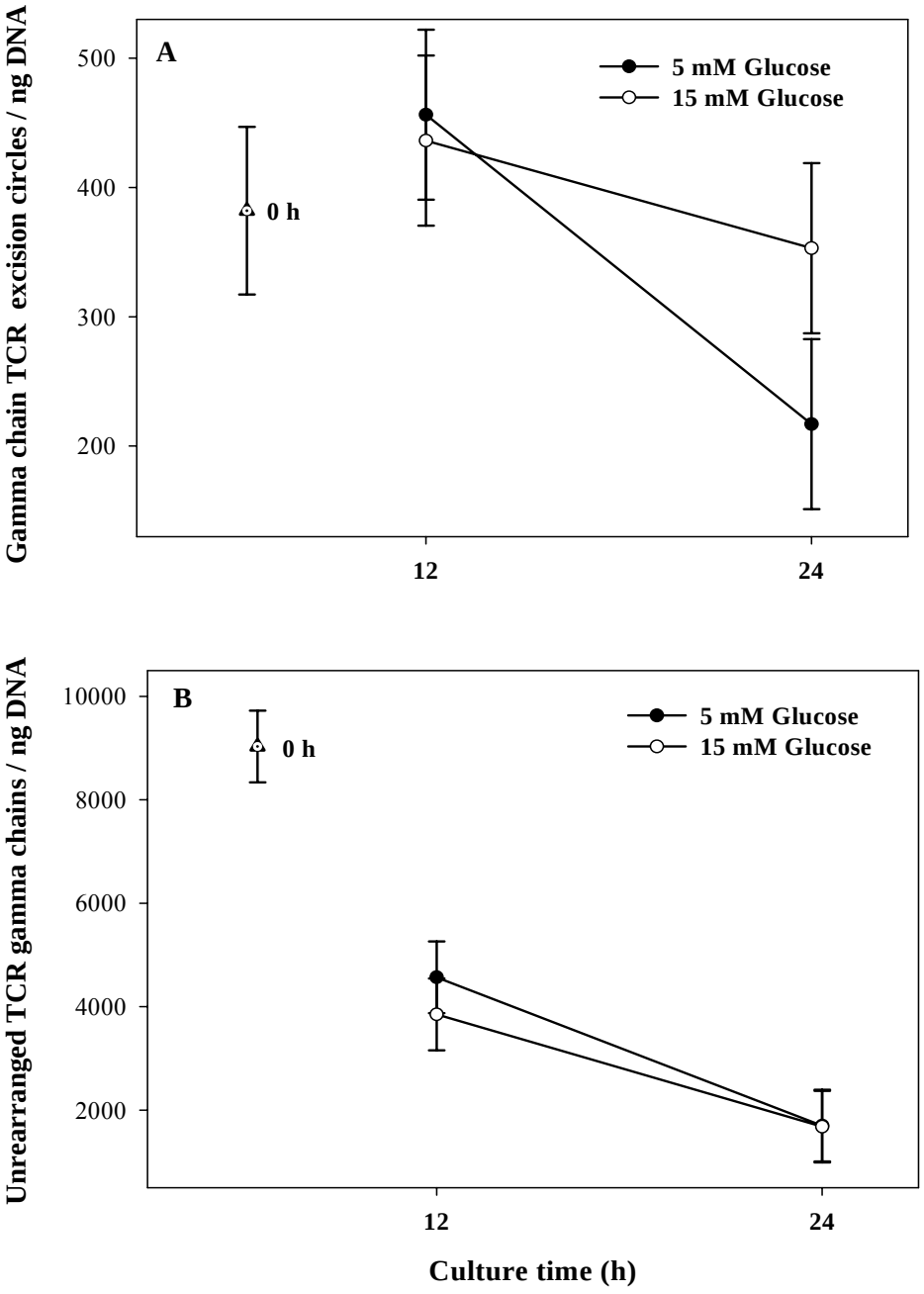

Figure 4.4 Effect of glucose availability on TCR γ rearrangement in thymocytes. (A) TCR γ excision circles; (B) unrearranged Vγ1 locus. Thymic lobes from embryonic day 15 were cultured in ETOC media containing 5 and 15 mM glucose. Thymocytes were isolated after 12 and 24 h of culture. Data are presented as number of TCR γ excision circles or unrearranged TCR γ per ng DNA. The average of 0 h time point is provided for reference. Values are means ± SEM, n = 6. P values for TCR γ excision circles: Time < 0.02. P values for unrearranged Vγ1 locus: Time < 0.01.

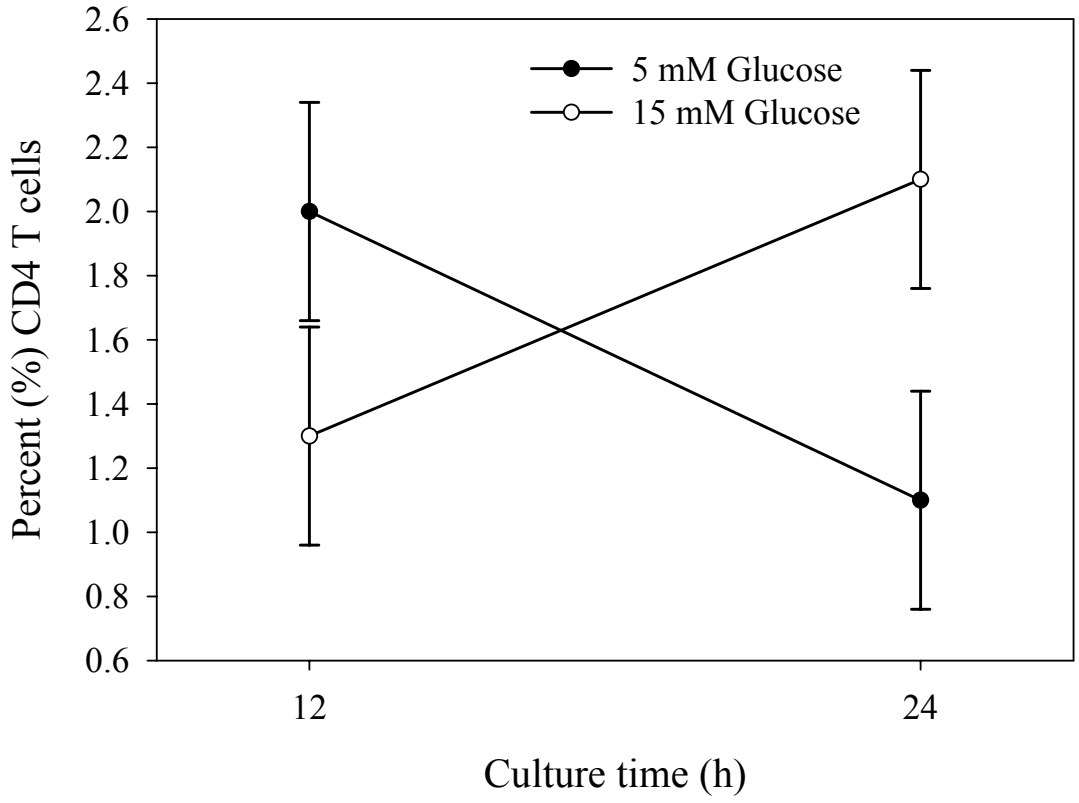

Figure 4.5 Effect of glucose availability on $CD4^+$ T cell population. Thymic lobes from embryonic day 15 were cultured in ETOC media containing 5 and 15 mM glucose. Thymocytes were isolated after 12 and 24 h of culture. Values are means ± SEM, n = 5. P values: Time X Glucose < 0.04.

Table 4.1 Primers used to quantify mRNA abundance or gene copy number by real-time PCR[1].

Gene	Primer sequence (5' → 3')	Orientation	Annealing (°C)	Product size (bp)
IL-7R[2]	TACACCACACTCACGGAAG	F	56	352
	CACCTCATACAACGCATCTG	R		
HPRT-1	GGGAAACAGAGAGGGTTGGAGTG	F	57	213
	TCCGCCCGTCTTTCTAACATCC	R		
GAPDH	GGTGCTGAGTATGTTGTGGAGTC	F	59	290
	GTCTTCTGTGTGGCTGTGATGG	R		
TBP	TTTAGCCCGATGATGCCGTATG	F	58	196
	CTGTGGTAAGAGTCTGTGAGTGG	R		
β_2-M	TGGAGCACGAGACCCTGAAG	F	59	161
	TTTGCCGTCATACCCAGAAGTG	R		
Vβ1-dβ TREC	GCAGAGAACAAGCAGAAGAC	F	57	300
	ACAGAGCAGTGGCAGAGGAA	R		
Vγ1-Jγ1 TREC	TCCTCAGTGTAAGCCTGTGG	F	57	381
	CCAAACAGCACTTACACTCT	R		
dβ locus[2]	GGGAATGAAAGCACCATGAC	F	57	318
	ATCATTGTGGATCCCCCTGT	R		
Vγ1 locus[2]	TGCAGTATTTACCTTGTGTGGA	F	57	305
	GACAAAGGAAGTTCAGCACCA	R		

[1]Abbreviations used: IL-7R, Interleukin-7 receptor; β_2-M, β_2-Microglobulin; GAPDH, Glyceraldehyde phosphate dehydrogenase; HPRT-1, Hypoxanthine phosphoribosyltransferase-1; TBP, TATA box binding protein; TREC, T cell receptor excision circles.
[2]Genbank accession number: IL-7R, NM_205209; HPRT-1, NM_204848; GAPDH, NM_204305; TBP, NM_205103; β_2-M; Z48921; dβ locus, AB092341; Vγ1 locus, U78216.

Table 4.2 Standard curves for absolute quantification of T cell receptor rearrangement by real-time PCR[1].

Gene	Correlation coefficient (r)	Slope	Intercept	PCR efficiency (%)[3]
Vβ1-dβ TREC	0.99	-3.27	39.21	102.4
Vγ1-Jγ1 TREC	0.99	-3.34	39.79	99.4
dβ locus[2]	0.98	-2.57	34.54	104.4
Vγ1 locus[2]	0.95	-3.17	38.18	106.8

[1]Abbreviations used: IL-7R, Interleukin-7 receptor; TREC, T cell receptor excision circles.
[2]Genbank accession number: dβ locus, AB092341; Vγ1 locus, U78216.
[3]PCR efficiency was provided by the iCycler iQ Real-Time PCR Detection System Software. This is calculated by regressing log fluorescence at each amplification cycle Vs cycle number

Table 4.3 Effect of glucose availability on $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^-CD8^+$ and $CD4^+CD8^-$ thymocyte population[1].

	$CD4^-CD8^-$ (%)[2]		$CD4^+CD8^+$ (%)[3]		$CD4^-CD8^+$ (%)[4]		$CD4^+CD8^-$ (%)[5]	
	12 h	24 h	12 h	24 h	12 h	24 h	12 h	24 h
5 mM glucose	76.6	72.4	8.7	10.2	12.7	16.3	2.0	1.1
15 mM glucose	78.1	72.3	7.3	7.7	13.3	17.9	1.3	2.1
Pooled SEM	2.6		1.59		1.64		0.34	
Glucose *P*-value	0.76		0.23		0.6		0.38	
Time *P*-value	0.07		0.51		0.03		0.73	
Time X Glucose *P*-value	0.70		0.77		0.74		0.04	

[1]Values are means ± SEM, n = 5.
[2]Thymocytes gated as negative for CD4 and CD8 expression.
[3]Thymocytes gated as positive for CD4 and CD8 expression.
[4]Thymocytes gated as negative for CD4 and positive for CD8 expression.
[5]Thymocytes gated as positive for CD4 and negative for CD8 expression.

SUMMARY, CONCLUSION AND POTENTIAL APPLICATION

Lymphocyte bioenergetic status is coordinately regulated by kinases, which integrate intracellular processes that consume energy with extracellular signals, such as cytokines, growth factors and nutrient availability (**Figure 5.1**). These signals coordinate energy-generating metabolic pathways to maintain an adequate bioenergetic status in lymphocytes, which has major implications for their survival, differentiation and proliferation. This underscores the importance of nutrition in lymphocyte biology. Glucose, a primary energy substrate in lymphocytes, metabolism and its intermediate metabolites have been shown to interact with proteins that regulate apoptosis (**Figure 5.2**) (Alves et al., 2006; Leist et al., 1997; Nutt et al., 2005; Pastorino et al., 2002; Zhao et al., 2007). In chickens, the primary energy supply is altered from lipids to carbohydrates following hatch. Therefore, the overarching goal of this project was to determine the effect of metabolic adaptations on lymphopoiesis.

Summary:

To summarize results of this project, altered macronutrient supply is one of the factors that induce metabolic adaptation in lymphocytes following hatch. The following are the summary points of each of the objectives : (a) both bursacytes and thymocytes adapt their energy metabolism by increasing glucose uptake and metabolism after hatch; (b) only bursacytes increase glutamine metabolism after hatch; (c) lipid metabolism was unaltered in both lymphocyte populations after hatch; (d) serum glucose and NEFA concentrations increase during the first week posthatch without any changes in glutamine

concentration; (e) some markers of lymphocyte glucose metabolism were positively correlated with the serum glucose concentration; (f) increased glucose availability induced thymocyte glucose metabolism and improved thymocyte mitochondria membrane potential and survival; (g) glucose availability modifies TCR β gene rearrangement, IL-7Rα gene expression and $CD4^+$ T cell development; (h) glucose availability does not affect TCR γ gene rearrangement, $CD4^-CD8^-$, $CD4^+CD8^+$ and $CD8^+$ T cell populations.

Conclusion and hypothesis:

Thymocytes develop in a low glucose environment and, based on these results, it is speculated that this might slow down thymocyte differentiation until hatch. It is hypothesized that improving the ability of thymocytes to utilize glucose *in ovo* and early neonatal life will advance their development in the thymus and exit into the periphery.

Potential application:

The following *in ovo* intervention strategies are proposed to manipulate the ability of thymocytes to utilize glucose and perhaps their development also: (a) nutritional supplements, such as glucose or carbohydrates; (b) recombinant cytokines, such as IL-7; or (c) a combination of nutritional supplements and recombinant cytokines. *In ovo* nutritional supplements increase the energy status of the embryo and tissue growth during the late embryogenesis and the first three week posthatch (Foye et al., 2006; Tako et al., 2004; Uni et al., 2005). Thymocytes constitute a small fraction of developing tissues in the embryo. It is likely that other developing tissues compete with thymocytes to utilize

energy substrates and hormones may provide favorable signals to large developing tissues, such as skeletal muscle, to transport and oxidize energy substrates. Recombinant cytokines, such as IL-7 (Okamoto et al., 2002) or the fusion IL-7 protein (Henson et al., 2005) have been used in mammals to enhance thymic function. Recombinant IL-7 increases the ability of thymocytes to acquire and metabolize glucose to generate energy, which improves their survival (Swainson et al., 2007); however, glucose is not abundantly available during embryogenesis. Therefore, the combination of nutritional supplements and recombinant cytokines hold more promise for the reason that recombinant cytokines provide inductive signals to thymocytes to utilize supplemented energy substrates.

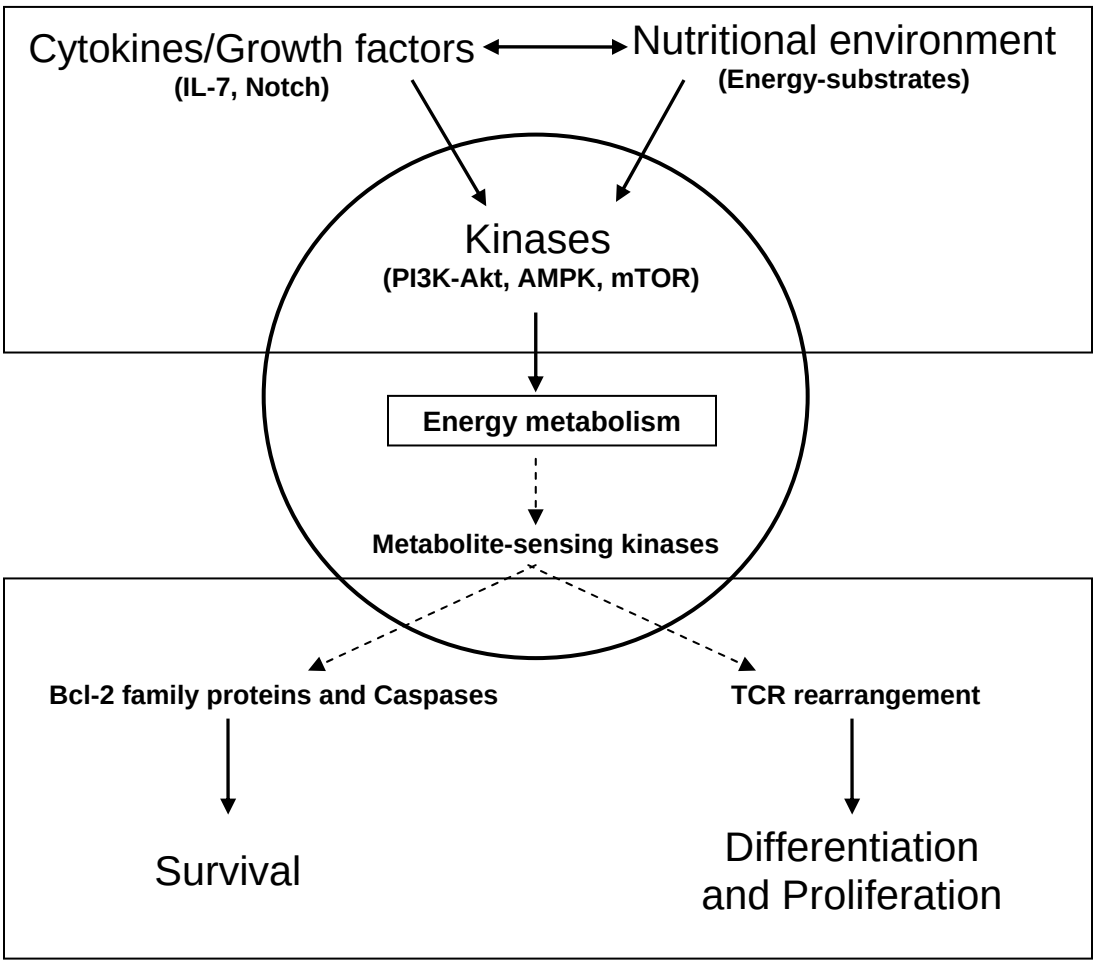

Figure 5.1 A schematic illustration to show the major extracellular factors and intracellular kinases involved in the regulation of energy metabolism in lymphocytes.

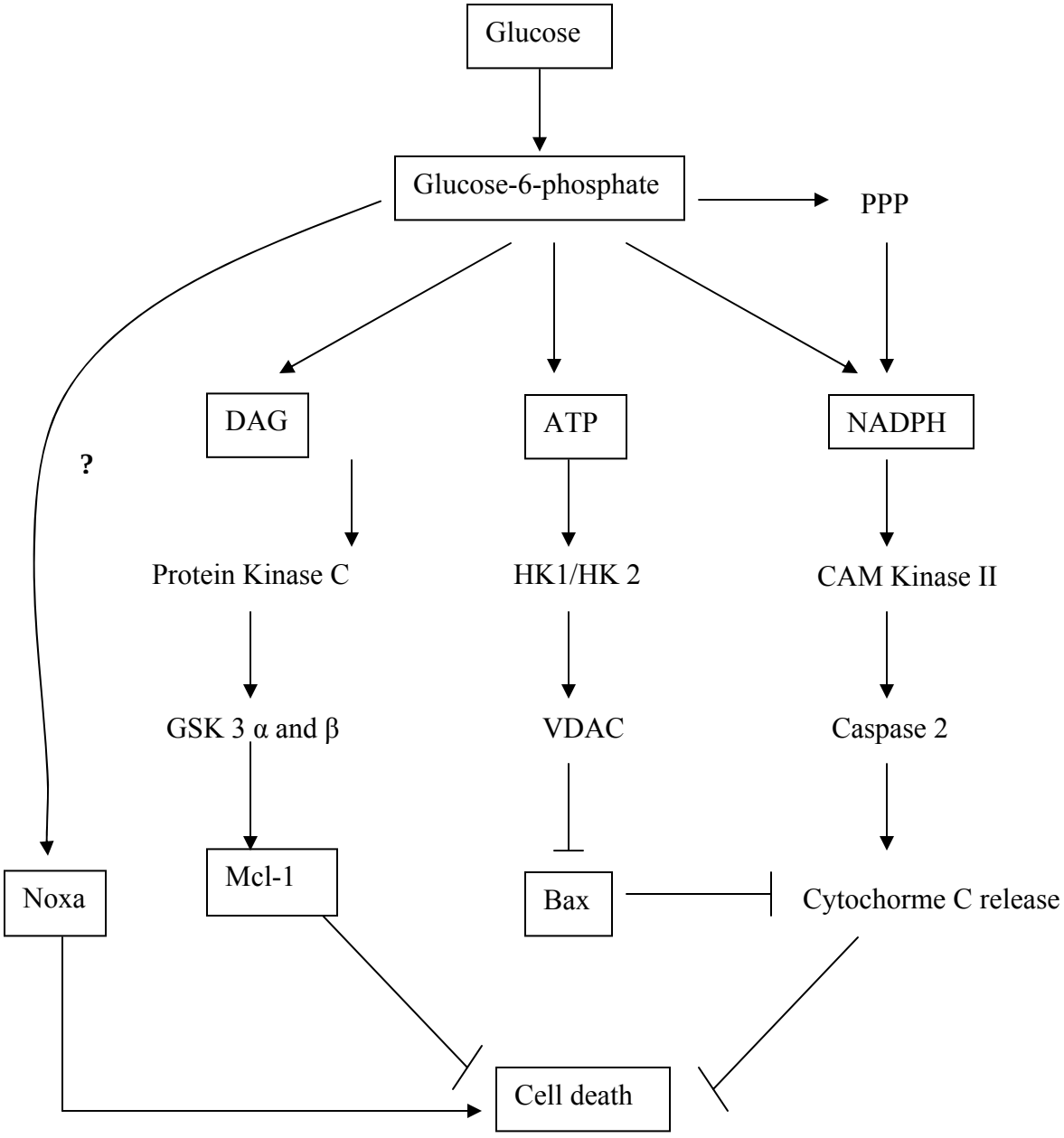

Figure 5.2 Schematic illustration of interaction of intermediatory metabolites of glucose metabolism and Bcl-2 family proteins to regulate cell death. Abbreviations: DAG, Di-acyl glycerol; HK1 and 2, Hexokinase 1 and 2; CAM Kinase II, Calmodulin Kinase II; GSK 3 α and β, Glycogen Synthase Kinase 3 α and β.

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